# MOLECULAR ANALYSIS OF GENE EXPRESSION IN MOUSE SALIVARY GLANDS

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## DEDICATION

This thesis is dedicated to my parents, especially Mum from whom, apparently, I inherited what little brains I have.

# ABBREVIATIONS

| | | |
|---|---|---|
| ACE | - | angiotensin-converting enzyme |
| ADH | - | anti-diuretic hormone |
| AI | - | angiotensin I |
| AII | - | angiotensin II |
| AIII | - | angiotensin III |
| Ap | - | ampicillin |
| A/S | - | antiserum |
| bp | - | base pairs |
| BP | - | blood pressure |
| cDNA | - | complementary deoxyribonucleic acid |
| DBM | - | diazobenzyloxymethyl |
| DEP | - | diethyl pyrocarbonate |
| DMF | - | dimethyl formamide |
| DMSO | - | dimethyl sulphoxide |
| DNA | - | deoxyribonucleic acid |
| DNAse | - | deoxyribonuclease |
| dpm | - | disintegrations per minute |
| DPM | - | dog pancreas membranes |
| dscDNA | - | double-stranded cDNA |
| DTT | - | dithiothreitol |
| EDTA | - | ethylenediaminetetra-acetic acid |
| EGF | - | epidermal growth factor |
| HMWt | - | high molecular weight |
| hnRNA | - | heteronuclear ribonucleic acid |
| hrs | - | hours |
| kd | - | kilodaltons |
| LMWt | - | low molecular weight |
| mins | - | minutes |
| mRNA | - | messenger ribonucleic acid |

| | | |
|---|---|---|
| M.Wt. | - | molecular weight |
| NGF | - | nerve growth factor |
| NRS | - | normal rabbit serum |
| OD | - | optical density |
| PMSF | - | phenyl methyl sulphonyl fluoride |
| PPO | - | 2,5-diphenyloxazole |
| psi | - | pounds per square inch |
| RAS | - | renin-angiotensin system |
| RNA | - | ribonucleic acid |
| RNAse | - | ribonuclease |
| rpm | - | revolutions per minute |
| RT | - | room temperature |
| SDS | - | sodium dodecyl sulphate |
| SL | - | sublingual |
| SMG | - | submaxillary gland |
| SSC | - | 0.15M sodium chloride, 0.015M sodium citrate |
| sscDNA | - | single-stranded cDNA |
| Tc | - | tetracycline |
| TCA | - | trichloracetic acid |
| TEMED | - | N,N,N',N'tetramethyl ethylenediamine |
| Tris | - | tris(hydroxymethyl)aminomethane |
| tRNA | - | transfer ribonucleic acid |
| (v/v) | - | (volume/volume) |
| (w/v) | - | (weight/volume) |
| X-gal | - | 5-bromo-4-chloro-3-indolyl β galactoside |
| MUP | - | Mouse Urinary Protein |
| VIP | - | Vasoactive Intestinal Polypeptide. |

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# CHAPTER 1

# INTRODUCTION

## 1.1 The salivary glands of the mouse

The salivary glands are amongst the most prolific secretory tissues of the body, producing and secreting, in varying quantities, a wide variety of hormones, enzymes and lubricating agents. Saliva is a mixture of the secretions from three major pairs of glands and in addition several minor glands which are at present poorly characterised. The secretions of each gland reach the mouth via separate ducts and mixing occurs in the oral cavity. It should therefore be possible to collect the secretions from individual salivary glands, but in practice this is a difficult procedure for small animals like the mouse. Of the three major pairs of glands the largest and most active are the submaxillary glands (SMG). Partly because of their size and ease of removal, the submaxillary glands have been by far the most intensively studied. In return, the majority of salivary gland products identified to date originate from this tissue (Barka, 1980). The one exception to this is α-amylase, whose primary site of synthesis in the salivary tissues is the parotid gland. The third pair of glands are the sublingual glands which, unlike the larger glands, have no well characterised products associated with them. They are known however to produce a high molecular weight glycoprotein or mucin, which has been isolated and its amino acid composition determined (Roukema *et al*, 1976). A summary of the proteins and peptides found in the salivary glands is given in Table 1. Very few of these are definitively products of the glands, of those that are, the best known are epidermal growth factor (EGF), nerve growth factor (NGF) and α-amylase.

The anatomical relationship of the salivary glands is shown in Figure 1 and indicates the substantial size difference between the submaxillary and sublingual glands. The morphology of the SMG is known to be androgen-

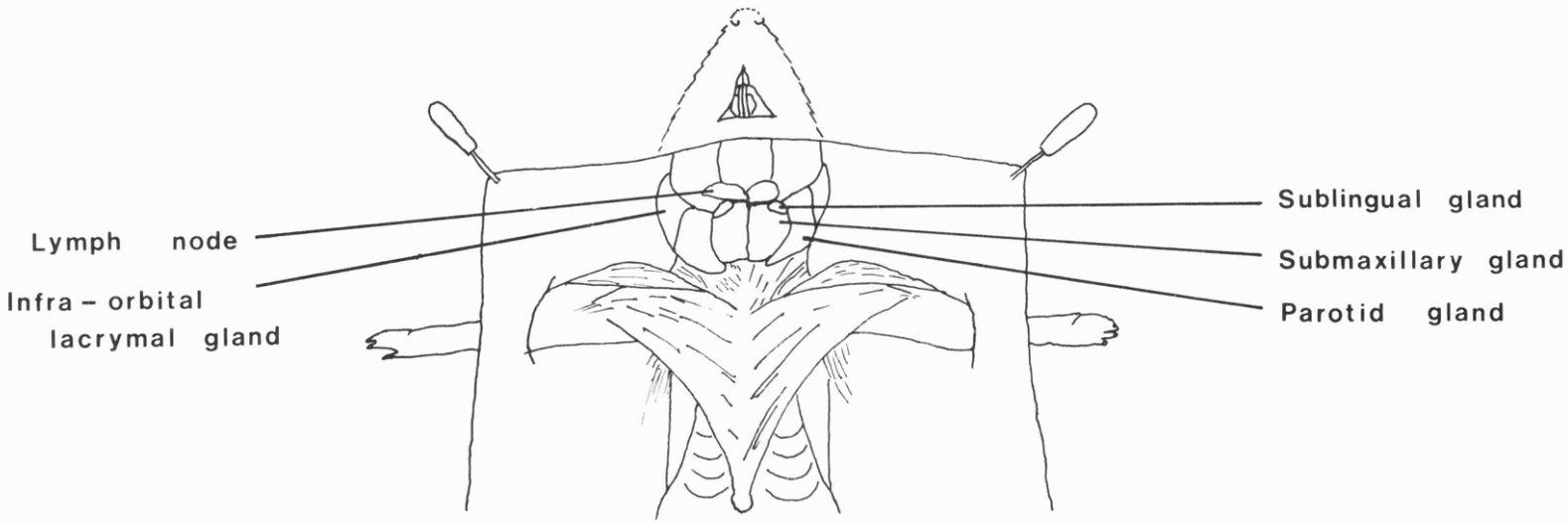



**Fig.1** - The salivary glands of the mouse

sensitive (Bhoola *et al*,1973). The overall size of the glands is slightly greater in males than females and the convoluted granular tubules are vastly more developed as shown in Fig.2. This is paralleled by the increased abundance in the male glands of several major products including EGF, NGF and RENIN.

**Table 1** - Salivary gland proteins

| PROTEIN | TISSUE | REFERENCE |
|---|---|---|
| α-,β-,γ-NGF | SM,SL | Barka,1980 (review) |
| EGF | SM | " |
| EGF binding-protein | SM | " |
| Kallikrein (β-NGF endopeptidase) | SM | " |
| Renin | SM | " |
| Esteroproteases | SM | " |
| α-amylase | P,SM | " |
| $AM_1$ (glycoprotein) | SM | Nieuw Amerongen *et al*,1977 |
| $AM_2$ (glycoprotein) | SM | Nieuw Amerongen *et al*,1978 |
| MUP | SM,SL,P | Shaw *et al*,1983 |
| VIP | SM | Polak and Bloom,1980 |
| Mucin | SM | Roukema *et al*,1976 |
| Mucin | SL | " |

The submaxillary gland is therefore an ideal tissue to study since it produces a large number of androgen-responsive products in a tissue specific manner, and these are two of the outstanding questions in the study of eukaryotic gene expression. As will be seen later in this chapter, certain products are also under genetic control. The major SMG products that have been extensively characterised are discussed in detail individually below.

## 1.2 Nerve growth factor

The discovery of a nerve cell growth-promoting activity in snake venom (Cohen, 1959) led to the observation that the submaxillary gland of the male mouse contained an extremely abundant protein with similar properties. The sublingual gland was also found to contain NGF but at only 1% of the level

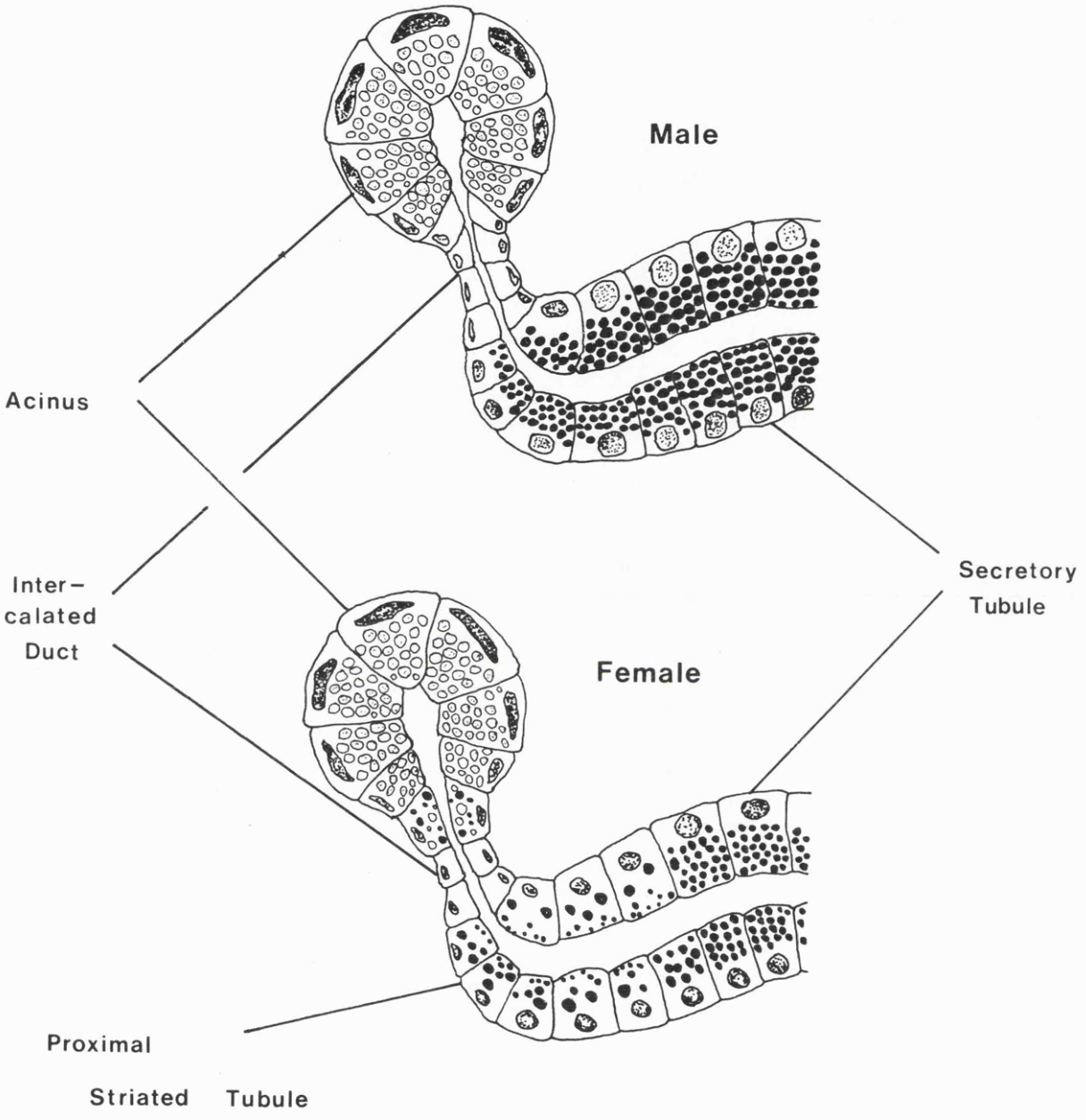



**Fig.2** - The morphology of male and female submaxillary glands

present in the submaxillary gland. The purification of the factor by Cohen (1960) allowed preliminary characterisation, and the factor was found to be a heat- and acid-labile protein, some ten-fold more active than its snake venom counterpart when assayed by its ability to promote the growth of nerve cells in tissue culture and in living chick embryos. On subcutaneous injection into mice, the isolate gave rise to a six-fold increase in the protein content of the superior cervical ganglion, accompanied by a two-fold increase in levels of DNA and RNA. When less pure NGF preparations were injected, gross anatomical changes were observed which ultimately led to the isolation of a new growth factor (see section 1.3). The injection of NGF antiserum into newborn mice resulted in the destruction of the nerve cells of the sympathetic chain ganglia whilst normal rabbit antiserum had no apparent effect (Cohen, 1960).

Two forms of NGF have been demonstrated, both of which have been isolated from the SMG, and are also found in SMG-saliva (Murphy *et al*, 1977a). The low molecular weight form (2.5S or β-NGF) is a dimer, each monomer having a molecular weight of 13,000 daltons (Angeletti *et al* 1973a,b). A high molecular weight complex is also found, consisting of one molecule of β-NGF and two molecules each of α- and γ-NGF. The biological role of the α-subunit is unknown but the γ-subunit possesses arginine esterase activity capable of specific cleavage of a 22,000 dalton precursor first seen by *in vitro* labelling of submaxillary products (Berger and Shooter,1977). The synthesis of β-NGF was shown to be under testosterone control, being present at low concentrations in the SMG prior to puberty at which stage the levels in the glands of males increased dramatically (Berger and Shooter,1978; Ishii and Shooter,1975). The 7S NGF complex was found to contain two Zn(II) ions per molecule and although the effect of these ions on the stability of the NGF complex is in doubt (Bothwell and Shooter,1978; Baker,1975) their removal irreversibly activates the protease function of the complex (Young and Koroly,1980). The carboxy-terminal arginine residue is essential in the maintenance of the overall complex

structure, and removal of this residue abolishes the binding of γ-NGF to the β-subunit (Moore *et al*,1974).

Although vast amounts of NGF are secreted into the saliva, early indications of serum NGF originating from the gland (Hendry and Iversen,1973) have not been substantiated (Murphy *et al*,1977b). Many of the properties and activities of NGF are reviewed by Bradshaw (1978) but recent observations by Orenstein *et al*(1978) and Boyle and Young (1982) have shown that the NGF complex is capable both of activating plasminogen and of complement C1-like activity:- it cleaved the zymogens C4 and C2 and was also sensitive to human C1 inactivator. The physiological relevance of these observations is unknown but, given the ability of 7S-NGF to accelerate wound healing (Li *et al*,1980), it has been suggested that these activities may be related as part of a general defence and repair system (Boyle and Young,1982).

Studies of NGF receptors has revealed the existence of high and low affinity receptors differing in their affinities by one hundred-fold. It was found that when β-NGF was added to the growth medium of PC12 cells, only low affinity receptors were initially observed. However, after a short lag phase high affinity receptors could also be detected (Landreth and Shooter,1980). If the exogenous NGF was removed after the initial binding then high affinity receptors were still produced at the expense of low affinity ones and associated with this conversion was a change from trypsin-sensitivity to a trypsin-insensitive state. This would imply either a conformational change or an interaction of the occupied receptor with another protein(s) in the plasma membrane.

The binding of NGF was found to vary with the cell cycle, being maximum in late $G_1$ and early S phases (Levi-Montalcini *et al*,1974) and its biological effects were seen to occur at very low concentrations ($10^{-12}$M). This concentration of NGF could potentially occupy only 0.1% of the low affinity receptors or 8% of the high affinity receptors. High affinity receptors originating at the cell surface have been found associated with

the nuclear membrane (Shooter *et al*,1981) and studies with monolayer and suspension cells suggest the involvement of the cytoskeleton in the translocation of NGF. This transport to the nucleus was found to be a requirement for the growth of neurites.

Recently the structure of the NGF precursor has been established by the cloning and nucleotide sequence analysis of a cDNA made from NGF mRNA (Scott *et al*,1983a). The results of that work are summarised in Figure 3. The cloning of NGF highlights both the power of molecular biology and also some of its limitations. The cDNA sequence has yielded the amino acid sequence of the precursor and provides a probe with which to ask questions relating to expression and its control. However, because the processing of NGF is relatively complex, involving both amino- and carboxy-terminal processing, the determination of cleavage sites is not unequivocal as alternative processing sites are available. Therefore a combination of traditional biosynthetic studies, amino acid sequencing and cloning technology is required to fully elucidate the pathway of NGF maturation.

## 1.3 Epidermal Growth Factor

As briefly mentioned in section 1.2, the injection of crude NGF preparations into newborn mice resulted in gross changes which were not observed with more highly purified samples (Cohen,1962). These effects included premature eyelid-opening and insissor-eruption and also thickening of the footpads, due to a direct stimulation of the proliferation and keratinisation of epidermal tissues, as determined by measuring the levels of protein, RNA and DNA in these tissues (reviewed by Hollenberg,1979).

The factor responsible was isolated by Cohen (1962) and termed epidermal growth factor (EGF). It was shown to exist as a high molecular weight complex (Taylor *et al*,1970), two molecules of EGF being associated with two molecules of an arginine protease called EGF binding-protein. In addition, like the NGF complex, it contains two zinc ions. The similarity extends further since the EGF binding-protein cleaves a peptide from the

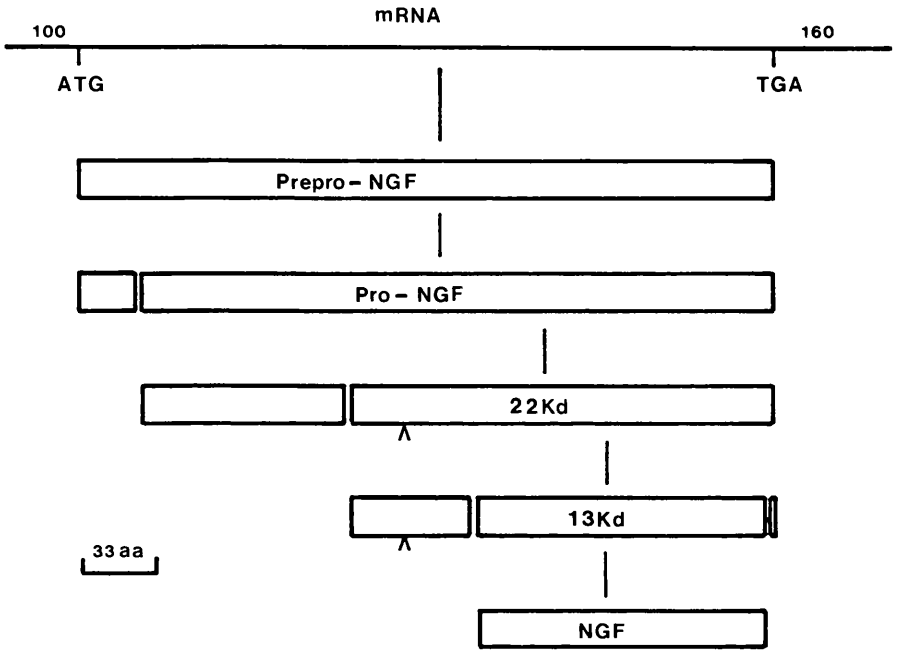



**Fig.3** - Maturation of NGF

After removal of the signal sequence three further cleavages take place, two at the $NH_2$-terminal side of NGF which yield a 22kd intermediate and then the mature 13kd NGF and one at the COOH- terminus which removes the dipeptide Arg-Gly. (Scott *et al*,1983; Berger and Shooter,1977)

carboxy-terminus of the precursor leaving a terminal arginine residue essential for maintaining the stability of the complex (Shooter *et al*,1981; Server *et al*,1976). This terminal residue is not required for biological activity, which is pleiotropic in nature, including cycloheximide-resistant stimulation of metabolite transport, showing that the hormone can elicit major changes without the need for the synthesis of new proteins. Other effects, such as the stimulation of ornithine decarboxylase activity, are dependent on protein synthesis. In keeping with this finding, administration of EGF prompted the formation of polysomes after 30 minutes contact and the levels of all species of RNA were increased 4-8 fold after 90 minutes contact. The effects of EGF are reviewed in detail by Hollenberg (1979).

As for NGF, the availability of pure EGF and its antibody have facilitated study of the hormone receptor(s) and Shechter *et al* (1978) demonstrated that ability of EGF to enhance DNA synthesis required a persistent occupation of high affinity receptors. The effect on DNA synthesis could be temporally separated from the internalisation, down regulation and lag phases of EGF binding, as it required the binding of hormone to receptor for over 8 hours before the effects were irreversible. Das (1980) found that EGF-dependent stimulation of quiescent nuclei was dependent on the intracellular generation of an unidentified protein.

The EGF receptor is internalised continuously, independent of EGF-binding, but should EGF-binding occur, the manner of this internalisation / degradation is altered. The binding of EGF to its receptor occurs via amino acids 20-31 (Komoriya *et al*,1984) and stimulates the phosphorylation of membrane and cytoplasmic proteins. Such phosphorylation is also observed with other growth factors and in a recent paper by Martin-Perez *et al* (1984) it was shown that EGF,$PGF_{2\alpha}$ and insulin induced the phosphorylation of the same target protein.

A protein structurally similar to mouse EGF was isolated from human urine by Gregory (1975) as an inhibitor of gastric acid secretion. This

protein, named urogastrone, when subjected to amino acid sequencing, was found to be very similar to mouse submaxillary gland EGF. A comparison of the amino acid sequences is given in Figure 4 and shows the conserved nature of the disulphide bridges. Unlike mouse EGF, the major site of synthesis is Brunner's gland near the duodenum, although it is also found in the human submaxillary glands (Elder *et al*,1978). The structural similarity between the two proteins prompted the discovery that mouse EGF, in addition to its mitogenic properties, was also capable of inhibiting gastric secretion (Bower *et al*,1975). Likewise, urogastrone or huEGF was found to have a mitogenic capacity (Starkey *et al*,1974).

EGF is more abundant in the submaxillary glands of male mice than female mice and the EGF content of the glands was found to parallel the development of the glands tubular system - castration leading to atrophy of the duct system and a decrease in the EGF content of the gland.

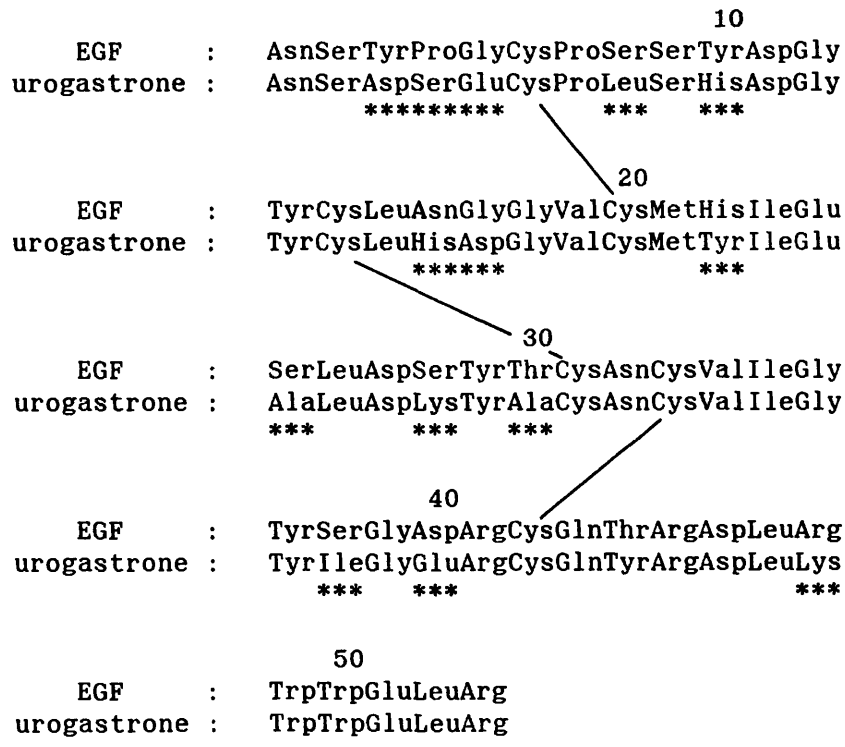



**Fig.4** - Comparison of amino acid sequences of EGF and urogastrone

The conserved disulphide bridges are indicated by solid lines. Sequence differences are underlined by the symbol ***

Confirmation that the SMG was the site of EGF synthesis came with organ

culture experiments in which EGF was found to be secreted into the medium (Frey *et al*,1979). *In vivo* EGF is secreted from the submaxillary gland into the saliva and serum, but the hormone is also found in milk and urine (Bynny *et al*,1974). The androgen-dependence of EGF was further demonstrated by Byyny *et al* (1972) when using a radioimmunoassay to determine the levels of EGF in the normal female and castrate male mouse. The levels present in these animals were too low to be detected by the traditional bioassay based on eyelid opening. The same workers showed that although testosterone stimulated the synthesis of EGF, it had no effect on its secretion, which was found to be under the control of the sympathetic nervous system.

Studies on the biosynthesis of EGF showed a 9 kd precursor (Frey *et al*,1979) and with apparently everything in their favour, several groups tried to isolate cDNA clones encoding the EGF precursor. Little success was achieved until two groups recently identified the EGF precursor as a 128-132 kd polypeptide by using sophisticated cDNA cloning procedures (Scott *et al*,1983b; Gray *et al*,1983). The peptide encoded by the cDNAs, contained seven potential EGF-like peptides in addition to EGF itself and did indeed have suitable cleavage sites to generate a 9 kd pro-EGF molecule. The precursor size difference reported by the two groups is due to nucleotide sequence differences which alter the reading frame at the 3' end.

## 1.4 Kallikrein

The submaxillary glands contain a large number of proteases which are often referred to collectively as the esteroproteases or the kallikreins. This terminology is misleading since the kallikreins are specific enzymes produced in the salivary glands, the pancreas and the kidney. In addition, there exists a plasma kallikrein having a very similar but distinct activity. The glandular kallikreins cleave both high molecular weight and low molecular weight kininogen to yield the decapeptide lysyl-bradykinin but are preferably active on the low molecular weight form. The plasma

kallikrein, however, only cleaves the high molecular weight kininogen yielding the nonapeptide, bradykinin (reviewed by Orstavik,1980). The kinins have many effects, amongst which is their vasodilatory activity. In parallel to the renin-angiotensin system in which the active peptide, angiotensin II, is cleaved by a specific enzyme (angiotensin converting enzyme), the kinins are degraded by two kininases (I and II). It has been shown that one of these, kininase II, is identical to angiotensin converting enzyme and, with the parallels already mentioned, the suggestion has been made that the renin-angiotensin and kallikrein-kinin systems act antagonistically in the control of blood pressure. Submaxillary gland kallikrein is capable of activating inactive renin *in vitro* some 10-fold more effectively than trypsin but whether this activation process occurs *in vivo* is not known (Sealey *et al*,1978). Submaxillary gland kallikrein appears enzymatically identical to β-NGF endopeptidase, a protease that removes an octapeptide from the amino-terminus of β-NGF. The peptide produced has no known biological role and its removal does not appear to change the properties of β-NGF. However, since the cleavage is very specific, there may be an unknown physiological reason for its occurrence. Bothwell *et al* (1979) compared the properties and available amino acid sequence data for SMG kallikrein, EGF-binding protein and γ-NGF, and concluded that the proteins were members of a closely related family. The same conclusion was made by Mason *et al* (1983) who found a family of related clones encoding kallikrein-like enzymes.

## 1.5 Renin

The discovery by Tiegerstedt and Bergman, in 1898, that the injection of an extract of dog kidney caused a potent increase in blood-pressure, led to the naming of the factor involved as renin. It was a considerable time before any success was achieved in the isolation and more detailed characterisation of this factor. The two main reasons for this inability to purify renin were its apparent instability and the low level at which it is

made. The instability was attributed to the presence of proteases, some of which were very similar to renin. The first major purification was carried out by Haas *et* al (1953) using hog kidney as a source of tissue. Using a combination of salt and solvent extraction procedures, they achieved a purification of 56,000 fold. This was not significantly improved upon until the development of modern chromatographic techniques, when use was made of the similarity of renin to other proteases by including a pepstatin affinity column in the isolation procedure. Once partial purification of renin had been achieved, antiserum was raised and used to improve separation techniques. In fact, since the antiserum contained antibodies to the impurities, the results were not as significant an improvement as had been hoped - the exception being the isolation of plasma renin. Inagami and Murakami (1977) reported the complete purification of hog renin using a combination of DEAE- and CM-cellulose chromatography and pepstatin affinity chromatography. This work allowed the first detailed characterisation of renin and showed it to be a 36 kd glycoprotein with a pH optimum of between 5.5 and 7.0. Unlike previous preparations, this purified renin was stable at -20$^{o}$C, suggesting that reports of instability were indeed due to contaminating proteases.

Although the availability of tissues such as hog kidney has facilitated the isolation of renins from several sources, the main interest has been in the isolation and study of human renin to define its role in the control of blood pressure. Human kidneys contain twenty-fold lower levels of renin than hog kidneys (or 100-fold lower than mouse kidney levels) which increases further the difficulty of purification. Recently, however, the human enzyme has been purified by three groups. Galen *et al* (1979) reported the purification of renin from a renin-secreting juxtaglomerular cell tumour that had a renin concentration 5000-fold higher than normal kidney tissue, permitting the isolation of pure renin after only a 40-fold purification which involved three steps: gel filtration, DEAE-cellulose chromatography and preparative isoelectric focussing. The gel filtration

step separated the major renin-containing fraction of ≃ 40 kd from two high molecular weight forms also possessing renin-like activity. Similar high molecular weight renins are described below.

The 40 kd renin fraction (97% of the total renin activity) was found to contain five species of renin separable by isoelectric focussing. The three major species had very similar specific activities and were heterogeneous when analysed by SDS-polyacrylamide gel electrophoresis. Each had peptides of 50 kd, 25 kd and 20 kd in size, the larger two being glycoproteins, and although they all possessed renin activity the relationship between them is not known. No details were reported for the two minor species even though one of them has a significantly higher specific activity than the three major fractions. The molecular weights of the major fractions were found to be 38-42 kd when determined by neutral polyacrylamide gel electrophoresis.

The heterogeneity of renin was also observed by Yokosawa *et al* (1980) who used a combination of pepstatin- and haemoglobin-affinity columns with CM-cellulose chromatography to purify human renin from normal kidney tissue (a 480,000-fold purification). The CM-cellulose column was introduced to replace an affinity step which utilised a synthetic octapeptide. The change resulted in the same level of purity but a greater yield (61%). The three renins isolated had identical specific activities and unlike the tumour renins of Galen *et al*, the major renin fraction was homogeneous when electrophoresed on SDS-polyacrylamide and IEF gels. The molecular weight of this renin was determined by gel filtration and sedimentation equilibrium to be 40-42 kd. Although human renin was a glycoprotein, the enzyme was distinct from the other purified kidney renins. It had a higher molecular weight - 40 kd compared to 36-37 kd for hog and mouse renins, a pI of 5.7 (4.5-5.4) and did not cross-react with antibody against other renins, whilst renins from other species cross-reacted inter-specifically. Lastly, the amino acid composition of most renins was similar but that of the human enzyme was found to be quite distinct. The occurrence of multiple forms of renin was suggested by Yokosawa *et al* to be due to partial proteolysis.

Although this may be true in the case of the kidney, there is good evidence that renin is truly heterogeneous in the submaxillary gland as will be discussed below.

The recent development of a new renin inhibitor, H77, has allowed the simplification of renin isolation, and M$^{C}$Intyre *et al* (1983) used it to purify human kidney renin in a simple procedure avoiding the much more complex method of Yokasawa *et al*. If the inhibitor is generally available, the method of M$^{C}$Intyre *et al* is obviously preferable.

In parallel to the purification of renal renin, interest grew with respect to extra-renal sources of renin activity. For some time these activities were controversially called iso-renins but purification from two major extra-renal sources has firmly established their existence. One major source of renin is the submaxillary gland of the mouse (Werle *et al*,1957). For reasons that are still unknown the gland produces enormous quantities of the enzyme, some three orders of magnitude higher than the levels produced by the kidney.

The submaxillary gland enzyme was partially purified by Cohen *et al* (1972) into five electrophoretically distinct fractions. The two major fractions (A and C) were shown to have molecular weights of 43 and 36-37 kd respectively. They had very similar amino acid compositions and, both fractions had prolonged hypertensive effects *in vivo* (rat). The abundance of SMG renin permitted the crystallisation of the major fraction - renin A - opening the way for X-ray crystallographic studies of the enzyme structure. The same group used purified renin to set up the first direct radioimmunoassay for the enzyme. This method was compared by Malling and Poulsen (1977a) to an alternative RIA method in which the enzyme activity was measured by angiotensin I production. The direct assay gave very similar data but was likely to include prorenin in the results. Using the direct radioimmunoassay Michelakis *et al* (1974) demonstrated that SMG renin activity correlated with the androgen status of the animal. The fact that kidney and plasma renins cross-reacted with the SMG renin antiserum enabled

the origin of plasma renin to be established. It was found that whilst removal of the salivary glands did not affect the concentration of plasma renin, the levels of renin in the plasma of nephrectomised male and female mice fell by approximately 75%. This strongly suggests that the kidney is the major source of plasma renin, and that there is no significant contribution from the SMG.

Although purified SMG renin possessed many of the features of an isozyme of kidney renin, it was still being referred to as a "renin-like substance" by Suzuki *et al* (1981) who developed a single-step purification procedure using pepstatin-aminohexyl agarose affinity chromatography. The preparation was electrophoretically homogeneous and was obtained at a very high yield (83%). Within this preparation were five active "renins" separable by CM-cellulose chromatography. As found previously the major fractions, A and C, were active *in vivo* but the minor fractions were inactive *in vivo* and may be cathepsin D or pseudorenin (Skeggs *et al*,1969).

Multiple forms of submaxillary gland renin were also found by Ho *et al* (1982), whose isolation procedure included several protease inhibitors. Together with amino acid sequencing data discussed later, there seems little doubt that SMG renin is a mixture of several isozymes.

The second extra-renal source of renin from which the enzyme has been purified is the brain. Because of the similarity with cathepsin D (Day and Reid, 1976) there were considerable doubts whether reports of renin activity truly represented the presence of an isozyme. Initial reports of renin in the brain (Fisher-Ferraro, 1971) and cerebospinal fluid (Finkielman *et al*, 1972) were strengthened by the identification of other renin-angiotensin system components in brain tissue, including angiotensin converting enzyme and angiotensin II. All the elements of the renin-angiotensin system were found to be present in neuroblastoma cell lines (Inagami,1982) but it was the ability to separate renin from cathepsin D (Inagami,1982; Hirose *et al*,1978) that confirmed the existence of a distinct brain renin isozyme. The highest concentrations of the enzyme

were found in the pineal and pituitary glands (Hirose *et al*,1982a). In a separate study, renin activity was localised to the synaptosomal fraction of brain homogenate (Husain *et al*,1981).

Attempts to isolate the brain isozyme have used several sources of brain tissue including mouse (Speck *et al*,1981) but the most successful purification has been from bovine pituitaries (Hirose *et al*,1982b) using the pituitaries from some 35,000 animals! The enzyme was found to have a molecular weight of 36 kd and an amino acid composition similar to those of rat, dog and hog kidney renins. The protein was not capable of binding to a concanavalin-A column indicating that it is not a glycoprotein in contrast to bovine renal renin, which would seem to imply that brain renin is not a sequestered form of the kidney enzyme, and is probably synthesised *in situ*.

## 1.6 Inactive renin

A second controversial side of renin biology is the nature of "inactive renin". The term is used to describe a range of poorly characterised 'renins' including precursor and high molecular weight forms of the enzyme. Some of these forms were recognised by radioimmunoassay (Malling and Poulsen, 1977b) and by the ability of various treatments to increase the renin activity of a particular sample. The three treatments commonly used to activate inactive renin are acid-treatment, cryoactivation and the use of proteases. It has recently been reported that renin itself can activate inactive renin (Inagaki *et al*,1983).

Inactive 'renins' of 200,000 and 70,000 kd occur in mouse plasma (Malling and Poulsen,1977b) and the former may be activated by either acid-treatment or by prolonged storage. This activation resulted in the release of a 40 kd polypeptide having renin activity. The 70 kd molecule was also acid-activatable but showed no change in molecular weight. Malling and Poulsen observed that when the SMG and kidneys were removed from mice the 40-50 kd plasma renin disappeared but high molecular weight forms of >200 kd and 70-140 kd remained. These high molecular weight forms had a much lower

specific activity than renin purified from the SMG, a tissue which does not appear to contain high molecular forms of renin (Nielsen *et al*,1979).

An inactive renin has now been purified from hog kidney by Murakami and coworkers. Their early characterisation (Murakami *et al*,1980) was carried out on material that was only purified 25-fold. Over the following two years this purification was improved by five orders of magnitude (Murakami *et al*,1981) suggesting caution when interpreting the earlier characterisation! The highly purified inactive form was found to be of higher molecular weight (44 kd) and is likely to be a precursor.

Human amniotic fluid contains a large amount of inactive renin (Morris and Lumbers,1972) but the first human inactive renin to be purified was from plasma by Inagami *et al* (1982) and could be activated by plasma kallikrein, plasmin and cathepsin D, but not by urinary kallikrein (unlike the rat system).

With the study of high molecular weight/inactive forms of renin came the discovery of renin binding-proteins. These were reported to be present in human and mouse plasmas (Poulsen *et al*,1979a) but to date there has been no follow-up work on these proteins. Once again, the hog kidney has proved interesting since it contains a 56 kd renin-binding protein which acts as a specific inhibitor (Ueno *et al*,1981). The ability to isolate the renin renin-binding protein complex should permit the purification and characterisation of this protein, and enable some of the questions surrounding the nature of "inactive renin" to be answered.

At the present time, therefore, there are no well characterised forms of inactive renin but the observations of inactive renin are likely to have at least four explanations: renin biosynthetic intermediates or precursors; mature and/or precursor forms bound to specific binding- or inactivating-proteins; renin bound unspecifically to plasma proteins; and finally some of these "renins" may in fact be other proteases whose specificity has been altered by the various treatments described.

## 1.7 The renin-angiotensin system

Having isolated and characterised the enzyme responsible for the original observations of Tigerstedt and Bergmann (1898) and discovered its enzymatic product, angiotensin I, and subsequent cleavage to angiotensin II by angiotensin converting enzyme the importance of renin and angiotensin in the control of blood pressure was realised. Although the system is still being intensively studied, many of the interactions of the elements so far identified are beginning to be understood.

The renin-angiotensin system (RAS) is one of the most central mechanisms by which blood pressure is controlled, not only in extreme conditions of salt depletion and fluid loss (Cowley *et al*,1971) but also under normal conditions (MacGregor *et al*,1981). It is a complex system whose components interact with other control systems such as the kallikrein-kinin system and the baroreceptor response. It cannot therefore be considered in isolation when discussing its role in conditions such as essential hypertension. This introduces a dilemma - many of the interactions known to occur are very poorly understood, for example the production of serotonin by the brain is known to affect the secretion of renin in the kidney, but the mechanism by which this occurs is still under study (Ganong,1982). The overall importance of the renin-angiotensin system has probably led to unfair expectations of its involvement in essential hypertension.

In this part of the introduction, I will first describe the elements of the renin-angiotensin system defined to date, their interactions and involvement in hypertension and its treatment. A summary of the renin-angiotensin system is shown in Figure 5.

Renin is stored in granules in the juxtaglomerular cells of the afferent arterioles of the kidney (Fig.6) and upon its release into the plasma the enzyme cleaves angiotensinogen, a 59 kd precursor produced under hormonal control by the liver (Dzau and Herrmann,1982). This cleavage removes a decapeptide, angiotensin I (AI) which is itself quickly cleaved

by angiotensin-converting enzyme (ACE) (Fig.7) which removes the two carboxy-terminus amino acids to generate the octapeptide, angiotensin II (AII). The fate or subsequent function of the remainder of the angiotensin precursor polypeptide is unknown. Whilst AI has no known biological activity its cleavage product AII is an extremely potent vasopressor (Cowley *et al*,1971). The rate-limiting step in this pathway is believed to be the concentration of angiotensinogen, and conditions which increase the levels of this polypeptide, such as Cushing's syndrome, hypothyroidism and oral contraception can result in elevated blood pressure.

Angiotensin II is the primary active product but removal of the amino-terminal aspartate residue of AII gives rise to a second active peptide, angiotensin III (AIII) which has the ability to stimulate aldosterone secretion. Angiotensin II has several known activities concerned directly or indirectly with blood pressure maintenance. Firstly it has a direct vasoconstrictive action on the arterioles giving rise to an elevation of pressure. It can also stimulate secretion of its primary precursor angiotensinogen, thereby enhancing the cascade. It has a negative feedback control on the secretion of renin but stimulates the secretion of aldosterone from the adrenal cortex (Lee,1981). Aldosterone in turn stimulates the retention of sodium ions leading indirectly to an increase in pressure. Aldosterone probably works via the synthesis of other proteins since in experimental systems the inhibition of transcription blocks the ability of aldosterone to transport sodium (reviewed by Edelman and Fimognari,1968). The effects of the renin-angiotensin system on blood-pressure is augmented

by other control mechanisms such as antidiuretic hormone (vasopressin) and serotonin which alter the fluid balance, and prostaglandins which are involved in the control of $Na^+/K^+$ balance and facilitating renal blood flow during an increase in blood pressure (Terragno,1981).

The effects of these homeostatic controls are detected in at least three ways: by osmolarity sensors, baroreceptors and the macula densa. The

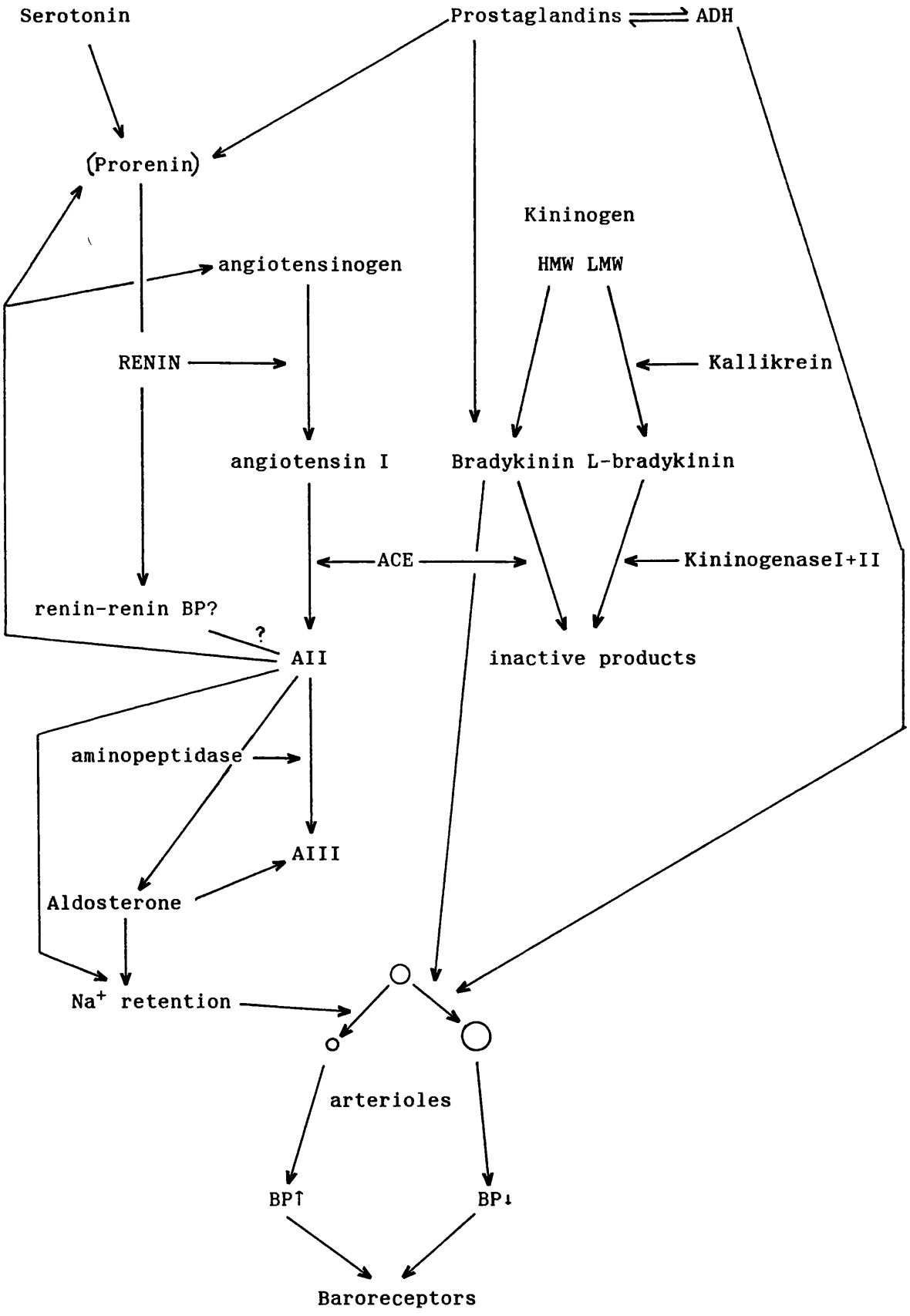



Fig.5 - The Renin-Angiotensin and Kallikrein-Kinin systems

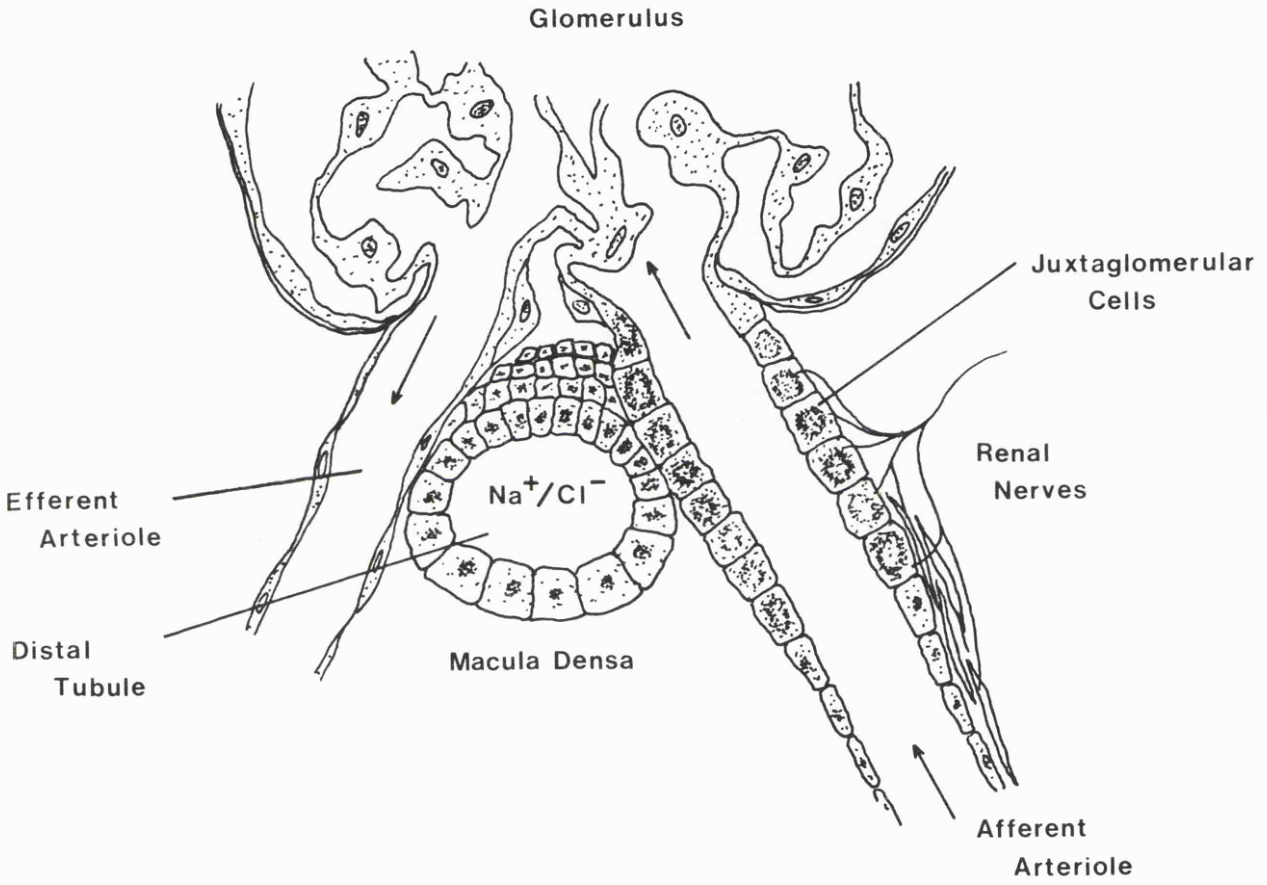



**Fig.6** - The juxtaglomerular apparatus

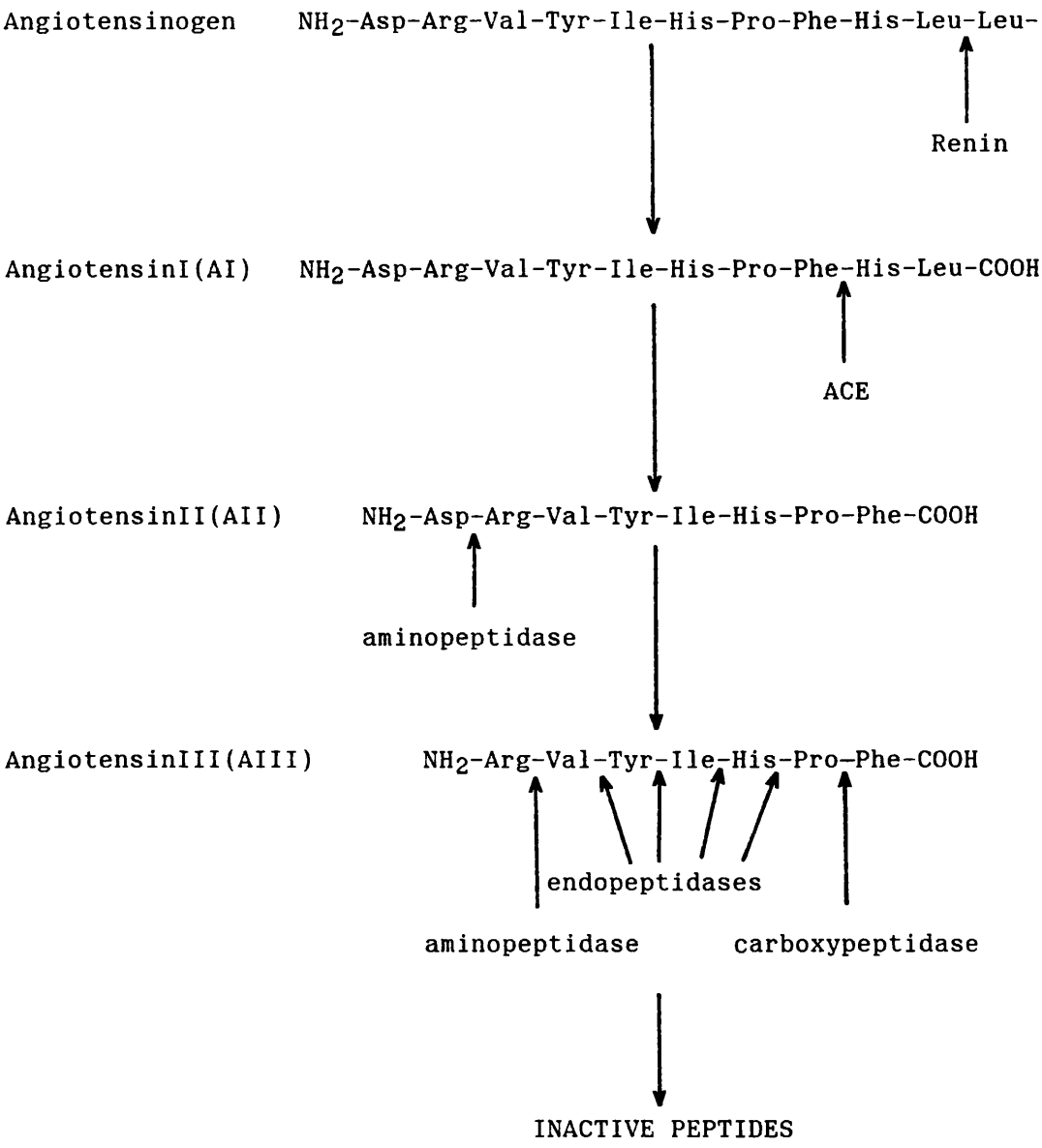



Fig.7 - Cleavage of angiotensinogen and the angiotensins

latter two methods are known to be directly linked to the renin-angiotensin system (Davis,1971). The baroreceptors detect stretch in the arterioles, implying an increase in blood pressure whilst the macula densa detects $Na^+/Cl^-$ balance. Renin secretion is controlled by both mechanisms as well as sympathetic nerve activity, AII and prostaglandins.

Studies on the secretion of isorenins indicate that the mechanisms by which secretion occurs are different to those of the kidney (Michelakis *et*

*al*,1976) and although there have been suggestions of isorenins being secreted into the blood (Menzie,1974) other work suggests that little if any of the circulating plasma renin is of extra-renal origin (Michelakis *et al*,1974). One exception to this is secretion from the extra-renal renin-producing tumours which can significantly elevate plasma renin levels (Ganten *et al*,1976).

One mechanism of blood pressure control which appears to be distinct but intimately related to the renin-angiotensin system is the kallikrein-kinin system. Whereas the RAS is involved in maintaining pressure, the kallikrein-kinin system releases the vasodilatory peptides bradykinin and lysyl-bradykinin. The relationship between the two systems is illustrated by the fact that ACE, which produces the vasoactive peptide AII, also cleaves and thereby inactivates bradykinin, nullifying the antagonistic effect of the vasodepressor system (Fig.5). Kallikrein, like renin is synthesised in the kidney but is not found in the juxtaglomerular cells (Orstavik and Inagami,1982).

As intimated earlier, blood pressure is under more general or widespread control than simply the RAS and kallikrein-kinin systems, but not only are other hormones involved but a distinctly different group of contributary factors determine blood pressure. The RAS and kallikrein-kinin systems etc. can be thought of as the "nuts and bolts" of blood pressure control. Overseeing the way that they interact are the "draftsman's plans" which in blood pressure terms refer to genetic background, diet, sex, race, lifestyle and body posture. The effects of these factors are becoming more clearly established as more and more surveys are carried out. One of the problems of hypertension research has been deciding at which level of blood pressure a person can be regarded as suffering from hypertension. There is no obvious cut-off point for normal pressure and although in general people with high blood pressure have a greater risk of heart attacks etc., this is not always the case. Childs (1983) has published a detailed account of factors contributing to essential hypertension and discusses the

difficulties of defining and studying the disease. The availability of animal models has confirmed the genetic basis of high blood pressure and it has demonstrated that the condition is a heterogeneous one, the cause(s) in each particular case being different. This heterogeneity is manifest in the fact that although renin is a central element in blood pressure control, hypertensive patients may be high, low or normal in their plasma renin activities! Currently, $Na^+$ and $K^+$ transport mechanisms are being studied with regard to essential hypertension (Hamlyn *et al*,1982). Although renin may not therefore be involved directly in the cause of hypertension, because of its place in maintaining blood pressure, the RAS has been the focus of treatment for the condition.

Inhibition of the renin-angiotensin system is possible at several of the steps outlined earlier. It is possible to use competitive inhibitors of AII such as the AII analogue 'Saralisin'. This agent not only prevents $Na^+$ reabsorption directly, by binding to AII receptors in the proximal tubule, but inhibits the secretion of aldosterone, and will prevent angiotensinogen release. Medical studies, however, revealed that whilst Saralisin was effective at reducing blood pressure when there was a high renin-angiotensin activity it had the opposite effect under conditions of low renin activity (Lee,1981). Besides competitive inhibition there have been attepts at direct enzyme inhibition of both ACE and renin. Specific inhibitors of renin have yet to yield useful pharmacological agents although significant work is being carried out towards that goal (Haber,1980). The X-ray crystallographic structure of renin is believed to be an important step towards the design of inhibitors and although at present only the structure of the mouse SMG enzyme is known, that of the human kidney is being actively studied.

To date the greatest success has been achieved using inhibitors of angiotensin converting enzyme. Initially, a lot of information was obtained from studies on naturally occurring peptide inhibitors isolated from snake venom (eg. teprotide). These studies aided the development of 'Captopril',

an inhibitor that could be administered orally, unlike teprotide. This has proved very effective in the treatment of hypertension (Giese and Rasmussen,1981; Heel *et al*,1980), even though side effects such as rashes and taste disturbances may result. One important point is that since Captopril inhibits the conversion of AI to AII there is a build-up of AI in the circulation combined with an elevation of renin levels. This means that a sudden removal of Captopril treatment results in a potentially dangerous surge in blood pressure.

The desire to obtain renin inhibitors is a strong influence on much of the renin research at present and information helpful to that end was a potential spin-off from the work undertaken in this thesis.

## 1.8 The Molecular Biology and Genetics of Mouse Renin

Although it had been known for some time that renin was very abundant in the submaxillary gland of the mouse, there was no direct evidence to suggest that it was being synthesised in this tissue until Poulsen *et al* (1979b) isolated and translated *in vitro* total mRNA from the male SMG. The translation products were immunoprecipitated with renin antiserum and the specific immunoprecipitation of a 50 kd polypeptide was observed. The size later proved to be overestimated but it gave the first glimpse at renin biosynthesis. The product identified was the primary translation product of renin mRNA. The same workers subsequently microinjected SMG mRNA into oocytes and observed a drop in molecular weight to 48 kd, and interpreted this change as the removal of the signal sequence. A similar drop was observed by Catanzaro *et al* (1983) when hybrid-selected SMG renin mRNA was translated *in vitro* in the presence of dog pancreas membranes which are capable of cleaving signal sequences *in vitro*.

The confirmation of the cleavage of a signal peptide completed the biosynthetic pathway of renin. Previously, pulse-chase experiments had detected a 44 kd precursor which was processed to 40 kd (Morris and Catanzaro,1981). On analysis of purified SMG renin the 38-40 kd molecule

was found to possess 33 and 5 kd polypeptide chains linked by a disulphide bridge (Morris *et al*,1983). Catanzaro *et al* (1983) found that both single chain 38 kd and the mature two-chain 38 kd renin were capable of binding to a pepstatin affinity column suggesting that the single-chain form may be enzymatically active. This was confirmed by Pratt *et al* (1983) who have shown that single chain renin is actually more active than the two chain form. This and the fact that both forms appear to be secreted, means that renin biosynthesis and secretion may be more complex than at first thought.

The *in vitro* precursor of mouse kidney renin is the same size as that of the SMG (Dykes *et al*,1980; Poulsen *et al*,1980) and the SMG renins of both high and low producer mice are also of similar size (J.J.M. unpublished data). This suggests that the difference in renin activity between high and low producer mice is not due to gross differences in protein structure.

After the initial discovery of high and low producer strains of mice (Bing *et al*,1967), Wilson *et al* (1977) suggested that the "high" and "low" phenotype is defined by a single regulatory gene (*Rnr*) located on chromosome 1 (Wilson *et al*,1978) whose activity varied between strains. At the time, there was no evidence that SMG and kidney renins were different proteins and the SMG renins of high and low producer mice appeared to differ quantitatively but not qualitatively. Although renin was already known to be induced by testosterone (Gecse *et al*,1976; Oliver and Gross, 1967) the high/low phenotype was found not to be due to variation in the levels of circulating testosterone (Wilson *et al*,1977) or to variation in androgen sensitivity (Wilson *et al*,1981).

The same workers went on to demonstrate that although high and low producer SMG renins were similar in their pH optima and substrate specificity, they differed in their heat stability. This fact was exploited and led to the observation that *Rnr* is closely linked to a gene determining renin structure. Given that there was now a clear difference between the two renins, the authors modified their suggestion of a regulatory gene to include the possibility that *Rnr* and the gene determining the structural

difference were identical (Wilson *et al*,1982a). In the light of this data they proposed three models to explain the control of SMG renin expression: firstly, that there were two distinct alleles, one responsible for a high renin activity and the second for a low renin activity; secondly, that the regulatory and structural genes each had two alleles; and thirdly, that there were two structural genes, the more active of which was a null allele in the low producer mouse. These models will be discussed later in the light of the work presented here and of work from other laboratories.

Studies by Wilson and coworkers demonstrated that the basal (i.e. female) level of renin is controlled by thyroxine and not androgen. In addition, the induction by androgen was shown to require a functional androgen receptor (Wilson *et al*,1982b).

In addition to the genetic analysis of renin by Wilson and co-workers, several laboratories have been studying the molecular biology of renin and have successfully cloned cDNA copies of renin mRNA (Rougeon *et al*,1981; Panthier *et al*,1982a; Mullins *et al*,1982; Morris *et al*,1982; Masuda *et al*,1982). The first major achievement was the determination of the complete amino acid sequence of the renin precursor (Panthier *et al*,1982a) but the availability of hybridisation probes facilitated the subsequent identification of human cDNA clones (Imai *et al*,1983; Soubrier *et al*,1983) and genomic clones both for human (Hobart F. personal communication) and mouse genes (Mullins *et al*,1982; Gross F. personal communication). Southern blot analysis revealed that high producer mouse strains possess two copies of the renin structural gene (Piccini *et al*,1982; Mullins *et al*,1982; Panthier *et al*,1982b) whereas low producers have a single copy (*Ren*1, also referred to as *Rn*1 and *Ren*A). Panthier *et al* (1982b) suggested the second copy (*Ren*2, or *Rn*2 or *Ren*B) arose by gene duplication, however the evidence does not uphold this hypothesis (see chapter 11). The existence of an additional renin gene in high producer mice is obviously not enough to account for the 100-200 fold difference in renin activity by gene dosage. The activity of the additional gene must be significantly higher than the

"original" gene, and a further model was proposed by Panthier and colleagues to explain renin expression (Table 2).

The successful cloning of a kidney renin cDNA from a high producer (SWR) mouse (Panthier *et al*,1983) confirmed that two renin genes were expressed in a tissue specific manner in these strains of mice.

One gross difference is observed between the two genes of the high-producer mouse :- downstream of *Ren*2 is found an additional 3 kb of DNA (Mullins *et al*,1982) and this accounts for the polymorphism seen by Piccini

| | allele | | |
|---|---|---|---|
| | $Rnr^b$ | $Rnr^s$ | |
| organ | *Ren*1 | *Ren*1 | *Ren*2 |
| SMG | +++ | +++ | +++++ |
| | + | + | + |
| KIDNEY | + | + | o |
| | + | + | o |

Table 2 - Model of renin gene expression proposed by Panthier *et al* (1982b)

*et al* (1982) which was associated with high renin levels in the SMG. Recent work in our laboratory has shown that this stretch of DNA has homology to a type A retrovirus (D.Burt, in preparation) and further discussion may be found in chapter 11.

In summary, there is still relatively little known about the molecular biology of mouse renin, and of what is known, virtually all has been discovered over the last four years, yet as a system for study it offers much, and should prove particulary useful for the analysis of androgen-

inducibility and tissue-specificity of mammalian gene expression.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 General methods

### Precautions against RNAse cotamination

Autoclaving of solutions and equipment was carried out at 15psi for 15 minutes. In the handling of RNA, gloves were always worn and solutions were prepared using DEP-treated water. This was prepared by dissolving DEP to a concentration of 0.1% in "super-Q" distilled water and then destroying residual DEP by autoclaving the solution.

### Siliconisation of glassware

Glassware was siliconised by either coating it directly with 2,4 dichlorodimethylsilane or by allowing the siliconising agent to vapourise under vacuum in a dessicator containing the glassware (for small items such as micropipettes). This procedure was carried out in the fume cupboard.

### Maintenance of bacterial and phage stocks

Stocks of bacterial strains were stored at -70°C as frozen cultures, which were prepared by mixing 1ml of a fresh overnight culture with 1ml of 80% glycerol. Stocks of λ and M13 recombinants were stored in λ buffer and TM buffer, respectively, at 4°C.

## 2.2 Sources of chemicals and biochemicals

All reagents and biochemicals were the best commercially available. The sources of certain chemicals felt to be critical in achieving the results, are listed below:

| | |
|---|---|
| formamide | - Fluka |
| urea | - BRL (Ultrapure) |
| acrylamide | - Serva |
| bis-acrylamide | - BRL or Cambridge Biolabs |
| dextran sulphate | - Sigma (M.wt. 5 x $10^5$) |
| diethylpyrocarbonate (DEP) | - Sigma |
| N(3-nitrobenzyloxymethyl-pyridinium chloride) | - Sigma |
| ammonium persulphate | - BRL (electrophoresis grade) |
| sucrose | - BRL (ultrapure) |
| CsCl | - Fisons |

All radiochemicals were supplied by Amersham International, Amersham, UK. Restriction endonucleases and DNA modifying enzymes, i.e. DNA polymerase etc (including $S_1$ nuclease) were supplied by BRL, with the exception of $T_4$ DNA ligase, which was obtained from P-L biochemicals, Miton Keynes. Micrococcal nuclease and creatine phosphokinase were supplied by P-L biochemicals and Sigma, respectively.Reverse transcriptase was the gift of Dr.F.Beard, or supplied by Life Sciences.

Dog pancreas membranes and human placental mRNA were obtained from New England Nuclear.

Wheat-germ S30 extract was the gift of Dr.B.R.G.Williams and testosterone pellets (30mgs) were the gift of Dr.G.Bulfield.

Nitrocellulose paper and filters were from Schleicher and Schull.
Two types of X-ray film were used: Fugi RX and Kodak Industrex CX. Stained gels were photographed using a Polaroid Land camera and either type 55 or type 57 polaroid film.

Animals were obtained from: Bantin and Kingman Ltd., Charles River UK Ltd., and Olac 1976 Ltd.

Sepharose G50 was obtained from Pharmacia, DE52 from Whatman and Biogel-A5M from Biorad.

## 2.3 Strains and plasmids

| Bacterial strains | | | Source |
|---|---|---|---|
| JA221 | - | *hsdR, lacY1, leuB6, recA, trpE5* | J.Windass |
| ED8910 | - | *hsdS, recB21, recC22, supE, supF* | D.Burt |
| JM101 | - | *Δlacpro, supE, thi,* F'*traD36, proAB,* $lacI^q$, *ZΔM15* | D.Burt |
| JM103 | - | *Δlacpro, supE, thi, strA, sbcB15, endA, hspR4,* F'*traD36, proAB,* $lacI^q$, *ZΔM15* | D.Burt |

| Phage strains | | | |
|---|---|---|---|
| M13mp10 | - | Messing, 1983 | BRL |
| M13mp11 | - | Messing, 1983 | BRL |
| λL47 | - | Loenen and Brammar, (1980) λ*sbh*I λ1°, *chiA*131, Δ(*s*RIλ1-2) *imm*434, *cI*,*s*RIλ4°; *nin5*, *s*RIλ5°, *s*hndIIIλ6° | D.Burt |
| λDB287 | - | D.Burt, unpublished λ*s*bam1°, *b*189(linker)*s*bam3$^+$(*s*RI3°)*s*bam4$^+$/ 3$^+$(*s*RI3°)*s*bam4$^+$(linker)KH54, *s*RI4°, *nin*5, *s*RI5° | D.Burt |

| Plasmids | | | | |
|---|---|---|---|---|
| pAT153 | - | $ApT^C$ | Twigg and Sheratt, (1980) | J.Windass |
| pBR322 | - | $ApT^C$ | Bolivar *et al*, (1977) | J.Windass |

## 2.4 Preparation of buffers and stock solutions

Buffers and stock solutions mentioned, but not detailed, in the methods described below, were made as follows:

TE - 10mM Tris-HCl, pH 7.5
1mM EDTA

TM - 100mM Tris-HCl, pH 7.5
50mM $MgCl_2$

PBS-azide - 17.53g NaCl
1.0g sodium azide
dissolved in 40mM sodium phosphate, pH 7.2 to 21

PBS-azide-formaldehyde -
39.5mls of formaldehyde (38%)
per litre of PBS-azide

*Staph.A* broth -

| | | |
|---|---|---|
| Antibiotic medium 3 | | 17.5g |
| Casitone | | 5.0g |
| Yeast extract | | 2.5g |
| DL-B-glycerophosphate | | 2.5g |
| $H_2O$ | -> | 1litre |

The broth was autoclaved at 15psi for 15 minutes, after which 1ml 4mg/ml nicotinic acid and 1ml 2mg/ml thiamine HCl were added.

Solid medium for the growth of *Staph.A.* -

| | | |
|---|---|---|
| Antibiotic medium 3 | | 2.625g |
| Difco-bacto agar | | 2.25g |
| $H_2O$ | -> | 150mls |

TLES - 0.2M Tris-HCl, pH 9.0
0.1M LiCl
25mM EDTA
0.1% SDS

TLE - as above, with no SDS

10 x $T_4$ DNA ligase buffer -

| | |
|---|---|
| 1M Tris-HCL, pH 7.5 | 66µl |
| 0.2M EDTA, pH 9.0 | 5µl |
| 1.0M $MgCl_2$ | 10µl |
| 1.0M 2-mercaptoethanol | 10µl |
| 0.1M ATP, pH 7.0 | 1µl |

λ buffer - 6mm Tris- HCl, pH 8.0
10mM $MgCl_2$
100mM NaCl
0.5mg/ml gelatine

SE buffer - 150mM NaCl
100mM EDTA, pH 8.0

Lysis buffer
2% SDS
8% Tris-isopropyl naphthalene sulphonate (dissolved in 12% butanol)

TNE - 50mM Tris-HCl, pH 7.5
0.1M NaCl
5mM EDTA

SSC - 0.15M NaCl
0.015M sodium citrate, pH 7.0

10 x Nick mix -
0.5M Tris-HCl, pH 7.8
50mM $MgCl_2$
0.1M 2-mercaptoethanol

10 x Quench mix -
2% SDS
50mM EDTA
10mM Tris-HCl, pH 7.5

5 x RT buffer -
250mM Tris-HCl, pH 8.3
250mM NaCl
40mM $MgCl_2$
5mM dATP
5mM dGTP
5mM dCTP
5mM dTTP

5 x RT-2 buffer -
250mM Tris-HCl, pH 8.3
40mM $MgCl_2$
100mM DTT
2mM dATP
2mM dGTP
2mM dCTP
2mM dTTP

Binding buffer -
0.4M NaCl
0.1M Tris-HCl, pH 7.6
0.02% SDS

Elution buffer -
0.001M EDTA
0.01M Tris-HCl, pH 7.6
0.02% SDS

Tris acetate electrophoresis buffer -
40mM Tris
20mM Na Acetate
0.2M EDTA
pH 7.8

TBE electrophoresis buffer -
0.9M Tris
0.9 M boric acid
0.025M EDTA, pH 8.3

Pronase buffer -

20mM Tris-HCl, pH 8.0
1mM EDTA, pH 8.0
0.1M NaCl
0.01% Triton X-100

L-agar - Difco Bacto Tryptone 10g
Difco Yeast extract 5g
NaCl 5g
$H_2O$ -> 1l

(after adjusting to pH 7.0, 17g of agar per litre were added.

L-broth - Difco Bacto Tryptone 10g
Difco Yeast extract 5g
NaCl 5g
glucose 10g
$H_2O$ -> 1l (pH 7.0)

Tris-glycine electrophoresis buffer -

Trizma base 30g
Glycine ($NH_4$ free) 144g
SDS 10g
$H_2O$ -> 1l

Immunoprecipitation buffer -

50mM Tris-HCl, pH 7.5
100mM NaCl
0.1% NP40
1mM PMSF (in DMSO)

Sample buffer - (for Laemmli gels)

125mM Tris-HCl, pH 6.8
4% SDS
40% glycerol
0.141M 2-mercaptoethanol
0.002% bromophenol blue

Sample buffer - (for agarose and acrylamide gels)

20% FICOLL
0.002% bromophenol blue

Sample buffer - (acrylamide-urea gels)

0.22g urea (BRL ultrapure)
200$\mu$l PPB dye (0.1% xylene cyanol,
0.1% bromophenol blue,
0.5% orange G)

Bacterial buffer -

$K_2HPO_4$ 3g
$Na_2HPO_4$ 7g
NaCl 4g
$MgSO_4.7H_2O$ 0.2g
$H_2O$ -> 1l

Minimal medium/glucose/B1 agar -

After autoclaving and cooling, the following solutions were mixed and used immediately:

(a) Difco minimal agar - 15g in 750mls
(b) 4 x salts - $K_2HPO_4$ 10.5g
$KH_2PO_4$ 4.5g
$(NH_4)_2SO_4$ 1g
Na citrate 0.5g
$H_2O$ -> 250mls
(c) 1ml 20% $MgSO_4.7H_2O$, (d) 0.5mls 1% B1 (thiamine hydrochloride)
(e) 10mls 20% glucose

## 2.5 Tissue isolation

### Dissection of salivary glands

Mice were etherised and then killed by cervical dislocation. The salivary glands were quickly removed by making a "T"-shaped incission in the neck running across the throat and up the chin. Fat deposits around the gland were removed and the two submaxillary glands teased apart. The blood vessels and secretory ducts were then cut and the submaxillary and sublingual glands separated and frozen in liquid nitrogen. When required, the parotid gland was removed by tracing it around the jaw and carefully extracting each of the lobes. Tissues were stored at -70°C prior to RNA isolation.

### Castration and testosterone implantation of mice

Castrate male mice were either castrated by the supplier or by Dr.D.Morton (animal house, Leicester University). When testosterone implants were required, the pellets were placed under the skin at the back of the neck (Dr.D.Morton).

## 2.6 RNA-related techniques

### Isolation of RNA from SMG and kidney tissue

Tissues were homogenised in a 1:1 mixture of phenol, chloroform, isoamylalcohol (50:48:2) and TLES buffer. Typically, 30mls of each was used with up to 3g of SMG or 10g of kidney. Homogenisation was carried out with

an MSE homogeniser using ten periods of 10 seconds at full speed with 15 second gaps between each period. The homogenate was then centrifuged at 5000 rpm for 15 minutes and the aqueous supernatant poured into a conical flask containing 20mls of the phenol, chloroform, isoamylalcohol mixture. The lower (organic) phase was re-extracted twice with 15mls TLES buffer and the supernatants pooled with the first. After shaking the conical flask containing the pooled supernatants vigorously for 10 minutes, the extract was centrifuged at 5000 rpm for 15 minutes and the aqueous phase removed to a sterile measuring cylinder. It was then carefully layered over 0.2 volumes of 5.7M CsCl, 0.1M EDTA, pH7.5 and the tubes balanced by the addition of TLES buffer. Ribonucleic acid was pelleted by centrifugation for 18 hrs at 25000 rpm, 25°C using a Sorvall AH627 rotor. The supernatant was removed slowly using an aspirator and taking particular care at the CsCl interphase where DNA was found to band. The tubes were inverted and the pellets drained of any residual CsCl. The translucent RNA pellet was resuspended in 1-2mls of DEP-treated water, ethanol precipitated and then re-dissolved in 2mls $H_2O$ and either stored at -70°C or used immediately for the isolation of polyA$^+$ RNA.

Purification of polyA$^+$ mRNA

Total RNA preparations were prepared for oligo-dT chromatography by the addition of an equal volume of 2 x binding buffer. It was then passed through an oligo-dT cellulose column (0.2g) equilibrated with binding buffer. Ten drops were run through every two minutes and in total the solution was passed through three times, after which the polyA$^-$ RNA was recovered by ethanol precipitation. The column was then rinsed through with 25mls of binding buffer and the polyA$^+$ RNA eluted with 5mls of elution buffer. It was recovered by ethanol precipitation and centrifugation (10000 rpm, -20°C for 20 mins, Sorvall HB4 rotor) and subjected to a second oligo-dT column as described above. Finally the polyA$^+$ RNA was diluted to a concentration of 1mg/ml and stored at -20°C.

Size fractionation of mRNA (Bedbrook *et al*,1980)

mRNA was size fractionated by gel electrophoresis using a vertical 1.5% agarose, 50% formamide gel (17cm long, 3mm thick). The buffer used was 30mM $NaH_2PO_4$, 36mM Tris, 1mM EDTA. After denaturing the samples by dilution (1 in 10) in sample buffer (80% formamide, 10% glycerol, 1 x electrophoresis buffer) and warming to 45°C for 30 minutes, they were electrophoresed for 4.5 hrs at 30mA. The sample tracks were then cut into 0.5cm slices, finely chopped and the mRNA eluted into 0.25 mls of elution buffer (10 mM Tris-HCl pH 7.6, 0.4% (w/v) SDS, 1mM EDTA, 400mM LiCl). The elution was carried out at 4°C in a shaking water bath for 20 hrs, during which time the buffer was changed twice. The three eluates were then pooled and the mRNA recovered by three rounds of ethanol precipitation to remove traces of formamide.

Preparation of rabbit reticulocyte lysate (Pelham and Jackson,1976)

Three New Zealand White rabbits (2-2.5Kg) were injected subcutaneously with a 2.5% solution of acrylphenylhydrazine, pH 7.0 over a five-day period as shown below.

| Day | Treatment |
|---|---|
| 1 | inject 1.0ml/rabbit |
| 2 | inject 0.8ml/rabbit |
| 3 | inject 0.6ml/rabbit |
| 4 | inject 0.8ml/rabbit |
| 5 | inject 1.0ml/rabbit |

On day 8, 30mls of blood were collected from the ear of each rabbit. The blood was collected into sterile corex tubes containing a drop of 1% heparin solution. On day 10 the rabbits were exsanguinated from the heart (carried out by Dr.D.Morton) and the blood was again collected into corex tubes containing heparin. During collection the blood was cooled on ice and when all the samples were collected, they were centrifuged at 2000 rpm for 7 minutes in a Sorval HB4 rotor at 0°C. The packed cells were resuspended in two volumes of resuspension medium (RS; 0.14M NaCl, 0.05M KCl, 5mM MgAc) using either a glass rod or a very gentle vortex. The cells were washed

twice in RS medium and the supernatants and "buffy-zone" (leucocyte layer which contains a large amount of RNAse) were removed and discarded. The reticulocytes were lysed by the addition of an equal volume of ice-cold DEP-treated water, and the lysate spun at 13000 rpm for 25 minutes at 0°C in the HB4 rotor. The supernatant was removed and aliqoted into 1ml fractions, frozen in dry ice and stored at -70°C prior to nuclease treatment.

(The induction of reticulocytes and bleeding from the ear were carried out under Home Office Licence N$^{O}$ SWI 3959).

Micrococcal nuclease treatment of reticulocyte lysate

1ml aliquots of lysate were thawed quickly into 40$\mu$l of haemin solution by rolling in the hand. When thawed, 10$\mu$l of 0.1M $CaCl_2$ and 20$\mu$l micrococcal nuclease (7,500 units/ml) were added and the lysate incubated at 24°C for 10 minutes. The enzyme was then inhibited by the addition of 10$\mu$l of 0.25M EGTA (to chelate the $Ca^{++}$ ions) and the lysate frozen in 100$\mu$l aliquots on dry ice and stored at -70°C. The haemin solution was prepared by dissolving 6.5mg haemin in 0.25mls 1M KOH and adding sequentially:

0.5mls 0.2M Tris-HCl, pH7.8
8.9mls ethylene glycol
0.19mls 1M HCl
0.04mls DEP-treated $H_2O$

It was then stored at -20$^{O}$C.

Optimisation of reticulocyte lysate *in-vitro* translation conditions

The rabbit reticulocyte lysate *in-vitro* translation system was optimised for the translation of salivary gland mRNA in three ways. Firstly, the optimum $K^+$ and $Mg^{++}$ concentrations were determined by using a series of cocktails containing different amounts of the two ions. Secondly, the optimum mRNA concentration was determined by translating a series of dilutions of SMG mRNA from 2$\mu$g/12.5$\mu$l reaction to 0.0625$\mu$g/12.5$\mu$l reaction. The optimum value sometimes varied from one mRNA sample to the next, but was generally 0.25$\mu$g/12.5$\mu$l reaction. Lastly, the ability of the system to translate was determined by removing aliquots from a single translation

over a period of 120 mins. The aliquots were split and one half was used to determine $^{35}S$ methionine incorporation whilst the remainder was analysed by polyacrylamide gel electrophoresis.

*In-vitro* translation using rabbit reticulocyte lysate

Translation reactions were set up as quickly as possible and where a large number of incubations were carried out simultaneously, a mix was prepared containing all the reaction components that were common to each incubation. This enabled more accurate volume measurements and greater speed.

More commonly, reactions were prepared individually. This was aided by the use of a cocktail containing most of the reaction components, and was prepared as follows:

| (5x) cocktail | | Amount | F.C. |
|---|---|---|---|
| Amino acid mix (1mM each,-Met) | | 200µl | 100µM |
| 100mM ATP, pH 7.0 | | 100µl | 5mM |
| 10mM GTP | | 200µl | 1mM |
| 4M KCl | | 200µl | 0.4M |
| 1M $MgCl_2$ | | 20µl | 0.01M |
| 500mM Tris HCl, pH 7.6 | | 200µl | 50mM |
| 200mM glucose | | 100µl | 10mM |
| Creatine phosphate | | 68mg | |
| $H_2O$ | -> | 2mls | |

The standard reaction volume was 12.5µl, sufficient for both TCA precipitation and gel analysis. For immunoprecipitations the reaction volume was increased accordingly and 20µl used for each immuno-precipitation. The standard reaction was prepared as follows:

| | | F.C. | |
|---|---|---|---|
| (5x) cocktail | | 2.5µl | (1x) |
| 1mg/ml tRNA (calf liver) | | 0.625µl | 0.05mg/ml |
| $^{35}S$ methionine (1200Ci/mmole) | | 1µl | 8-12µCi |
| lysate (+CPK) | | 6.25µl | 50% |
| $polyA^+$ RNA | | 1µl | 0.1-1.0µg |
| $H_2O$ | | 1.125µl | |
| | TOTAL | 12.5µl | |

and incubation was at 30°C for 90 minutes. Creatine phosphokinase (CPK) was added to the reticulocyte lysate immediately before use (2µl of a 5mg/ml

solution per 100μl of lysate). All stock solutions were stored at -20°C with the exception of the reticulocyte lysate and $^{35}S$ methionine which were stored at -70°C.

*In-vitro* translation using the wheat-germ S30 extract

Like the reticulocyte lysate system, a series of concentrated mixes were prepared to save time in preparing reactions. A 20x translation buffer and 20x energy mix were prepared as follows:

(a) 20 x translation buffer
0.4M Hepes pH7.6
0.7mg/ml spermidine
0.48M DTT

(b) 20 x energy mix
0.02M ATP (pH 7.0)
0.4mM GTP
0.19 M creatine phosphate

In addition, a 20 x amino acid mix was used (0.4mM each amino acid except methionine). These stock solutions were used to prepare a 'cocktail' to which the S30 extract and mRNA were added:

(c) Reaction cocktail

| | | |
|---|---|---|
| 20 x translation buffer | | 2.5μl |
| 20 x energy mix | | 2.5μl |
| 20 x amino acid mix | | 2.5μl |
| 0.1M MgAc | | 2.5μl |
| 1M KAc | | 5.0μl |
| | TOTAL | 15 μl |

| | | |
|---|---|---|
| mRNA | | 1μl (1μg) |
| S30 extract | | 20μl |
| $H_2O$ | | 9μl |
| | TOTAL | 45μl |

The translations were carried out at 25°C for 60 minutes, and the products analysed as for the translation products made using the reticulocyte lysate.

Measurement of $^{35}S$-methionine incorporation during *in-vitro* translation

Early translations were assayed by spotting 1$\mu$l of the translation reaction onto a Whatman GF/C 2.5cm filter paper disc and placing the filter into 10% TCA at 90$^{o}$C for 5 mins. It was then washed in absolute alcohol for 5 mins and rinsed briefly in 50% alcohol, 50% acetone before drying and counting in a non-aqueous scintillant. This method was found to result in a relatively high background and a second method was used. This involved adding 1$\mu$l of the reaction to 0.5mls 1M NaOH in 30% $H_2O_2$ and incubating the mixture at 37°C for 10 minutes. It was then placed on ice and 2mls of 2% casein hydrolysate in 25% TCA were added. After a further 30 mins on ice, the TCA precipitate was filtered through a pre-wetted glass fibre disc. The precipitate was washed with 10mls of ice-cold 8% TCA, the filter dried and the sample counted in 5mls of non-aqueous scintillant.

Acrylamide-SDS gels for protein separation

The products of *in-vitro* translations and subsequent immuno-precipitations were separated electrophoretically according to size by the method of Laemmli (1970)

Fluorography of polyacrylamide-SDS gels

Gels were fixed for 1hr in a solution of 10% acetic acid, 25% isopropanol and then immersed in DMSO and gently rocked at room temperature for 1hr. The DMSO was then replaced and the gels kept immersed for a further hour after which they were placed into a 20% solution of 2,4,diphenyl-oxazole (PPO) in DMSO. After 1.5hrs in the PPO solution, they were placed into water for 45 minutes and then dried using a Biorad gel drier.

Preparation of fixed *Staphylococcus aureas* cells

The *Staphylococcus aureas* strain used was Cowan serotype I (National Collection of Type Cultures #NCTC8530) and all equipment used was routinely soaked in 1% hycolin after use.

A 20ml culture was grown overnight at 3°C after inoculation with a fresh colony, 0.5ml 4mg/ml niacin and 0.5ml 2mg/ml thiamine HCl were added to 500ml broth which was then inoculated with 10mls of the overnight culture.

This large culture was then incubated at 37°C overnight and the cells harvested by centrifugation at 10000 rpm, 20°C for 10 minutes. (The $OD_{680}$ of the culture should be ≥8.0 when measured against a water blank). The pellet was resuspended by addition of 10mls of PBS-azide and vigorous stirring/ aspiration. The volume was made up to 200mls and the cells recovered by centrifugation. This washing step was repeated and the cells were then resuspended to approximately 10% ($^{W}/_{V}$) in PBS-azide-formaldehyde and shaken at 20°C for 1.5 hours. The cells were harvested, washed and resuspended in PBS-azide (10% ($^{W}/_{V}$)). The cells were placed in a 1litre Erlenmeyer flask and killed by swirling the flask rapidly in an 80°C water-bath for 5 minutes and then quickly cooling them in an ice-water bath. The cells were again harvested and washed once with 100mls PBS-azide. The final cell-pellet was resuspended in PBS-azide 10%($^{W}/_{V}$), the pellet being measured using a graduated conical tube. The suspension was aliquoted and stored at -70°C prior to use.

Immunoprecipitation of *in-vitro* translation products

Immunoprecipitations were started immediately at the end of a translation reaction (usually 90 minutes). Twenty microlitres of the reaction mix were added to 45μl of immunoprecipitation buffer at 4°C, followed by 5μl of either normal rabbit serum or antiserum. The precipitation reactions were incubated overnight at 4°C and the following morning, 20μl of a 10% *Staphylococcus aureas* (*Staph.A)* cell suspension were mixed with each reaction, at room temperature, using a rotating wheel. After 30 mins the cells were recovered by brief centrifugation (< 1 min) and washed six times by vigorous resuspension in 1ml of immunoprecipitation buffer and re-centrifugation. The pellets were finally resuspended in 1 x protein-gel sample-buffer and the *Staph.A* cells pelleted and discarded. The

supernatants were then analysed by polyacrylamide gel electrophoresis.

Double-labelling of *in-vitro* translation products

An amino acid-depleted reticulocyte lysate was used and found to have optimal activity when KAc was added to a final concentration of 125mM - addition of MgAc did not enhance the activity. Two amino acid stock solutions were made, one being deficient in methionine and serine, and the other deficient in methionine and alanine. A stock reaction mix was made for the methionine/serine and methionine/alanine labellings as listed below:

| | Met/Ser | Met/Ala |
|---|---|---|
| Depleted lysate | 43.75µl | 43.75µl |
| amino acid mix (-met,-ser) | 3.125µl | - |
| amino acid mix (-met,-ala) | - | 3.125µl |
| 2M KAc | 3.9µl | 3.9µl |
| $^{35}S$ methionine | 2.5µl(25µCi) | 2.5µl |
| $^{3}H$ serine (dried down) | 50µCi | - |
| $^{3}H$ alanine (dried down) | - | 50µCi |
| $H_2O$ | 1.72µl | 1.72µl |
| TOTAL | 55µl | 55µl |

From these mixes, 11µl were added to 0.25µg (1.5µl) of the following mRNAs: male DBA/2 sublingual, bovine pituitary gland, human placenta and a minus RNA control (1.5µl DEP-treated $H_2O$). The reactions were incubated at 30°C for 90 minutes and terminated by the addition of 12.5µl of 2 x sample buffer. The translation products were then separated on a 17.5% polyacrylamide-SDS gel from which a Western blot was prepared.

Peptide mapping

Peptide mapping of preprorenin(s) was carried out as described by Cleveland *et al*,(1977) and the products separated by electrophoresis on a 12.5% acrylamide-SDS gel and visualised by fluorography.

Isoelectric focusing of preprorenins

Samples were prepared and electrophoresed on 1.5mm thick vertical slab gels according to the method of O'Farrell (1975) and after focusing, the pH gradient was determined by measuring the pH of the gel at 0.5cm intervals using a flat-bed pH electrode.

Western blotting (Towbin *et al*,1979; Burnette,1981)

Western-blots were prepared on nitrocellulose using the Biorad Trans-blot system. For the transfer of *in-vitro* translation products from 17.5% acrylamide gels a 20% methanol, 192mM ($NH_3$-free) glycine, 25mM Tris-HCl buffer (pH 8.3) was used and electrophoresis was for 4hrs at 60V(0.2A). After transfer, the blot was air-dried, covered in clingfilm and autoradiographed.

Isolation of rRNA species for use as size markers

An 8-20% sucrose gradient was prepared from 8mls of 8% and 8mls of 20% sucrose stock solutions containing 100mM KCl, 10mM Tris HCl pH 7.6, 1mM EDTA. 1ml (3.3mgs) of polyA$^-$ RNA (from IF-induced C243 cells) was layered on top of the gradient and centrifuged for 18hrs at 4°C and 23000 rpm in a Sorval AH627 rotor. Fractions were collected (0.5ml) and their $OD_{260}$ measured. The 18S and 28S peaks were pooled and ethanol precipitated, and checked by electrophoresis on an agarose-urea gel. The yields of 18S and 28S rRNAs were 0.8 and 1.5mgs respectively. The markers were aliquoted and stored at -70°C.

Preparation of diazobenzyloxymethyl (DBM)-celllose (Alwine *et al*,1980)

The preparation of this activated paper was carried out exactly as described by Alwine *et al*. It was stored as the nitrobenzyloxymethyl form (NBM) at 4°C and activated immediately before use.

Northern blotting of RNA

RNA samples were denatured in 90% DMSO, 9% glycerol, 0.01M citrate (Na) pH 3.4, at 45°C for 30 minutes before loading on a low-pH agarose-urea gel made as follows:

| | | F.C. |
|---|---|---|
| agarose | 1.875g | 1.5% |
| 9M urea | 83.32mls | 6M |
| 1M citrate pH3.4 | 3.12mls | 25mM |
| 0.3M NaIAc | 6.25mls | 15mM |
| DEP $H_2O$ | 32.31mls | |

The gel was allowed to set at 4°C for 4hrs and was then immersed in electrophoresis buffer in a gel tank. The buffer contained 6M urea, 25mM citrate pH3.4, 1mM NaIAc. After loading the samples, the gel was electrophoresed at 100V for 14hrs at 4°C. If the gel was not to be blotted, then the RNA was visualised by staining with ethidium bromide (1μg/ml) for 15 mins.

To prepare the gel for blotting, it was rocked gently at room temperature for 40 minutes in 200mls of 50mM NaOH, 5mM 2-mercaptoethanol. It was then washed twice in 200mls of 25mM sodium phosphate pH 6.0, 7mM iodoacetic acid, each wash being for 10 minutes. Finally, the gel was washed twice in 25mM sodium phosphate,pH 6.0 (the transfer buffer) and placed onto the blotting system (as for the Southern blot). The gel was covered with DBM paper (activated during the gel preparation) and 20 sheets of Whatman 3MM filter paper, on top of which were 2-3 dozen tissues and a 1kg weight. The blotting was carried out overnight in the cold-room. The transfer was checked by staining the gel in ethidium bromide, and the blot was dried and stored at -20°C or probed immediately.

Dot blot analysis of RNA

Appropriate dilutions of RNA samples were made in a 0.25 mg/ml solution of tRNA prepared in TE. 4μl of each dilution were spotted onto a nitrocellulose filter which had been pre-soaked in 20 x SSC and air-dried. After baking at 80°C for 2 hrs, the filters were hybridised as described below.

Hybridisation of Northern blots and mRNA dot-blots

Northern blots and mRNA dot-blots were pre-hybridised, hybridised and washed as described by Thomas (1980). They were stored dry at -20°C before and after probing. Dot-blots were dried very carefully as it was found that formamide caused nitrocellulose filters to shrink markedly in size.

## 2.7 cDNA- and DNA-related techniques

Preparation of dscDNA

For preparative synthesis 10μg of polyA$^+$ RNA was used. The standard first-strand reaction was as follows:

| | |
|---|---|
| $^3$H dCTP | 10μCi (dried down) |
| 0.1M DTT | 10μl |
| 5 x RT buffer | 20μl |
| 1mg/ml oligo-dT | 10μl |
| 1mg/ml polyA$^+$ RNA | 10μl |
| reverse transcriptase | 4μl (52 units) |
| $H_2O$ | 36μl |
| TOTAL | 100μl |

The synthesis was carried out at 42°C for 60 minutes, and then the mixture diluted with an equal volume of RT dilution buffer (5mM DTT, 5mM Tris-HCl, pH 8.3) and a further 52 units of reverse transcriptase were added. The reaction was then incubated at 47°C for 30 minutes and finally stopped by the addition of 2μl 0.4M EDTA, 2μl 10% SDS and 100μl Tris-saturated phenol. After vortex-mixing and centrifugation, the aqueous phase was removed and cDNA separated from $^3$H dCTP by passage over a G50 column. The relevant fractions were pooled and 2.5 volumes of 1M NaOH were added. The RNA was alkali-digested at 70°C for 15 minutes, then the reaction neutralised by an equivalent amount of 1M HCL and diluted by an equal volume of 1M Tris-HCl, pH 7.5. The single-stranded cDNA was then recovered by ethanol-precipitation and used for the synthesis of dscDNA as follows:

| | |
|---|---|
| $\alpha^{32}$P dCTP | 1μl (≈5μCi) |
| 5 x RT-2 buffer | 100μl |
| sscDNA | 30μl (≈1μg) |
| reverse transcriptase | 15μl (200 units) |
| $H_2O$ | 354μl |
| TOTAL = | 500μl |

The reaction was incubated at 46°C for 4hrs and then stopped and phenol extracted as described for the first-strand synthesis. After ethanol-precipitation, the dscDNA was separated from free $\alpha^{32}P$ dCTP by a second passage over G50, and recovered by ethanol precipitation.

In later preparations of cDNA the RNAse inhibitor, "RNAsin", was included (to a final concentration of 1 unit/$\mu$l) in the first-strand reaction and was seen to allow the synthesis of longer reverse-transcripts.

Preparation of competent cells (JA221, JM101/103)

Cultures were grown in L-broth at 37°C until the $OD_{650}$ was 0.6. They were harvested in round-bottomed universal tubes by centrifugation at 5000 rpm, 4°C for 10 minutes and resuspended in 0.1M $MgCl_2$ to the original volume and left on ice for 5 minutes. The cells were recovered once more by centrifugation and then resuspended in 0.1M $CaCl_2$ to half the original volume. After 20 minutes on ice, the cells were harvested and resuspended in 0.1M $CaCl_2$ to $^1/_{20}$ of the original volume and stored at 4°C.

Transformation of competent cells (JA221)

The DNA to be used in transformation (5$\mu$l of solution containing less than 1$\mu$g DNA) was diluted to a volume of 100$\mu$l by the addition of 95$\mu$l 1 x SSC, and mixed with 200$\mu$l of competent cells. The cells were left on ice for 30 mins, then given a 2 minute heat-shock at 42°C and returned to ice for 20 minutes. One millilitre of L-broth was added and the cells incubated at 37°C for 45 minutes. After centrifugation at 5000 rpm, 4°C for 10 minutes, the cell pellet was resuspended in 100$\mu$l bacterial buffer and plated onto an L-agar plate containing a suitable selection.

Screening of colony- and phage-banks

The screening procedures of Young and Hogness (1977) and Benton and Davis (1977) were used for colony- and phage($\lambda$ and M13)-screening respectively and were carried out as described by the authors.

Preparation of plasmid DNA

Using a suitable selection (usually 10mg/ml tetracycline), 250ml overnight cultures were grown and the cells harvested by centrifugation at 6000 rpm for 10 mins at 4°C. The cell pellets were resuspended in 3mls of 25% sucrose, 0.05M Tris, pH 8.0 and treated with 0.5mls of 10mg/ml lysozyme at 4°C for 15 minutes. After the addition of 1ml of 0.25M EDTA, and a further 15 minutes at 4°C, the cells were lysed with 4mls of Triton lysis buffer (2% Triton X-100, 0.05M Tris-HCL,pH 8.0, 0.0025M EDTA) and the lysate cleared by centrifugation at 18000 rpm, 4°C for 30 minutes. To 7.4mls of cleared lysate, 7.1g of CsCl were added and dissolved. Ethidium bromide was then added (0.2ml 10mg/ml solution) and the tubes topped-up with paraffin oil and sealed. Samples were centrifuged for 60hrs at 40000 rpm, 20°C in a Beckman 50Ti rotor.

Plasmid bands were harvested after removal of the chromosomal DNA band (upper band) using a hypodermic syringe and needle. The ethidium bromide was removed by 2-3 extractions with CsCl-saturated, TE equilibrated isopropanol, and the solution was then dialysed against TE overnight (3 changes). The DNA preparation was phenol-extracted and residual phenol removed by ether-extraction, after which the DNA was recovered by ethanol precipitation and resuspended in sterile water. Plasmids were either stored frozen at -20°C or over 5-10$\mu$l of chloroform at 4°C.

Purification of plasmid DNA using A5M (cellulose)

Plasmid DNA samples containing large amounts of RNA were purified by passage over a 30 x 1 cm column of Biorad Biogel A5M. The column was equilibrated with 50mM Tris HCl,pH 8.0, 1mM EDTA, 500mM NaCl and after loading the sample 100-drop fractions were collected, using a Gilson fraction-collector and their OD's measured. Typically, plasmid DNA was recovered in fractions 5 to 7 and RNA was found in fractions 9 to 14.

Agarose gel electrophoresis

DNA samples were analysed by electrophoresis on various strengths of agarose gel, depending on the size of fragments expected. Generally, 0.6%-1.0% gels were used for restriction digests and for sizing M13 recombinants. To size partial *Sau*3A digests of genomic DNA and test their ability to religate, 0.2% agarose gels were used. These were poured onto a 0.8% agarose base for support. The buffer used was 1 x Tris acetate gel buffer and conditions of electrophoresis varied from 25V overnight to 200V for 2hrs.

Acrylamide gel electrophoresis for the separation of DNA fragments

Acrylamide gels were used both analytically and preparatively. In general, preparative gels were made and used in the same way as analytical gels except that they were thicker (3mm) and had larger sample wells. A stock solution of 29:1 acrylamide:bis-acrylamide was stored at 4°C and diluted to the required concentration when needed. For analysis of restriction digests, gels of 5-12% were used and the running buffer was 1 x TBE.

Primer-extension products, cDNA and protected fragments obtained during $S_1$-mapping were analysed on denaturing acrylamide gels. The recipe for a 5% denaturing gel is shown below:

| | | |
|---|---|---|
| acrylamide | | 2.5g |
| bis-acrylamide | | 0.083g |
| urea | | 21g |
| 10 x TBE | | 2.5mls |
| $H_2O$ | -> | 50mls |
| TEMED | | 160µl |
| 10% APS | | 75µl |

Before addition of the APS and TEMED, the gel solution was filtered and degassed. The running buffer was 0.5 x TBE.

The products of DNA sequencing were also analysed on acrylamide-urea denaturing gels. These were prepared as described by Maxam and Gilbert (1980)

Isolation of DNA restriction fragments

Restriction fragments were separated by polyacrylamide gel electrophoresis and visualised by ethidium bromide staining. The restriction fragments were then removed by cutting out a gel slice. This was placed into dialysis tubing with 1-2mls of 0.1 x TBE and electroeluted at 200V for 1hr. The DNA was then released from the wall of the dialysis tubing by reversing the polarity for 30 seconds. A column of DE52 cellulose (equilibrated with 0.1 x TBE) was prepared in a large Gilson tip plugged with repelcoated glass wool. The column was washed with 0.1 x TBE and the DNA solution passed through it four times. It was then washed again with 0.1 x TBE and the DNA eluted from the column with 500µl of 1M NaCl, 50mM Tris-HCl, pH 7.5, 1mM EDTA. The DNA was recovered by ethanol precipitation, redissolved in sterile water and stored at -20°C.

Nick-translation of DNA

Nick-translations were carried out in a standard reaction prepared as follows:

| | |
|---|---|
| DNA (0.1-0.2µg) | 5µl |
| 10 x Nick mix | 2.5µl |
| 50µM dATP | 2µl |
| 50µM dTTP | 2µl |
| 50µM dGTP | 2µl |
| $H_2O$ | 8.5µl |
| 8ng/ml DNAseI | 1µl |
| $\alpha^{32}$P dCTP | 2µl (20µCi) |
| *E.coli* DNA polymerase I | 1µl (4 units) |

and incubated at 15°C for 60 minutes. The reaction was stopped by addition of 25µl of quench mix and the DNA recovered by phenol-extraction and ethanol precipitation in the presence of 100µg of denatured salmon sperm carrier DNA. After a second ethanol precipitation, the DNA was dissolved in 500µl 10mM Tris-HCL, pH 7.5 and its specific activity determined.

Smith-Birnsteil mapping (Smith and Birnsteil, 1976)

In Chapter 4 reference is made to the use of Smith-Birnsteil mapping to confirm the existence of small *Hae*III fragments. This was performed by digesting the cDNA insert from 20μg of pSMG199 (end-labelled) in the presence of 5μg of pAT153 DNA using 3 units of *Hae*III. Aliquots (10μl from 110μl reaction) were removed at the following times: 0, 15, 30 seconds, 1, 5, 20 and 90 minutes and the reaction stopped by placing the aliquots into pre-heated 70°C tubes for 5 minutes. The samples were analysed by electrophoresis on a 5% acrylamide gel and exposure to film.

Hybrid-selection of mRNA

A stock hybridisation solution was prepared and stored at -70°C in 1ml aliquots. The formamide was deionised before use and the solution was made as follows:

| | | F.C. (during hybridisation) |
|---|---|---|
| formamide | 20mls | 50% |
| 5M NaCl | 3.2mls | 0.4M |
| 0.1M Pipes-NaOH pH6.4 | 4.0mls | 10mM |
| 0.5M EDTA pH8.0 | 0.32mls | 4mM |
| 10mg/ml calf liver tRNA | 2mls | 0.5mg/ml |

From this stock, the following two solutions were made as required:

(1) pre-hybridisation solution:

0.295mls STOCK SOLUTION
0.105mls $H_2O$

(2) hybridisation reaction solution:

0.295mls STOCK SOLUTION
0.105mls mRNA solution

For hybrid-selection screening of clones, 10μg of each plasmid were lyophilised and resuspended in 5μl of 1M NaCl, 0.5M NaOH, 10mM EDTA. The samples were incubated at 65°C for 20 minutes, and then spotted and dried onto a gridded nitrocellulose filter, each sample being numbered. The filter was rinsed three times in 3 x SSC for a total of two minutes, dried and baked at 80°C for 4hrs. It was then washed in sterile water at room

temperature (4 x 30 seconds) and at 80°C (2 x 2 minutes). The individual samples were cut out on small squares of filter using a scalpel-blade, and placed into 400μl of pre-hybridisation solution at 41°C for 3hrs. The solution was changed and the filters prehybridised for a further 2hrs, before placing them into 400μl of hybridisation solution and incubating them overnight at 41°C. The solution was removed and stored at -20°C. The filters were then washed sequentially as follows:

| WASH SOLUTION | TEMPERATURE | N$^{O}$ OF WASHES |
|---|---|---|
| (1) 1 x SSC,0.5% SDS | R.T. | 4 x 5mls |
| (2) 0.1 x SSC,0.1% SDS | R.T. | 6 x 5mls |
| (3) 0.1 x SSC,0.1% SDS | 50°C | 2 x 5mls |
| (4) TE buffer | 50°C | 4 x 5mls |

After the last wash each filter was placed into a large Eppendorf tube with 150μl of DEP-treated water and heated at 80°C for 5 mins. The water (now containing any selected mRNA) was removed and stored on ice. Any remaining mRNA was recovered with a further 100μl of water and the two solutions were pooled and the mRNA precipitated with the addition of 4μl of 1mg/ml tRNA, 25μl 2M NaAc pH 5.0 and 550μl ethanol. The RNA pellets were dissolved in 5μl of water and 2μl were used for *in-vitro* translation.

Preparation of template DNA from M13 recombinants

Template DNAs were always prepared using fresh plaques, and a fresh overnight culture of JM101 or JM103. One drop (50μl) of the overnight culture was placed into 25mls of L-Broth, which was divided into 1ml aliquots in small universal bottles. The required plaques were picked using 200μl micropipettes, placed into the 1ml aliquots of JM101/3 culture and incubated on a roller at 37°C for 5-6 hours.

The cultures were transferred to 1.5ml Eppendorf tubes and microfuged for 3 minutes, after which 800μl of each supernatant was transferred to a fresh tube. Two hundred microlitres of 2.5M NaCl,10% PEG 6000 were then added, mixed and the phage allowed to precipitate at room temperature for 30

minutes. The phage pellet was harvested by centrifugation for 5 minutes (minifuge), and the supernatant removed carefully. The pellet was resuspended in 100$\mu$l of 1.1M NaOAc,pH 7.0, and 50$\mu$l of Tris-saturated phenol and 50$\mu$l of $CHCl_3$:isoamylalcohol (50:1) were added and mixed. The extractions were allowed to stand for 5 minutes and after a second vigorous mixing the phases were separated by centrifugation for 1 minute. The aqueous (upper) layer was placed into a fresh tube and remaining phenol removed by extraction with 60$\mu$l $CHCl_3$:isoamylalcohol (50:1). Template DNAs were precipitated from the aqueous phase by the addition of 250$\mu$l ethanol and cooling in a dry ice/ethanol bath for 15 minutes. After centrifugation, the DNA pellets were rinsed in 70% ethanol, resuspended in 20$\mu$l TE and stored at -20°C.

DNA sequencing

DNA sequence determinations were carried out by the chemical cleavage method of Maxam and Gilbert (1980) and the dideoxynucleotide chain-termination method of Sanger *et al* (1977).

Transformation of JM101/103 with recombinant M13 DNA

Competent cells were prepared as for JA221 (above) and the transformation carried out in the same way. 500$\mu$l of exponentially growing cells were then added followed by 25$\mu$l of 100mM IPTG and 125$\mu$l of 2% X-Gal (in DMF). Finally, 7.5mls of BBL top agar were added and the mixture poured onto 1% minimal medium, glucose, $B_1$ agar plates and incubated overnight at 37°C.

Preparation of cDNA libraries in the vectors M13 mp10/11

Double-stranded cDNA was prepared as previously described and treated with 1 unit/$\mu$l of $S_1$ nuclease at 37°C for 1 minute. The $S_1$ resistant dscDNA was phenol extracted and ethanol precipitated, then redissolved in 20$\mu$l of water. It was then treated with $T_4$ polymerase by the addition of 2.5$\mu$l of 10 x TM buffer, 2.5$\mu$l of a dNTP mix (0.25mM each) and 1$\mu$l (8 units) of $T_4$ DNA

x TM buffer, 2.5μl of a dNTP mix (0.25mM each) and 1μl (8 units) of $T_4$ DNA polymerase (BRL). The reaction was incubated at 10°C for 1 hour and then phenol extracted and the cDNA ethanol precipitated.

The blunt-ended cDNA was then ligated to phosphatased, *Sma*I-cut M13mp11 (or mp10) in a reaction set up as follows:

| | | |
|---|---|---|
| phosphatased, *Sma*I cut M13mp10 | | 2μl (40pg) |
| 10 x $T_4$ DNA ligase buffer | | 1μl |
| ds cDNA | | 0.5-1.5μl |
| $T_4$ DNA ligase (PL) | | 1μl (4.5 units) |
| $H_2O$ | -> | 10μl |

and incubated at 15°C overnight. The ligated DNA was then transformed into JM101 or JM103 competent cells.

Dot-blot analysis of template DNAs from M13 recombinants

One microlitre of each template preparation was spotted onto a gridded nitrocellulose filter; 1μl of 0.5M NaOH, 1M NaCl was immediately spotted on top of each sample and after drying at room temperature, the filter was baked at 80°C for 2 hours. The samples were then probed as for colony-screening.

Extraction of DNA from tissue

Tissues were thawed into 5 volumes of 1 x SE buffer, chopped up and then gently homogenised using a motor-driven teflon homogeniser. One volume of lysis buffer was added and mixed gently, after which lysis was allowed to continue 'on ice' for 5 minutes until the mixture was 'gloopy'. At this point, 0.25 volumes of 5M $NaClO_4$ and 0.5 volumes of phenol:$CHCl_3$ (50:50) were added and mixed to form an emulsion. The phases were separated by centrifugation in siliconised Corex tubes using a Sorvall HB4 rotor at 10000 rpm, 4°C for 10 minutes. The supernatant (aqueous phase) was removed carefully using a broken Pasteur pipette and 2 volumes of ethanol added and mixed gently to precipitate the DNA. The supernatant was poured off, the DNA rinsed in 70% ethanol and redissolved in a small volume of 0.1 x TNE. To this was added 0.005 volumes of 20 mg/mL pancreatic RNAse (heated to 90°C for

10 minutes to destroy DNAse) and after incubation at 37$^{o}$C for 15 minutes the following were added: 0.1 volumes 10% SDS, 0.05 volumes 20 x TNE and 0.005 volumes 20mg/ml pronase. The mixture was reincubated at 37°C for a further 15 minutes and was then poured into a conical flask and mixed gently with 0.5 volumes of phenol/chloroform to form an emulsion. The aqueous phase was separated by centrifugation at 10K, 4$^{o}$C for 10 minutes and the DNA precipitated by addition of 0.1 volumes 2M NaAc,pH 5.6 and 2 volumes of ethanol. The precipitate was dissolved in 10mM Tris-HCl, pH7.5, reprecipitated and taken up again in 10mM Tris-HCl, pH 7.5. In early DNA preparations, an extraction with 2-methoxyethanol was carried out to remove any carbohydrate which might inhibit the activity of restriction enzymes, but this was found not to be necessary and therefore, after the second step, the DNA was ready for use

Southern blotting of restriction digests of DNA (Southern,1980)

DNA samples (10-20$\mu$g) were mixed with an equal volume of loading buffer (made by mixing 1ml of agarose beads, 5$\mu$l of 5mg/ml ethidium bromide and 400$\mu$l 1.5M NaOH, 0.1M EDTA). The samples were allowed to stand at room temperature for 5minutes before being loaded on a 0.8% agarose gel. They were left for a further 5 minutes in their wells and then electrophoresed at 50V for 30 minutes. The gel was then run at 150V for 2hrs. It was soaked for 20 minutes in 20 x SSC and placed on the transfer apparatus, ensuring that no air bubbles were trapped between the gel and the filter paper. A sheet of nitrocellulose paper was cut to the size and soaked in 3 x SSC. The nitrocellulose paper was carefully placed on top of the gel and covered with a sheet of Whatman 3MM filter paper (also soaked in 3 x SSC). Several sheets of dry 3MM paper were then placed on top, followed by 4-6" of absorbant tissues and a 1kg weight. The transfer was carried out overnight in the cold-room, using 20 x SSC as transfer buffer. The DNA samples could be located on the filter using an ultra-violet lamp and if necessary the filter

was cut into manageable strips at this stage. The filter(s) were baked at 80°C for 2hrs and were then ready for hybridisation.

Hybridisation of Southern blots (Jeffreys *et al*,1980)

Southern-blot hybridisation conditions were those of Jeffreys *et al* except that hybridisations were carried out in plastic bags, and the final wash conditions were 0.5 x SSC at 65°C.

Size fractionation of partial restriction digests of genomic DNA

Genomic DNA, partially digested by the enzyme *Sau*3A, was phenol extracted, ethanol precipitated and redissolved in TE. It was then layered over a 1.25-5M NaCl gradient (17mls) and centrifuged at 18000 rpm 20°C for 17hrs in a Kontron 40.1 Ti rotor. A maximum of 150μg of DNA was loaded per gradient. The DNA was harvested by collecting 0.25ml fractions into 1.5ml Eppendorf tubes containing 0.25ml of TE. The DNA in each fraction was precipitated by the addition of 1ml ethanol and storage overnight at -20°C. After centrifugation in a minifuge for 15 minutes the supernatants were discarded and the DNA pellets rinsed with 70% ethanol. They were each lyophilised and resuspended in 50μl of TE, of which 1μl was analysed on a 0.2% agarose gel to determine the spread of fragment size in each fraction.

Ligation and packaging of λ recombinants

Sized human DNA was ligated to λ arms (DB287) in a reaction made as follows:

| | | |
|---|---|---|
| Human DNA (3μg) | 14μl | |
| λ arms (14μg) | 28μl | |
| 10 x $T_4$ DNA ligase buffer | 9μl | |
| $T_4$ DNA ligase | 4μl | (6 units) |
| $H_2O$ | 34μl | |
| TOTAL | 90μl | |

and incubated overnight at 15°C. The ligated DNA was then packaged using the Promega "Packagene" kit available from P&S Biochemicals Ltd (Liverpool). The recombinant phage particles were then diluted with λ buffer and

purified by centrifugation on a CsCl block gradient.

Plaque purification of λ recombinants

Plaques giving positive hybridisation signals were picked using sterile glass tubes and the phage eluted from the agar by vortexing in 1ml of λ buffer. The agar was separated from the phage by centrifugation and dilutions of the phage plated out for the next round of screening. With primary screens, a large area around the putative positive was picked to ensure it was not missed. With subsequent rounds of plaque purification a more precise agar "plug" was taken until finally a single plaque was picked.

Preparation of phage DNA

Phage were grown by the liquid lysate or "Blattner" method (Blattner *et al*,1977). A single plaque was picked, suspended in 1ml of λ buffer and 0.1-0.2mls mixed with 0.1ml of plating cells (prepared from 10mls of an overnight culture of ED8910 (*Rec*BC$^-$,RK$^-$) by spinning the cells down and resuspending them in 10mls of 10mM $MgSO_4$). After absorbtion for 10 minutes at room temperature, the mixture was diluted into 200mls L-broth, containing 20mM $MgSO_4$, in a non-baffled flask and shaken overnight at 37°C. When the culture had cleared, 0.5mls of chloroform were added and the cell debris was pelleted by centrifugation at 8000 rpm for 10 mins at 4°C. The phage were precipitated by the addition of 4g NaCl to 200mls of lysate (4°C, 1hr) and 20g of PEG 6000 (4°C, overnight). After pelleting at 4000 rpm for 5 mins (4°C), the precipitate was resuspended in 1ml of λ buffer and purified by centrifugation on a CsCl block gradient. The phage band was removed using a hypodermic syringe and needle and dialysed against 2l of TE at 4°C for 1hr. Any remaining RNA was digested by the addition of 20μg/ml heat-treated RNAse and continued dialysis at room temperature for 1hr. Protein was then digested with 1mg/ml pronase at 37°C for 1hr and dialysis was continued against pronase buffer during this incubation. Residual protein was removed

by two phenol-extractions and traces of phenol were then removed by dialysis against TE buffer at 4°C. If necessary, the DNA was then concentrated by ethanol precipitation.

Caesium chloride block gradient purification of phage

Prior to plating, recombinant phage were purified by centrifugation through a CsCl block gradient made by sequencially underlaying 4mls of 1.3g/cm$^3$ CsCl with 4mls of 1.5g.cm$^3$ CsCl and then 4mls of 1.7g/cm$^3$ CsCl. The resuspended PEG precipitate of the phage was gently layered on top and the gradient centrifuged at 37000 rpm, 20°C for one hour in a Kontron TST 41.14 rotor (alternatively, 20°C, 24000 rpm for 4hrs using a Sorvall AH627 rotor). The phage band, located in the 1.5g/cm$^3$ layer, was removed using a hypodermic syringe and needle. The purified phage preparation was then dialysed overnight against λ buffer to remove CsCl.

$S_1$ mapping

$S_1$ mapping was carried out according to the method of Favaloro *et al* (1980). Typically, 2μg of DBA/2 mRNA or 40μg of C57BL/10 mRNA were used per reaction. Protected fragments were recovered by ethanol precipitation and were analysed on denaturing acrylamide-urea gels.

Primer extension-analysis

Early primer-extensions were carried out in the presence of $\alpha^{32}$P dCTP and synthesised either in a standard cDNA synthesis at 42°C or by the method described below. Best results were obtained by using kinase-labelled primers and all subsequent extensions, except where indicated, were labelled in this manner. For a typical reaction, 10 pmoles of primer were labelled and added to 2μl of 5 x RT buffer (-nucleotides), 1μl (1μg) of polyA$^+$ RNA and the volume made up to 7μl with DEP treated water. The mixture was sealed in a siliconised 50μl micropipette and boiled for 1 minute in a water bath containing DEP-treated water. The whole bath was then placed

into a 65°C water-bath which was turned down to 42°C, and allowed to cool to 42°C over a 45 minute period. The capillary tube was then broken and the contents added to the following prewarmed reaction mix:

| | |
|---|---|
| 5 x RT ( - nucleotides) | 2μl |
| 10mM dTTP | 2μl |
| 10mM dCTP | 2μl |
| 10mM dATP | 2μl |
| 10mM dGTP | 2μl |
| 0.1M DTT | 2μl |
| Reverse transcriptase | 0.8μl (10 units) |

and incubated at 42°C for 1 hour. The reaction was stopped by the addition of 1μl 0.5M EDTA, 1μl 10% SDS, 1μl 10mg/ml yeast tRNA and 40μl TE buffer, phenol and ether extracted and ethanol precipitated. The extension products were resuspended in 5μl TE and mixed with 5μl 0.2M NaOH, 2mM EDTA. After the addition of 10μl loading buffer, samples were boiled for 1 minute and loaded on a denaturing gel.

Primer-extensions terminated by dideoxynucleotides were carried out in the same way except that the deoxynucleotide was replaced by half the concentration of the dideoxynucleotide.

Recovery of primer-extension products from gels prior to sequencing

The species to be sequenced were located using an autoradiograph and their positions in the gel marked by piercing the autoradiograph and gel using a needle. The products were then excised using a sterile scalpel-blade and mascerated individually in Eppendorf tubes with enough elution buffer to cover the gel slice. (Elution buffer: 500mM $NH_4Ac$, 10mM MgAc, 1mM EDTA, 0.1% SDS). Ten micrograms of calf liver tRNA were added as carrier and the fragments were allowed to elute at 37°C for 1 hour. The eluate was filtered through siliconised glass-wool using a blue Gilson tip and the fragments recovered by the addition of 2.5 volumes of ethanol and cooling in a dry ice/ethanol bath for 15 minutes.

# CHAPTER 3

## ISOLATION AND *IN VITRO* TRANSLATION OF SALIVARY GLAND MRNA

### 3.1 Extraction of total cellular RNA

In the isolation of mRNA from tissue, three protein denaturants are commonly used: guanidine HCl, lithium chloride and phenol. The guanidine methods are the most effective when the source is rich in ribonucleases, (Deeley *et al*,1977), but they involve many steps including exchange of the sodium salt for the guanidinium salt of the RNA, and in my hands, gave a low yield of RNA which was not very active in *in-vitro* translation. Barlow *et al* (1963) found that 2M LiCl was not only effective in isolating ribosomal proteins but could also be used to specifically precipitate RNA from a crude lysate. No degradation was observed but their tissue (reticulocytes) contained very little RNAse activity. This method has been developed for extraction of RNA but has the disadvantage of taking longer and consequently increasing exposure to ribonucleases. The simplest and most general methods use phenol extraction, first described by Kirby (1956), who developed a method for extracting rat liver RNA by homogenisation of the tissue in phenol/water at room temperature. Centrifugation gave an aqueous phase containing RNA and polysaccharide, a cloudy interface consisting of insoluble protein and a phenol phase containing protein and DNA-protein complexes. Initially the use of phenol gave rise to some RNA degradation, due in part to the use of unbuffered, non-redistilled phenol, since the addition of 8-hydroxy- quinoline improved the yield (Kirby,1968), and also to the fact that early extractions either took no account of ribonucleases or used bentonite as an inhibitor and this has since been shown to be relatively ineffective (Williamson *et al*,1973;Payne *et al*,1970).

The discovery of polyA sequences in eucaryotic RNA (section 3.3) led to the observation that polyA$^+$ mRNA, and also synthetic polymers, behaved unexpectedly during phenol extraction. Much of the polyA was found in the

phenol phase and could, during extraction, be removed from the main mRNA molecule by a process requiring the participation of polysomal proteins (Perry *et al* 1972). This process was found to be dependent on both salt concentration and pH of the extraction buffer. Lee *et al* (1971) optimised the pH to 9.0 and found very little RNA recovered at neutral pH, even in the presence of low salt concentrations. It is, however, possible to recover polyA$^+$ RNA at neutral pH if a 1:1 mixture of phenol/chloroform is used instead of phenol, the mixture giving more effective deproteinisation of polyA$^-$ protein complexes (Perry *et al* 1972).

Most of the phenol methods currently in use combine these observations and extract in a low salt, alkaline buffer, using a 1:1 mixture of phenol and chloroform, often with a small amount of isoamyl alcohol added as a defoaming agent (Marmur 1963). Such a method is described Noyes *et al* (1979) and includes SDS as a strong protein denaturant and RNAse inhibitor, and EDTA to destabilise RNA-protein interactions.

The method of Noyes *et al* was found to give very good yields, both qualitatively and quantitavely, as described below, and was the method routinely used for isolation of RNA from mouse tissues.

## 3.2 The use of Diethylpyrocarbonate during mRNA isolation

One of the most important considerations when isolating RNA is the prevention of degradation, particularly by ribonucleases, which are both ubiquitous and resistant to simple heat-inactivation. A variety of inhibitors have been used (reviewed by Taylor,1979) including bentonite, heparin, vanadyl chloride, naturally occurring inhibitors from human placenta and rat liver, and D.E.P. (reviewed by Ehrenberg *et al*,1976). In the procedure of Noyes *et al*, the combination of phenol/SDS is sufficient for tissue that does not have excessive amounts of ribonuclease, and mRNA prepared from salivary glands has shown no signs of degradation.

Glassware and solutions, which are major sources of contamination, are best treated with DEP, which was originally developed as a

food-preservative ("Baycovin") and has several properties that make it useful in nucleic acid extraction:

1) moderate solubility in aqueous environment where it is rapidly converted to $CO_2$ and ethanol (half-life of 28 minutes at 25°C).

2) inactivates RNAse by carbethoxylation of three histidine residues, one of which is at the active centre.

3) responsible for the formation of polymers of ribonucleases and also other proteins giving a second method of inactivation.

These properties and its ease of use make it ideal for ensuring that all glassware and solutions are free from ribonucleases. DEP has been used directly as a protein denaturant in the extraction of several types of nucleic acid from many different sources (Ehrenberg *et al*,1976), but it is not commonly used for mRNA since it can react with nucleic acid, rendering it biologically inactive. However the reaction with RNA is much slower than that with protein and since for analytical purposes, such as gel electrophoresis and hybridisation, a small amount of alteration is acceptable, DEP has been used successfully either in addition to or in place of phenol. For mRNA extraction, where biological activity is a priority, such alteration could mean reduced translational efficiency and either smaller cDNA products due to termination or the introduction of sequence artifacts.

### 3.3 Purification of mRNA

The discovery of polyA tracts has made the isolation of mRNA much simpler. Messenger RNA or 'rapidly labelled RNA' from a variety of sources has been shown to contain polyadenylate tracts of 150-200 residues in length (Kates and Beeson,1970; Lee *et al*,1971; Lim and Canelakis,1970; Darnell, 1971a).Originally identified as adenylate-rich, RNAse resistant stretches, subsequent work has shown that these tracts are covalently bound at the extreme 3' ends of mRNA molecules (Sheldon 1972, Molloy 1972). The presence of polyA in both the RNA and mRNA helped to establish the precursor relationship of the nuclear hnRNA (Edmonds *et al*,1971, Molloy 1973).

Despite early suggestion to the contrary (Kates and Beeson,1970), polyadenylation has been shown to be a post-transcriptional event (Darnell 1971b) that takes place in the nucleus prior to transport into the cytoplasm. Elongation of polyA also takes place in the cytoplasm probably via a different enzyme to the one involved in *de-novo* addition in the nucleus (Edmonds,1982). The recognition of polyA provided a possible role for the polyA polymerase (RNA terminal riboadenylate transferase) found several years earlier by Edmonds and Abrams (1960,1963), but there is still no direct evidence for this. Since the discovery of the calf thymus polyA polymerase, similar enzymes have been purified and characterised (reviewed by Edmonds,1982) including two different polymerases from the same cell type.

Sequence comparison of mRNA 3' ends revealed a strongly conserved sequence, AAUAAA, 13-20 nucleotides from the polyA tract, independent of the length of the 3' non-coding region, and this has been called the "polyadenylation signal" (Proudfoot and Brownlee,1976).

Addition of polyA is common to the vast majority of eukaryotic mRNA, but the classic exception is histone mRNA (Adesnik *et al*,1972a,b, Schochetman *et al*,1972). These non-polyadenylated mRNAs appear to enter the cytoplasm quicker than the majority of mRNA, and it is possible that these properties may have explanations in the fact that the histone genes are only expressed at one particular stage of the cell-division cycle. Histone mRNA has a very short lifetime when injected into the cytoplasm of HeLa cells, but can be stabilised by polyadenylation *in-vitro* prior to injection (Nevins,1983). Experiments such as these have suggested that the role of the polyA tract is to stabilise mRNA in the cytoplasm (see below).

Several other polyA$^-$ mRNAs have been reported (Moffett and Doyle,1981) and some predominantly polyA$^+$ RNAs, such as those for albumin and actin, have a minor polyA$^-$ fraction associated with them. It is not clear from the literature if these RNAs are truly polyA$^-$, since the assays are based either on hybridisation to polyU, *or* inability to bind to oligo-dT columns, or

possible to rule out the presence of a small number of adenosine residues which may be attached by a mechanism not inhibited by cordecepin. In addition, histone messengers may not be an absolute exception, since the oocyte histone messengers of *Xenopus laevis* are polyA$^+$ (Levenson and Marcus,1976).

Recent work on transcription and polyadenylation shows that transcription occurs beyond the last nucleotide of the mature 3' non-coding sequence, but no precise termination site has been defined Salditt-Georgieff and Darnell,1983). The mature RNA is generated by subsequent endonucleolytic cleavage and polyA addition. This mechanism is at present poorly understood, as is the function of the polyA tract itself. There is good evidence for an age-dependent reduction in length of the polyA tail (Sheiness and Darnell,1973) and, as mentioned, increasing stability is the only known function of the polyA tract. Darnell and Nevins (1982) report that over half of the total polymerase II products are not polyadenylated and do not reach the cytoplasm, suggesting that a quantitatively significant mechanism of gene control could lie at the level of processing via the polyA-addition step. Wallace and Edmonds (1983) have described the presence of 2'-5' branches in nuclear polyA RNA, that may be intermediates either in polyadenylation or splicing.

The occurrence of polyA provided a convenient method for the purification of mRNA. It was found that the homopolymeric tract facilitated the retention of mRNA on nitrocellulose filters at high ionic strength (Lee *et al*,1971). This was improved by the use of short thymidine oligomers covalently attached to cellulose (Aviv and Leder,1972), to which mRNA (polyA$^+$ RNA) could be hybridised and then recovered relatively free from other RNA species. Bantle *et al* (1976) studied the specificity of oligo dT for isolating polyA$^+$ RNA and found significant aggregation of rRNA/mRNA complexes resulting in indirect binding of rRNA to oligo dT. This can be overcome by treating the RNA with DMSO and heating to 65$^o$C before running on the oligo dT column. No study was made of the effect of such treatment on

the biological activity of the RNA and such steps are not in common use. As a result, significant amounts of rRNA can remain in polyA$^+$ RNA preparations.

## 3.4 *In-vitro* translation systems

Many different *in-vitro* translation systems have been used, including mouse ascites, HeLa, yeast, L-cell, liver, wheat germ and rabbit reticulocyte lysate. The two most commonly used are the rabbit reticulocyte lysate and the wheat germ. The reticulocyte lysate was the first cell-free protein synthesising system ever used, and following the introduction of nuclease-treatment (Pelham and Jackson,1976) is now the most widely used system.

Reticulocytes are immature, but enucleated, red blood cells, and they retain the ability to synthesise haemoglobin *in-vitro* using existing mRNA, both as intact cells (Kruh *et al*,1956) and in a cell-free extract (Schweet *et al*,1958). Lockhard and Lingrel (1969) and Stavnezer and Huang (1971) used the system to translate exogenous RNA and it has now been shown to synthesise products faithfully using mRNA from a wide variety of sources. In addition to mammalian RNA, viral and prokaryotic mRNAs have also been translated. After an unfractionated system was shown to work almost as well as intact cells, Adamson *et al* (1968) optimised the conditions for using the reticulocyte system: in particular they showed that the presence of haemin was essential for sustained protein synthesis. Farrell *et al* (1977) demonstrated that this premature cut-off in activity was due to the inhibition of met-tRNA$_f$ / 40S subunit binding caused by phosphorylation of eIF2$\alpha$. This occurs in the absence of haemin when the protein kinase of a specific inhibitor called the haemin-controlled inhibitor (HCI) or haemin-controlled repressor (HCR) becomes activated.

The main advantages of the reticulocyte system for *in-vitro* translation are its high translational activity, the fact that it can faithfully

translate polypeptides of up to 250,000 daltons with few incomplete chains, and that it can be fractionated to remove large pools of endogenous amino acids. The main disadvantage is the large amount of endogenous globin which makes analysis of products in the 8-14 kd region difficult. Endogenous globin mRNA can be removed by a controlled nuclease digestion (Pelham and Jackson,1976), but small amounts remain which, when translated, can obscure co-migrating products. In addition, the unlabelled pool of endogenous globin smears during polyacrylamide gel electrophoresis, affecting the mobility of similarly sized polypeptides being studied.

This problem can be overcome by complementing the reticulocyte lysate with the wheatgerm system. This is not as active as the reticulocyte lysate, and suffers from premature termination giving so called "early quitters", but provides a better analysis of low molecular weight products and has smaller pools of amino acids, allowing a wider choice of label without fractionation. The wheatgerm system, unlike the reticulocyte lysate, is a naturally mRNA-dependent system (Marcus and Feeley,1964), which like the lysate, has been optimised for *in-vitro* translation (Roberts and Paterson,1973; Marcu and Dudock,1974).

### 3.5 Modification of *in-vitro* translation products

Primary translation products undergo many co- and post-translational modifications, depending on what type of protein is being made. These include proteolytic cleavage, the formation of disulphide bridges, methylation and glycosylation. Many of these require a defined sequence of events which tend to be compartmentalised, for example, signal-cleavage in the rough endoplasmic reticulum and glycosylation in the Golgi. As a result, *in-vitro* systems, often made from high speed supernatants of cell extracts, cannot be expected to retain these functions. It is possible, however, to supplement the system with purified subcellular components responsible for specific modifications. Blobel and Dobberstein (1975) showed that a preparation of rough microsomes was capable of cleaving the

signal sequence from secretory proteins. Such microsomes also contain the enzymes involved in core glycosylation (Czichi and Lennarz,1977; Heath *et al*,1979) and can carry out these modifications in both wheatgerm and reticulocyte systems (Das *et al*,1980; Katz *et al*,1977). *In-vitro* systems, therefore, are also useful in studying protein maturation, but only to a limited extent.

### 3.6 Isolation of polyA$^+$ RNA from salivary glands

The phenol-based method of Noyes *et al* (1979) was found to be very effective for the isolation of salivary gland RNA. After centrifugation through a CsCl pad a pellet of RNA was obtained which rarely contained any DNA. Typical yields of total cellular RNA and polyA$^+$ RNA are shown in table 3. The accompanying figure (Fig.8) shows the spectra of two RNA samples and the $A_{260}/A_{280}$ ratio reveals very little protein present in the preparations.

TABLE 3 - Yield of polyA$^+$ RNA from salivary tissue

| PREP | TISSUE WET WEIGHT(g) | TOTAL RNA(mg) | POLYA$^+$ RNA($\mu$g) | % TOTAL |
|---|---|---|---|---|
| 1 | 1.26 | 7.9 | 131 | 1.6 |
| 2 | 2.20 | 9.6 | 126 | 1.3 |
| 3 | 1.60 | 12.0 | 155 | 1.3 |
| 4 | 3.00 | 7.9 | 137 | 1.7 |
| 5 | 2.00 | 13.5 | 156 | 1.2 |
| 6 | 2.90 | 8.6 | 97 | 1.1 |
| 7 | 1.50 | 8.1 | 114 | 1.4 |
| 8 | 2.60 | 17.0 | 169 | 1.0 |
| 9 | 2.10 | 9.4 | 145 | 1.5 |
| 10 | 2.20 | 8.8 | 123 | 1.4 |
| 11 | 3.50 | 13.5 | 203 | 1.5 |
| 12 | 4.20 | 14.4 | 290 | 2.0 |
| 13 | 3.30 | 9.6 | 118 | 1.2 |
| 14 | 9.80 | 28.0 | 537 | 1.9 |

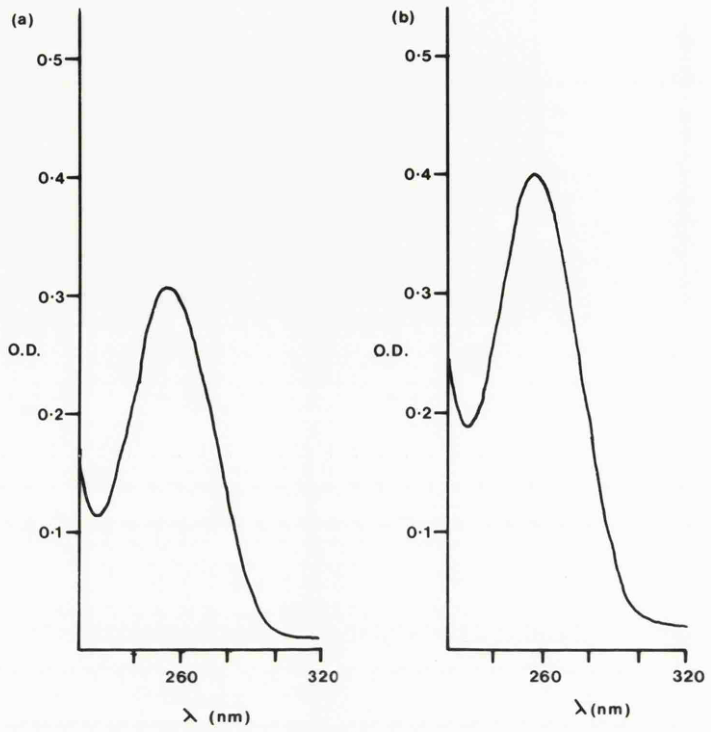
Samples of RNA were run on a denaturing urea-agarose gel (Fig.9), which gave very good resolution of RNA species. The two major bands are 28S and 18S ribosomal RNA and show that in a single passage over oligo dT significant rRNA remains in the polyA$^+$ fraction due to aggregation as discussed above. Many discrete mRNA species are visible and it is possible

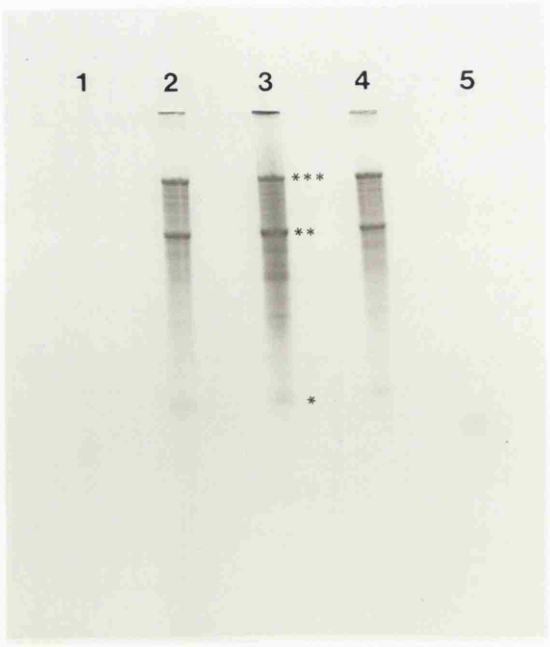
Fig.8 - Spectra of mRNA

The spectra of two typical mRNA preparations.
Their $A_{260}/A_{280}$ ratios are (a) 2.3 and (b) 2.2.

Fig.9 - Agarose-urea gel electrophoresis of mRNA

mRNA samples were denatured and electrophoresed as described in chapter 2. The gel was stained with ethidium bromide to reveal mRNA. Lanes 1 and 5: yeast tRNA; lanes 2 and 4: 6$\mu$g polyA$^+$ SMG mRNA; lane 3: 9$\mu$g polyA$^+$ SMG mRNA.
(*** = 28S rRNA, ** = 18S rRNA, * = 5S tRNA)


(a)
0·5
0·4
0·3
O.D.
0·2
0·1
260
320
λ (nm)
(b)
0·5
0·4
0·3
O.D.
0·2
0·1
260
320
λ (nm)


1
2
3
4
5
***
**
*

to isolate these from the gel and translate them *in-vitro*. However, translational activity is easily affected by agarose- or urea-contamination and a better gel system for fractionating mRNA is described by Bedbrook *et al* (1980)

3.7 Preparation of rabbit reticulocte lysate

Although *in-vitro* translation systems, notably the reticulocyte lysate, are commercially available, such preparations have the disadvantage of being relatively inflexible to alterations in the concentrations of various components. Secondly, although they are very active systems their activities are averages of a group of animals, and even better activities may be obtained by individually testing each rabbit and discarding the low activity lysates.

Reticulocyte lysates were therefore prepared from three New Zealand White rabbits, which were bled twice with two days between the bleedings. (Activity does not correlate directly with the haematocrit and activities can vary significantly during reticulocytosis).

After preparation, the lysates were stored at -70°C in 1ml aliquots which were nuclease-treated when required and then refrozen in 100µl aliquots. Each lysate was then tested for its ability to translate salivary gland mRNA by removing aliquots of translations after 0,10,20,30,60 and 90 minutes incubation and measuring incorporation of $^{35}S$ methionine into protein by TCA-precipitation. Two aliquots for each time point were taken in order to compare two different TCA-precipitation procedures, one in common laboratory use involving a hot TCA step and the second suggested by Amersham International (see materials and methods).

The results are shown graphically in Figure 10, each translation being shown relative to its own -RNA control translation. From these data it is clear that the rabbits vary enormously in their ability to produce an active lysate, and secondly there are significant differences between the bleedings of a single rabbit. The kinetics of each translation reaction appear slightly different but this may not be a significant result since the

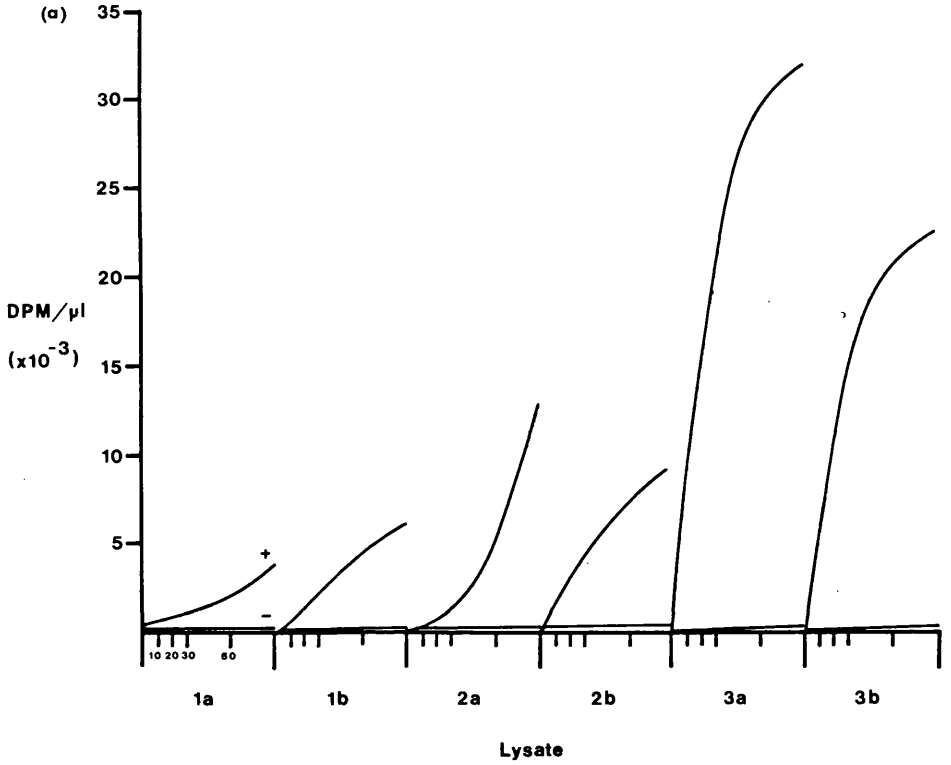
Fig.10 - Determination of reticulocyte lysate activity

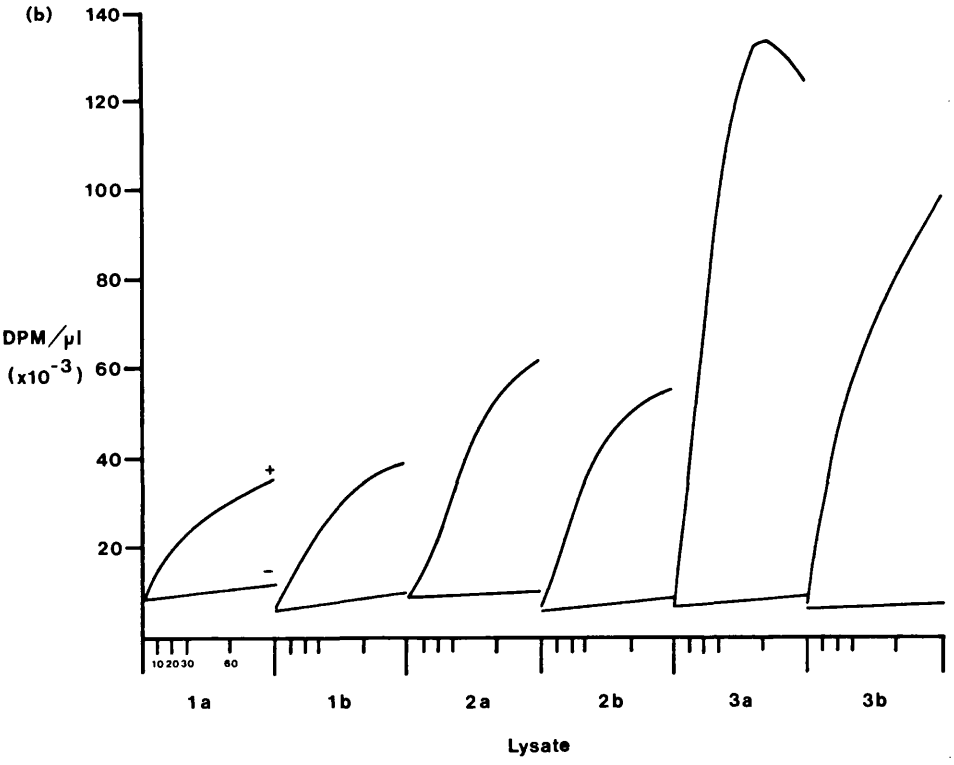
Two lysates from each of the three rabbits (eg. 1a: first bleeding, 1b: second bleeding) were tested for their ability to translate SMG mRNA *in vitro*. TCA precipitation assays were carried out after 10, 20, 30, 60 and 90 minutes incubation and the results plotted (+) against control (-mRNA) translations of the respective lysate (-).
Graph (a) results by the "Amersham" method.
Graph (b) results by the "hot TCA" method.

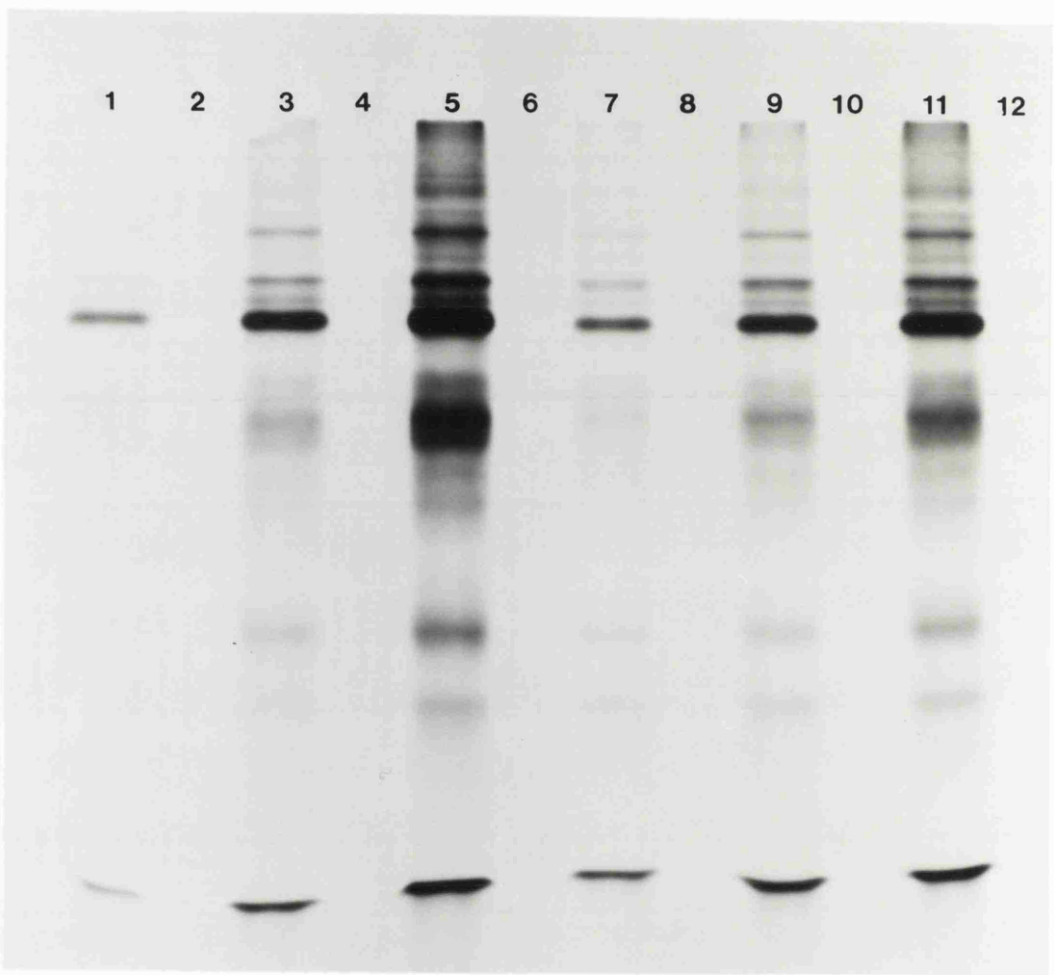
**Fig.11** - SDS-polyacrylamide gel electrophoresis of the translation products obtained after 90' incubation of the test lysates. **(overleaf)**

5µl of each translation were electrophoresed through a 12.5% gel.

| Lane | Lysate | mRNA |
|---|---|---|
| 1 | 1a | + |
| 2 | 1a | - |
| 3 | 2a | + |
| 4 | 2a | - |
| 5 | 3a | + |
| 6 | 3a | - |
| 7 | 1b | + |
| 8 | 1b | - |
| 9 | 2b | + |
| 10 | 2b | - |
| 11 | 3b | + |
| 12 | 3b | - |


(a)
35
30
25
20
15
10
5
DPM/µl
(x10-3)
+
-
10 20 30
60
1a
1b
2a
2b
3a
3b
Lysate


(b)
140
120
100
80
60
40
20
DPM/µl
(x10-3)
+
-
10 20 30
60
1a
1b
2a
2b
3a
3b
Lysate


1
2
3
4
5
6
7
8
9
10
11
12

differences appear between the same sample when counted by two different methods. In comparing these procedures, the hot TCA method gives significantly higher values for $^{35}S$ methionine incorporation but not when quoted as stimulation over background, the most commonly used criterion. The background with this method is unrealistically high (see Fig.11), casting similar doubt on the absolute values for the translations.

The method used by Amersham was found to be a much better reflection of what is seen when the products are analysed by gel electrophoresis, and was therefore used in preference. SDS-polyacrylamide gel electrophoresis of each translation (after 90 minutes) is shown in Figure 11.

Both lysates of rabbit #3 were extremely active, incorporating more than twice as much $^{35}S$ methionine into TCA-precipitable material as the reticulocyte system available from Amersham under equivalent conditions. The lysates of rabbit #2 were also active enough to be routinely used.

### 3.8 Optimisation of the reticulocyte system for translation of salivary gland RNA

One of the advantages of preparing an *in-vitro* system "in house" is that it can be optimised for the particular mRNA to be translated. $K^+$ and $Mg^{++}$ concentration curves were made for the translation of salivary gland mRNA and these are shown in Figure 12. The $K^+$ curve displays a steep decline in activity for potassium ion concentrations greater than the peak of 80mM and such a profile is not unusual (see the Amersham publication "Protein synthesis in cell-free systems"). The magnesium ion concentration curve shows that very little, if any, magnesium ion addition is necessary for translation of salivary mRNA.( This result is confirmed in Chapter 9 where Amersham's amino acid-depleted lysate is optimised for translation of submaxillary gland mRNA). The translation was also optimised for mRNA concentration and this revealed how active this source of mRNA is. The most efficient concentration of RNA was found to be 0.25$\mu$l/12.5$\mu$l reaction, at which concentration, $^{35}S$ incorporation was comparable to the viral

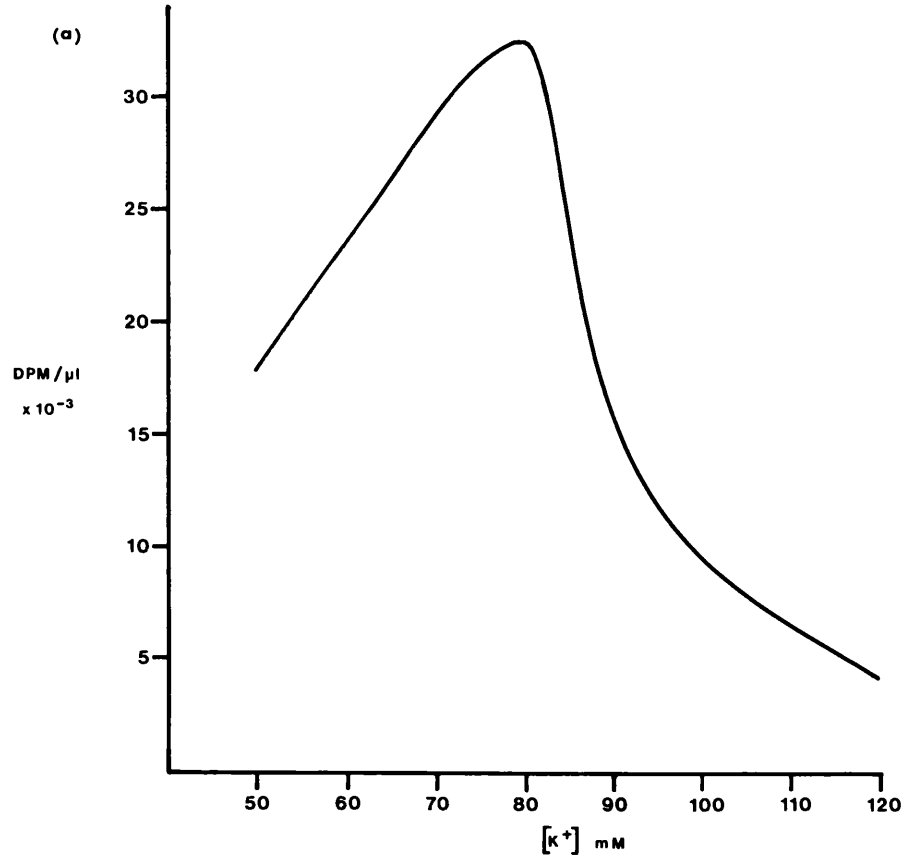
Fig.12 - (a) Determination of $K^+$ requirement
(b) Determination of $Mg^{++}$ requirement

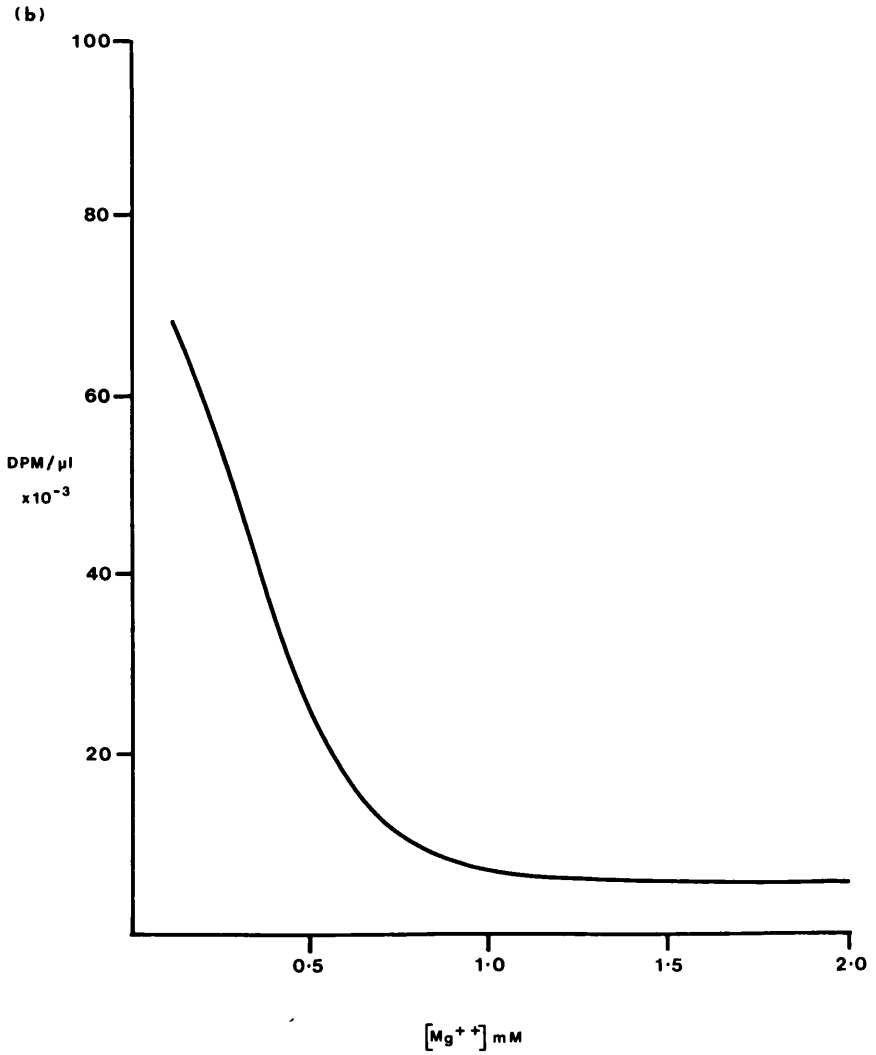
The $K^+$ and $Mg^{++}$ concentrations indicated refer to the concentrations present in the 5 x cocktail used in the standard reaction (see chapter 2).

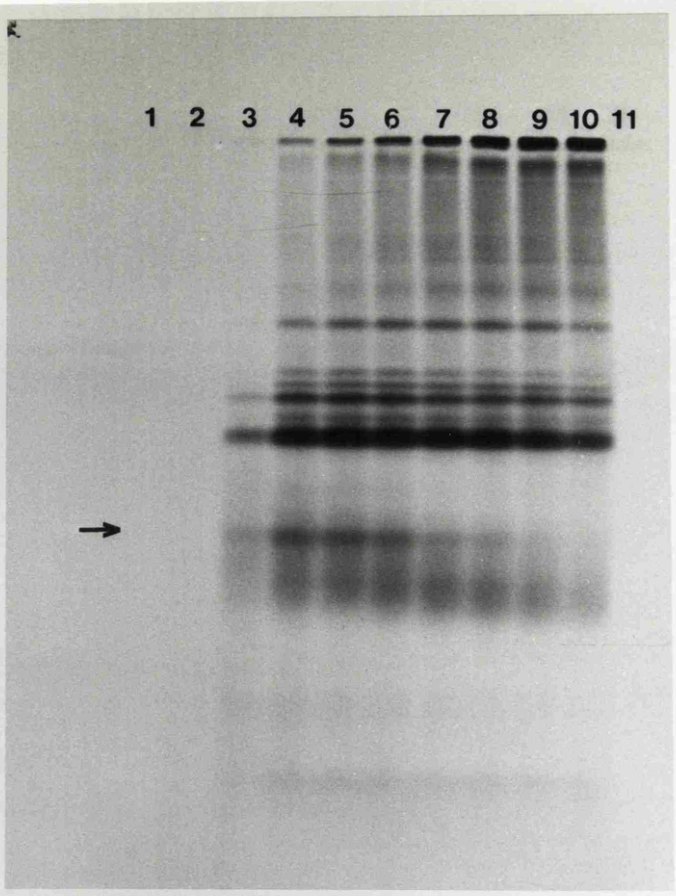
The $Mg^{++}$ assay was carried out at a $K^+$ concentration of 75mM and the $K^+$ assay was carried out at a $Mg^{++}$ concentration of 0.5mM.

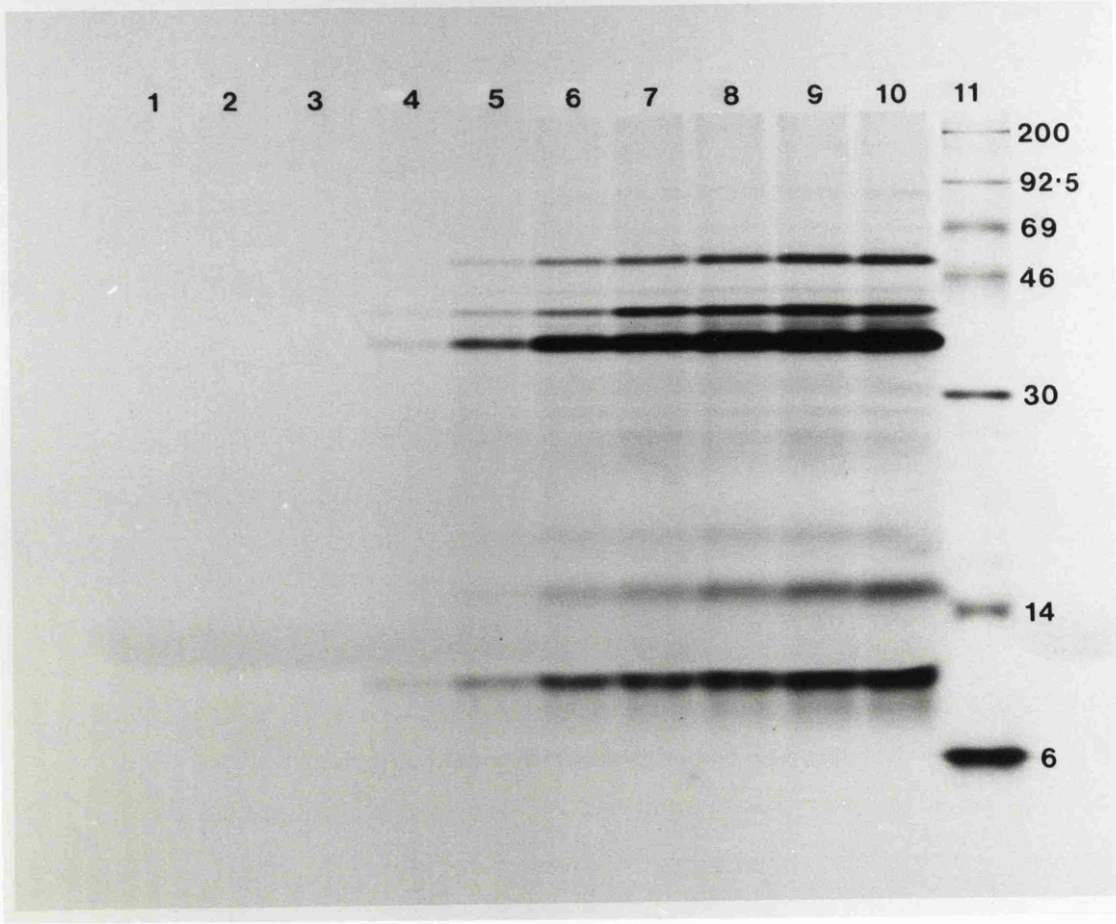
Fig.13 - Translation time-course for male (a) and female (b) SMG mRNAs (overleaf)
2$\mu$l aliquots from the translations of male (top) and female (bottom) mRNAs were taken at the times indicated and analysed by SDS polyacrylamide gel electrophoresis (12.5%). The arrow in the top figure indicates a major translation product (29 kd) whose abundance decreases after 15 minutes of translation.

| Lane | top | bottom |
|---|---|---|
| 1 | 0 mins | - RNA |
| 2 | 1 " | mins |
| 3 | 2 " | " |
| 4 | 5 " | " |
| 5 | 10 " | " |
| 6 | 15 " | " |
| 7 | 30 " | " |
| 8 | 60 " | " |
| 9 | 90 " | " |
| 10 | 120 " | " |
| 11 | - RNA | $^{14}C$ M.Wt.markers |


(a)
30
25
20
DPM/µl
x 10⁻³
15
10
5
50
60
70
80
90
100
110
120
[K⁺] mM


(b)
100
80
60
DPM/µl
x10⁻³
40
20
0·5
1·0
1·5
2·0
[Mg⁺⁺] mM


1 2 3 4 5 6 7 8 9 10 11


1 2 3 4 5 6 7 8 9 10 11
200
92·5
69
46
30
14
6

templates used to assess commercial *in-vitro* systems.

To characterise completely the translation of salivary gland mRNA, aliquots were taken at several times during translation, and run on an SDS-polyacrylamide gel (Fig.13). This qualitative time-course showed that, although overall protein synthesis peaked around 90 minutes, in agreement with the data presented earlier, there were some polypeptides which appeared and then disappeared during translation. The most obvious example is a male-specific product of about 29kd in size. Such products must either be sensitive to a protease present in the lysate or degraded by a specific protease being synthesised from the exogenous mRNA during translation.

A purified protease from the mouse submaxillary gland is available commercially (Boerhinger Mannheim) but addition of this protease to 60 minute translations failed to enhance the disappearance of the 29kd polypeptide.

3.9 Translation of salivary gland mRNA using a wheatgerm extract

As seen from Figures 11 and 13 the endogenous globin mRNA is extremely low in this lysate; however there is some smearing and compression in the lower part of the gel due to the large pool of unlabelled globin present in the lysate. This was a potential problem and to provide an alternative a wheatgerm extract was used (a gift from Dr. B.R.G.Williams). The conditions for using the extract were optimised and the translation profile compared with that obtained using the rabbit reticulocyte lysate. Although the resolution of products in the size range 8-14kd was improved, because of its lower activity the wheatgerm system was not used routinely.

3.10 Comparison of the *in-vitro* translation products of submaxillary, sublingual and parotid mRNAs

For the optimisations described above, mRNA was isolated from a mixed population of mice donated by Dr.G.Bulfield and K.Moore, and this source of mRNA was also used for routine screening, for example filter selection (see Chapter 4).

The salivary tissue isolated above consisted of the submaxillary gland, the sublingual gland and part of the parotid. This complex is very easily dissected and can be frozen within seconds of killing the animal. When referring to the submaxillary gland, most people include by implication , the other tissues (J.Scott, personal communication). In contrast, the three glands were dissected out individually, frozen within 1-2 minutes and mRNA prepared from them separately.

The translation of total salivary gland mRNA is compared to those of the three separate mRNAs in Figure 14, which shows an unusual profile of products in the sublingual gland. Between 80 and 90% of the mRNA of this tissue appears to code for one major product. This was subsequently found to be a family of peptides, which are studied in more detail in Chapter 9. As expected from the sizes of the glands, most of the products of the mixed mRNA originate in the submaxillary gland, and those that do not are diluted out by the relatively large amount of submaxillary products.

### 3.11 Translation of male and female DBA/2 submaxillary mRNA

Many submaxillary gland products are known to be androgen-regulated (Barka,1980) and this is reflected in translations of mRNAs derived from male and female mice (Fig.15). The most obvious differences are a group of peptides between 26 and 29 kd in size corresponding to the androgen-dependent esteroproteases such as EGF-binding protein,$\gamma$-NGF and kallikrein (Pratt *et al*,1981). In addition, there is a male-specific product of 45 kd (preprorenin) and an unidentified female-specific polypeptide of about 30 kd in size. The other major products of the submaxillary gland appear not to be sex-related.

### 3.12 Processing of *in-vitro* translation products by dog pancreas membranes

The salivary glands are prolific secretory tissues, secreting products into the mouth through ducts and directly into the blood. Therefore, many of the products seen during *in-vitro* translation are likely to be precursors

Fig.14 - Translation products of SMG, sublingual and parotid gland mRNAs.

| Lane | mRNA translated |
|---|---|
| Mixed | total salivary glands |
| – RNA | no added mRNA |
| Submax. | submaxillary gland |
| Subling. | sublingual gland |
| Parotid | parotid gland |
| $^{14}C$ | $^{14}C$ M.Wt. markers |

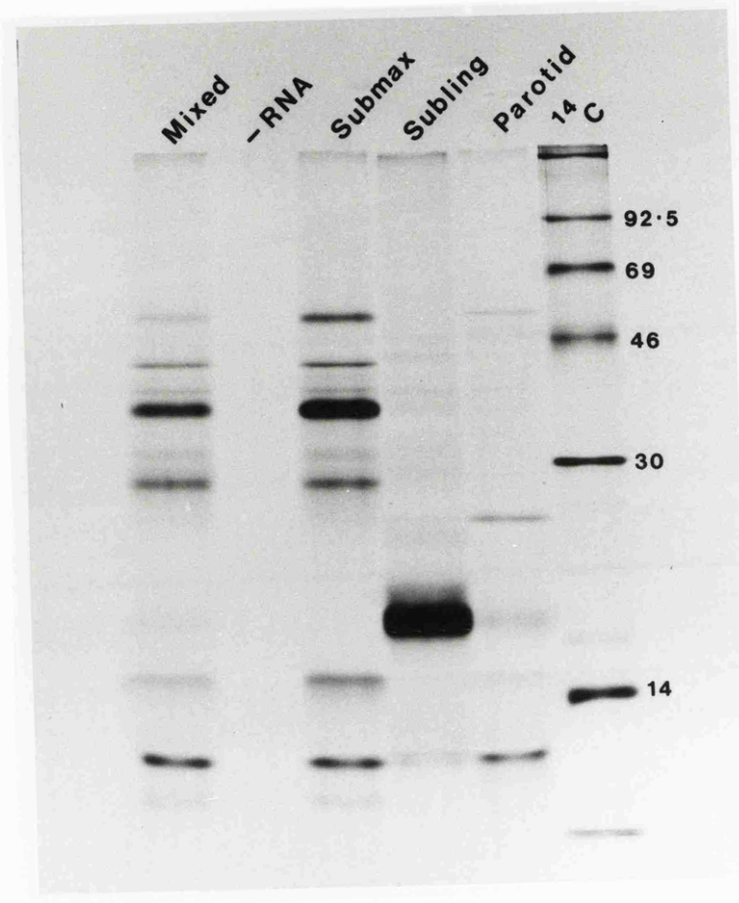

Mixed
-RNA
Submax
Subling
Parotid
14 C
92·5
69
46
30
14

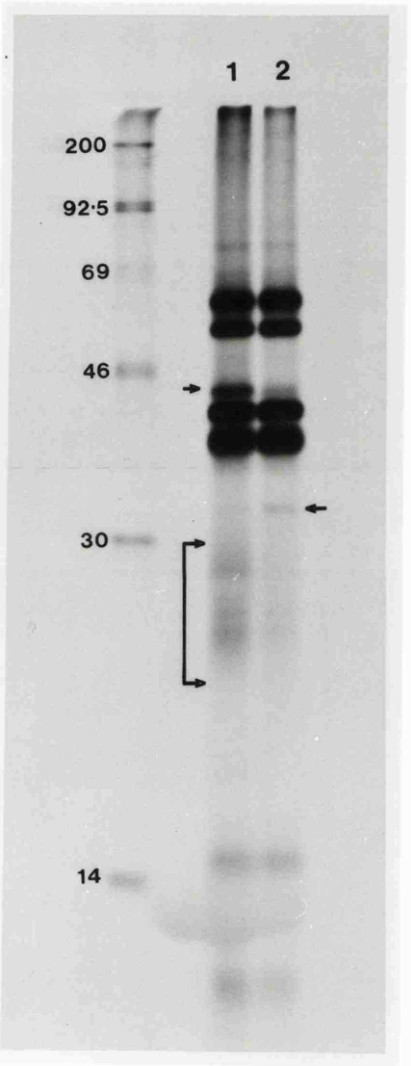
Fig.15 - Comparison of the translation products of male and female DBA/2 SMG mRNA.

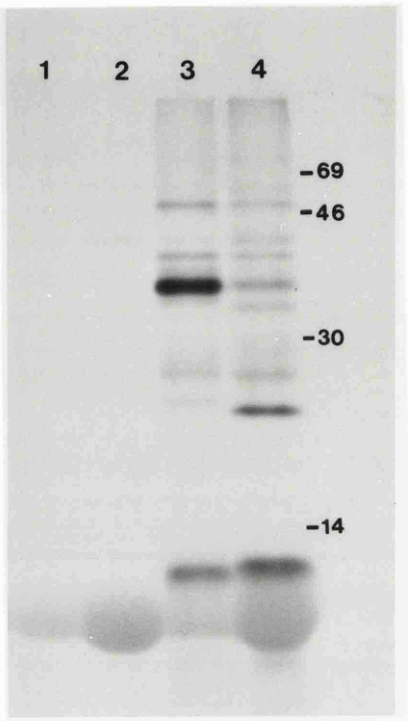
The translation products of mRNA isolated from the SMG of male and female mice were analysed on a 12.5% SDS-polyacrylamide gel. Lane 1 - male; lane 2 - female. The presence of male-abundant products is shown by arrows on the left of the figure and the occurrence of a female-abundant product is indicated by an arrow on the right hand side. M.Wt markers are given on the left of the figure.

**Fig.16** - *In vitro* processing of SMG mRNA translation products **(overleaf)**

| Lane | Sample |
|---|---|
| 1 | - RNA control translation |
| 2 | - RNA " in presence of dog pancreas membranes |
| 3 | translation of male DBA/2 SMG mRNA |
| 4 | as (3) but in presence of dog pancreas membranes |

$^{14}C$ molecular weight markers are indicated on the right of the figure.


1
2
200
92·5
69
46
30
14


1
2
3
4
-69
-46
-30
-14

for secretory proteins.

The inclusion of dog pancreas membranes during translation demonstrated that several major products were processed to new sizes (Fig.16). Since such membranes are not capable of complete glycosylation and are relatively poor at core glycosylation (Heath *et al*,1979), these differences reflect the removal of signal sequences. This makes the dramatic change in mobility of the 36 kd species (which by relative abundances must be the precursor of the 26 kd processed product) difficult to explain, since signal sequences are commonly only 1-3 kd in size. Glycosylated proteins can run anomalously on SDS-polyacrylamide gels but this is not a possible explanation for this product. This protein has been the subject of work by other people in the laboratory and is discussed in more detail by Windass *et al*,(1984).

Membranes prepared from rat liver by the method of Das *et al* (1980) were found to be active, but were not as efficient as commercially available membranes and the latter were used in preference for these and later studies.

# CHAPTER 4

# IDENTIFICATION AND ISOLATION OF SMG CDNA CLONES CODING FOR AN EGF-LIKE POLYPEPTIDE AND RENIN

## 4.1 Introduction

As described in the introduction, the salivary glands contain and synthesise a large number of secretory products, including many peptide hormones and enzymes. At the start of the work described in this thesis, the focus of interest in the mouse submaxillary gland centred around EGF, NGF and, to a lesser extent, renin. The primary sequences of mature EGF and β-NGF had been reported (Savage *et al* 1972, Angeletti *et al* 1973a,b) but, although intermediates had been recognised, their primary biosynthetic precursors were unknown. In the case of EGF a 9000 dalton intermediate was identified by pulse-labelling techniques (Frey *et al* 1979), and the same methodology was used to reveal a 22,000 molecular weight precursor to β-NGF (Berger and Shooter 1977).

A second classical method for identifying the precursors of such proteins involves *in vitro* translation of their mRNAs and immunoprecipitation of the translation products using antiserum to the mature protein. There was no report of this approach being used successfully for either EGF or NGF, and to date this is still the case.

Secretory proteins and many enzymes are made as precursors and as such may not be recognised by antibodies raised to the mature protein. Polyclonal antibody preparations, such as antisera raised in a rabbit or guinea-pig, provide the best chance for immune-complex formation since antibodies are present to many different determinants. To increase the probability of detecting a precursor, antisera from different animals are often used in parallel. The nature of the precursor and the type of mature protein being made are reflected in the relative ease or difficulty of

detecting precursors by this method. If, for example, the mature protein is either significaantly smaller or very heavily glycosylated, then the chances of immunoprecipitating the precursor are greatly reduced.

4.2. Use of *Staphylococcus aureus* protein A as a second antibody.

Protein A from *Staphyloccus aureus* has become widely used over the last ten years as a "second antibody" for immunoprecipitation reactions. The 42,000 dalton protein is a major cell wall constituent of the organism and is responsible for the agglutination of *S.aureus* cells by normal human serum. Although it was originally believed (Forsgren and Sjoquist,1966) that all human sera contained natural antibodies to the protein, it was soon demonstrated that the reaction between protein A and the antibody molecule did not involve the antigen binding site, but instead was localised to the Fc fragment (Forsgren and Sojquist,1966,1967).

Protein A binds almost exclusively to IgG and often only to certain subgroups of this class of antibody (Kronvall *et al* 1970, Mackenzie *et al* 1978). In some species, other antibody classes will also react, but usually only to a limited extent (Surolia *et al* 1982). Sjoquist *et al* (1972) purified the protein and found a binding ratio with IgG of 1:2. Sequence analysis (Sjodahl 1977) revealed four highly homologous Fc binding regions of 58-62 amino-acids and a C-terminal 150 amino acid region that did not bind to the Fc fragment, and was responsible for anchorage of the protein in the cell wall. These four regions have extensive homology and can be subdivided into further regions of internal homology (Sjodhal,1977). Lancet *et al* (1978) localised the binding region of the Fc fragment to the junction between the $C_H2$ and $C_H3$ domains; only when this is intact is Fc binding possible.

Because of its location in the bacterial cell wall, and the ability of the purified protein to be bound to sepharose, protein A makes an ideal "second antibody" for immunoprecipitation. Titration of antisera to obtain an insoluble complex is not necessary since the antigen- antibody-protein A

complex can be recovered as a cell pellet, and the antigen then released from the complex.

4.3 Immunoprecipitation of *in-vitro* translations of submaxillary gland mRNA with EGF and renin antisera

Male submaxillary gland RNA was translated in-vitro using the rabbit reticulocyte system, and 20$\mu$l aliquots were immunoprecipitated with either normal rabbit serum or antiserum to mouse EGF or mouse submaxillary gland renin. EGF antisera raised in four different rabbits were kindly provided by Dr.H.Gregory (ICI,PLC,Alderley Park) and rabbit anti-mouse renin antiserum was the gift of Dr.K.Poulson (Royal Dutch College, Copenhagen, Denmark). Normal (pre-immune) rabbit serum (NRS) was obtained both from Dr.Gregory and also from Dr.M.Blair. During early immunoprecipitation experiments the NRS given by Dr.Gregory precipitated a large number of polypeptides. These could not be removed by extensive washing of the pellet, and when compared to the peptides precipitated by the NRS supplied by Dr.Blair (Fig.17) it was clear that either it was of a very poor quality, or was not in fact a normal or non-immune serum. In all future immunoprecipitations, NRS refers to the use of Dr.Blair's sample of rabbit serum.

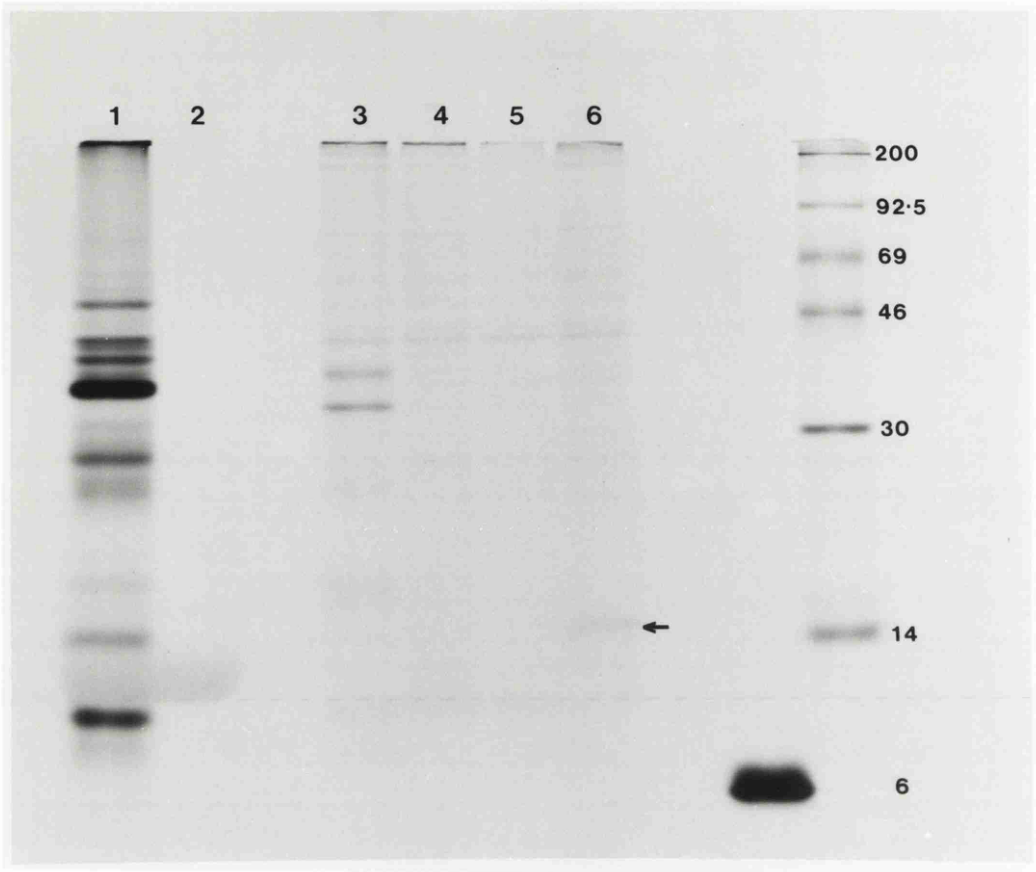
(1) Anti-EGF antiserum

Figure 17 shows the immunoprecipitation of submaxillary gland RNA translation products with the two normal sera described above and with two EGF antisera. The result shows the precipitation of a 14 kd peptide with one of the antisera, which is not seen with the normal serum or the second antiserum. This peptide was also detectable with the two other EGF antisera but not with commercially available antisera (Collaborative Research). For the reasons already described, the variability with different antisera preparations is not surprising and this peptide therefore appeared to be a potential EGF precursor.

**Fig.17** - Immunoprecipitation of SMG mRNA translation products with normal and anti-EGF antisera

| Lane | Sample |
|---|---|
| 1 | Translation products of SMG mRNA |
| 2 | - RNA control translation |
| 3 | Immunoprecipitation of (1) with NRS (Dr.H.Gregory) |
| 4 | " " (1) with NRS (Dr.M.Blair) |
| 5 | " " (1) with EGF A/S #3 |
| 6 | " " (1) with EGF A/S #4 |

M.wt. markers are shown on the right of the figure.
The precipitation of the 14 kd peptide is arrowed.


1
2
3
4
5
6
200
92·5
69
46
30
14
6

In an alternative experiment, using an immunoprecipitation procedure suggested by Dr.Blair which involved spinning the immune complex through a sucrose pad, the translation products were precipitated with either antiserum #4 or NRS. The supernatant from the NRS precipitation was then re-precipitated with antiserum #4. The result (Fig.18) shows the immunoprecipitation of two peptides after pre-absorbtion with NRS, one of 45 kd and the 14 kd peptide seen above. In this experiment no significant difference was seen between NRS and EGF antiserum, a puzzling result in view of the previous precipitation and the result of the double precipitation. If there was no difference between the NRS and antiserum results one would have expected the double precipitation to contain either no peptides or a reduced amount of the same peptide profile. The 45 and 14 kd species have also been seen by Dr.J.Scott (personal communication) using a different EGF antiserum.

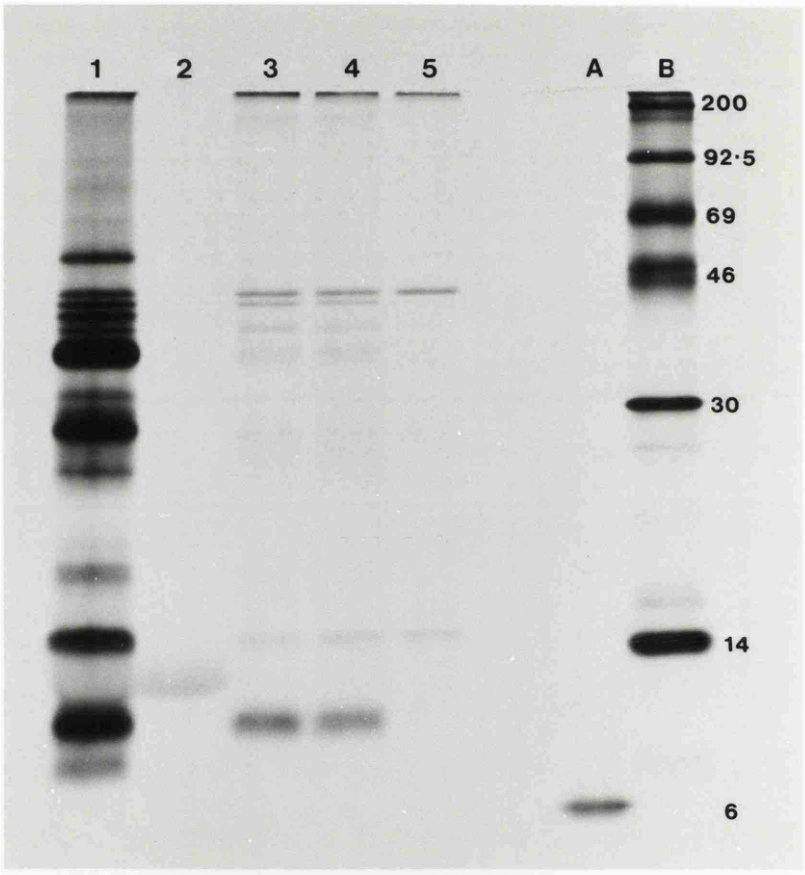
To try to confirm the identity of the 14kd species, an immuno-precipitation using the former method was carried out in the presence of unlabelled, purified EGF (supplied by Dr.Gregory), to compete out the 14kd species. The result showed that the addition of significant quantities of EGF did not reduce the amount of immunoprecipitable 14kd peptide, indicating that it may well not be a precursor to the growth factor, as originally thought.

(2) Anti-renin antiserum

Poulsen *et al* (1979b,1980) showed that the *in-vitro* precursors, of both mouse submaxillary and mouse kidney renins, had a molecular weight of 50kd, some 12kd larger than the mature protein. The strain of mouse originally chosen by members of this laboratory for the cloning of submaxillary products was DBA/2. Fortuitously, this strain had been shown by Wilson *et al*(1978) to produce high levels of submaxillary renin. Renin antiserum was therefore used in an attempt to show the presence of an abundant 50kd renin precursor in the translation products of male DBA/2 mRNA. The result is

**Fig.18** - Double immunoprecipitation of *in vitro* translation products of SMG mRNA using normal rabbit serum and anti-EGF antiserum.

| Lane | |
|---|---|
| 1 | Translation products of male SMG mRNA |
| 2 | - RNA control translation |
| 3 | Immunoprecipitation of (1) with NRS |
| 4 | Immunoprecipitation of (1) with EGF A/S |
| 5 | Immunoprecipitation of supernatant from (3) with EGF A/S |
| A | $^{14}C$ insulin |
| B | $^{14}C$ M.Wt markers |


1
2
3
4
5
A
B
200
92·5
69
46
30
14
6

shown in Figure 22(a) (later this chapter) and reveals the specific immunoprecipitation of a 44kd peptide with renin antiserum, and its absence in the control immunoprecipitation with NRS. To ensure that the size of this renin precursor was not strain specific, and its abundance correlated with the known biology, mRNA was prepared from the submaxillary glands of a second high producer strain, SWR, and a low producer strain, CBA/Ca. These mRNAs were translated *in-vitro*, and subsequent immunoprecipitation revealed that both these strains also had renin precursors of 44kd (Fig.19). In addition, the submaxillary glands of SWR mice either contained more renin mRNA than those of the CBA/Ca strain, or translated it more efficiently. Subsequent work, both by myself and others (Rougeon *et al*,1981; Panthier and Rougeon, 1983; Piccini *et al*,1982) have confirmed that the primary translation products of submaxillary and kidney mRNAs are 44kd and that the sizes determined by Poulsen were overestimated.

## 4.4 Isolation of cDNA clones corresponding to mRNAs encoding the EGF-like 14kd polypeptide and the 44kd renin precursor

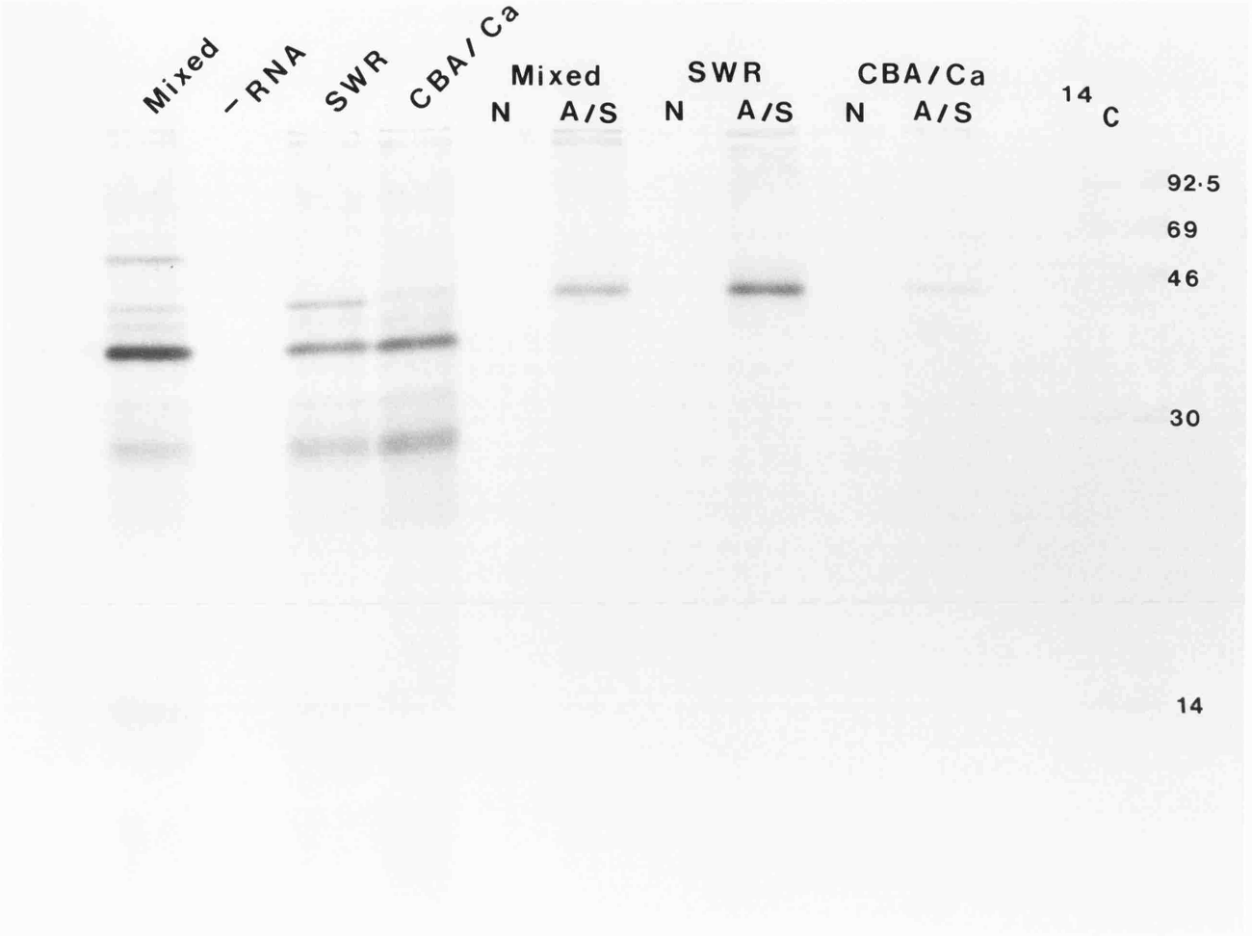
Several cDNA libraries had been made by Dr.J.Windass and others in the laboratory using mRNA isolated from adult male DBA/2 salivary glands. These had been probed with (1) total submaxillary gland cDNA; (2) pSMG3 - a cDNA clone corresponding to the major mRNA species of the gland ( encoding the 36kd polypeptide described in Chapter 3) and (3) cDNA made from RNA fractions isolated using the method of Bedbrook *et al*(1980) (see Fig.20). The result of this screening was a large number of clones corresponding to abundant salivary mRNAs, other than pSMG3. These clones were screened using the hybrid-selection method of Wood *et al*(1982), in which individual (or pooled) plasmid cDNA clones were nicked, denatured and baked onto nitro-cellulose filters. After prehybridisation, the immobilised DNAs were hybridised overnight to total submaxillary gland mRNA, and then washed in a series of increasingly stringent rinses. Finally the mRNA(s) hybridising to the cDNA (or to the vector DNA control) was melted away, precipitated and

Fig.19 - Immunoprecipitation of high and low producer renin precursors

| Lane | | Sample |
|---|---|---|
| Mixed(1) | | Translation of SMG mRNA from wild mice |
| - RNA | | Control translation with no added RNA |
| SWR(2) | | Translation of SMG mRNA from male SWR mice |
| CBA/Ca(3) | | " " " " " " CBA/Ca mice |
| MIXED | N | Immunoprecipitation of (1) with NRS |
| | A/S | " " (1) " renin A/S |
| SWR | N | " " (2) " NRS |
| | A/S | " " (2) " renin A/S |
| CBA/Ca | N | " " (3) " NRS |
| | A/S | " " (3) " renin A/S |
| | $^{14}C$ | $^{14}C$ molecular weight markers |

Fig.20 - Translation of SMG mRNA fractionated by agarose-formamide gel electrophoresis

| Lane | |
|---|---|
| A | translation of male SMG mRNA |
| B | - mRNA control translation |
| 1-21 | Translation products of mRNA recovered from 0.5cm gel slices (1 = top of gel) |
| C | $^{14}C$ molecular weight markers |


Mixed
- RNA
SWR
CBA/Ca
Mixed
N
A/S
SWR
N
A/S
CBA/Ca
N
A/S
14C
92·5
69
46
30
14

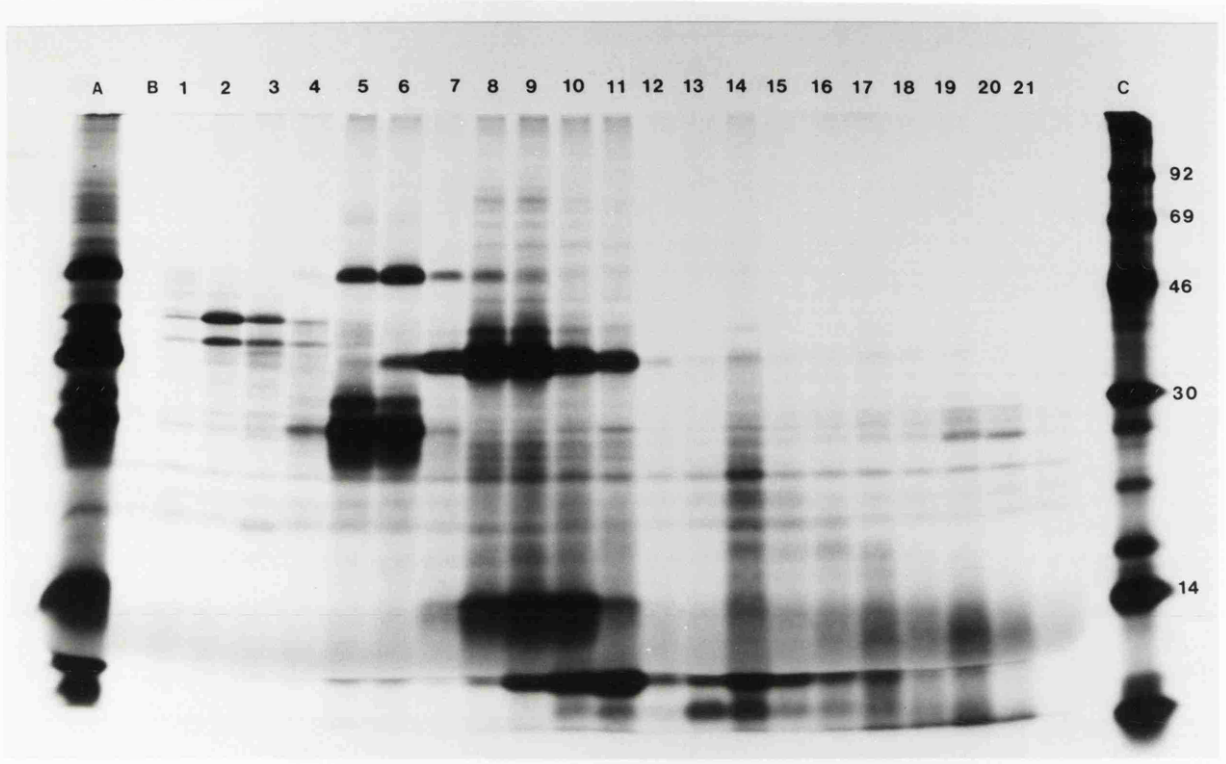
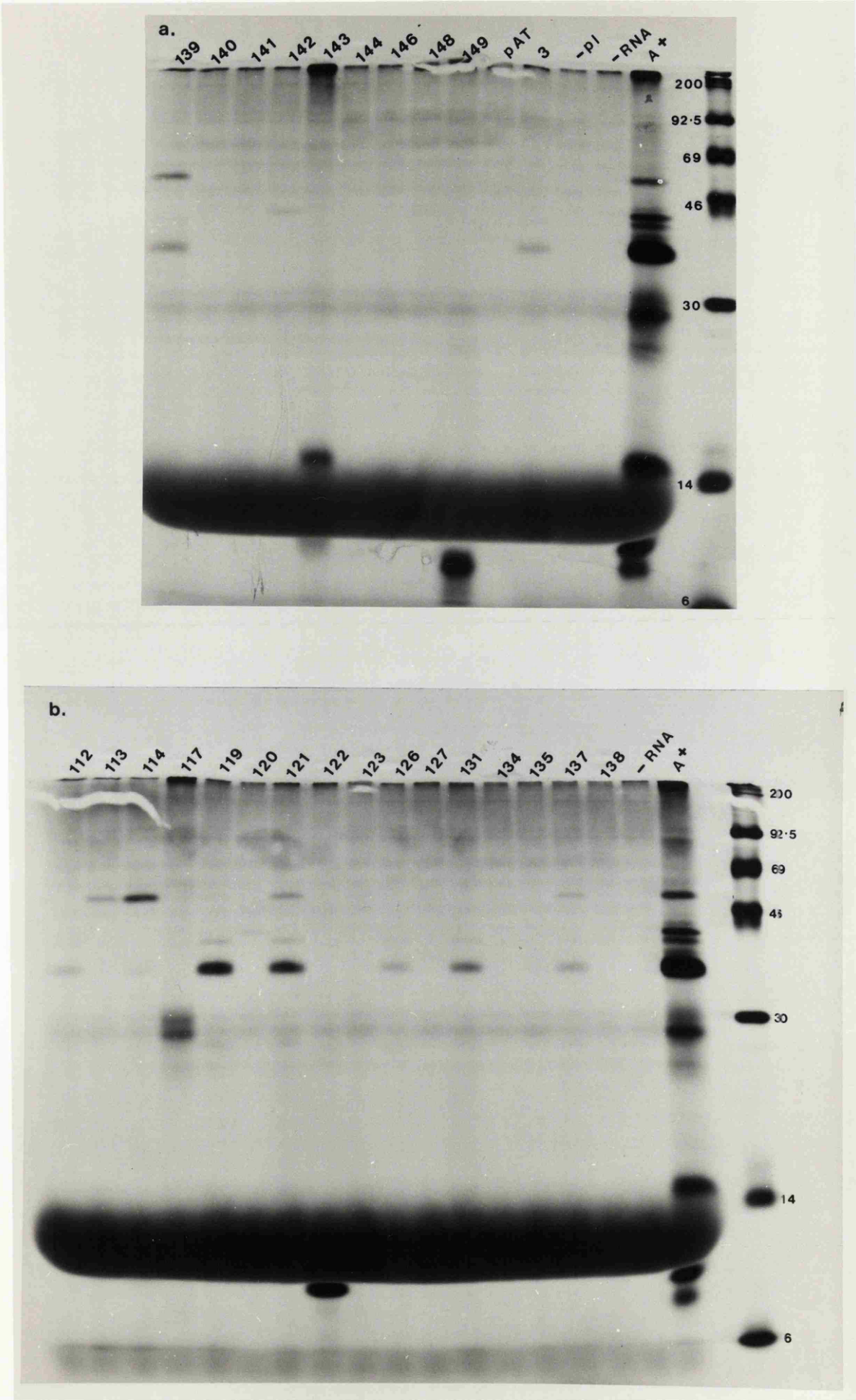

A
B
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
C
92
69
46
30
14

**Fig.21 (a) and (b)** - Hybrid-selection of SMG mRNA

Plasmid DNAs prepared from isolated salivary gland cDNA clones were used to hybrid-select SMG mRNA species. These were then translated and the products analysed by SDS-polyacrylamide gel electrophoresis.
The lane numbers refer to clone isolation numbers and the other headings are as follows:

pAT = pAT153 'control' plasmid
− RNA = no mRNA control translation
$A^+$ = translation of total SMG mRNA
− Pl = no plasmid


a.
139
140
141
142
143
144
146
148
149
pAT
3
-pl
-RNA
A+
200
92·5
69
46
30
14
6
b.
112
113
114
117
119
120
121
122
123
126
127
131
134
135
137
138
-RNA
A+
200
92·5
69
46
30
14
6

translated *in-vitro*.

Typical hybrid selections are shown in Figure 21a and b. The plasmid vector pAT153 (Twigg and Sherratt,1980) into which the cDNAs were cloned was used as a negative control. Some clones selected mRNA that translated to give a single polypeptide species, for example pSMG113 and pSMG122 (Fig.21b). The clones pSMG142 and pSMG143 selected RNAs that translated to give single polypeptide species of 44kd and 14kd respectively and it was therefore possible that these cDNA clones were derived from the mRNAs for renin (pSMG142) and EGF (pSMG143).

In total, 92 cDNA clones were screened by hybrid-selection and Table 4 lists the polypeptide(s) encoded or partially encoded by these clones. As shown, a small subset of these clones selected cleanly eight of the major salivary mRNAs.

Some clones such as pSMG124 and128 do not appear to select mRNA. This may have been due to the presence of too little plasmid DNA in the selection (due to a heavily contaminated plasmid preparation). Alternatively, a minor mRNA species may have been selected which was translated to give a small amount of product undetectable by the assay. Other clones e.g. pSMG121,119 and 137 selected more than one species of mRNA. Again the reason for this could vary; firstly the cDNA could have had limited homology to more than one mRNA species; secondly, the plasmid preparation could have been mixed or contained more than one insert. Thirdly, the cDNA could have been derived from one member of a family of mRNAs encoding closely related proteins. Probable examples of this are pSMG117 and 155 which select mRNAs for at least two polypeptides of 26-29 kd which correspond to the size of the kallikreins, known to be produced by the submaxillary gland and to have extensive homology (Mason *et al*,1983).

## 4.5 Confirmation of the identities of cDNA clones pSMG142 and pSMG143

Thus far, clones have been isolated that may correspond to the precursors of renin and EGF. To confirm that pSMG142 was a renin cDNA

TABLE 4 - Summary of hybrid-selection data

| PLASMID No. | | PEPTIDES SELECTED | PLASMID No. | | PEPTIDES SELECTED |
|---|---|---|---|---|---|
| pSMG98 | | 36,29,37 | pSMG155 | | 29,27,60,36 |
| pSMG103 | | 36,29,9,(14) | pSMG156 | * | 55 |
| pSMG111 | | 36,55,9,40 | pSMG157 | | 36,37,28,25 |
| pSMG112 | | 36,40 | pSMG158 | | 29,27,36,37 |
| pSMG113 | * | 55 | pSMG159 | | 36,28,25,16 |
| pSMG114 | | 55,36 | pSMG160 | * | 14 |
| pSMG115 | | 36,40,28,(16,18) | pSMG161 | | - |
| pSMG116 | | - | pSMG162 | | - |
| pSMG117 | | 29,30,(27) | pSMG163 | | 36,37,28,25,16 |
| pSMG118 | | - | pSMG164 | | - |
| pSMG119 | | 36,40,(28,27) | pSMG165 | | - |
| pSMG120 | * | 44 | pSMG166 | * | 14 |
| pSMG121 | | 36,40,55,(28,27) | pSMG167 | | - |
| pSMG122 | * | 9 | pSMG168 | | - |
| pSMG123 | | - | pSMG175 | | - |
| pSMG124 | | - | pSMG176 | | - |
| pSMG125 | | (36) | pSMG177 | | - |
| pSMG126 | * | 36 | pSMG178 | | - |
| pSMG127 | | - | pSMG179 | | - |
| pSMG128 | | (36) | pSMG180 | | - |
| pSMG129 | * | 20 | pSMG181 | * | 20 |
| pSMG130 | | (36) | pSMG182 | | - |
| pSMG131 | | 36,40 | pSMG183 | | - |
| pSMG132 | * | 9,(55) | pSMG196 | * | 44 |
| pSMG133 | | 6,9,27,28 | pSMG197 | * | 44 |
| pSMG134 | | - | pSMG198 | * | 44 |
| pSMG135 | | - | pSMG199 | * | 44 |
| pSMG136 | | 40,29,26,32,(80) | pSMG200 | * | 44 |
| pSMG137 | | 36,55 | pSMG201 | | - |
| pSMG138 | | - | pSMG202 | | - |
| pSMG139 | | 36,55,40 | pSMG203 | * | 44 |
| pSMG140 | | - | pSMG204 | * | 44 |
| pSMG141 | | - | pSMG205 | * | 44 |
| pSMG142 | * | 44 | pSMG208 | * | 44 |
| pSMG143 | | 14,9 | pSMG209 | | - |
| pSMG144 | | - | pSMG210 | * | 44 |
| pSMG145 | | 36,45,28,47 | pSMG211 | * | 44 |
| pSMG146 | | - | pSMG212 | * | 44 |
| pSMG147 | * | 20 | pSMG213 | * | 44 |
| pSMG148 | * | 44 | pSMG214 | | - |
| pSMG149 | * | 6 | pSMG215 | | - |
| pSMG150 | | 9,(6),29 | pSMG216 | | - |
| pSMG151 | | 55,36,37 | pSMG217 | | - |
| pSMG152 | | - | pSMG218 | | - |
| pSMG153 | | 55,36 | pSMG219 | | - |
| pSMG154 | | 55,36 | pSMG220 | | - |

* clones apparently selecting single mRNA species

clone, a large-scale hybrid-selection was carried out in which the only plasmid used was pSMG142. The mRNA isolated was then translated in a 50μl reaction, the products immunoprecipitated with NRS and renin antiserum, and the precipitates electrophoresed on a 12.5% SDS polyacrylamide gel (Fig 22a). This confirmed that the 44 Kd peptide translated from pSMG142 selected mRNA was precipitable with renin antiserum, and therefore this clone contained a renin cDNA.

The remainder of the mRNA selected by pSMG143 in the initial screening was also translated in a 50μl reaction, and 25μl aliquots were immunoprecipitated with either NRS or EGF antiserum 4. Figure 22b shows the autoradiograph from SDS-PAGE analysis of these immunoprecipitates and confirms the identity of pSMG143 as a cDNA clone encoding, or partially encoding, a 14Kd polypeptide immunoprecipitable with EGF antiserum.

Further analysis of pSMG143 and related clones reported elsewhere (Windass *et al*,1984) showed that the clone had no significant homology to the EGF cDNA sequence recently determined by J. Scott *et al* (1983b) and Gray *et al* (1983). The peptide encoded by pSMG143 has no extensive homology either to EGF or to the other EGF-like sequences encoded in the EGF mRNA. The peptide does have the sequence Arg-Cys-Leu-Cys present in one of the EGF-like peptides at the conserved Cys-X-Cys position. A search of the Doolittle database (Doolittle, 1981), carried out by Dr Peter Stockwell, revealed no homology to any listed sequences.

Since the antiserum used in the above experiments was made using a very pure preparation of EGF, and other workers have seen similar results, the observation is puzzling and the basis of the immunoreactivity remains unknown.

## 4.6 Identification of pSMG142-related cDNA clones

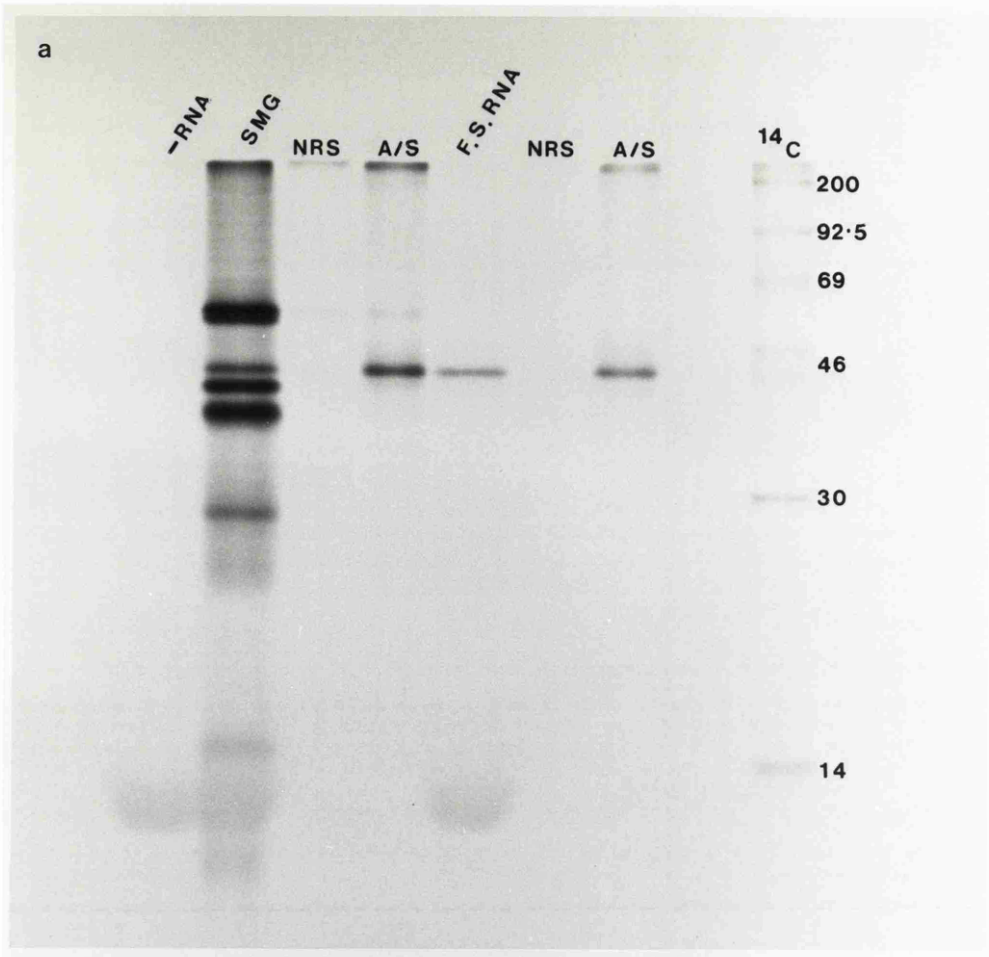
After confirmation of pSMG142 as a renin cDNA clone the cDNA insert was used as a hybridisation probe to screen the cDNA library, using the method of Young and Hogness (1977). This screening yielded ten related clones and

**Fig.22** - (a) Confirmation of pSMG142 as a renin cDNA clone

| Lane | Sample |
|---|---|
| - RNA | No mRNA control translation |
| SMG (1) | translation products of male DBA/2 SMG mRNA |
| NRS | immunoprecipitation of (1) with NRS |
| A/S | " " (1) with renin A/S |
| FSRNA(2) | translation products of mRNA hybrid-selected by pSMG142 |
| NRS | immunoprecipitation of (2) with NRS |
| A/S | " " (2) with renin A/S |
| $^{14}C$ | $^{14}C$ molecular weight markers |

(b) Confirmation of pSMG143 as an EGF-like cDNA clone

| Lane | Sample |
|---|---|
| 1 | immunoprecipitation of the translation products directed by pSMG143 hybrid-selected mRNA with NRS |
| 2 | immunoprecipitation of the translation products directed by pSMG143 hybrid-selected mRNA with EGF A/S |


a
-RNA
SMG
NRS
A/S
F.S. RNA
NRS
A/S
14C
200
92·5
69
46
30
14


b
1
2
14kd

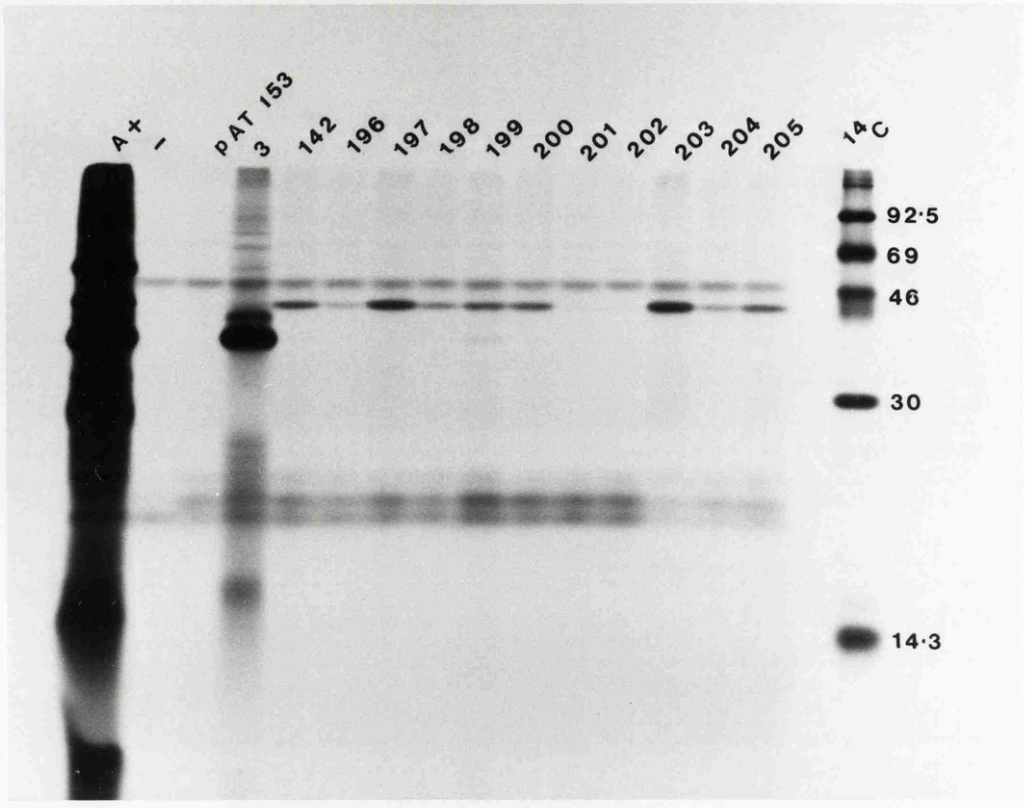
**Fig.23** - Hybrid-selection of pSMG142-like clones

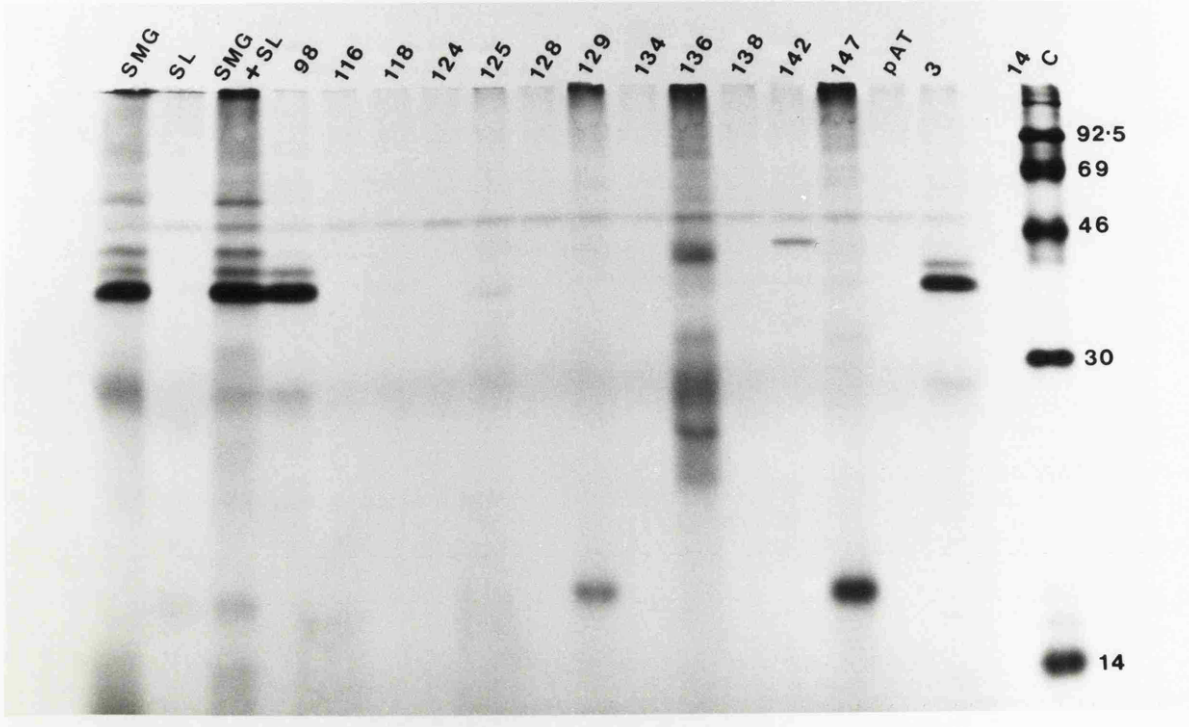
In addition to the clone identification numbers, the following symbols are used:

$A^+$ = translation of total SMG mRNA
– = no mRNA control translation
pAT153 = control hybrid-selection using pAT153
$^{14}C$ = $^{14}C$ M.wt. markers

**Fig.24** - Hybrid-selection of SMG and sublingual gland mRNAs

SMG = translation of SMG mRNA
SL = " " sublingual gland mRNA
SMG+SL = " " mixture of the two mRNA preps
pAT = control hybrid-selection using pAT153
$^{14}C$ = $^{14}C$ M.Wt.markers


A+
–
pAT 153
3
142
196
197
198
199
200
201
202
203
204
205
14C
92·5
69
46
30
14·3


SMG
SL
SMG +SL
98
116
118
124
125
128
129
134
136
138
142
147
pAT
3
14C
92·5
69
46
30
14

to further characterise these plasmid preparations were made and used in hybrid-selection experiments of the type already described. Eight of the clones (Fig.23) selected mRNA coding for the 44 kd peptide and were used for further study. In addition to the clones mentioned above a further five cDNA clones were isolated from a second cDNA library giving a total of fifteen renin clones.

4.7 Isolation of cDNA clones coding for the major sublingual protein

During the hybrid-selection screening, some clones were assayed using a mixture (1:10) of sublingual gland and submaxillary gland mRNAs. As shown in Figure 24 two clones, pSMG129 and pSMG147, selected mRNA coding for the major 16kd peptide described in Chapter 3. The insert from pSMG129 was used as a hybridisation probe, and screening of the cDNA library resulted in one additional clone, pSMG181.

The work described in the subsequent chapters will concentrate primarily on renin, but will return in chapter 9 to give a more detailed analysis of the major sublingual gland product and the clones mentioned above.

# CHAPTER 5

## RESTRICTION MAPPING OF RENIN CDNA CLONES

### 5.1 Estimation of insert size and the purification of mixed clones

Plasmid DNA was prepared from the following cDNA clones identified in the last chapter; pSMG120,142,196,197,198,199,200,203,204,205,208,211,212 and 213. Using the triton lysis method of Katz *et al*(1973) plasmid yields varied from 75 to 250 μg per 200mls of overnight culture (not amplified). The amount of RNA present in these preparations was determined visually by ethidium bromide staining after gel electrophoresis of the sample, and varied widely from undetectable levels to greater than 50% of the sample. Those with high levels of RNA were purified further by passage over a Bio-gel A5M column which separates plasmid DNA from RNA on the basis of size. The optical density of column fractions was measured (260nm) and the first peak recovered by ethanol-precipitation. The profile of pSMG199 DNA treated in this way is shown in Figure 25. When such DNA samples were re-assayed by gel electrophoresis they were found to be RNA free. The removal of contaminating RNA is necessary to obtain an accurate measurement of the DNA concentration but more important, since RNA will bind ethidium bromide, its presence can mask the existence of restriction fragments less than 80bp in size, during polyacrylamide gel analysis.

The cDNA library had been made using the G-C tailing method of Deng *et al* (1981) which reconstructs sites for the restiction enzyme *Pst*I at either end of the cDNA (Fig.26). This allows the separation of vector DNA from cDNA by a simple cleavage with *Pst*I.

To determine the sizes of the inserts in the renin cDNA clones, 1μg of each of the above DNAs was digested by *Pst*I and then electrophoresed through a 5% polyacrylamide gel (Fig.27). The lengths of the inserts were determined graphically and are listed in Table 5.

Plasmids pSMG142 and pSMG199 were seen to contain three and two *Pst*I

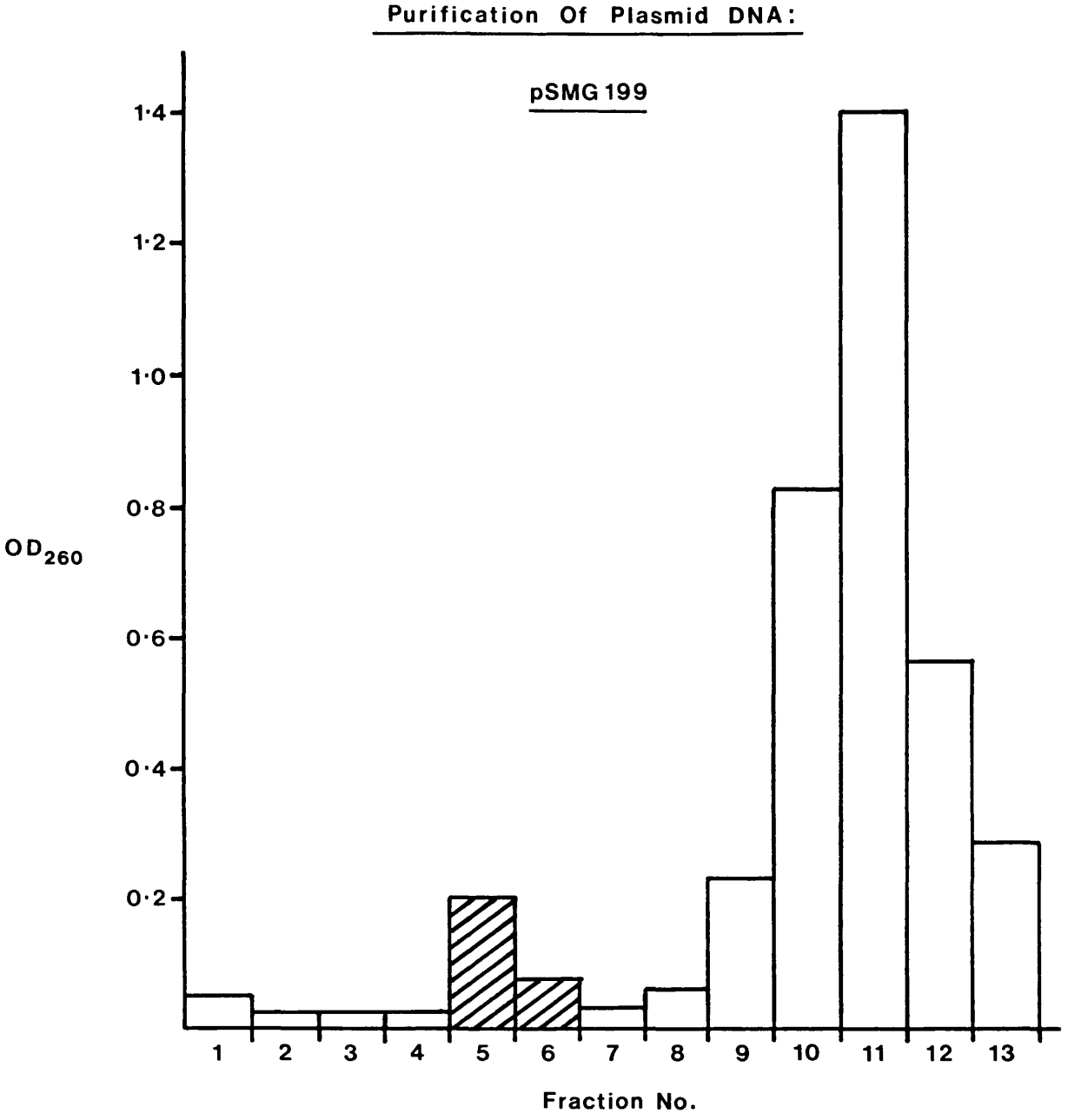



**Fig.25** - Absorbance profile of the eluate from an A5M column loaded with an RNA-contaminated plasmid sample (pSMG199)

The elution of the plasmid DNA is indicated by shading.

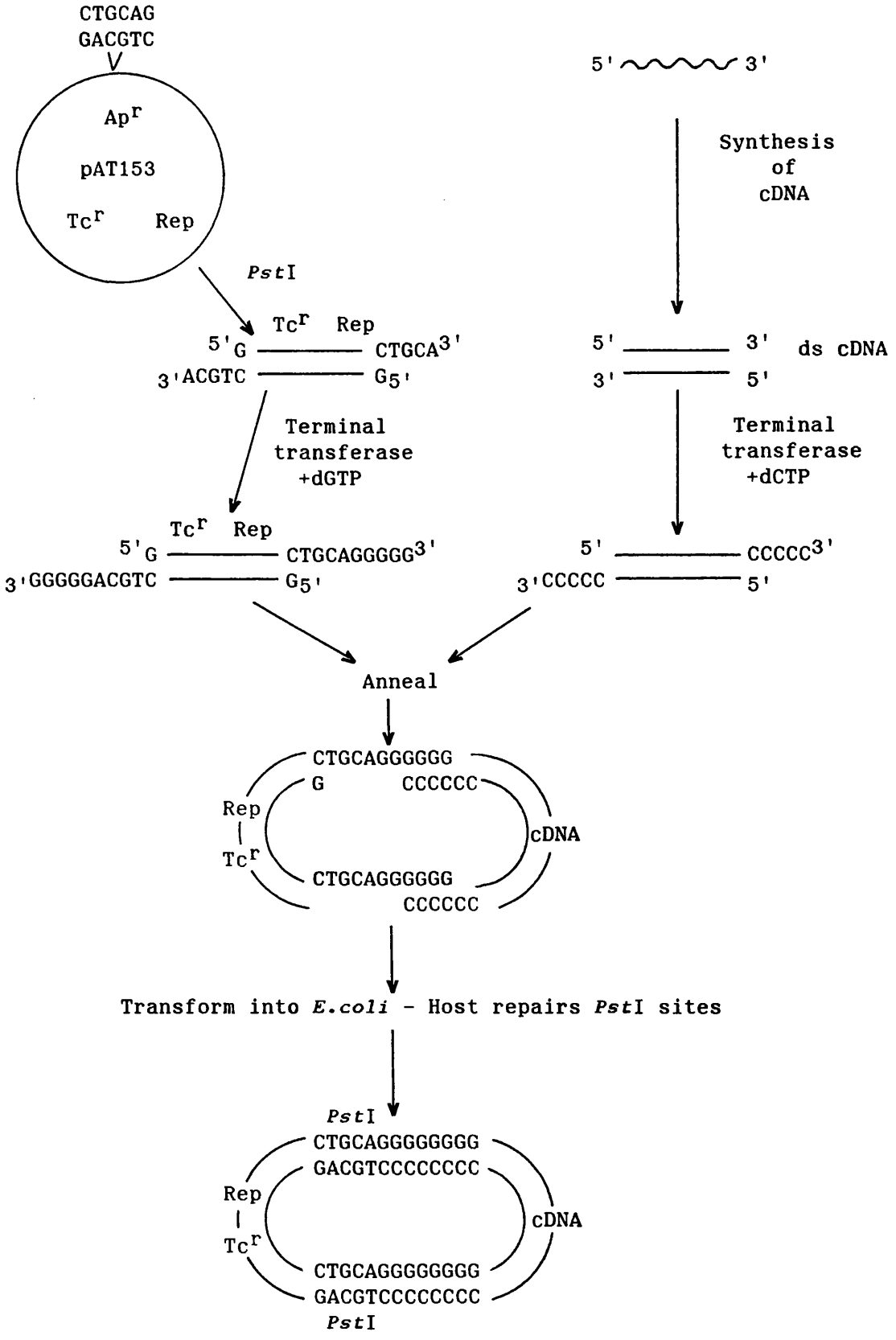


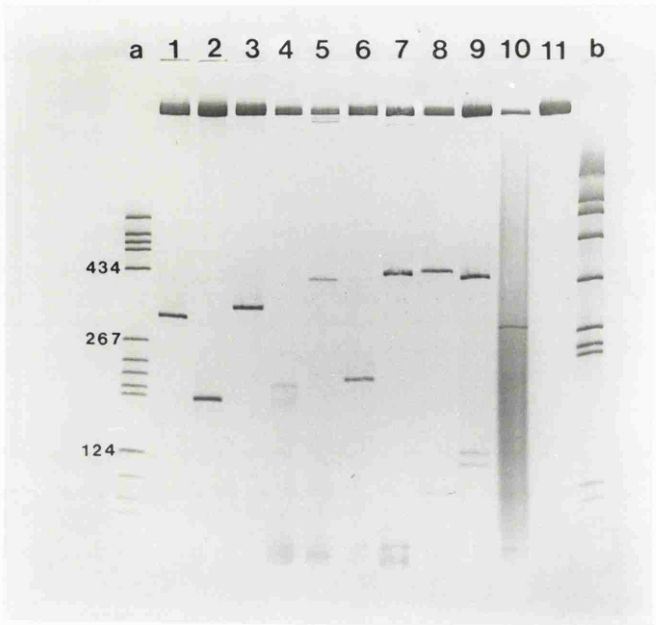

Fig.26 - Reconstruction of *Pst*I restriction sites during G-C tailing and cloning procedures

**Fig.27** - Restriction digests of recombinant plasmids with the enzyme *Pst*I

| Lane | Sample |
|---|---|
| a | pBR322 x *Hae*III |
| 1 | pSMG205 |
| 2 | pSMG204 |
| 3 | pSMG203 |
| 4 | pSMG200 |
| 5 | pSMG199 |
| 6 | pSMG198 |
| 7 | pSMG197 |
| 8 | pSMG196 |
| 9 | pSMG142 |
| 10 | pSMG120 |
| 11 | pAT153 |
| 12 | pBR322 x *Alu*I |

**Fig.28** - Restriction digests of retransformed DNA preps of pSMG196 and 142 with the enzyme *Pst*I

| Lane | Sample |
|---|---|
| a | pBR322 x *Alu*I |
| 1 | |
| 2 | pSMG196 isolate numbers |
| 3 | |
| 4 | |
| 1 | |
| 2 | pSMG142 isolate numbers |
| 3 | |
| 4 | |
| b | pAT153 |
| c | pBR322 x *Hae*III |


a 1 2 3 4 5 6 7 8 9 10 11 b
434
267
124

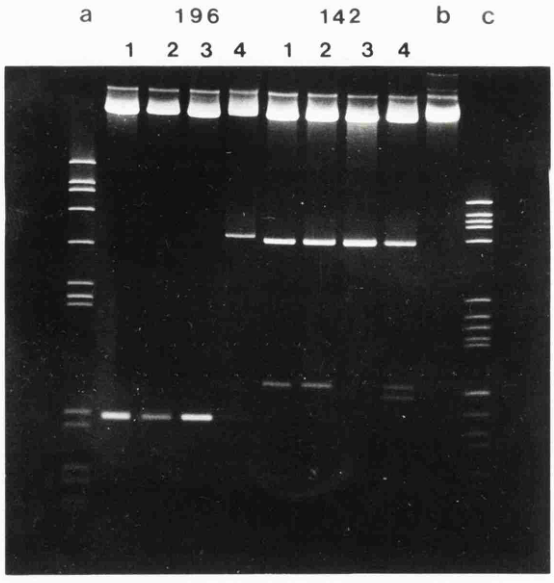

a
196
142
b c
1 2 3 4 1 2 3 4

TABLE 5 - Sizes of cDNA clone inserts

| Clone | size (bp) |
|---|---|
| pSMG120 | 285 |
| pSMG142 | 400 |
| pSMG196 | 410 |
| pSMG197 | 405 |
| pSMG198 | 200 |
| pSMG199 | 385 |
| pSMG200 | 195 |
| pSMG203 | 320 |
| pSMG204 | 175 |
| pSMG205 | 305 |
| pSMG208 | 250 |
| pSMG211 | 250 |
| pSMG212 | 400 |
| pSMG213 | 600 |

fragments respectively, in addition to the linearised vector. None of the other clones possessed potential internal *Pst*I sites and it was therefore likely that these represented mixed clones. There are several possibilities that could account for this: firstly, a single recombinant plasmid could contain two cDNA inserts due to a cloning artefact; secondly, the recombinant plasmid may be a dimer containing both two vector molecules and two cDNA inserts; lastly, during the isolation of the clone, more than one bacterial colony was grown in the same culture.(This is not a likely explanation since a single colony from a fresh plate was always used to start a liquid culture).

To try to separate the cDNA inserts the *E.coli* strain JA221 was transformed to tetracycline-resistance by either pSMG142 or pSMG196 at very high dilution (1μg/ml).Transformants were selected by their ability to grow in the presence of 10μg/ml tetracycline, and plasmid DNA was prepared individually from three pSMG142-derived transformants and three pSMG196-derived transformants. These plasmid DNAs were cut with *Pst*I and analysed by electrophoresis. Of the three pSMG142 related clones, two had lost the smallest *Pst*I fragment (pSMG142(1) and pSMG142(2)) and the third only contained the largest *Pst*I insert (400bp) having lost both small fragments (Fig.28). The plasmid pSMG142(3) retained the ability to hybrid-

select renin mRNA and was used for restriction mapping and later experiments. In Chapter 4 it was the large *Pst*I fragment of pSMG142 that was used as the hybridisation probe. At that time it was chosen simply because of its size, in retrospect a fortuitous choice! Had this fragment not been capable of selecting renin mRNA, and likewise if the related clones were not capable of doing so, then the other two fragments would have been tested. This could have been done by recloning the isolated fragments and using the new plasmids for hybrid-selection.

Digestion of pSMG196 with *Pst*I revealed a 600bp insert and a much less abundant 100bp fragment. The attempt to separate these inserts resulted in three clones that contained only the small insert.

The ease of separation of these *PstI* fragments and those of pSMG142 confirms that they do not reflect the existence of internal *Pst*I sites within single cDNA inserts. The contaminant present in the pSMG196 preparation was such a minor component that it did not prevent the use of this DNA to prepare a restriction map of the 600bp insert (see below). Subsequent work in this laboratory has shown that DBA/2 submaxillary renin cDNA, like that of SWR (Panthier *et al*,1982a) does not contain sites for the enzyme *Pst*I.

## 5.2 Restriction mapping of renin cDNA inserts

The renin cDNA clones listed in Table 5 were mapped by single and double digests with the following enzymes: *Hae*III, *Hinf*I, *Sau*3A, *Pst*I, *Taq*I, *Msp*I, *EcoR*I, *Hha*I and *Tha*I. Figure 29 summarises the results of this mapping.

Digestion of clones pSMG199, 142, 120, 197, 203 and 205 with the enzyme *Hae*III predicted insert sizes approximately 50bp shorter than those determined by digestion with *Pst*I. The most likely explanation was the presence of very small internal *Hae*III fragments which were not detected with the gel system used. It was predicted from the mapping already done that these fragments should occur consecutively, and immediately to the right of the 80bp *Hae*III fragment in Figure 29.

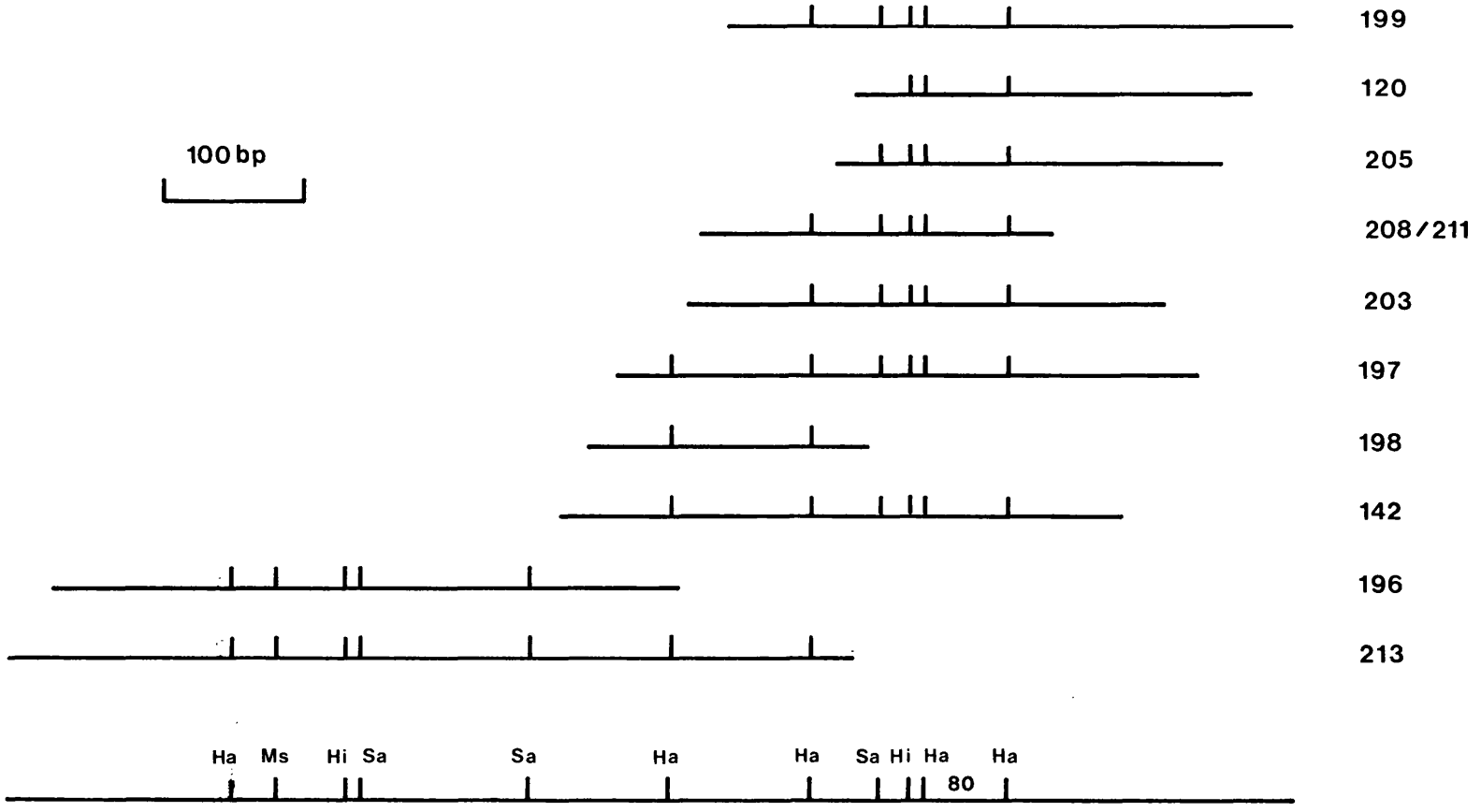



**Fig.29** - Restriction maps of DBA/2 SMG Renin cDNA clones

Ha : *Hae*III; Ms : *Msp*I; Hi : *Hinf*I; Sa : *Sau*3A

To confirm the existence of these additional *Hae*III sites the mapping technique of Smith and Birnsteil (1976) was used, in which an end-labelled restriction fragment containing the sites to be mapped is partially digested with the enzyme in question. The digestion products are then sized by gel electrophoresis and visualised by autoradiography. Partial digestion products reflect the internal sites, which can be mapped accurately from the labelled end of the initial restriction fragment. To locate potential *Hae*III sites the cDNA insert of pSMG199 was labelled at both ends using $^{32}$P-cordecepin and then digested by *Hinf*I to produce fragments of 110bp and 290bp in length which were labelled only at one end. These fragments were separated by gel electrophoresis and the 290bp fragment recovered by electroelution from the excised gel slice, and passage over DE52 cellulose to remove acrylamide contaminants. After ethanol precipitation the fragment was partially digested with *Hae*III, in a reaction that included 5$\mu$g of pAT153 carrier DNA, and half the required amount of enzyme to give complete digestion after 60 mins. incubation. Aliquots were taken after 0, 15 secs., 30 secs., 1, 5, 20 and 90 mins., the digestion products were analysed by electrophoresis through a 30cm vertical 5% acrylamide gel and visualised by autoradiography. The two predominant species were the 290bp parental fragment and the final 210bp *Pst*I/*Hae*III digestion product. Between these two fragments intermediates of 230bp and 275bp in length could be seen and thus there was an additional *Hae*III site as shown in Figure 30 (see text below).

## 5.3 Comparison of clones pSMG199, 142 and 213 with the renin cDNA sequence determined by Panthier *et al* (1982a)

Since the cloning methodoloogy used leads to preferential cloning of cDNAs from the 3' end of the mRNA it was likely that the orientation of the clones was from right to left (5'→3') in Figure 29. If the polyA tail was present in some of the clones it would have been possible to orientate them by direct sequence analysis. This approach became unnecessary with the

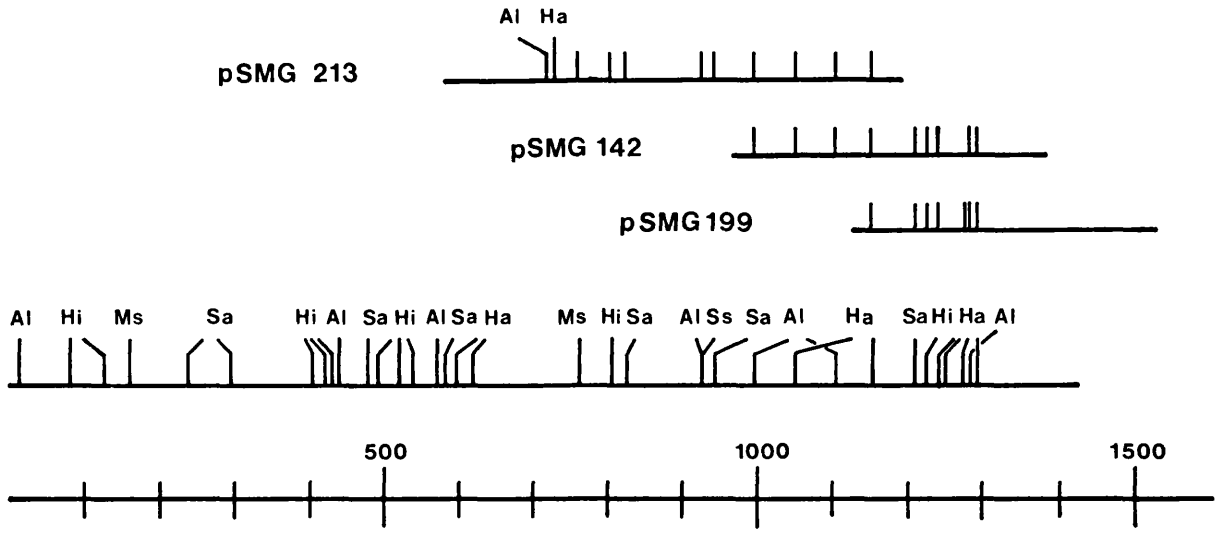



Fig.30 - Comparison of clones pSMG199, 142 and 213 to a restriction map generated from the sequence data of Panthier *et al*(1982)

Al : *Alu*I; Hi : *Hinf*I; Ms : *Msp*I; Sa : *Sau*3A;
Ha : *Hae*III; Ss : *Sst*I

publication by Panthier *et al* (1982a) of the sequences of the two mouse submaxillary renin cDNAs from the high producer Swiss strain. This sequence was searched for restriction enzyme sites using the SUBMIT program written by Chris Boyd. From this analysis sites were predicted for the enzymes *Alu*I and *Sst*I. Clones pSMG142, 213 and 199, which together cover the entire cloned region, were mapped for *Alu*I and *Sst*I restyriction sites. The computer-predicted sites were confirmed and are indicated in Figure 30 which compares clones 142, 199 and 213 to the clones of Panthier *et al*. The additional *Hae*III site found by Smith and Birnsteil mapping is present in the sequence of the published cDNA. There is also a further site not seen in the mapping experiment. This site is only 10bp away from one of the *Hae*III sites mentioned above and was not detected, but its presence was confirmed by DNA sequence analysis of related clones (D.Pioli, unpublished result).

5.4 Determination of renin mRNA length

To establish the proportion of renin mRNA covered by pSMG142, 199 and 213 the size of the mRNA was determined by Northern blot analysis. Unlabelled rRNA size markers were prepared from poly $A^-$ RNA, by sucrose gradient centrifugation as shown in Figure 31(a). In addition to 28S and 18S rRNA, yeast tRNA (4S) was used, and Figure 31(b) shows a typical marker profile after gel electrophoresis and staining with ethidium bromide. Five micrograms of male DBA/2 submaxillary gland RNA and 6$\mu$g of each marker were electrophoresed on a 1% urea-agarose gel and the submaxillary RNA track blotted onto diazobenzyloxymethyl paper prepared as described in Chapter 2 by Alwine *et al* (1974). The blot was then hybridised to a $^{32}$P-labelled probe made by nick-translating the *Pst*I insert of pSMG142. The hybridisation conditions were those described by Thomas (1980), which include several agents for blocking non-specific sites and 50% formamide allowing a lower hybridisation temperature (42°C) and preferential formation of RNA-DNA hybrids.

Autoradiography of the Northern blot (Fig.32) showed that renin mRNA

**Fig.31** (a) Separation of rRNA species by sucrose gradient centrifugation

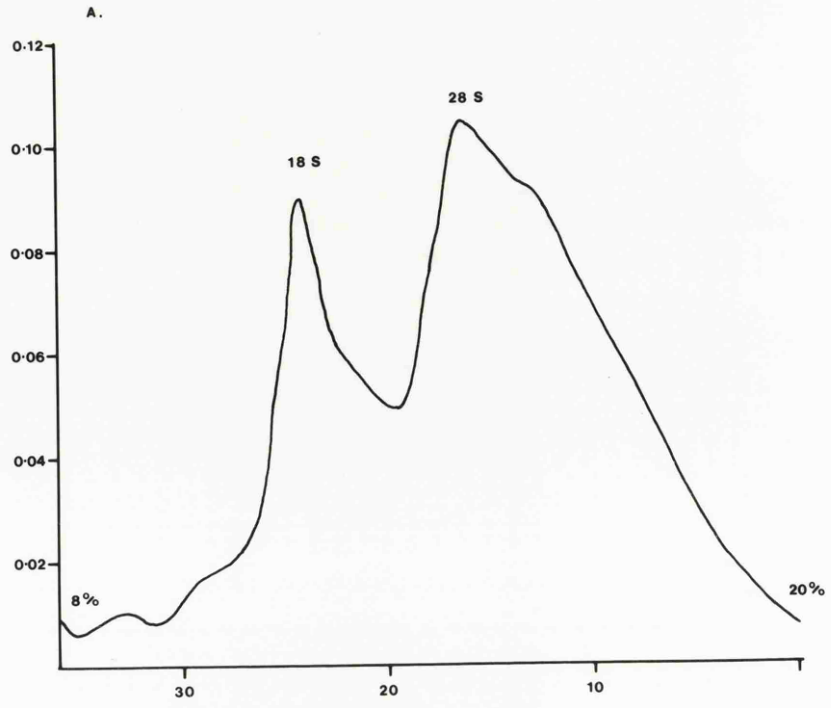
After harvesting, an aliquot of each fraction was diluted 100-fold and its $OD_{260}$ determined. The location of the 28S and 18S rRNA species and the fractions required for pooling were determined graphically.
The ordinate represents $OD_{260}$ and the abscissa indicates the fraction number of the sample.

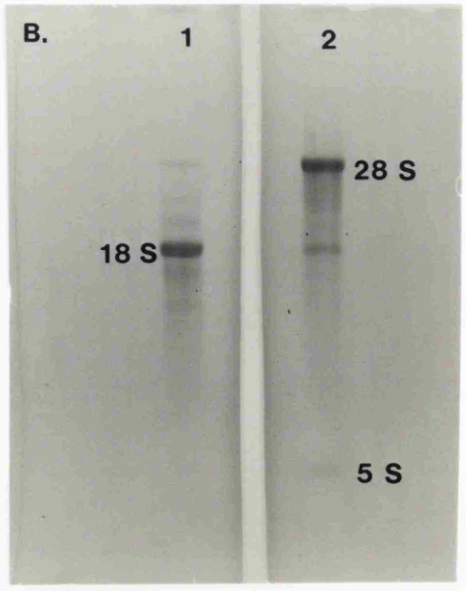
(b) Gel electrophoresis of rRNA size markers

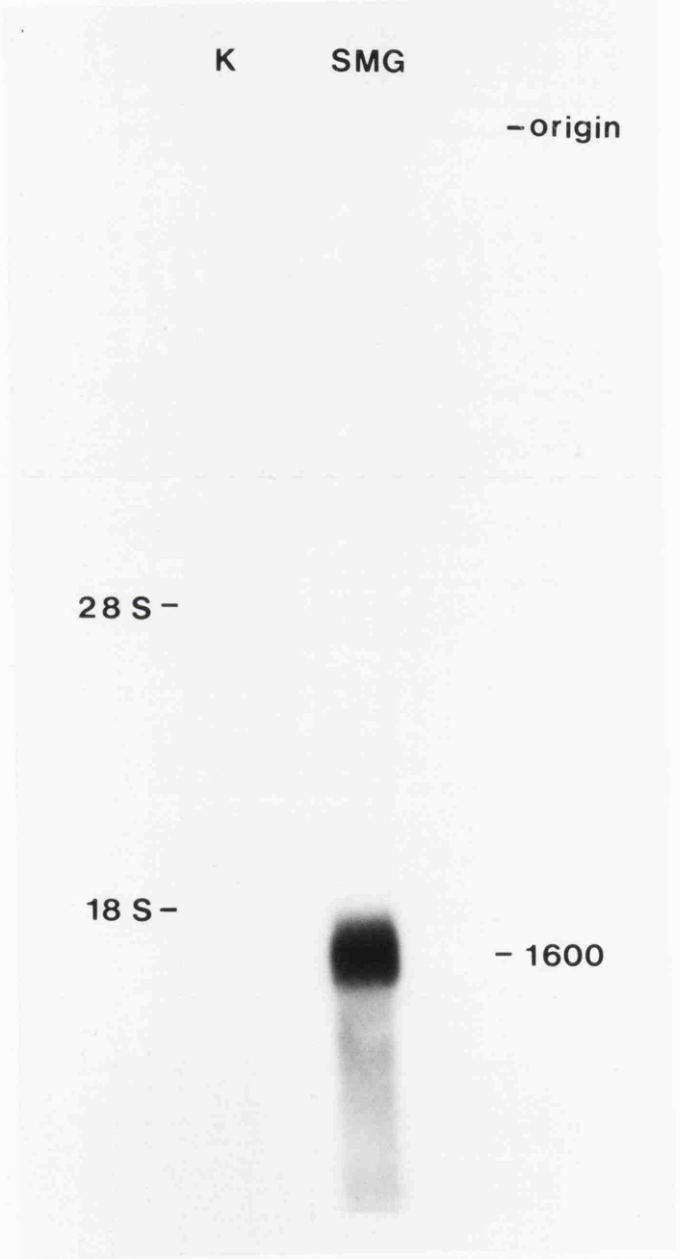
The quality of the rRNA markers was determined by electrophoresis on a urea-agarose gel as described (chapter 2).
Lane 1 - 9μg 18S rRNA; lane 2 - 9μg 28S rRNA.

**Fig.32** - Northern blot of male DBA/2 SMG and kidney mRNAs **(overleaf)**

10μg each of male DBA/2 SMG and kidney mRNAs were electrophoresed and blotted onto DBM paper. The blot was then probed with nick-translated pSMG142 DNA. The positions of size markers are indicated. The size of renin mRNA was determined both by extrapolation from these markers and with the aid of two similar blots which had additional tRNA markers.


A.
0·12
0·10
0·08
0·06
0·04
0·02
18 S
28 S
8%
20%
30
20
10


B.
1
2
28 S
18 S
5 S


K
SMG
-origin
28 S -
18 S -
- 1600

was 1600 nucleotides in length and the clones pSMG142, 199 and 213 therefore covered approximately half of the renin messenger-RNA. In addition to the predominant RNA species there are also less abundant higher molecular weight species. The most obvious is an RNA of 3000 nucleotides which presumably represents an intermediate in renin mRNA biosynthesis.

# CHAPTER 6

# RENIN BIOSYNTHESIS AND THE DISCOVERY OF TWO RENIN GENES IN THE HIGH PRODUCER MOUSE

## 6.1 The use of renin cDNA clones as probes to study the biosynthesis of mouse renin

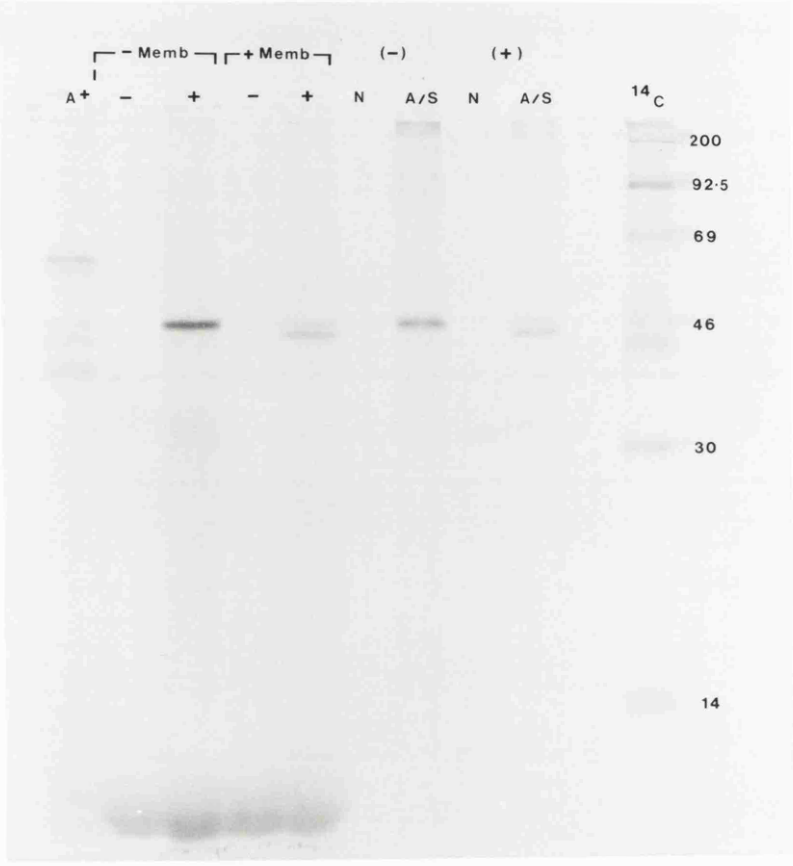
Although it had been determined that mature renin was significantly smaller than the proenzyme (Poulsen *et al*,1979b), the structures of precursors and of mature renin were not known. The isolation of a renin cDNA clone provided a tool for looking at the first step of renin biosynthesis and perhaps confirming the likely presence of a signal sequence.

The clone pSMG142 was used to hybrid-select, preparatively, renin mRNA. Fifteen micrograms of plasmid DNA were denatured, bound to nitrocellulose filters and used to select renin mRNA from 50$\mu$g of male DBA/2 submaxillary gland poly $A^+$ RNA. This renin mRNA was divided into two equal aliquots and translated *in-vitro* in either the presence or the absence of dog pancreatic membranes (see chapter 3). The resulting translation products were again split into two equal aliquots and immunoprecipitated with either normal rabbit serum or anti-renin antiserum. Translation of the hybrid-selected mRNA, as previously seen, gives a single major product of 44-45kd. When membranes are included during translation the predominant species is a 43kd single chain polypeptide, showing the removal of approximately 2kd from the primary translation product and revealing the presence of a signal sequence (Fig.33). This result completes for the first time the biosynthetic pathway of submaxillary gland renin, bringing together data from several groups that have identified and sized the primary translation product and work by Morris and Catanzaro (1981) and others showing that the first detectable *in-vivo* renin precursor is smaller than the *in-vitro* translation product.

As Figure 33 shows, not all of the renin precursor is processed. Dog pancreas membrane preparations are inhibitory to *in-vitro* translation

Fig.33 - Processing of preprorenin *in vitro*

| Lane | | Sample |
|---|---|---|
| | $A^+$ | Translation of male SMG mRNA |
| – memb | | No FS renin mRNA; no dog pancreas membranes |
| | + (a) | + FS renin mRNA; no dog pancreas membranes |
| + memb | | No FS renin mRNA; + dog pancreas membranes |
| | + (b) | + FS renin mRNA; + dog pancreas membranes |
| – memb | N | Immunoprecipitation of (a) with normal rabbit serum |
| | A/S | " " (a) " renin antiserum |
| + memb | N | Immunoprecipitation of (b) with normal rabbit serum |
| | A/S | " " (b) " renin antiserum |
| | $^{14}C$ | $^{14}C$ M.Wt markers |


– Memb
+ Memb
(–)
(+)
A+
–
+
–
+
N
A/S
N
A/S
14C
200
92·5
69
46
30
14

systems and in this type of analysis a balance has to be found between inhibiting the system and maximum processing activity. In this experiment the optimal concentration of membranes was 4 $A_{260}$ units/ml.

With the publication of the amino-acid sequence of mouse submaxillary gland renin, determined both by reverse-translation of cDNA sequence (Panthier *et al*,1982a) and by amino-acid sequencing of the purified protein (Misono *et al*,1982), the primary structure of the precursors of renin became known (Fig.34a). The positions of cleavage sites were assigned on the basis of known proteolytic cleavage sites and from amino-terminal sequencing of the two chains of the protein. The amino-acid sequence originally published by Panthier *et al* has since been changed at several positions in view of the amino-acid sequence of Misono *et al*. Some of these changes are pointed out in a more recent paper (Soubrier *et al*,1983) and a comparison of the sequences of the two groups is given in Figure 34b. The corrections made in the sequence of Panthier *et al* do not alter the sequence differences pointed out by Mullins *et al* (1982) which must be due to strain differences.

Although the maturation cleavage points of prepro- and prorenin have now been determined, nothing is yet known about the enzyme(s) that carry out these steps and this will be discussed further in Chapter 11 where possibilities for tackling these problems are considered.

## 6.2 Study of renin mRNA levels in high and low producer mice using translation/immunoprecipitation and Northern blotting

Wilson *et al* (1977) showed that mouse submaxillary gland renin activity is genetically determined and that the level in the uninduced female gland varies 100 fold between strains described as low producers (e.g. C57BL/10) and those that have higher activity (e.g. DBA/2,SWR). These levels are under the control of the *Rnr* locus which is tightly linked if not coincident with the renin structural gene located near the *Pep*3 locus on chromosome 1 (Wilson *et al*,1978).

**Fig.34** - (a) Structure of renin and its precursor

The structures shown in the figure (Panthier *et al*,1982a) were derived from amino acid sequence analysis and nucleotide sequence of renin cDNA clones.

(b) Mouse SMG renin amino acid sequence **(overleaf)**

A comparison of the amino acid sequences of mouse SMG renin determined by amino acid sequencing (Misono *et al*,1982)(above) and nucleotide sequencing of mouse SMG renin cDNAs (Panthier *et al*, 1982a)(below).

**Fig.35** - Translation and immunoprecipitation of renin from high (DBA/2) and low (C57BL/6) producer mice **(overleaf ff.)**

(a) Lane

| | | |
|---|---|---|
| | | Translation of male DBA/2 SMG mRNA |
| | N | Immunoprecipitation of male translation products with NRS |
| SMG | A/S | " " " " " with renin A/S |
| | | Translation of female DBA/2 SMG mRNA |
| | N | Immunoprecipitation of female translation products with NRS |
| | A/S | " " " " " with renin A/S |
| | | Translation of male DBA/2 kidney mRNA |
| | N | Immunoprecipitation of male translation products with NRS |
| | A/S | " " " " " with renin A/S |
| Kidney | | Translation of female DBA/2 kidney mRNA |
| | N | Immunoprecipitation of female translation products with NRS |
| | A/S | " " " " " with renin A/S |

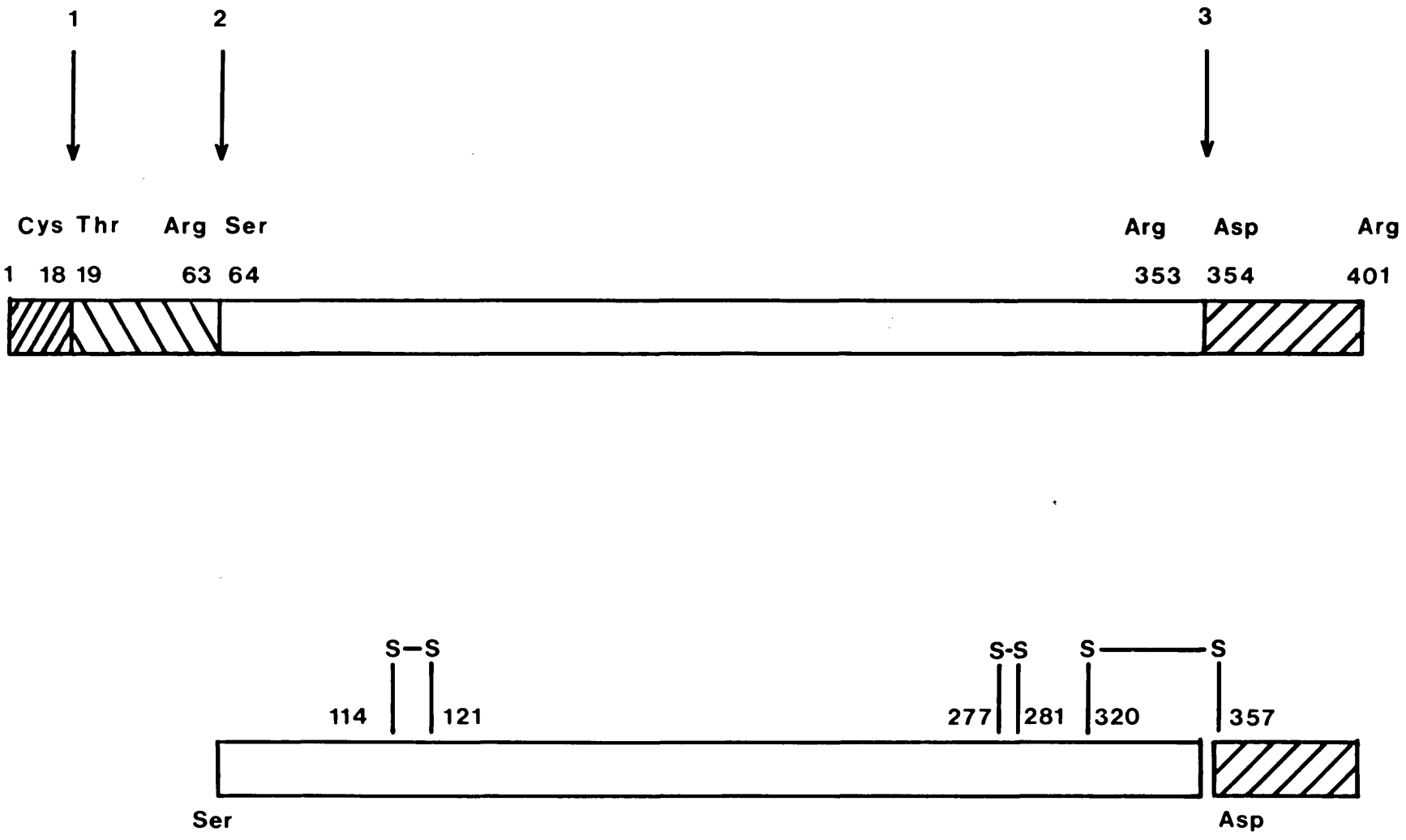
Molecular weight markers are shown on the right hand side of the figure

(b) Lane

| | | |
|---|---|---|
| | | Translation of male C57BL/6 SMG mRNA |
| | N | Immunoprecipitation of male translation products with NRS |
| | A/S | " " " " " with renin A/S |
| SMG | | Translation of female C57BL/6 SMG mRNA |
| | N | Immunoprecipitation of female translation products with NRS |
| | A/S | " " " " " with renin A/S |
| | | Translation of male C57BL/6 kidney mRNA |
| | N | Immunoprecipitation of male translation products with NRS |
| | A/S | " " " " " with renin A/S |
| Kidney | | Translation of female C57BL/6 kidney mRNA |
| | N | Immunoprecipitation of female translation products with NRS |
| | A/S | " " " " " with renin A/S |

Molecular weight markers are shown on the left hand side of the figure

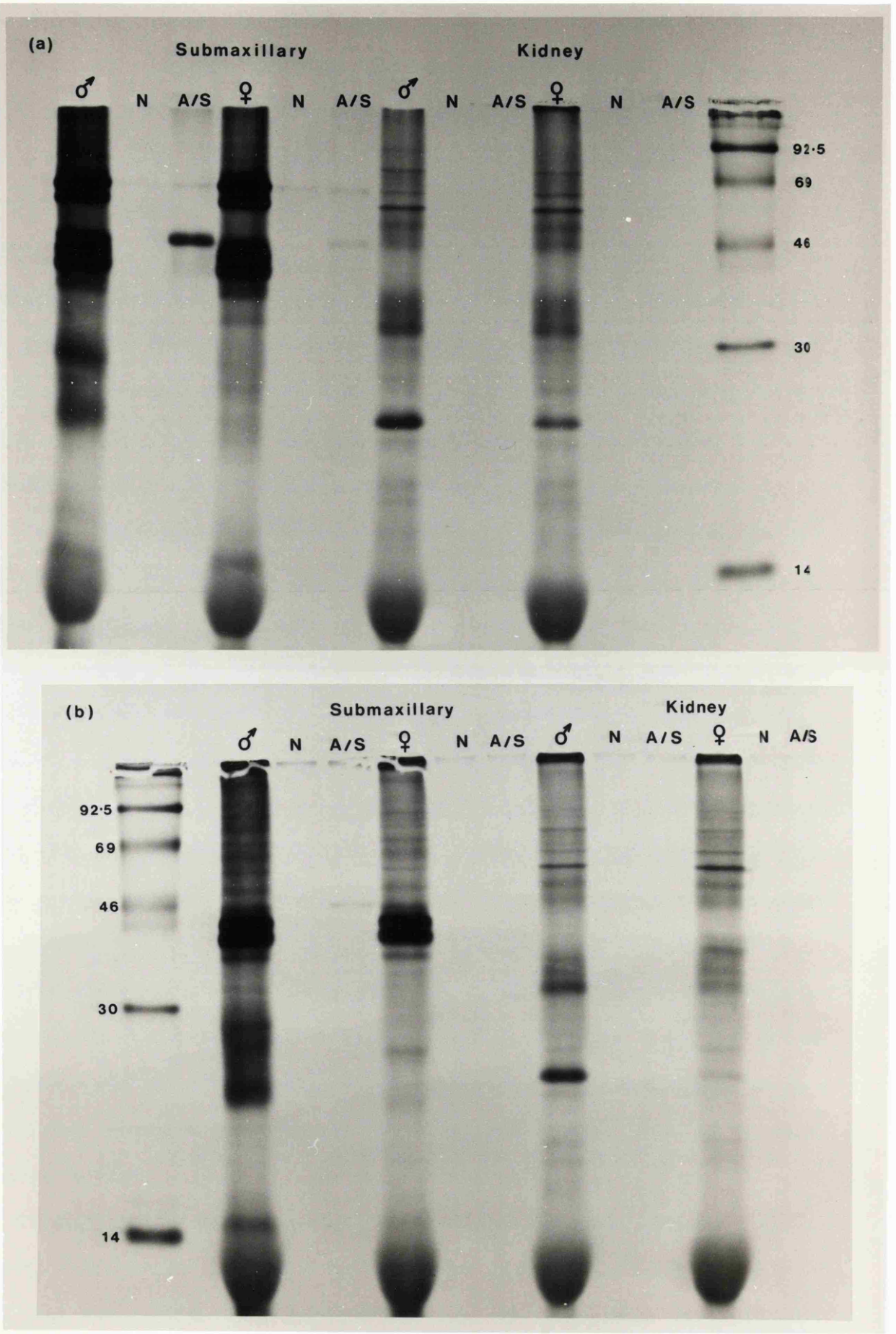
A.


1
2
3
Cys Thr
Arg Ser
Arg
Asp
Arg
1 18 19
63 64
353 354
401
S—S
S-S
S——S
114
121
277
281
320
357
Ser
Asp
A Chain
B Chain

**B.**

```
-> MetAspArgArgArgMetProLeuTrpAlaLeuLeuLeuTrpSerProCysThrPhe ->
   ATGGACAGGAGGAGGATGCCTCTCTGGGCACTCTTGTTGCTCTGGAGTCCTTGCACCTTC
           10         20         30         40         50         60

-> SerLeuProThrGlyThrThrPheGluArgIleProLeuLysLysMetProSerValArg ->
   AGTCTCCCAACGGGCACCACCTTTGAACGAATCCCACTCAAGAAAATGCCCTCTGTCCGG
           70         80         90        100        110        120

-> GluIleLeuGluGluArgGlyValAspMetThrArgLeuSerAlaGluTrpAspValPhe ->
   GAAATCCTGGAGGAGCGGGGAGTGGACATGACCAGGCTCAGTGCTGAATGGGACGTATTC
          130        140        150        160        170        180
             SerSerLeuThrAspLeuIleSerProValValLeuThrAsnTyrLeuAsn ->
 -> ThrLysArgSerSerLeuThrAspLeuIleSerProValValLeuThrAsnTyrLeuAsn ->
    ACAAAGAGGTCTTCCTTGACTGATCTTATCTCCCCCGTGGTCCTCACCAACTACCTGAAT
           190        200        210        220        230        240
 -> SerGlnTyrTyrGlyGluIleGlyIleGlyThrProProGlnThrPheLysValIlePhe ->
 -> SerGlnTyrTyrGlyGluIleGlyIleGlyThrProProGlnThrPheLysValIlePhe ->
    AGCCAGTACTATGGCGAGATCGGCATTGGTACCCCACCCCAGACCTTCAAAGTCATGTTT
           250        260        270        280        290        300
 -> AspThrGlySerAlaAsnLeuTrpValProSerThrLysCysSerArgLeuTyrLeuAla ->
 -> AspThrGlySerAlaAsnLeuTrpValProSerThrLysCysSerArgLeuTyrLeuAla ->
    GACACGGGCTCCGCCAACCTCTGGGTGCCCTCCACCAAGTGCAGCCGCCTCTACCTTGCT
           310        320        330        340        350        360
 -> CysGlyIleHisSerLeuTyrGluSerSerAspSerSerSerTyrMetGluAsnGlyAsp ->
 -> CysGlyIleHisSerLeuTyrGluSerSerAspSerSerSerTyrMetGluAsnGlyAsp ->
    TGTGGGATTCACAGCCTCTATGAGTCCTCTGACTCCTCCAGCTACATGGAGAATGGAGCC
           370        380        390        400        410        420
 -> AspPheThrIleHisTyrGlySerGlyArgValLysGlyPheLeuSerGlnAspSerVal ->
 -> AspPheThrIleHisTyrGlySerGlyArgValLysGlyPheLeuSerGlnAspSerVal ->
    GACTTCACCATCCACTACGGATCAGGGAGAGTCAAAGGTTTCCTCAGCCAGGACTCGGTG
           430        440        450        460        470        480
 -> ThrValGlyGlyIleThrValThrGlnThrPheGlyGluValThrGluLeuProLeuIle ->
 -> ThrValGlyGlyIleThrValThrGlnThrPheGlyGluValThrGluLeuProLeuIle ->
    ACTGTGGGTGGAATCACTGTGACACAGACCTTTGGAGAGGTCACCGAGCTGCCCCTGATC
           490        500        510        520        530        540
 -> ProPheMetLeuAlaGlnPheAspGlyValLeuGlyMetGlyPheProAlaGlnAlaVal ->
 -> ProPheMetLeuAlaGlnPheAspGlyValLeuGlyMetGlyPheProAlaGlnAlaVal ->
    CCTTTCATGCTGGCCCAGTTTGACGGGGTTCTAGGCATGGGCCTTTCCCGCTCAGCCGTC
           550        560        570        580        590        600
 -> GlyGlyValThrProValPheAspHisIleLeuSerGlnGlyValLeuLysGluLysVal ->
 -> GlyGlyValThrProValPheAspHisIleLeuSerGlnGlyValLeuLysGluLysVal ->
    GGCGGGGTCACCCCTGTCTTTGACCACATTCTCTCCCAGGGGGTGCTGAAGGAGAAAGTG
           610        620        630        640        650        660
 -> PheSerValTyrTyrAsnArgGlyProHisLeuLeuGlyGlyGluValValLeuGlyGly ->
 -> PheSerValTyrTyrAsnArgGlyProHisLeuLeuGlyGlyGluValValLeuGlyGly ->
    TTCTCTGTCTACTACAACAGGGGTCCCCACCTGCTGGGGGGCGAGGTGGTGCTAGGAGGC
           670        680        690        700        710        720
 -> SerAspProGluHisTyrGlnGlyAspPheHisTyrValSerLeuSerLysThrAspSer ->
 -> SerAspProGluHisTyrGlnGlyAspPheHisTyrValSerLeuSerLysThrAspSer ->
    AGCGACCCGGAGCATTACCAAGGCGATTTTCACTATGTGAGCCTCAGCAAGACTGATTCC
           730        740        750        760        770        780
 -> TrpGlnIleThrMetLysGlyValSerValGlySerSerThrLeuLeuCysGluGluGly ->
 -> TrpGlnIleThrMetLysGlyValSerValGlySerSerThrLeuLeuCysGluGluGly ->
    TGGCAGATCACAATGAAGGGGGTGTCTGTGGGGTCTTCCACCCTACTGTGTGAAGAAGGC
```

```
          790        800        810        820        830        840
 -> CysGluValValValAspThrGlySerSerPheIleSerAlaProThrSerSerLeuLys ->
 -> CysGluValValValAspThrGlySerSerPheIleSerAlaProThrSerSerLeuLys ->
    TGTGAGGTAGTGGTGGACACTGGTTCATCCTTTATCTCGGCTCCTACGAGCTCCCTGAAG
          850        860        870        880        890        900
 -> LeuIleMetGlnAlaLeuGlyAlaLysGluLysArgLeuHisGluTyrValValSerCys ->
 -> LeuIleMetGlnAlaLeuGlyAlaLysGluLysArgLeuHisGluTyrValValSerCys ->
    TTGATCATGCAAGCCCTGGGAGCCAAGGAGAAGAGACTACATGAATATGTTGTGAGCTGT
          910        920        930        940        950        960
 -> SerGlnValProThrLeuProAspIleSerPheAsnLeuGlyGlyArgAlaTyrThrLeu ->
 -> SerGlnValProThrLeuProAspIleSerPheAsnLeuGlyGlyArgAlaTyrThrLeu ->
    AGCCAGGTGCCCACCCTCCCCGACATCTCCTTCAACCTGGGAGGCAGGGCCTACACACTC
          970        980        990       1000       1010       1020
 -> SerSerThrAspTyrValLeuGlnTyrProAsn      AspLysLeuCysThrValAla ->
 -> SerSerThrAspTyrValLeuGlnTyrProAsnArgArgAspLysLeuCysThrValAla ->
    AGCAGTACGGACTACGTGCTACAGTATCCCAACAGGAGAGACAAGCTGTGCACAGTGGCT
         1030       1040       1050       1060       1070       1080
 -> LeuHisAlaMetAspIleProProProThrGlyProValTrpValLeuGlyAlaThrPhe ->
 -> LeuHisAlaMetAspIleProProProThrGlyProValTrpValLeuGlyAlaThrPhe ->
    CTCCATGCCATGGACATCCCACCACCCACTGGGCCTGTCTGGGTGCTGGGTGCCACCTTC
         1090       1100       1110       1120       1130       1140
 -> IleArgLysPheTyrThrGluPheAspArgHisAsnAsnArgIleGlyPheAlaLeuAla ->
 -> IleArgLysPheTyrThrGluPheAspArgHisAsnASnArgIleGlyPheAlaLeuAla ->
    ATCCGCAAGTTCTATACAGAGTTTGATCGGCATAACAATCGCATTGGATTCGCCTTGGCC
         1150       1160       1170       1180       1190       1200
->  Arg
->  Arg
    CGCTAA
```


(a)
Submaxillary
Kidney
♂ N A/S ♀ N A/S ♂ N A/S ♀ N A/S
92·5
69
46
30
14
(b)
Submaxillary
Kidney
♂ N A/S ♀ N A/S ♂ N A/S ♀ N A/S
92·5
69
46
30
14

It was not known from these genetic studies whether this variation in activity was a result of mRNA concentration and/or the efficiency of translation of the respective mRNAs, or whether the differences were due to specific activity or stability of the protein. To test the former possibility, messenger RNA was prepared from the SMG and kidneys of male and female DBA/2 and C57BL/10 mice and after translation and immunoprecipitation the results shown in Figure 35 a and b were observed. Figure 35a shows the immunoprecipitates from DBA/2 mRNA translations and shows detectable amounts of translatable renin mRNA in the female submaxillary gland. The levels in the kidneys of both male and female mice were not detected by this assay even after a long exposure of the fluorograph.

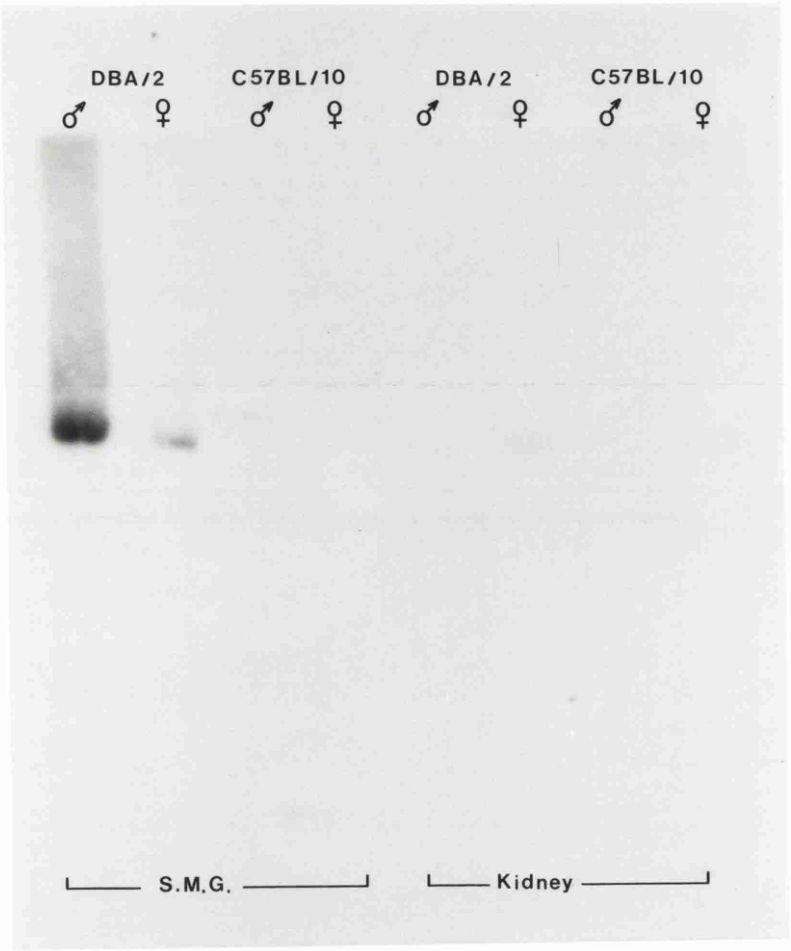
The results for C57BL/10 under identical experimental conditions were qualitatively the same as for DBA/2 (Fig.35b). The size of the C57BL/10 *in-vitro* precursor was the same as DBA/2 preprorenin and there was a similar drop in abundance in the translation products of female mRNA compared to those seen with male mRNA. The preprorenin translated from mRNA from female C57BL/10 submaxillary gland could just be detected on the fluorograph after a 3-week exposure and, as expected, the translation of C57BL/10 kidney renin mRNA was not visible in this system. Further purification of the renin mRNA is essential before detection of the translation product is possible (see below).

From these results one can conclude that there is a major difference in submaxillary gland renin mRNA between the two strains and that if the difference was not one of mRNA concentration then there would have to be a significant difference in either the stability of the mRNAs or in their translatability.

To distinguish between these possibilities the mRNA preparations were run on a denaturing gel and a Northern blot prepared using DBM paper. The blot was then probed with pSMG213 DNA and the subsequent autoradiograph revealed parallel differences in renin mRNA levels to the strain and sex differences seen in the immunoprecipitation experiment (Fig.36). There is

Fig.36 - Northern blot of submaxillary and kidney renin mRNAs

The figure shows the autoradiograph of a northern blot of SMG and kidney mRNAs from high (DBA/2) and low (C57BL/10) producer mice probed with the renin cDNA pSMG142.
♂ : male; ♀ : female.


DBA/2
C57BL/10
DBA/2
C57BL/10
♂
♀
♂
♀
♂
♀
♂
♀
S.M.G.
Kidney

considerably more renin mRNA in the glands of male and female DBA/2 mice than in their C57BL/10 counterparts, and the male *versus* female difference in translation product is reflected in renin mRNA levels. With this assay the kidney renin mRNA levels are detectable and show quite clearly that in both DBA/2 and C57BL/10 there is more renin mRNA in the female kidney than in the male kidney.

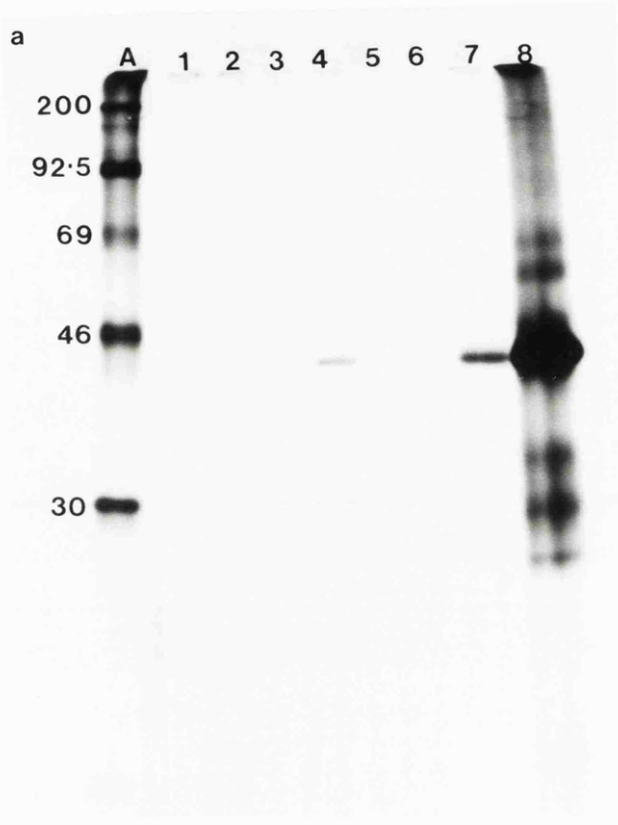
This latter result was surprising, since renin was not believed to be under androgen control in kidney, but similar results have been reported by Rougeon *et al* (1981). This finding is the subject of a more detailed series of experiments described in Chapter 8. The size of renin mRNA was the same for both strains and for the two tissues, as was the size of their respective translation products. It had been shown by Wilson *et al* (1982a) that the renins of high and low producers differed in their thermostability and immunological properties and were therefore likely to be physically different proteins or isozymes. In addition, high producer kidney renin resembled low producer submaxillary and kidney renins immunologically. To try and demonstrate this difference physically and to show that high-producer kidney renin was similar to low producer isozyme, renin mRNA was purified by hybrid-selection from male DBA/2, C57BL/10 and CBA/Ca (a second low producer strain) submaxillary glands, and male DBA/2 kidney poly $A^+$ RNAs. Each selected mRNA was translated and the products immuno-precipitated as previously described. One half of each immunoprecipitation was electrophoresed on a 12.5% polyacrylamide-SDS gel (Fig.37a) to check the purity of the respective renin precursors, prior to running the remainder of each sample on an isoelectric focussing gel. As can be seen, renin precursors were obtained in relatively pure form from the three submaxillary gland translations. The DBA/2 kidney renin precursor sample showed the presence of a second major polypeptide of approximately 38kd. At first this was assumed to be a contaminant because of the difficulty of obtaining pure renin mRNA from a tissue in which it comprises probably only 0.05% of the total mRNA, but a close comparison of the translation products

Fig.37 - (a) Immunoprecipitation of translation products from hybrid-selection purified mRNAs

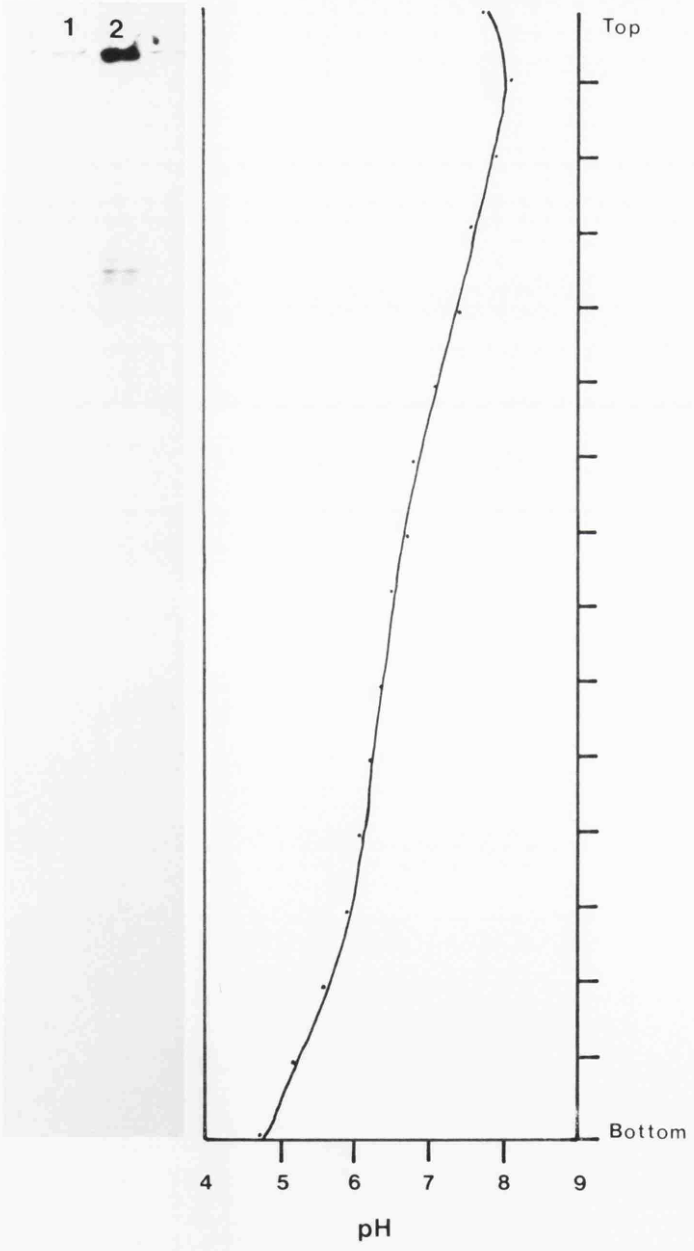
| Lane | Sample | Serum |
|---|---|---|
| A | $^{14}C$ M.Wt.markers | – |
| 1 | DBA/2 kidney | NRS |
| 2 | DBA/2 kidney | A/S |
| 3 | CBA/Ca SMG | NRS |
| 4 | CBA/Ca SMG | A/S |
| 5 | C57BL/10 SMG | NRS |
| 6 | C57BL/10 SMG | A/S |
| 7 | DBA/2 SMG | NRS |
| 8 | DBA/2 SMG | A/S |

(b) Isoelectric focussing of immunoprecipitates from the translation of hybrid selection purified male DBA/2 SMG mRNA (overleaf)

| Lane | Sample |
|---|---|
| 1 | NRS immunoprecipitation products |
| 2 | A/S immunoprecipitation products |


a
A
1
2
3
4
5
6
7
8
200
92·5
69
46
30

b



of both polyA$^+$ kidney RNA and hybrid-selected kidney RNA (still heavily contaminated with other mRNAs) reveals no very abundant 38kd product that could be, by virtue of its abundance, a contaminant. Indeed, the 'major' kidney protein of approximately 25kd, which one might expect to be a problem, is not seen at all in the final immunoprecipitation. The question remaining is whether the 38kd peptide could be related to renin. This question was recently emphasised with the publication of partial kidney renin cDNA sequence by Panthier *et al* (1983). In this paper they show the translation products of renin mRNA purified by centrifugation on a sucrose gradient. Although the autoradiograph is under-exposed it shows that very good purification of mRNA has been achieved, since the renin precursor could be immunoprecipitated cleanly from the translation products. A close look at the autoradiograph, however, shows the presence of an additional 38kd peptide! Because of the method used to purify their mRNA this peptide could be more easily dismissed as a contaminant, except that its presence means it has now been seen in independent experiments with slightly different methodology. In addition, the antisera used by Panthier *et al* and myself were different, leaving only two explanations: firstly that there is a protein which co-purifies with submaxillary gland renin and is produced in the kidney (or that antibody to this co-purifying protein cross reacts with a kidney protein); secondly, that the 38kd peptide is related to renin. It is worth noting that single chain renin, before cleavage of the A and B chains has a molecular weight of 38kd.

Preliminary attempts to investigate the relationship between the 45kd and 38kd polypeptides using peptide mapping were unsuccessful due to shortage of material.

The remaining aliquots of the pure submaxillary gland preprorenins were analysed on a 1.5mm vertical slab gel prepared according to the recipe of O'Farrell *et al* (1975), containing 1.2mls of ampholines pH 4-6 and 0.6mls of ampholines pH 3.5-10 (per 60ml). At the end of the experiment a good pH gradient was obtained, which provided a wide margin either side of the pI of

mature renin (pI=6.2). However, after extensive autoradiography, the only samples that were visible were those of the male submaxillary gland (Fig.37b). If the polypeptide species at pI 7.5 really are renin then clearly there is heterogeneity in the submaxillary gland renin precursor, perhaps due to a small amount of proteolysis. After several attempts to distinguish these renins by isoelectric focusing the approach was abandoned for reasons of technical difficulty and economy!

As mentioned above, Panthier *et al* (1983) have now isolated a kidney renin cDNA. The implications of its sequence will be described later in this chapter where its relevance will be obvious, but together with data from our laboratory it demonstrated that high and low producer renins *were* different proteins, and that the IEF approach attempted above would no longer yield new information.

## 6.3 Southern blotting of DBA/2 genomic DNA - identification of two renin genes

Since evidence had been growing that high and low producer renins were different proteins, two of the most likely explanations were that the genes coding for the enzymes were quite different alleles of the renin gene, or were distinct genes in their own right. Rougeon *et al* (1981) had suggested that there was only one structural renin gene in the high producer mouse from hybridisation studies between kidney polyA$^+$ RNA and a submaxillary renin cDNA clone. However, their evidence was not unequivocal and it was still possible that the two strains differed in the number of renin genes.

To test this hypothesis, a Southern blot of DBA/2 genomic DNA (provided by S.Adams) was probed with nick-translated pSMG142. The result (Fig.38) showed two restriction fragments hybridising for each single or double restriction digest. It proved impossible to map all the data as a single large DNA fragment and the two maps derived from the data are shown in Figure 39.

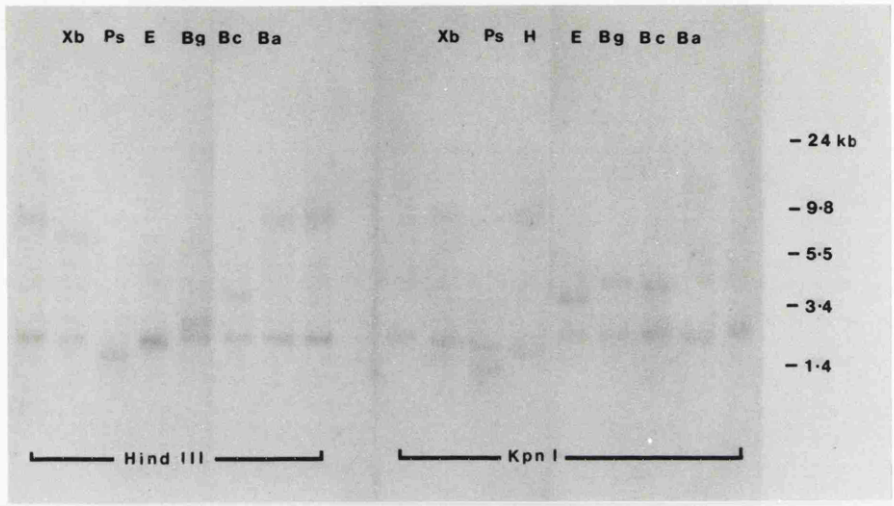
Three interpretations are possible: firstly, that the probe contained

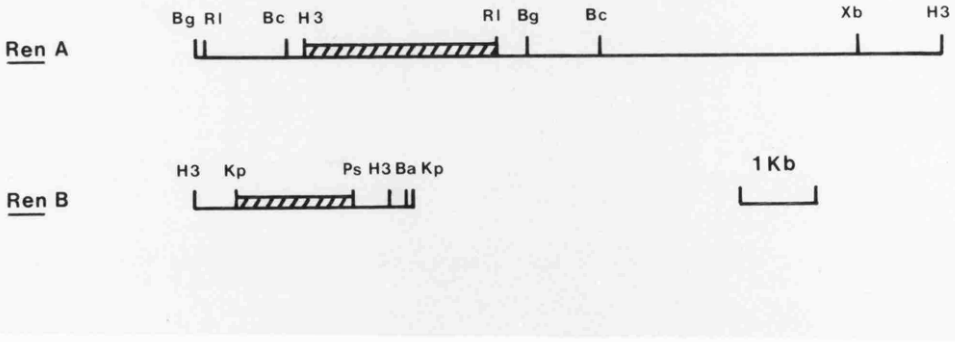
**Fig.38** - Southern blot of DBA/2 genomic DNA

The southern blot was probed with nick-translated pSMG142 DNA and washed finally with 0.5 x SSC.
Xb = *Xba*I; Ps = *Pst*I; E = *Eco*RI; Bg = *Bgl*II; Bc = *Bcl*I;
Ba = *Bam*HI; H = *Hin*dIII.
Size markers are from λ DNA standards.

**Fig.39** - Restriction maps derived from the data obtained from the Southern blot shown in Fig.39

Symbols for restriction enzymes are as listed in the legend to Fig.38


Xb Ps E Bg Bc Ba
Xb Ps H E Bg Bc Ba
– 24 kb
– 9·8
– 5·5
– 3·4
– 1·4
Hind III
Kpn I


Ren A
Bg Rl Bc H3 Rl Bg Bc Xb H3
Ren B
H3 Kp Ps H3 Ba Kp
1 Kb

both *Kpn*I and *Hind*III restriction sites, implying that the equivalent genomic region would also possess these sites, and would therefore present two restriction fragments for hybridisation to pSMG142 when cut with one of these enzymes; secondly, there is a single renin gene containing a large intron in that part of the gene corresponding to the probe; or, thirdly, that there are two distinct renin genes.

The absence of *Hind*III and *Kpn*I restriction sites within the cDNA insert of pSMG142, was shown by single digests of pSMG142 and the vector plasmid pAT153 with these two enzymes. Subsequent gel analysis revealed that both plasmids had been linearised by cleavage at the single sites for each enzyme within the vector, but there were no internal sites in the cDNA insert.

The possibility of the corresponding genomic region being split by an intron implies from the Southern blotting data that such an intron would be a very large one and must contain many hexanucleotide restriction sites.

The third explanation, the existence of two genes, seemed the simplest and so to test this possibility, genomic DNA was prepared from DBA/2 spleen tissue and 30μg was digested with *Hind*III, an enzyme that gave restriction fragments of 8.4 and 2.4 Kb which hybridised to pSMG142. This DNA was divided into three equal aliquots which were electrophoresed in parallel tracks on the same agarose gel, and then transferred by Southern blotting to a nitrocellulose filter. The three tracks were located and the filter cut lengthways to separate them. After prehybridisation, one track was hybridised to the cDNA insert of pSMG142, isolated as a *Pst*I restriction fragment. As shown in Figure 29 the insert of pSMG142 contains a single restriction site for the enzyme *Sau*3A, and a *Pst*I/*Sau*3A double digest of pSMG142 allowed the preparative isolation of the two *Pst*I/*Sau*3A fragments comprising the insert of pSMG142. These fragments were called S (180bp) and L (235bp) and after separate nick-translation, each was hybridised to one of the remaining Southern blots. After overnight hybridisation, rinsing and a final wash in 0.5 x SSC the blots were placed on film. The result is shown

in Figure 40 and reveals that both *Pst*I/*Sau*3A fragments hybridised to the 8.4kb and 2.4kb *Hind*III fragments. The possible results of this experiment are summarised in Table 6, which shows that the result obtained can only be interpreted as meaning the presence of two renin genes.

TABLE 6 - Possible outcomes of probing southern blots of *Hind*III digested DBA/2 DNA with fragments of cDNA clone pSMG142

| HYPOTHESIS | FRAGMENT SIZE | EXPECTED RESULTS S | L | | S | L |
|---|---|---|---|---|---|---|
| 1 gene; intron within genomic sequence corresponding to S | 8.4 | + | + | | + | - |
| | | | | or | | |
| | 2.4 | + | - | | + | + |
| 1 gene; intron within genomic sequence corresponding to L | 8.4 | + | + | | - | + |
| | | | | or | | |
| | 2.4 | - | + | | + | + |
| 1 gene; intron very close to S/L junction | 8.4 | + | - | | - | + |
| | | | | or | | |
| | 2.4 | - | + | | + | - |
| 2 genes; | 8.4 | + | + | | | |
| | 2.4 | + | + | | | |

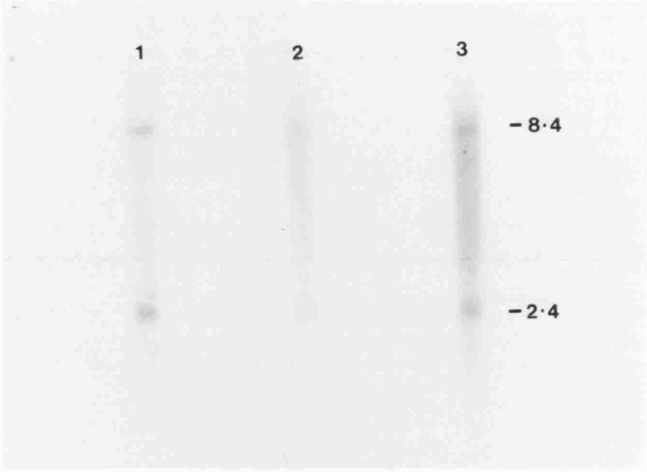
The 8.4kb and 2.4kb restriction fragments were of equal abundance and although one cannot rule out the possibility of more than two renin genes, the stoichiometry of hybridisation would indicate equivalent numbers of genes related to each of the two genes identified above.

To extend the initial observation, cloning of the two genes was a logical step and therefore 300μg of genomic DNA was cut partially with the enzyme *Sau*3A, to generate a random set of restriction fragments ranging in size from <2kb to >50kb, with most of the DNA in the 10-25kb range. This DNA was sized by centrifugation on a 10-40% sucrose gradient. After harvesting 0.25ml fractions, the DNA in each fraction was recovered and sized by gel electrophoresis. Fractions containing DNA in the size range 10-20kb were

**Fig.40** - Southern blots of *Hind*III digested DBA/2 DNA probe with fragments of the renin cDNA clone pSMG142

| Lane | Probe |
|---|---|
| 1 | pSMG142 *Pst*I insert |
| 2 | pSMG142 small *Pst*I/*Sau*3A fragment |
| 3 | pSMG142 large *Pst*I/*Sau*3A fragment |

The sizes of the hybridisation fragments are indicated on the right of the figure.


1
2
3
– 8·4
– 2·4

pooled and used by D.Burt to construct a DBA/2 genomic library in the λ vector L47 (Loenen and Brammar,1980).The *Pst*I insert of pSMG213 was isolated, cleaned by DE52 chromatography and nick translated for use as a hybridisation probe to detect renin genomic clones. After the identification and purification of these clones, further detailed analysis was carried out by D.Burt and others in the laboratory. Figure 41, taken from Mullins *et al* (1982) summarises these results, and shows that the clones obtained could be divided into two groups on the basis of their restriction maps, confirming the presence of two distinct DBA/2 renin genes, arbitrarily called *Ren*-A and *Ren*-B. This nomenclature has since changed to help standardise the names given to renin genes (C.Wilson, personal communication). In the new system *Ren*-A becomes *Ren*-1 and *Ren*-B becomes *Ren*-2.

From the work mentioned above the orientation of the two genes was determined and a 3', 3kb insertion/ deletion in *Ren*-2 identified. The restriction maps presented for *Ren*-1 and *Ren*-2 clones showed complete identity within their group, making it unlikely that the *Ren*-1 and *Ren*-2 clones represented sets of closely related genes. The restriction maps drawn from Southern blotting data are identical to the corresponding maps from the genomic clones. In Figure 40, map 1 relates to *Ren*-1 and map 2 to *Ren*-2.

The identification and characterisation of two DBA/2 renin genes coincided with work by two other groups (Piccini *et al*,1982; Panthier *et al*, 1982b) who had used Southern blotting to show that high producer strains have an additional renin gene. Low producers appear to have a single renin gene whilst high producers, such as DBA/2, have an extra copy. From a comparison of the Southern blot data the single gene found in low producer strains was identical to the *Ren*-1 gene found in the high producer strains. *Ren*-2 was therefore responsible for the extremely high expression of renin in the submaxillary gland of strains possessing this copy. This higher expression cannot be accounted for by gene dosage since the single extra

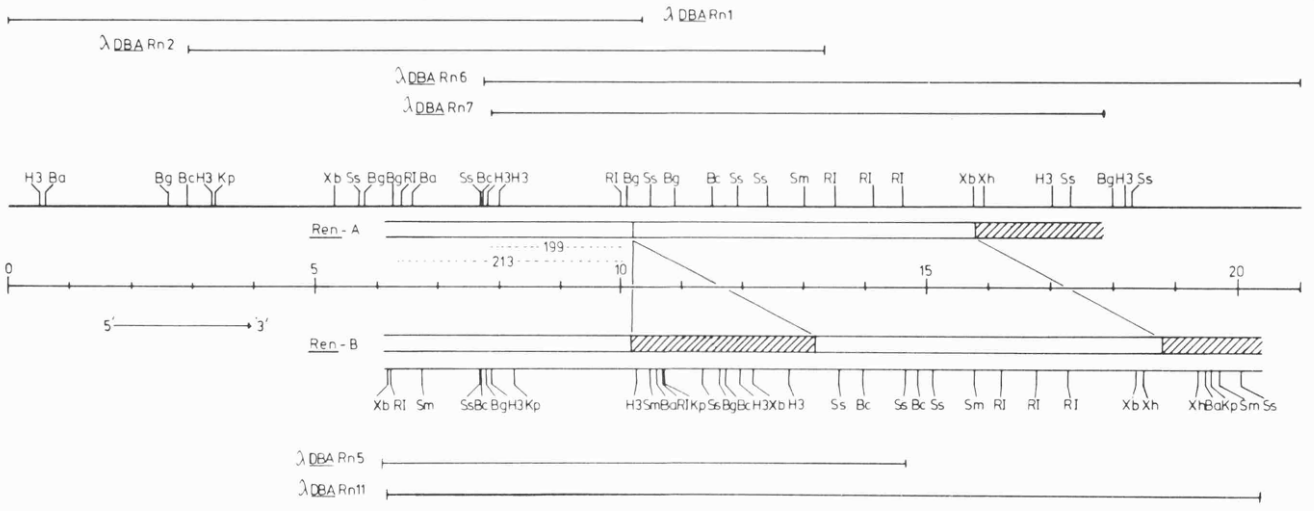



**Fig.41** - Restriction maps of DBA/2 genomic renin clones

H3 = *Hin*dIII; Ba = *Bam*HI; Bg = *Bgl*II; Bc = *Bcl*I
Kp = *Kpn*I; Xb = *Xba*I; Ss = *Sst*I; RI = *Eco*RI;
Sm = *Sma*I; Xh = *Xho*I.

copy would appear to increase the amount of renin by up to one hundred fold. There must therefore be elements contained within the *Ren*-2 gene that confer high expression.

The fact that low producer strains have only one renin gene implies that low producer submaxillary gland and kidney renins must be the products of the same gene. Therefore, unless alternative splicing of primary transcripts occurs as in the case of α-amylase (Hagenbuchle *et al*,1980), the two proteins will be translated from identical mRNAs and would have the same primary sequence.

It is an obvious extrapolation, when the thermostability and immunological studies of Wilson *et al* (1982a) are considered, that *Ren*-1 would be the primary gene expressed in the high producer kidney and that if *Ren*-2 is expressed, then it is at a very low level. This extrapolation was recently confirmed by Panthier *et al* (1983) by partial sequence data of a renin cDNA clone that was synthesised from high producer (SWR) kidney mRNA, and showed complete sequence identity to the sequence of *Ren*-1 (DBA/2) published by Mullins *et al* (1982). In terms of renin gene expression, therefore, the high producer submaxillary gland is the exception - although it is possible that *Ren*-1 is expressed in this tissue, the primary gene expressed is *Ren*-2. These arguments will be re-introduced when more detailed questions are asked in Chapter 8 as to the expression of these genes in the submaxillary gland and the kidney.

Thus, having identified submaxillary gland renin cDNA clones, questions relating to renin gene structure and expression can be addressed. Whilst the fine structure of the mouse renin genes was being determined by others in the laboratory, my attention focussed on determining the start of transcription of the renin genes by primer extension and $S_1$ mapping techniques.

# CHAPTER 7

## PRIMER EXTENSION AND $S_1$ MAPPING OF RENIN GENES

### 7.1 Primer extension of renin mRNAs

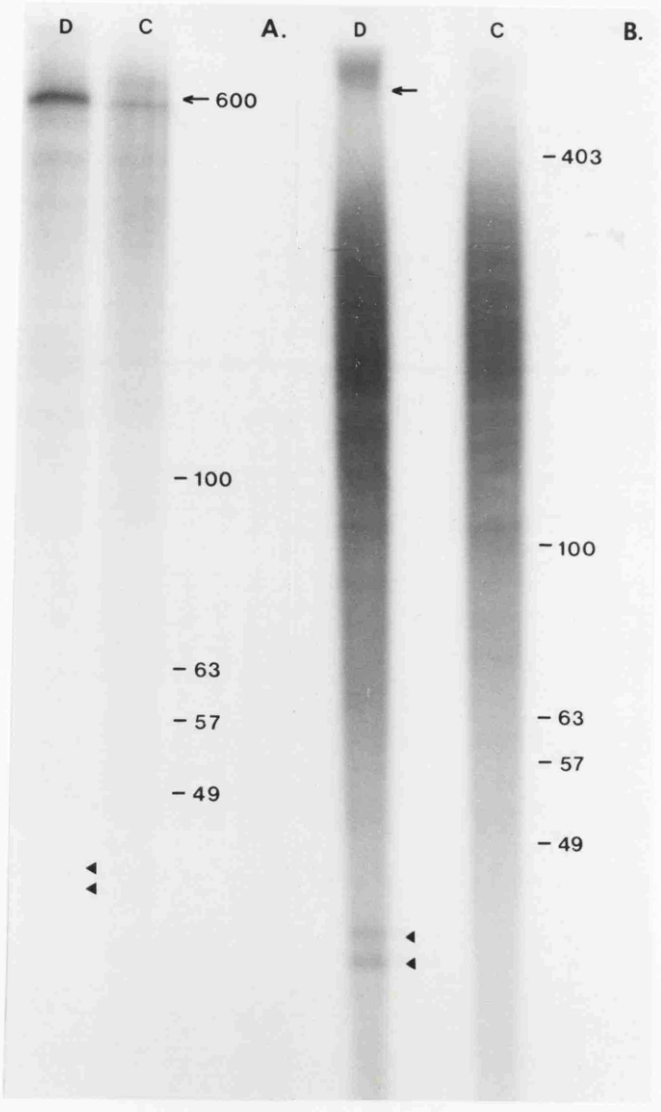
Synthetic oligonucleotides are now used extensively to ask specific questions about gene expression, and are being used both as tailor-made primers (Das *et al*,1983) and as the basis of introducing specific mutations (Norris *et al*,1983) to ask more complex questions about gene regulation.

Before such questions can be asked a detailed structure of the gene(s) in question must be available. Most of the elements identified as regulating gene expression are located 5' to the start of transcription, and therefore an accurate determination of such transcription start points is essential prior to further analysis of a particular gene. In the case of renin the cDNA sequence published by Panthier *et al* (1982a) included 38bp of the 5' non-coding region. It was therefore possible to design an oligonucleotide which could be used both as a probe to identify the 5'ends of *Ren*-1 and *Ren*-2 by hybridisation to genomic subclones, and as a primer to direct the synthesis of cDNA whose length should locate the transcription start point. The sequence chosen is shown in Figure 46. The primer, Mu Ren, is a 19mer oligonucleotide which spans the ATG initiation codon and runs 10bp into the 5' non-coding region. From the cDNA sequence published by Panthier *et al* we would therefore expect an extension product of ≥48 nucleotides. The first attempt at primer extension involved a standard cDNA reaction in which the primer was substituted for oligo-dT, and the reaction was allowed to proceed at 42°C for 1 hour. The products were labelled by incorporation of $\alpha^{32}$P dCTP and recovered by ethanol precipitation and then sized by electrophoresis through a denaturing acrylamide-urea gel. The synthesis was carried out in parallel using male DBA/2 and male C57BL/10 submaxillary gland RNA. The autoradiograph (Fig.42a) indicated that the primer hybridised to many mRNA species resulting in a background of cDNA

**Fig.42** - Primer extension of MuRen

(a) extension products of male DBA/2 and C57BL/10 SMG mRNAs primed by MuRen without prior annealing. Molecular weight markers are indicated on the right hand side of the figure and the relative position of the two 40-45 nucleotide extension products seen in (b) are indicated by ◀

(b) extension products of male DBA/2 and C57BL/10 SMG mRNAs primed by MuRen after annealing of the primer. Molecular weight markers are indicated on the right of the figure and the position where a 600 nucleotide extension product would be is arrowed. The symbol highlights discrete products of 40-45 nucleotides in size.


D
C
A.
600
100
63
57
49
D
C
B.
403
100
63
57
49

products but the predominant product for both samples was a 600 nucleotide cDNA. This was significantly larger than expected and theoretically not possible if the primer was initiating at the correct position since the renin mRNA is approximately 1600 nucleotides in length. The fact that the product was much more abundant in the DBA/2 sample than in the C57BL/10, however, suggests that it might be renin related and a computer search of the renin cDNA sequence of Panthier *et al* revealed other potential sequences to which the primer could hybridise. These are listed in Table 7. One potential complementary sequence is a particularly good match. Between residues 589 and 608, 14 of 19 residues can be correctly base-paired to the primer including the nucleotides at the 3' end of MuRen. Extension from this position to the end of the published sequence would produce a cDNA of 608 nucleotides in length and is almost certainly the reason for the observed result.

**TABLE 7** - Potential alternative priming sites for the primer MuRen within renin mRNA

| No | SEQUENCE | COORDINATE | HOMOLOGY |
|---|---|---|---|
| 1 | GCTGCACTTGGTGGAGGGC | -386 | >12 |
| 2 | ACTGGGCCAGCATGAAAGG | -600 | >12 |
| 3 | GCTGGCCCAGTTTGACGGG | +589 | >14 |
| 4 | GCAGATCACAATGAAGGGG | +823 | >11 |
| 5 | GTTGAAGGAGATGTCCGGG | -1037 | >12 |
| 6 | CTTCAACCTGGGAGGCAGG | +1030 | >11 |
| 7 | TCCCAACAGGAGAGACAAG | +1087 | >11 |

On close inspection of the autoradiograph reproduced in Figure 42a it was possible to see two bands of about 40 nucleotides in length present in the DBA/2 track but not visible in the C57BL/10 track. In order to make the synthesis more specific, the hybridisation of the oligonucleotide to the mRNA was carried out by boiling the reactants for one minute and then

placing the sample into a 65°C water bath which was turned down to 42°C and allowed to cool (45 mins). After reaching 42°C, the remaining components, including the nucleotides, were prewarmed and added to the mRNA-primer mix. The extension reaction was then carried out and analysed by denaturing gel electrophoresis. As seen from Figure 42b hybridisation of MuRen between nucleotides 589 and 608 appears to have been prevented and standing out from the heavy background are two products of 40-45 nucleotides in length.

The high background is unrepresentative of the relative amounts of product being made since they are being labelled in direct proportion to their size. To overcome this problem and obtain a more quantitative picture of the products being synthesised, MuRen was kinased (oligonucleotides are synthesised with both 5' and 3' hydroxyl groups) and then used in an identical manner to that described immediately above. Using the same conditions of electrophoresis, the extension products seen in Figure 43 were observed. There are two major products of 40-45 nucleotides in length with very little background synthesis, showing the specificity of the primer under these conditions. As expected these cDNAs are considerably more abundant in the DBA/2 sample than in the C57BL/10, helping to confirm their renin identity.

## 7.2 Sequence analysis of primer-extension products

In order to determine (a) that these products were definitely renin cDNAs and (b) their exact termination points, the extension products from DBA/2 mRNA were made preparatively (10μg of mRNA) and separated on a 12% acrylamide-urea gel. As can be seen from Figure 44, which shows the autoradiograph used to excise the products (3hrs exposure), there are four species of extension product, seen previously (Fig.43) as doublets. These were excised individually and recovered by electroelution and passage over DE52 cellulose followed by ethanol precipitation.

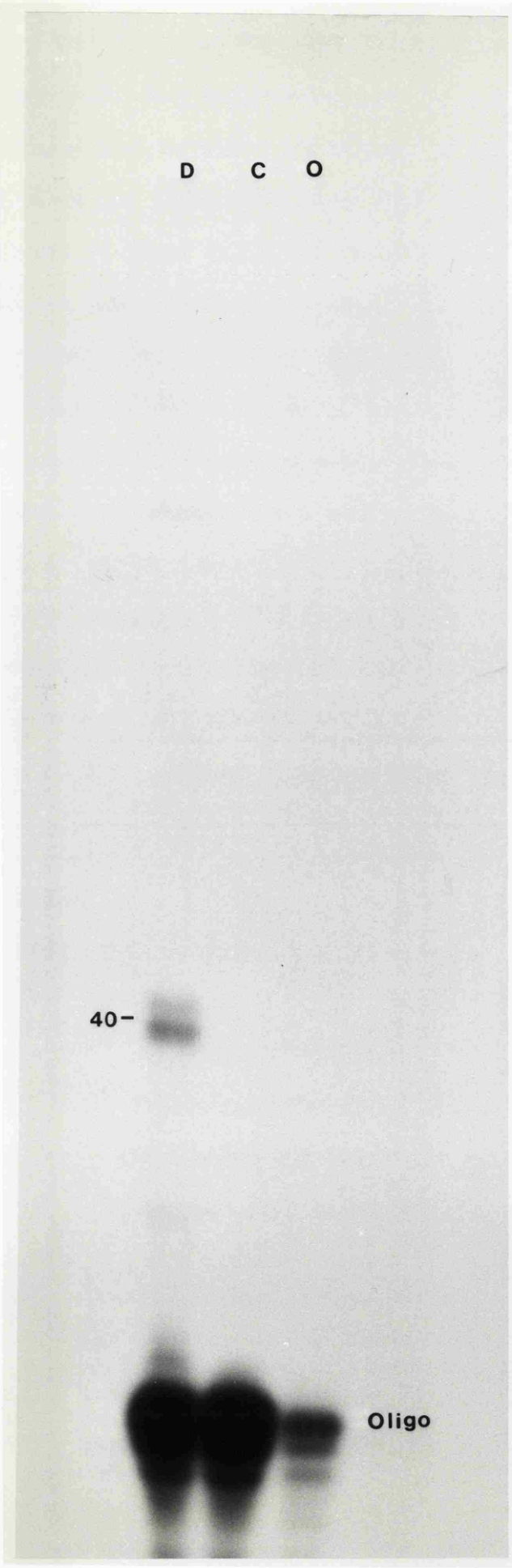
Each cDNA was subjected to sequencing chemistry according to the method of Maxam and Gilbert (1980) and the cleavage products separated out on a

**Fig.43** - Primer extension of MuRen using kinased primer as a source of label **(left)**

| Lane | | Sample |
|---|---|---|
| D | ♂ | DBA/2 mRNA products |
| C | ♂ | C57BL/10 mRNA products |
| O | | kinased oligonucleotide marker |

**Fig.44** - Preparative primer extension using male DBA/2 SMG mRNA **(right)**

A preparative primer-extension (D(P)) was carried out (see text) and the products separated by denaturing gel electrophoresis. The autoradiograph shown here reveals four products. Marks on the autoradiograph show the positions of location points for excision of the products from the gel.


D
C
O
40–
Oligo


D(p)
40 –
1
2
3
4

0.4mm thick acrylamide-urea gel run at 1600V to keep the fragments denatured (for details, see Chapter 2). The gel was wrapped in "clingfilm" and placed on high-sensitivity film for four weeks. The autoradiograph of that gel is shown in Figure 45 and reveals that the sequences of the four products are identical, except that each differs in length from its neighbour(s) by a single nucleotide. These sequences can be read with confidence to within a single base of their 5'ends and are compared to the published cDNA sequence (Fig.46). This indicates that the longest extension product terminates some 7-8 nucleotides from the 5'end of the cDNA clone of Panthier *et al* (1982a). The extreme 5' seven nucleotides of that cDNA sequence are present as a direct repeat some 70 nucleotides from the 5' end (nucleotides 70-77). Similar features have been seen in an aberrant cDNA clone made in this laboratory and it was not impossible, therefore, that the 5'end of the published clone was an artifact.

```
  TCTGGGC                                                    TCTGGGC
5'                                        --->                        3'
  TCTGGGCTACACAGCTCTTAGAAAGCCTTGGCTGAACCAGATGGACAGGAGGAGGATGCCTTCTGGGC

                                  CGACTTGGTCTACCTGTCC  MuRen

                GTCGAGAATCTTTCGGAACCGACTTGGTCTACCTGTCC  cDNA 1

               TGTCGAGAATCTTTCGGAACCGACTTGGTCTACCTGTCC  cDNA 2

              GTGTCGAGAATCTTTCGGAACCGACTTGGTCTACCTGTCC  cDNA 3

             TGTGTCGAGAATCTTTCGGAACCGACTTGGTCTACCTGTCC  cDNA 4
```

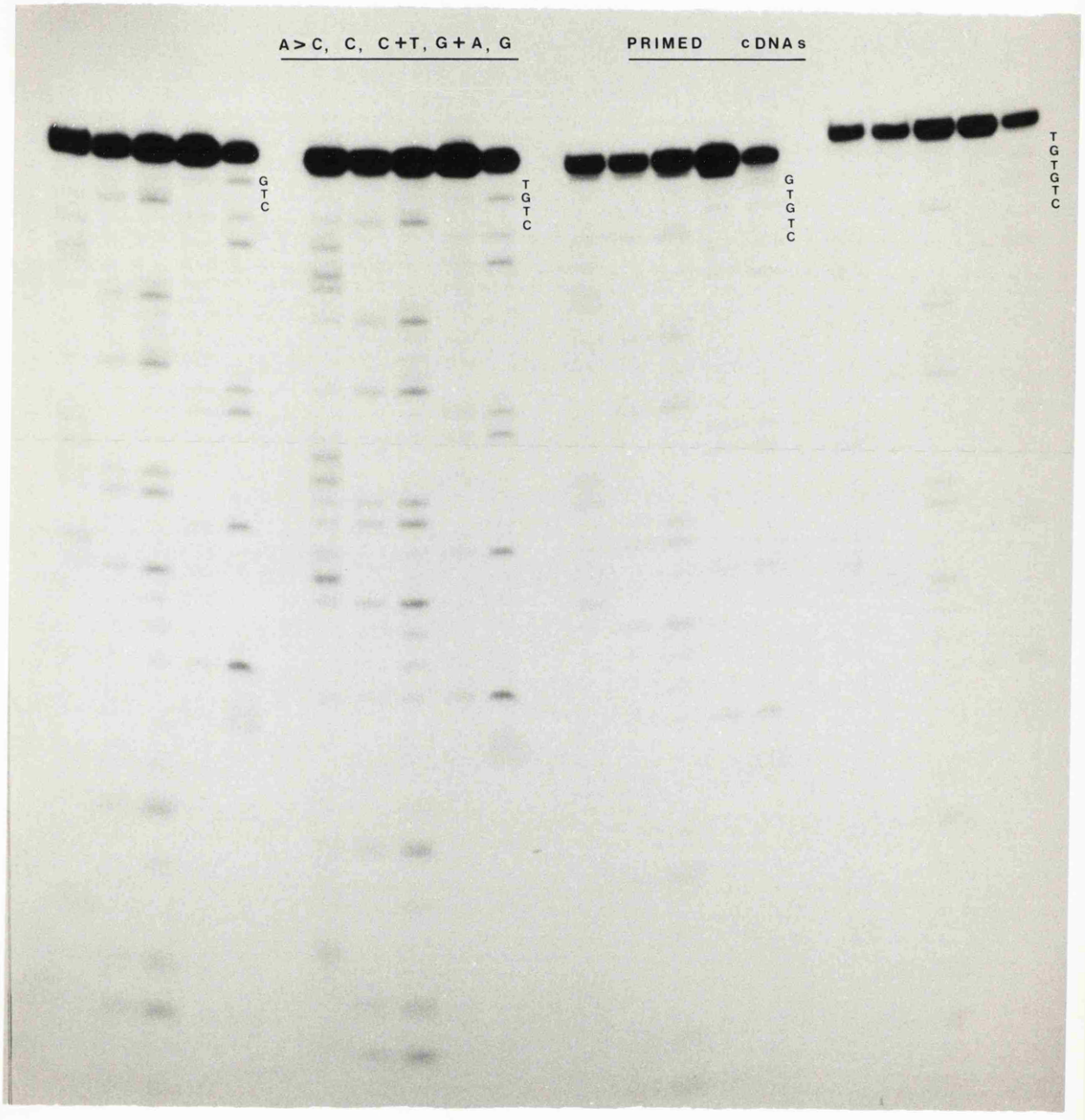
**Fig.46** - Nucleotide sequences of MuRen primed extension products

The sequence of the primer MuRen and the four extension products obtained by reverse transcription of DBA/2 mRNA are listed with respect to the sequence at the 5' end of the renin cDNA clone of Panthier *et al*(1982a). The repetition of the sequence TCTGGC is shown at the top of the figure (see text). ---> = ATG initiation codon.

Primer extensions of this type usually give rise to two products of closely similar size and this is believed to be caused by the presence of $m^7G^{5'}ppp^{5'}N$, the so called "cap" structure, present at the 5'ends of

Fig.45 - Sequencing of MuRen extension products

The four extension products shown in figure 41 were subjected to 'Maxam and Gilbert' sequencing chemistry and analysed electrophoretically. The autoradiograph shows each of the sequences with that of the largest product on the right. The sequencing reactions used were: A>C, C, C+T, G+A and G


A>C, C, C+T, G+A, G
PRIMED cDNAs
GTC
TGTC
GTGTC
TGTGTC

eukaryotic mRNAs (Shatkin *et al*,1979). The presence of four such products does not necessarily mean two transcription start points, as reports of two products are usually based on gel analysis of the type shown in Figure 43. Sequencing of the 5'end of the *Ren*-1 gene by D.Burt and others in this laboratory agreed with *Ren*-2 cDNA sequence published by Panthier *et al*, including the extreme 5'end, indicating that the seven 5' nucleotides were not an artefact. The possibility was therefore considered that secondary structure may block reverse-transcription and that the four products represented a stuttering effect. Such secondary structure would be aided by the slow annealing used in the synthesis. Computer analysis of the 5' sequence revealed no good potential hairpin loops that would block synthesis. In addition, there is no run of guanosine residues which are known to cause reverse-transcriptase to fall away from the molecule.

Further cDNA cloning in the laboratory yielded a DBA/2 SMG renin cDNA clone spanning from nucleotide 99 of the published sequence to the polyA tail, allowing the isolation of restiction fragments for use as probes and primers. In particular, a 33bp *Hinf*1 restriction fragment covering residues 100-133 of the published sequence was isolated and after phosphatase treatment (using the enzyme calf intestinal alkaline phosphatase) the DNA was kinase labelled. This fragment was then used for primer-extension with male DBA/2 and C57BL/10 mRNAs and also mRNA isolated from testosterone-treated female DBA/2 SMG (see chapter 8). Both DBA/2 mRNA samples gave rise to extension products of 105 nucleotides in length. This cDNA was not detectable in the C57BL/10 track. The length of this cDNA predicts a termination point at the same position as determined by extension of MuRen.

MuRen-directed extension products of 40-45 nucleotides are also seen with other methods including that of Das *et al* (1983) (observations of L.Beecroft and JJM). Therefore, in the absence of evidence to suggest premature termination, these data must define a major start of transcription for the *Ren*-2 gene. In view of the published cDNA sequence, it

is not the only transcription start point, but extension products larger than 40-45 nucleotides are not easily detected even when the primer is in excess. When the concentration of primer is increased still further, from 1.4pmoles to 7pmoles per annealing reaction (7μl)(Fig.47), many higher molecular weight species are seen. These reflect increasing background as the primer becomes more abundant.

Since MuRen was in excess at 1.4pmoles per μg mRNA, and the background obtained at this concentration was very low, an attempt was made to look for any cDNAs greater than 45 nucleotides in length. Seven pmoles of the primer were kinased and divided into three equal aliquots. Two of these were boiled for 1 minute with 1μg of male DBA/2 SMG mRNA, and the third was denatured in a similar manner with 1μg male C57BL/10 mRNA. The C57BL/10 sample and one of the DBA/2 samples were "flash-cooled" by placing the tubes into a dry-ice methanol bath (a method suggested by Dr.M.Edge) and then subjected to the extension reaction. The second DBA/2 sample was treated in the normal way. The extension products from the three reactions are shown in Figure 48, which shows a long exposure of the 12% acrylamide-urea gel used to analyse the samples. The quick-cooling method was found to generate a very high background which was identical in the C57BL/10 and DBA/2 samples, indicating that these species were not renin-related. This background is also identical to that obtained in conditions of vast primer excess (Fig.47). The 40-45 nucleotide,*Ren*-2 mRNA directed extension products are clearly visible as a group of four over-exposed bands. In addition to these species, two larger products are seen in both DBA/2 tracks, which are absent from the C57BL/10 track. Using the normal method (Fig.48;lane3) these species can be seen over a very clean background. Their sizes are 75 and 77 nucleotides and they therefore predict one or two transcription starts some 27-29bp upstream of the published cDNA sequence. To confirm that these species are derived from renin mRNA, the best approach would be to use a primer that would anneal 5' to the transcription start point predicted by MuRen, and carry out an extension experiment which should yield a

**Fig.47** - Primer extension using excess primer

| Lane | Sample |
|---|---|
| 1 | 33 bp marker |
| 2 | 0.2 pmoles primer |
| 3 | 1.4 pmoles primer |
| 4 | 7.0 pmoles primer |
| 5 | 58 bp marker |

Molecular weight markers are shown on the left hand side of the figure.

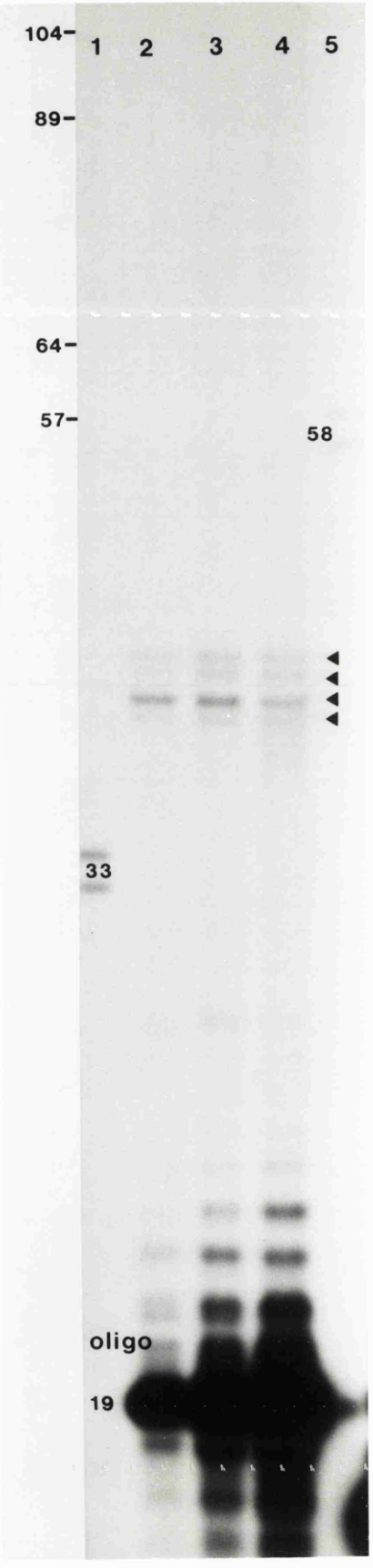
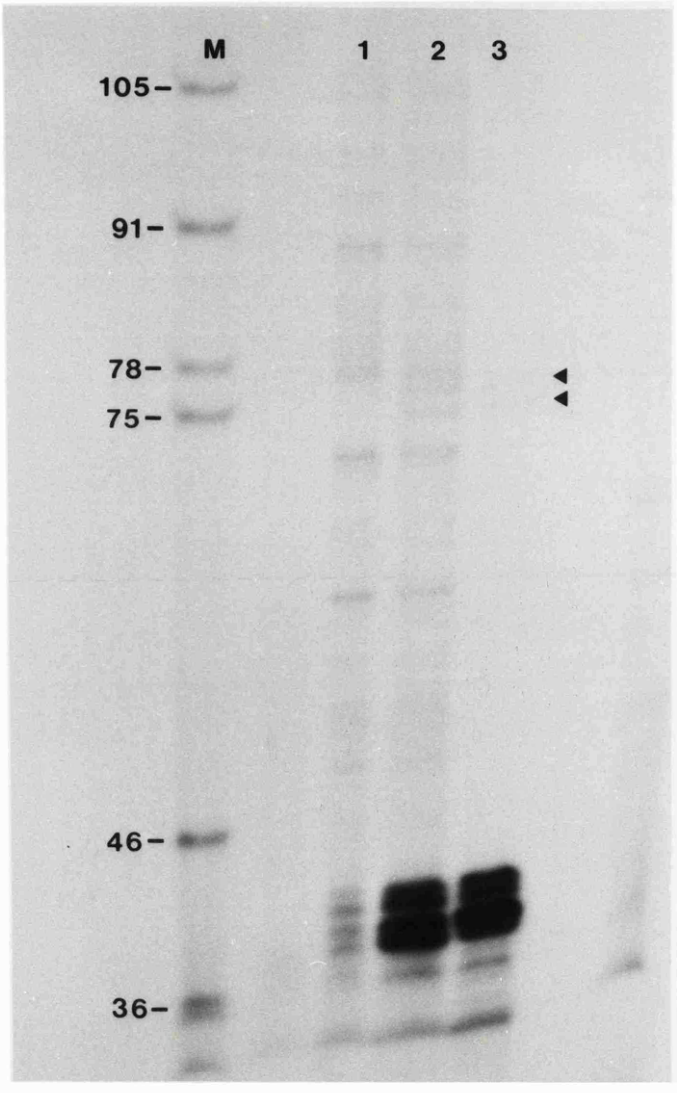

104
89
64
57
1
2
3
4
5
58
33
oligo
19

**Fig.48** - Primer extension of MuRen to locate further 5' termini

| Lane | | Sample |
|---|---|---|
| M | | molecular weight markers |
| 1 | ♂ | C57BL/10 SMG mRNA, quick cool annealing |
| 2 | ♂ | DBA/2 SMG mRNA, quick cool annealing |
| 3 | ♂ | DBA/2 SMG mRNA, slow annealing |


M
1
2
3
105–
91–
78–
75–
46–
36–

product(s) predicting an identical start point.

7.3 $S_1$ mapping of the *Ren*-1 gene using DBA/2 SMG mRNA

The enzyme $S_1$ nuclease isolated from *Aspergillus oryzae* is a single-strand specific endonuclease widely used to remove single stranded DNA from a mixture of single and double-stranded DNA. One of its most common applications is in the removal of single-stranded hairpin loops during the synthesis of cDNA clones.

A second major use of $S_1$ nuclease is the removal of the single-stranded DNA from an mRNA-DNA hybrid. Having approximately located the 5'end of a gene, a specific restriction fragment containing the potential transcription start(s) can be isolated, labelled and either strand-separated or denatured, and then hybridised to mRNA(s) originating from the gene in question. After suitable hybridisation, the reaction is quickly cooled and then treated with $S_1$ nuclease to remove both the non-complementary strand of the restriction fragment and also any complementary strand not hybridising to the mRNA. After denaturation, the product of this reaction is a single-stranded DNA fragment from whose length it is possible to extrapolate the start of transcription. The technique may also be used to determine intron-exon boundaries and the 3'ends of mRNAs.

Genomic subcloning led to the determination of the 5' nucleotide sequence of the *Ren*-1 gene (Fig.49)(Burt *et al*, in preparation), and allowed the prediction of a suitable restriction fragment for use in $S_1$ mapping. The fragment chosen was the 239bp *Hinf*I fragment between nucleotides 541 and 779 in Figure 49. The fragment was isolated and the DNA phosphatased, kinased and then denatured (80% formamide) prior to hybridisation to SMG mRNA (total polyA$^+$ RNA). Because of the much lower amounts of renin mRNA in the C57BL/10 SMG, male DBA/2 SMG mRNA was used initially to optimise the technique. After hybridisation of male DBA/2 SMG mRNA to the 239 nucleotide *Hinf*I fragment the resultant hybrid was treated with $S_1$ nuclease and analysed by denaturing gel electrophoresis. Figure 50

Fig.49 - 5' nucleotide sequence of the DBA/2 *Ren*-1 gene - origin of $S_1$ mapping probe and putative transcription start points

The nucleotide sequence of the 815 bp *Xba*I fragment containing the 5' end of the *Ren*-1 gene. The figure shows the origin of the 239 bp *Hinf*I fragment used in the $S_1$ studies. To orientate the relative position of this fragment, the translational initiation codon (MetAspArg->) and putative cleavage point for the signal sequence are indicated (X). The splice site between Exon-1 and Intron-A is shown at co-ordinate 486.
P1, P2 and P3 represent potential transcription start points derived by $S_1$ mapping (see text).
*1 indicates the 5' extent of published cDNA sequences.

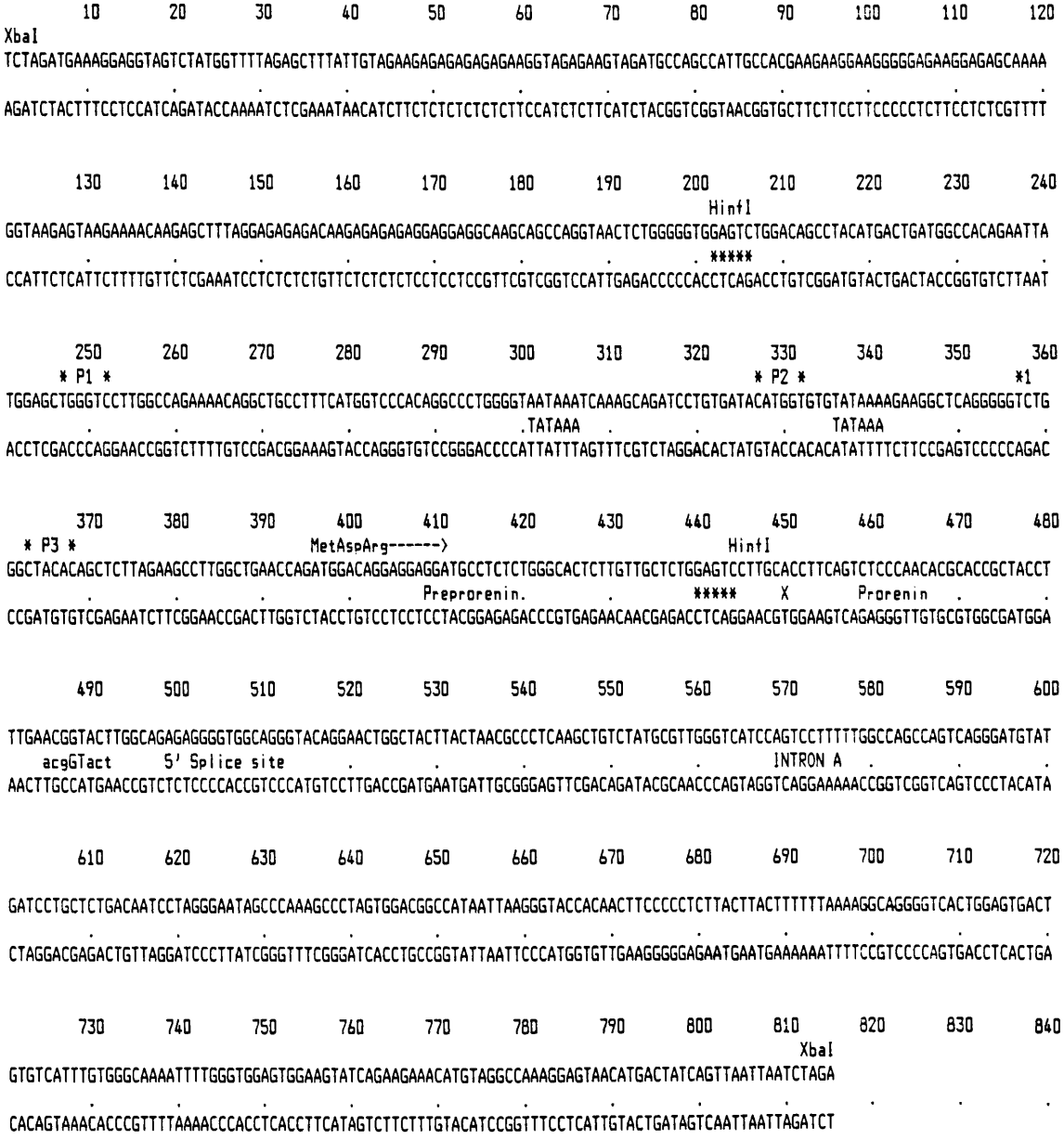
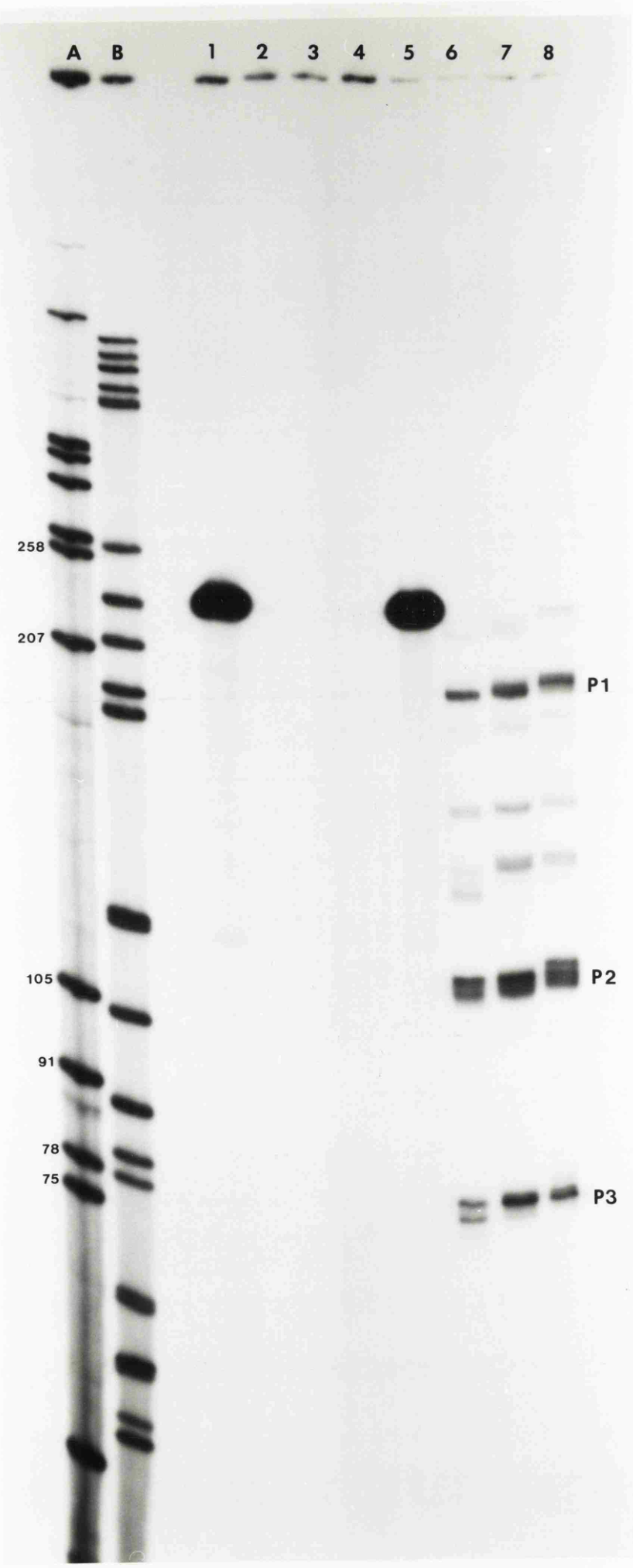

10 20 30 40 50 60 70 80 90 100 110 120
XbaI
TCTAGATGAAAGGAGGTAGTCTATGGTTTTAGAGCTTTATTGTAGAAGAGAGAGAGAAGGTAGAGAAGTAGATGCCAGCCATTGCCACGAAGAAGGAAGGGGGAGAAGGAGAGCAAAA
AGATCTACTTTCCTCCATCAGATACCAAAATCTCGAAATAACATCTTCTCTCTCTCTCTTCCATCTCTTCATCTACGGTCGGTAACGGTGCTTCTTCCTTCCCCCTCTTCCTCTCGTTTT
130 140 150 160 170 180 190 200 210 220 230 240
HintI
GGTAAGAGTAAGAAAACAAGAGCTTTAGGAGAGAGACAAGAGAGAGAGGAGGAGGCAAGCAGCCAGGTAACTCTGGGGGTGGAGTCTGGACAGCCTACATGACTGATGGCCACAGAATTA
***** .
CCATTCTCATTCTTTTGTTCTCGAAATCCTCTCTCTGTTCTCTCTCTCCTCCTCCGTTCGTCGGTCCATTGAGACCCCCACCTCAGACCTGTCGGATGTACTGACTACCGGTGTCTTAAT
250 260 270 280 290 300 310 320 330 340 350 360
* P1 * * P2 * *1
TGGAGCTGGGTCCTTGGCCAGAAAACAGGCTGCCTTTCATGGTCCCACAGGCCCTGGGGTAATAAATCAAAGCAGATCCTGTGATACATGGTGTGTATAAAAGAAGGCTCAGGGGGTCTG
.TATAAA TATAAA
ACCTCGACCCAGGAACCGGTCTTTTGTCCGACGGAAAGTACCAGGGTGTCCGGGACCCCATTATTTAGTTTCGTCTAGGACACTATGTACCACACATATTTTCTTCCGAGTCCCCCAGAC
370 380 390 400 410 420 430 440 450 460 470 480
* P3 * MetAspArg------> HintI
GGCTACACAGCTCTTAGAAGCCTTGGCTGAACCAGATGGACAGGAGGAGGATGCCTCTCTGGGCACTCTTGTTGCTCTGGAGTCCTTGCACCTTCAGTCTCCCAACACGCACCGCTACCT
Preprorenin. ***** X Prorenin
CCGATGTGTCGAGAATCTTCGGAACCGACTTGGTCTACCTGTCCTCCTCCTACGGAGAGACCCGTGAGAACAACGAGACCTCAGGAACGTGGAAGTCAGAGGGTTGTGCGTGGCGATGGA
490 500 510 520 530 540 550 560 570 580 590 600
TTGAACGGTACTTGGCAGAGAGGGGTGGCAGGGTACAGGAACTGGCTACTTACTAACGCCCTCAAGCTGTCTATGCGTTGGGTCATCCAGTCCTTTTTGGCCAGCCAGTCAGGGATGTAT
acgGTact 5' Splice site INTRON A
AACTTGCCATGAACCGTCTCTCCCCACCGTCCCATGTCCTTGACCGATGAATGATTGCGGGAGTTCGACAGATACGCAACCCAGTAGGTCAGGAAAAACCGGTCGGTCAGTCCCTACATA
610 620 630 640 650 660 670 680 690 700 710 720
GATCCTGCTCTGACAATCCTAGGGAATAGCCCAAAGCCCTAGTGGACGGCCATAATTAAGGGTACCACAACTTCCCCCTCTTACTTACTTTTTTAAAAGGCAGGGGTCACTGGAGTGACT
CTAGGACGAGACTGTTAGGATCCCTTATCGGGTTTCGGGATCACCTGCCGGTATTAATTCCCATGGTGTTGAAGGGGGAGAATGAATGAAAAAATTTTCCGTCCCCAGTGACCTCACTGA
730 740 750 760 770 780 790 800 810 820 830 840
XbaI
GTGTCATTTGTGGGCAAAATTTTGGGTGGAGTGGAAGTATCAGAAGAAACATGTAGGCCAAAGGAGTAACATGACTATCAGTTAATTAATCTAGA
CACAGTAAACACCCGTTTTAAAACCCACCTCACCTTCATAGTCTTCTTTGTACATCCGGTTTCCTCATTGTACTGATAGTCAATTAATTAGATCT

**Fig.50** - $S_1$ mapping of DBA/2 SMG renin mRNA

| Lane | Sample | | |
|---|---|---|---|
| A | pBR322 X *Sau*3A | | |
| B | pBR322 X *Hae*III | | |
| | 240 bp *Hinf*I probe | mRNA | $S_1$ |
| 1 | + | - | - |
| 2 | + | - | 12 units/ml |
| 3 | + | - | 96 units/ml |
| 4 | + | - | 800 units/ml |
| 5 | + | + | - |
| 6 | + | + | 12 units/ml |
| 7 | + | + | 96 units/ml |
| 8 | + | + | 800 units/ml |


A
B
1
2
3
4
5
6
7
8
258
207
105
91
78
75
P1
P2
P3

shows that the result is not simple, and that there are three major groups of protected fragments of approximately 198, 112 and 75 nucleotides in length. All three potentially represent starts of transcription and to distinguish between them they have been called P1, P2 and P3 respectively. As Figure 53 shows, P2 appears quantitatively the most important of the three. When mapped within the sequence of the *Hinf*I restriction fragment, P2 coincides well with a potential "TATAA box" (Fig.49). However, 35bp downstream is a second potential "TATAA box" and P3 maps to a position from which one would expect transcription to begin if this second TATAA box was being used. The third position, P1, defined by the 198 nucleotide fragment has no potential TATAA sequence. None of these putative start points possess the "CAAT" consensus sequence. This feature is not unique since the genes coding for the C1 and C2 peptides of rat prostate steroid binding protein (Parker *et al*,1982) and human myoglobin (Weller *et al*,1984) also lack this sequence.

The position of P3 is interesting since it maps accurately to the predicted start of transcription determined by primer-extension using MuRen. It was therefore possible that P3 represented the start of transcription of the most active gene, *Ren*-2. To extend the $S_1$ study the reaction was carried out using three different concentrations of male DBA/2 SMG mRNA (8, 2 and 0.5$\mu$g) in the standard reaction (annealing at 52°C; 130 units/ml $S_1$ nuclease, 30 minutes at 37°C). The protected fragments were qualitatively the same but the amount of 75 nucleotide fragment (P3) varied in a different manner to the other fragments. Increasing the mRNA concentration four-fold from 0.5$\mu$g per 10$\mu$l to 2$\mu$g per 10$\mu$l resulted in a similar increase in all the protected fragments, indicating that the *Hinf*1 fragment is present in excess. When the mRNA concentration was increased a further four-fold (8$\mu$g/10$\mu$l) the 198 and 112 nucleotide fragments, as well as the other minor species, once again increased in abundance. The 75 nucleotide species (P3), however, showed a sharp decrease in abundance (Fig.51a). The point is being reached where the DNA is no longer in excess

**Fig.51** - Effect of varying $S_1$ mapping conditions

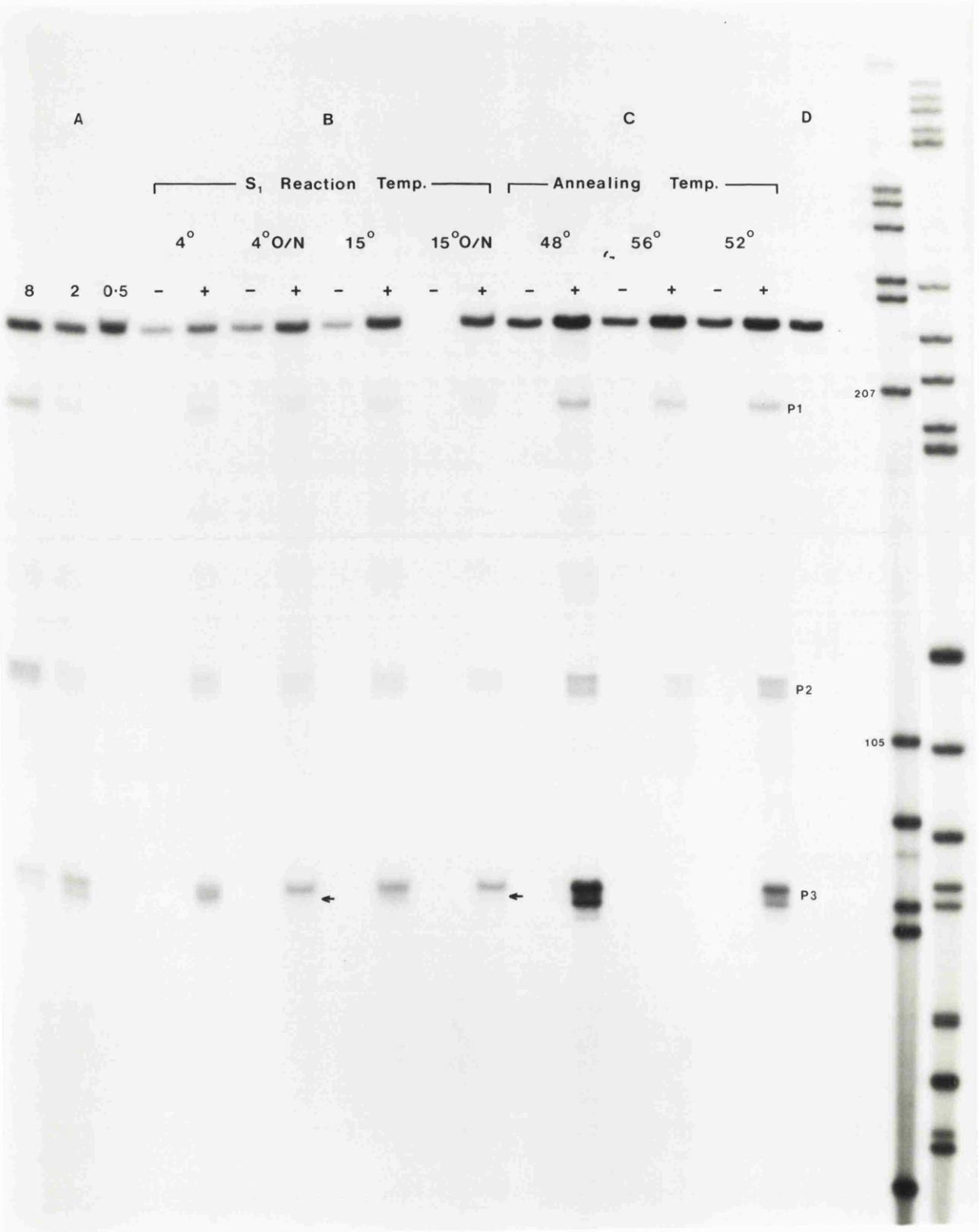
(A) Lane

| | |
|---|---|
| 8 | 8μg DBA/2 SMG mRNA |
| 2 | 2μg DBA/2 SMG mRNA |
| 0.5 | 0.5μg DBA/2 SMG mRNA |

(B) After annealing at 52°C, hybrids were digested with 130 units/ml of $S_1$ nuclease (+) at either 4°C or 15°C for 30 minutes or overnight (O/N) as indicated. In addition, control digestions were carried out in the absence of $S_1$ nuclease (−). The position of a protected fragment which is affected by the digestion conditions is arrowed.

(C) DBA/2 mRNA was annealed to the probe at 48°C, 52°C and 56°C. The hybrids were then split and either digested with 130 units/ml of $S_1$ nuclease (+) or incubated in the absence of the enzyme (−).

(D) $S_1$ mapping of 2μg male C57BL/10 SMG mRNA using the same probe. (52°C annealing, 130 units/ml $S_1$ nuclease).


A
B
C
D
S1 Reaction Temp.
Annealing Temp.
4°
4°O/N
15°
15°O/N
48°
56°
52°
8
2
0·5
– + – + – + – + – + – + – +
P1
P2
P3
207
105

and the mRNA species themselves compete for hybridisation. Titrations of this kind should be carried out in order to avoid misleading results that may be obtained if the DNA fragment is not in excess.

One explanation for the presence of so many minor protected fragments is the possibility of mismatches between the sequence of the 239bp *Hinf*I *Ren*-1 restriction fragment and the mRNA originating from the *Ren*-2 gene. Such mismatches could result in cleavage of the hybrid yielding shorter protected fragments. This may be studied by analysing samples on both a denaturing gel and a non-denaturing gel and comparing the two sets of data. In addition, if the $S_1$ reaction is carried out at a temperature of 0°-20°C cleavage of single-stranded DNA opposite mismatches is reduced (Maniatis *et al*,1983). If the $S_1$ reaction is carried out at 37°C and such nicking occurs, then its presence is revealed in the type of gel comparison mentioned above. This is possible due to the enzyme's inability to cleave a ribophosphodiester bond opposite a nick in the DNA strand of an mRNA-DNA duplex (Beard *et al*,1973).

The annealing temperature was also varied and proved to have a dramatic effect on the results obtained. To observe the effect of reaction temperature, a DBA/2 SMG mRNA, *Hinf*I, "239" hybrid was made as described above. A control annealing which contained no mRNA was used in parallel. Each was split into four equal aliquots and $S_1$ nuclease was added to a concentration of 130 units/ml. Two aliquots were incubated at 15°C and the remaining pair were incubated at 4°C. After one hour, one reaction from each pair was terminated and the protected fragments recovered. The remaining two samples were incubated overnight (20hrs) at their respective temperatures and then processed for gel analysis (Fig.51b).

In a separate experiment, three identical hybridisations were prepared and after denaturation at 82°C one was annealed at 48°C, the second at 52°C and the third at 56°C. After their annealings, the hybrids formed were treated with 130 units/ml of $S_1$ nuclease for 30min at 37°C and the protected fragments recovered and analysed in the normal way. Figure 51b shows the

protected fragments generated during $S_1$ treatment of hybrids at 4°C and 15°C, and Figure 51c compares the fragments obtained after annealing at different temperatures.

Two observations can be made from these data. Firstly, the temperature under which the $S_1$ nuclease reaction is carried out makes no significant difference to the complexity of protected fragments. The only noticeable change is found in the smaller of the two ≈75 nucleotide species. This is arrowed in Figure 51b and is reduced in abundance after overnight exposure to $S_1$ nuclease, but at present the significance of this $S_1$ sensitivity is not clear. Secondly, whilst making no difference to the relative amounts of P1 and P2 type protection fragments, the temperature of the annealing is critical for the presence/absence of P3-related species. At 52°C, the standard temperature used in these experiments, the 75 nucleotide P3-related species are major fragments. If the hybridisation temperature is lowered to 48°C these species become significantly more abundant and appear to be the major product of the reaction. Increasing the temperature to 56°C, whilst having no effect on other species, results in a virtual abolition of the P3-related species (Fig.51c).

The samples used in the above experiments were also analysed on a non-denaturing acrylamide gel and showed the same pattern of protection as Figure 51(a-c) but it was more diffuse than the denaturing gel, presumably due to secondary structure and heterogeneity of fragment size. It showed P1, P2, P3 and the minor fragments, which, taken together with the results from denaturing gel analysis of the products obtained at different $S_1$ reaction temperatures, means that P2, P3 and possibly many of the minor fragments are not the result of cleavage by $S_1$ nuclease at regions of mismatch.

The disappearance of P3-type species caused by mRNA excess is very similar to the results obtained with polyomavirus late mRNAs (Favaloro *et al*,1980), in which the smallest and most abundant fragment is reduced in quantity relative to the two larger species. This reduction, like that of

the P3-type fragments, occurs over an increase in mRNA concentration of 2-4 fold. The effect of increasing mRNA concentration is paralleled by an increase in the annealing temperature, and the two observations are related: the introduction of competition between mRNA species results in the more stable mRNAs being dominant, and less stable mRNAs (e.g. those possessing shorter 5' sequences) are only seen quantitatively under conditions of DNA excess or the mildest possible hybridisation conditions.

## 7.4 $S_1$ mapping of the *Ren*-1 gene using C57BL/10 SMG mRNA

Having studied several parameters for the $S_1$ nuclease reaction using DBA/2 SMG mRNA and found quite a complex group of protected fragments, it was decided to use the *Hinf*I 239bp fragment with male C57BL/10 SMG mRNA. Because of the much lower mRNA concentration, 40$\mu$g of C57BL/10 mRNA was used in each reaction in contrast to the normal 2$\mu$g of DBA/2 mRNA used in the above experiments. The annealings were carried out at 52°C and the hybrids were digested with either 130 or 520 units/ml of $S_1$ nuclease at 37°C for 30mins, then analysed as previously described. The autoradiograph of the subsequent denaturing gel is reproduced in Figure 52 and the major group of protected fragments is 195-200 nucleotides in length,corresponding very well to the P1-type fragments seen with DBA/2 mRNA. When 130 units of $S_1$ nuclease were used, many other species were seen including minor ones of ≈75 nucleotides in length. These are not seen when only 2$\mu$g of C57BL/10 mRNA is used (Fig.51d), and therefore do not mean that an mRNA excess is hiding P3 activity. A summary of the data generated from primer extension, $S_1$ mapping and *in vitro* transcription is shown in Figure 53.

In discussing these results at the present time, an assumption must be made : the low producer renin gene and the *Ren*-2 gene must both have very similar but not identical 5' structures to that of the *Ren*-1 gene. Given the data presented in this chapter, one can then postulate that *Ren*-2 is transcribed primarily from P3 but also from P2. The low producer gene is transcribed from P1, and the homologous *Ren*-1 gene is active in the high

**Fig.52** - (a) $S_1$ mapping of male C57BL/10 SMG mRNA

| Lane | Treatment |
|---|---|
| 1 | 130 units/ml $S_1$ |
| 2 | 550 units/ml $S_1$ |

M.Wt. markers are shown on the left of the figure.

(b) Summary of $S_1$ protection data

**Fig.53** - Transcription of renin **(overleaf)**

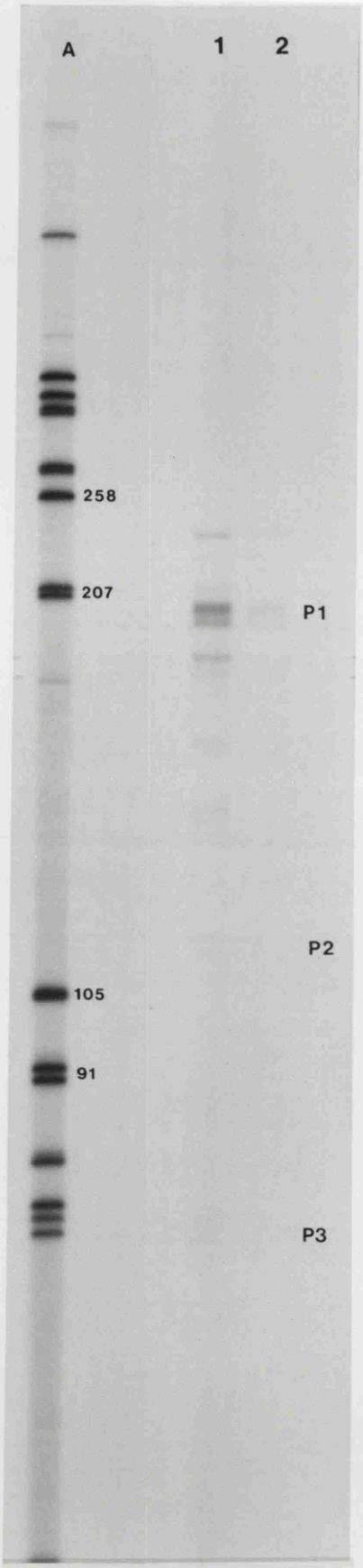
This figure summarises the data from several experiments to locate potential transcription start points.
The number of arrows is not directly related to abundance of product but is intended as a guide within each individual experiment.

IVT = *in vitro* transcription of *Ren*1 (L.Beecroft, personal comm.)

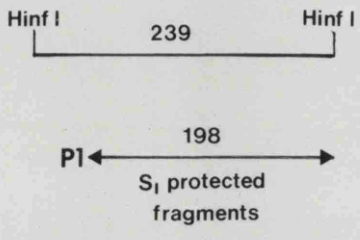
PRIMEXT = primer extension of male DBA/2 SMG mRNA

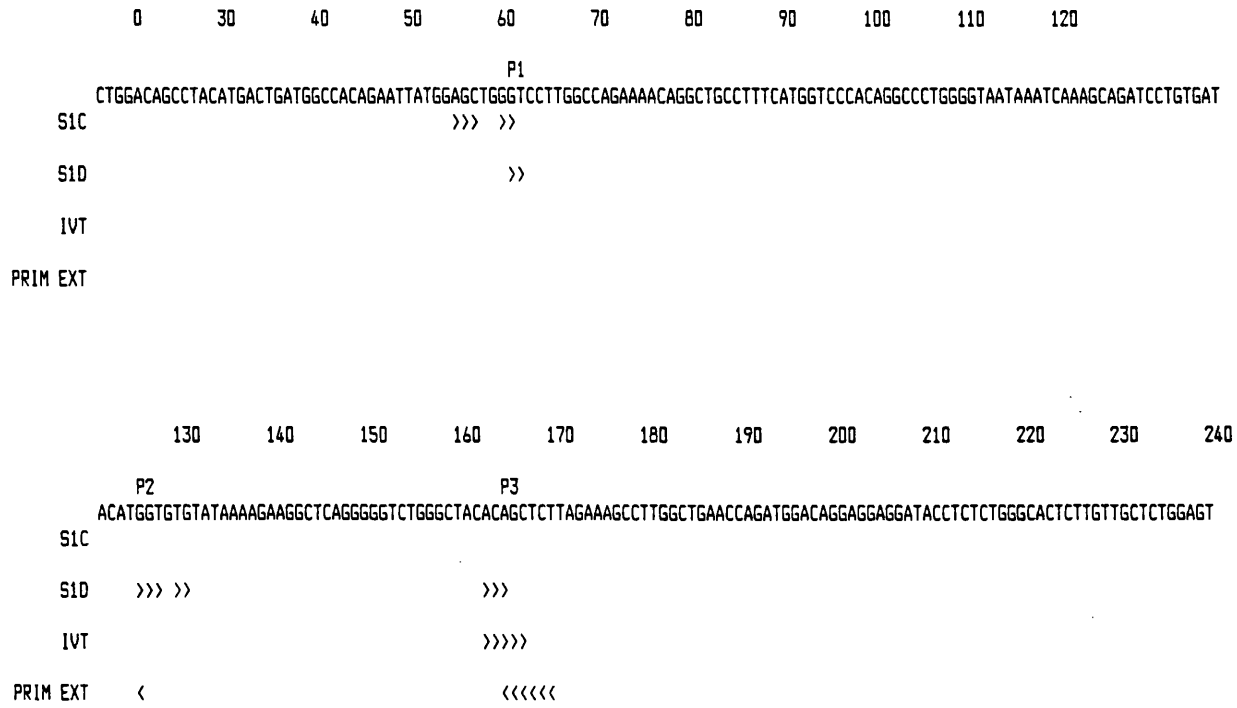
$S_1C$ = $S_1$ mapping of male C57BL/10 SMG mRNA

$S_1D$ = $S_1$ mapping of male DBA/2 SMG mRNA


A
1
2
258
207
P1
P2
105
91
P3

b


Hinf I
239
Hinf I
198
P1
S1 protected
fragments


0 30 40 50 60 70 80 90 100 110 120
P1
CTGGACAGCCTACATGACTGATGGCCACAGAATTATGGAGCTGGGTCCTTGGCCAGAAAACAGGCTGCCTTTCATGGTCCCACAGGCCCTGGGGTAATAAATCAAAGCAGATCCTGTGAT
S1C
S1D
IVT
PRIM EXT
130 140 150 160 170 180 190 200 210 220 230 240
P2
P3
ACATGGTGTGTATAAAAGAAGGCTCAGGGGGTCTGGGCTACACAGCTCTTAGAAAGCCTTGGCTGAACCAGATGGACAGGAGGAGGATACCTCTCTGGGCACTCTTGTTGCTCTGGAGT
S1C
S1D
IVT
PRIM EXT

producer mouse SMG in addition to *Ren*-2. The last point is speculative, however, since *Ren*-2 may be partially or entirely responsible for the P1-type fragments seen with DBA/2 mRNA and this question is studied in more detail in Chapter 8.

# CHAPTER 8

## THE EFFECT OF TESTOSTERONE ON RENIN GENE EXPRESSION

### 8.1 Effect Of Testosterone On Total SMG And Kidney Renin mRNA Concentrations.

Renin expression in the SMG of both high- and low-producer strains of mice is known to be under the control of androgens and thyroxine. The basal level is controlled by thyroxine and can be induced by up to 20 fold after treatment with androgens (Wilson *et al*,1981). In the same paper Wilson *et al* commented that no sexual dimorphism has been found in kidney renin activity in any inbred strains. This is not the situation one would predict from the Northern blotting data described in Chapter 6. The results of those experiments confirmed and extended the observations of Rougeon *et al* (1981) that there is more renin mRNA in the kidneys of female mice than in those of males. This was seen to be true of both high (DBA/2) and low (C57BL/6) producer strains.

It was decided to try and confirm this result in a more quantitative manner. Dot-hybridisation has become widely used as a method for quantifying changes in the levels of specific mRNAs (*e.g.* Piccini *et* al,1982), and one method is to probe a series of dilutions of the mRNA samples and use the limit of detection of each series as an end-point to quantify one sample relative to the next. A protocol for this technique was obtained from Dr.G.Birnie (Beatson Institute, Glasgow) and it was tested using mRNA prepared from male and female DBA/2, and male C57BL/6 submaxillary glands. The concentrations of the mRNA samples were determined accurately and 2$\mu$g of each was diluted with 18$\mu$l of 0.05mg/ml calf-liver tRNA in TE. Twelve one-in-two serial dilutions of each DBA/2 mRNA and six dilutions of the male C57BL/6 mRNA were then made, the diluent being the same as above. Two samples of each dilution, 2$\mu$l (0.2$\mu$g) and 4$\mu$l (0.4$\mu$g), were then spotted onto a gridded nitrocellulose filter and dried. After

baking at 80°C for 2hrs the filter was prehybridised for 4hrs in a buffer containing 50% formamide as detailed in Materials and Methods. The hybridisation solution was then changed and the probes, prepared by nick-translating the isolated cDNA inserts of pSMG213 and pSMG142 (specific activity: $4x10^7$ cpm/$\mu$g), were denatured, added, and hybridised overnight at 42°C. The filters were then washed six times in hybridisation wash solution, and blotted dry. After autoradiography for 12hrs the difference between SMG renin mRNA levels in male and female mice could be clearly seen (Fig.54), and longer exposure also allowed the male C57BL/6 renin mRNA to be detected. By comparing the end-points of detection in these hybridisations it was found that there was four times more renin mRNA in the DBA/2 male SMG than in the female SMG, a figure that parallels published data on the relative renin activities. In addition there was ≈200 fold less renin mRNA in the male C57BL/6 SMG as compared to male DBA/2 mRNA. Again this is in agreement with published renin activity figures (Wilson *et al*,1981).

A large-scale experiment was prepared in which the same methodology was used to determine the relative levels of renin mRNA in the SMG and kidney of male and female DBA/2 and C57BL/6 mice. In addition to the control mice,

**Fig.55** - Outline of testosterone treatment experiment

| | MALE | | | FEMALE | |
|---|---|---|---|---|---|
| WEEK | CONTROL | CASTRATE | CASTRATE (C) +TESTOSTERONE | CONTROL | +TESTOSTERONE (T) |
| | | | C | | T |
| 1 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 2 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 3 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 4 | ↓ | ↓ | ↓ | T | |
| 5 | | | ↓ | | |
| 6 | | | ↓ | | |
| 7 | | | ↓ | | |

mRNA was isolated from the SMG and kidneys of castrated males, control-castrates, which were given testosterone inplants, and testosterone-treated

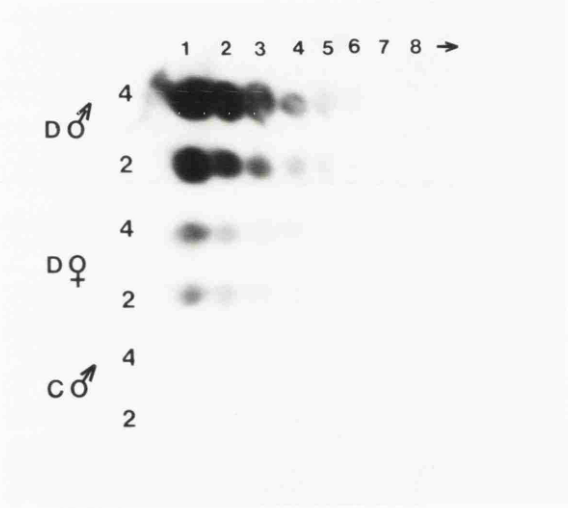
**Fig.54** - Pilot RNA dot blot

D ♂ : male DBA/2 SMG mRNA

D ♀ : female DBA/2 SMG mRNA

C ♂ : male C57BL/6 SMG mRNA

1-8 –> : 1 in 2 serial dilutions of mRNA samples


1 2 3 4 5 6 7 8 →
D ♂
4
2
D ♀
4
2
C ♂
4
2

female mice. Ten mice were used for each experimental group and the timescales involved are summarised in figure 55. The lengths of treatment were determined from studies by Wilson *et al* (1981), Bhoola *et al* (1973) and discussions with Drs.G.Bulfield and D.Morton. Messenger RNA was prepared as previously described and was diluted to a concentration of 1mg/ml. Filters were prepared on which were spotted the dilutions of each sample, and these were then treated and probed as in the pilot experiment. The samples were named according to Table 8 and the symbols were used in the following order: strain, tissue, treatment *e.g.* DSN = DBA/2, SMG, no treatment.

TABLE 8 - mRNA sample nomenclature

| STRAIN | TISSUE | TREATMENT |
|---|---|---|
| C: C57BL/6 | S: SMG | N: No Treatment |
| D: DBA/2 | K: Kidney | C: Castration |
| | | T: Testosterone |
| | | C/T: Castration + Testosterone |

In addition to the test samples a "control mRNA" was used which contained a dilution of male SWR SMG mRNA. This enabled a direct comparison between different filters. The levels of renin mRNA in the various samples are summarised in Table 9, and are quoted with respect to the level present in female DBA/2 SMG. Figure 56 represents the autoradiograph of filter #2 containing the male DBA/2 kidney samples, which gave a surprising result. Analysis of these samples strongly suggests that renin is testosterone-repressed in the kidney of male DBA/2 mice. To summarise the data presented in Table 9, in the male, *Ren*-1 would appear to be testosterone-repressed, and *Ren*-2, where active, is testosterone-inducible. In the female both *Ren*-1 and *Ren*-2 are testosterone inducible in the SMG but appear indifferent to the hormone in the kidney, possibly implying the involvement

Fig.56 - RNA dot blot filter #2

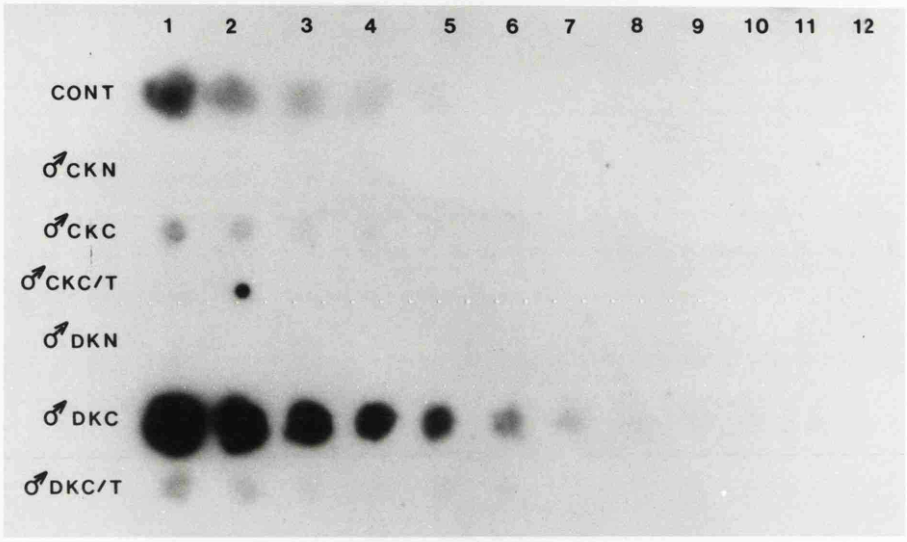
Sample nomenclature as listed in Table 7.

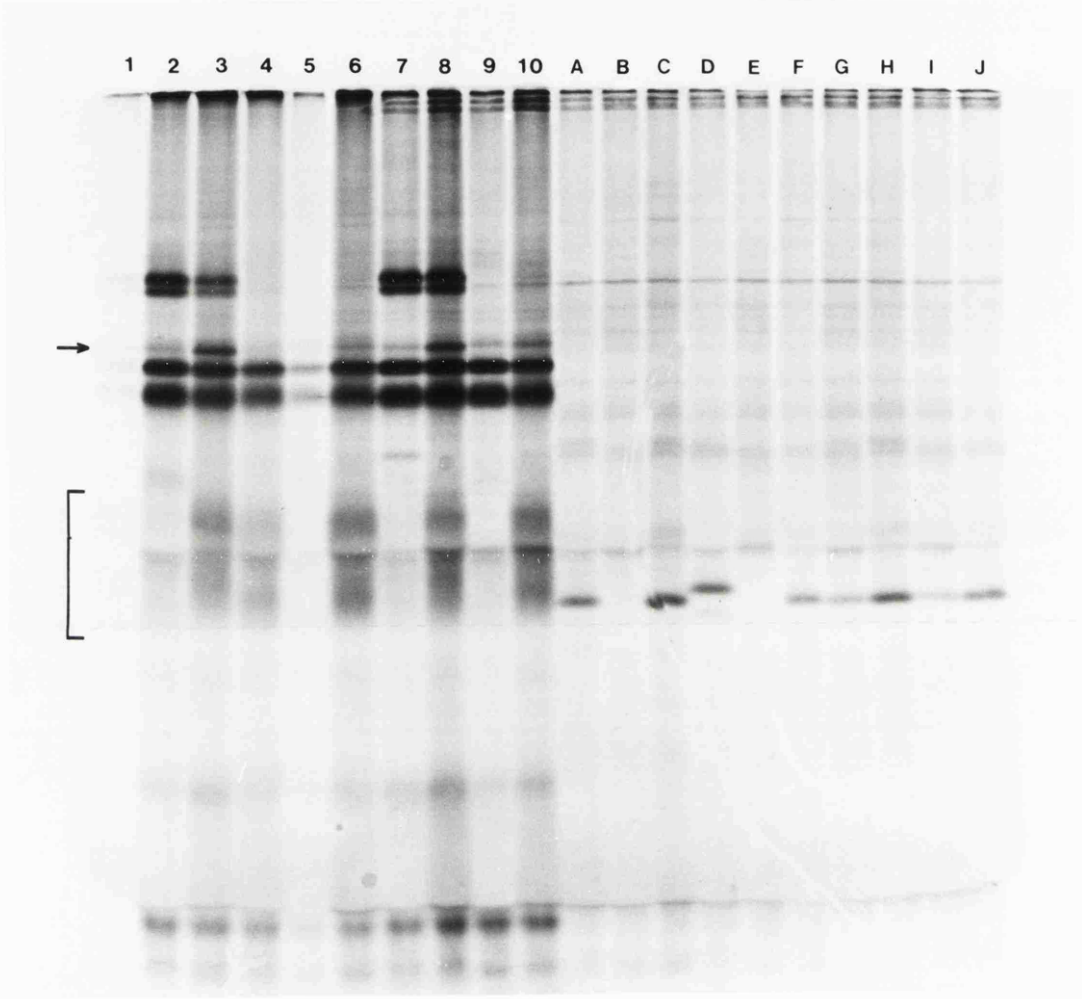
1-12 : 1 in 2 serial dilutions of mRNA samples.

Fig.57 - Translation products of experimental mRNAs (overleaf)

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| 1 | ♂ DSN | A | ♂ DKN |
| 2 | ♂ DSC | B | ♂ DKC |
| 3 | ♂ DSC/T | C | ♂ DKC/T |
| 4 | ♂ CSN | D | ♂ CKN |
| 5 | ♂ CSC | E | ♂ CKC |
| 6 | ♂ CSC/T | F | ♂ CKC/T |
| 7 | ♀ DSN | G | ♀ DKN |
| 8 | ♀ DST | H | ♀ DKT |
| 9 | ♀ CSN | I | ♀ CKN |
| 10 | ♀ CST | J | ♀ CKT |

The position of the renin precursor is arrowed. The esteroproteases, an internal control as to the effect of castration and/or testosterone treatment, are bracketed at the left side of the figure.


1
2
3
4
5
6
7
8
9
10
11
12
CONT
♂CKN
♂CKC
♂CKC/T
♂DKN
♂DKC
♂DKC/T


1 2 3 4 5 6 7 8 9 10 A B C D E F G H I J

of female hormone(s).

**Table 9** - Relative renin mRNA levels present in mRNA preparations

| DBA/2: | MALE | | FEMALE |
|---|---|---|---|
| DSN | 16 | DSN | 1 |
| DSC | 1 | DST | 8 |
| DSC/T | 16 | DKN | 0.5 |
| DKN | <0.016 | DKT | 0.5 |
| DKC | 16 | | |
| DKC/T | 0.5 | | |

| C57BL/6: | MALE | | FEMALE |
|---|---|---|---|
| CSN | <0.016 | CSN | 0.125 |
| CSC | 4 | CST | 0.125 |
| CSC/T | 0.06 | CKN | 0.125 |
| CKN | 0.016 | CKT | 0.125 |
| CKC | 0.5 | | |
| CKC/T | 0.016 | | |

When the mRNAs where translated *in-vitro* however (Fig.57) there was no evidence of the male castrate tissues containing highly elevated levels of renin mRNA. To try and solve this discrepancy three approaches were taken; firstly, the filters were rehybridised to a cDNA clone (pSMG3) corresponding to the major *in-vitro* translation product of SMG mRNA, a polypeptide which is not androgen-dependant and is not produced in the kidney (Windass *et al*, 1984). The result of this screening was that all the SMG mRNA samples, from whatever source, contained similar levels of pSMG3-type mRNA, whilst the kidney samples did not contain detectable levels of the message. The second approach was to determine if there were significant quantities of prematurely terminated transcripts being made which, although able to hybridise, would not produce detectable renin precursors *in-vitro*. To do this, probes were prepared from restriction fragments of the DBA/2 SMG renin cDNA clone 59/13 (which was isolated by Dr. D.Pioli and spans from nucleotide 99 to the polyA tail with respect to the sequence of Panthier *et al*,1982a). The fragments were kinase-labelled and

used to probe dot-blots of the following mRNAs: male DSC/T (in place of male DSN as there was very little of the latter mRNA sample available); male DKC; and male CSN. The origin of the probes and the hybridisation results obtained are shown in figure 58(a and b). What can be seen is that whether the probe is 5', central, or 3' the same hybridisation pattern is obtained and conflicts with the data from the main experiment. One would have expected, from the previous data, that the levels present in male DSC/T and male DKC would be the same, in fact the kidney level was very much lower than the SMG.The level in the castrate DBA/2 kidney, however, did appear to be higher than in the male C57BL/6 SMG.

The best way of solving the problem was to repeat the main experiment and this was done on a larger scale (20 mice/group) so as to provide enough mRNA for more extensive analysis. The mice were treated in exactly the same manner but for the second series of experiments the animals were nine weeks old (renin levels reach a plateau at maturity:-8 weeks), and C57BL/10 mice were used instead of C57BL/6 due to supply problems. Both strains are virtually identical with respect to renin expression and their genetic backgrounds are very closely related.

Messenger RNA was isolated from each tissue sample, their concentrations carefully measured and 0.5mg/ml solutions of each were made. The dot hybridisations were carried out as for the previous experiment and figure 59a shows the autoradiograph of this set of samples. One can immediately see that the results differ from those of the previous experiment insofar as the mRNAs from castrate males do not show enormously elevated levels of renin mRNA. The relative amounts of renin mRNA in these samples are much more consistent with the data obtained from *in-vitro* translation studies. Hybridisation of duplicate filters with 5' and 3' cDNA probes gave identical results to those shown in figure 59a.

The second series of mRNAs were also translated *in-vitro* and the translation products were immunoprecipitated with normal rabbit serum and anti-renin antiserum. The results (figs 60(a and b) parallelled the earlier

Fig.58 - RNA dot blots probed with renin cDNA restriction fragments

The origin of the probes and the results obtained using them are summarised. (H = *Hinf*I; D = *Dde*I; S = *Sau*3A).Numbers indicate the size (bp) of the restriction fragments.

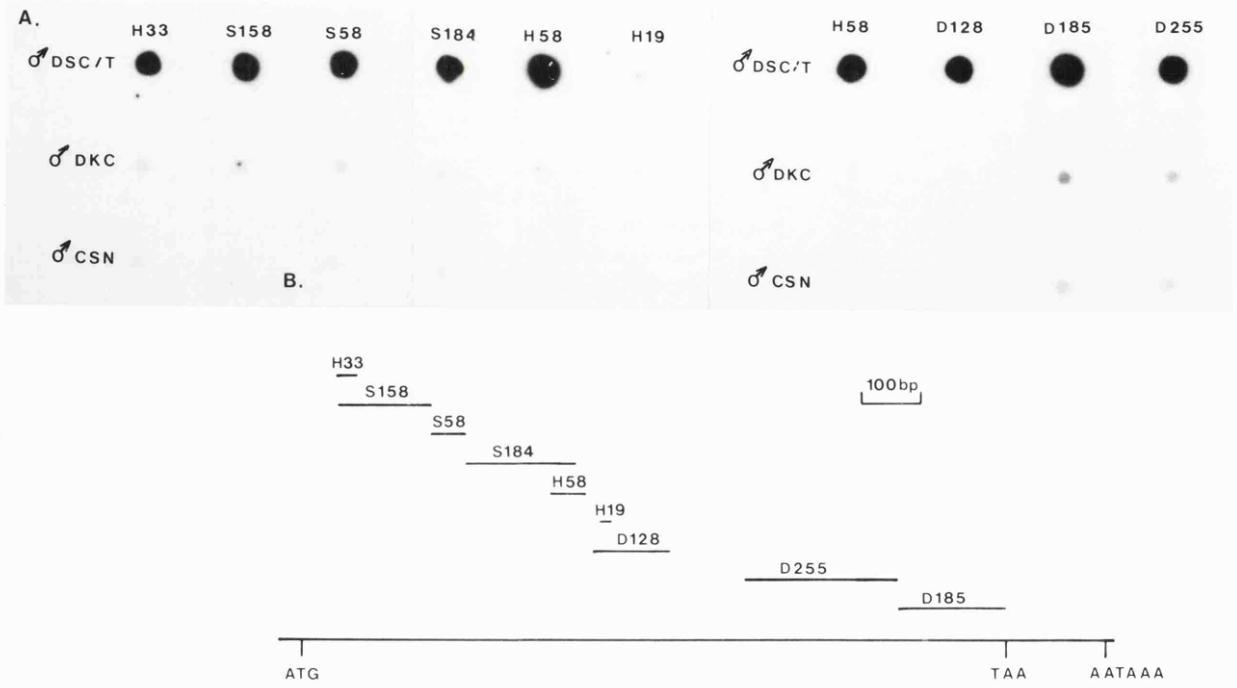

A.
H33
S158
S58
S184
H58
H19
♂DSC/T
♂DKC
♂CSN
B.
H58
D128
D185
D255
♂DSC/T
♂DKC
♂CSN
H33
S158
S58
S184
H58
H19
D128
D255
D185
100bp
ATG
TAA
AATAAA

**Fig.59** - (a) RNA dot blot of mRNA samples from the second hormone experiment

Four serial dilutions of each mRNA sample were probed. Sample nomenclature as previously described (Table 7) except that C57BL/10 mice were used instead of C57BL/6.

(b) Reprobing original mRNA samples

SMG and kidney mRNAs from DBA/2 and C57BL/6 animals (±testosterone) were probed with pSMG142.

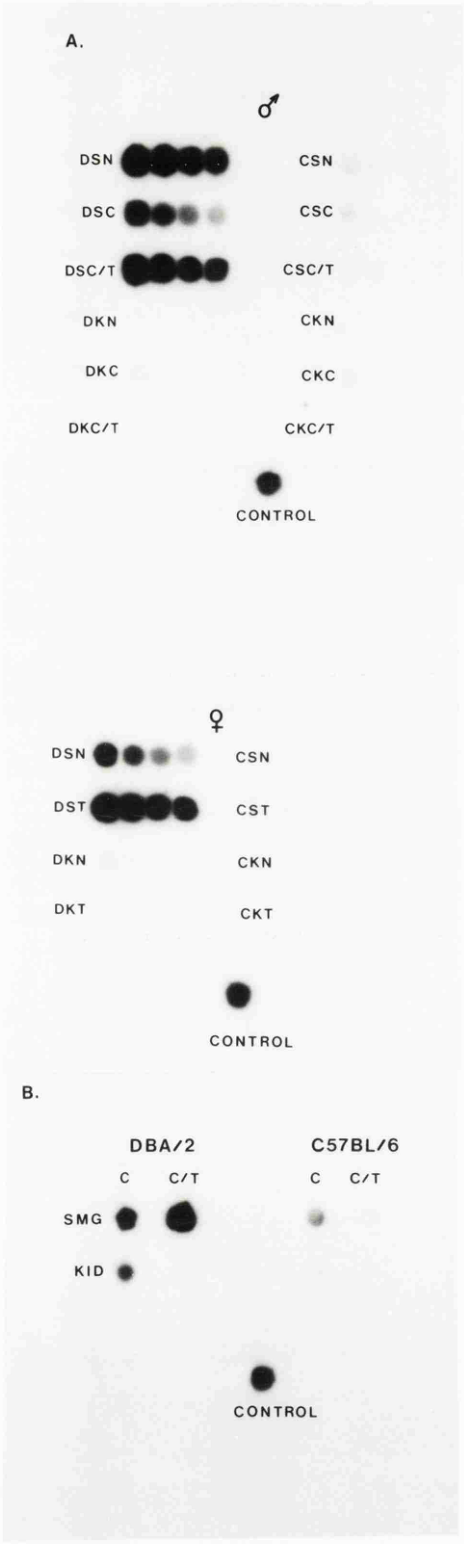

A.
♂
DSN
CSN
DSC
CSC
DSC/T
CSC/T
DKN
CKN
DKC
CKC
DKC/T
CKC/T
CONTROL
♀
DSN
CSN
DST
CST
DKN
CKN
DKT
CKT
CONTROL
B.
DBA/2
C57BL/6
C
C/T
C
C/T
SMG
KID
CONTROL

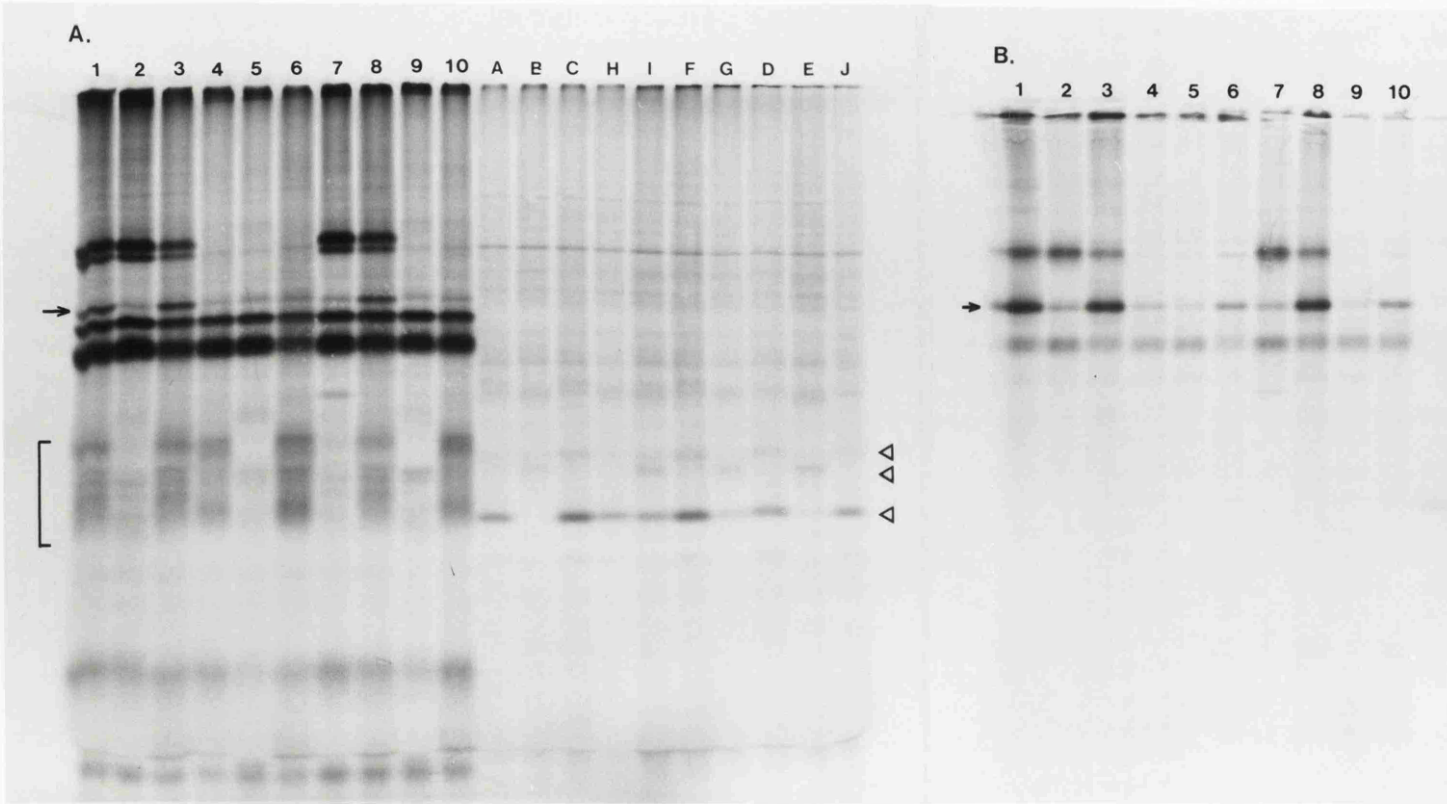
**Fig.60** - (a) Translation products of experimental mRNAs II

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| 1 | ♂ DSN | A | ♂ DKN |
| 2 | ♂ DSC | B | ♂ DKC |
| 3 | ♂ DSC/T | C | ♂ DKC/T |
| 4 | ♂ CSN | D | ♂ CKN |
| 5 | ♂ CSC | E | ♂ CKC |
| 6 | ♂ CSC/T | F | ♂ CKC/T |
| 7 | ♀ DSN | G | ♀ DKN |
| 8 | ♀ DST | H | ♀ DKT |
| 9 | ♀ CSN | I | ♀ CKN |
| 10 | ♀ CST | J | ♀ CKT |

(As for figure 59(a) the C57 strain used was C57BL/10)
The position of the renin precursor is arrowed and the esteroproteases are bracketed. The symbols (◁) indicate three major kidney peptides that fluctuate with testosterone levels.

(b) Immunoprecipitation of renin precursor from SMG mRNA translations shown in Fig.60(a)

The position of the renin precursor is arrowed. The sample numbers correspond to those in Fig.60(a)


A.
1 2 3 4 5 6 7 8 9 10 A B C H I F G D E J
B.
1 2 3 4 5 6 7 8 9 10

translation data in which the level of preprorenin was seen to change in response to the presence/removal of testosterone. The effect was visible for both male and female DBA/2 samples and also those of female C57BL/10 mice, but was less pronounced in the translations of male C57BL/10 mRNAs. Renin mRNA levels in all the kidney samples were too low to permit detection of the *in-vitro* translation product even after immunoprecipitation. In the repeat experiment the hybridisation and translation/immunoprecipitation data were compatible.

As part of this experiment some pertinent mRNAs from the first experiment were reprobed in parallel with the new RNAs. The samples chosen were the following (all male): DSC; DSC/T; DKC; DKC/T; CSC; CSC/T; CKC; CKC/T. As shown in Figure 59b the result confirmed the original data *i.e.* the castrate male DBA/2 kidney mRNA gave a much more intense hybridisation signal than would be expected, and the same was true for the castrate C57BL/10 SMG and kidney samples. At present this data is puzzling and plans are being made to repeat selected parts of the experiment. In both experiments, the translation data confirmed that the treatments had been effective: not only did renin respond but the levels of the esteroproteases, also known to be androgen-dependent, rose and fell according to the presence/removal of testosterone. In the kidney, two characteristic peptides of 25-30 Kd also appeared and disappeared as the treatment changed.

Given the sequence differences between the *Ren*-1 and *Ren*-2 genes, it should be possible to differentiate between *Ren*-1 and *Ren*-2 mRNAs using oligonucleotide probes, and ask questions as to their relative abundances under such hormone treatments.

## 8.2 Use of oligonucleotide probes to differentiate between *Ren*-1 and *Ren*-2 expression under different hormonal treatments

One outstanding question concerning renin expression in the mouse is whether *Ren*-1 is expressed co-ordinately with *Ren*-2 in the high producer

SMG. The following experiments were carried out to answer that question and also to provide an alternative way of determining the relative levels of *Ren*-1 and *Ren*-2 expression in the same tissue. An oligonucleotide primer was designed such that it would prime the synthesis of both *Ren*-1 and *Ren*-2 cDNAs. The choice was such that 5' of where the oligonucleotide would hybridise was a *Ren*-1->*Ren*-2 base change. Incorporation of the appropriate dideoxynucleotide would terminate one of the cDNAs at this point but allow the other to proceed to the next complimentary nucleotide. The primer REI/REII (Fig.61) was chosen for two reasons: (a) using dideoxy-GTP (ddGTP) there would be a significant difference (17 bases) in the predicted termination products of *Ren*-1 and *Ren*-2 cDNAs and (b) *Ren*-2 cDNA would terminate first. The latter point is important since *Ren*-1 termination, if it occurred first, would be hidden amongst the premature termination products from the much more abundant *Ren*-2 cDNA. Primer extensions were carried out as previously described except that dGTP was replaced by ddGTP.

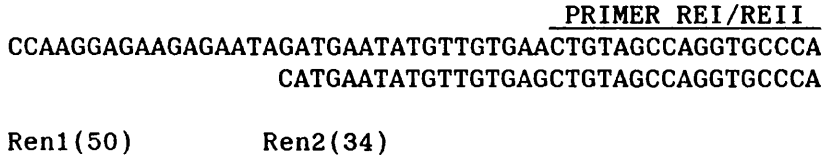



**Fig. 61** - Termination points for REI/REII primer extension

Because of the large amount of primer used and the results obtained in pilot experiments, the products were labelled during synthesis instead of using kinase-labelled primer. Figure 62 shows the extension products obtained with male DBA/2 and female C57BL/10 SMG mRNAs. In addition to the slow cooling method of primer annealing, a duplicate series of reactions was carried out in which the oligonucleotide was added and the incubation started immediately at 42°C. The addition of cold dCTP to 'chase' the label ($^{32}$PαdCTP) is seen to have no effect. *Ren*-2 cDNA is expected to terminate at 34 bases in length and this is confirmed in Figure 62. *Ren*-1 mRNA should

Fig.62 - Primer extensions of REI/REII

Extensions were performed either without prior annealing (42°) or after cooling the primer/mRNA from 100°C to 42°C (100° -> 42°)

D ♂ = male DBA/2 SMG
C ♀ = female C57BL/10 SMG
− = no dATP chase (15')
+ = dATP chase (15')

Molecular weight markers are shown in lane A and in addition, 58 bp and 33 bp markers were run alongside.

—▷ *Ren*2 extension product
—> unknown extension product
—▶ termination point for *Ren*1 extension product

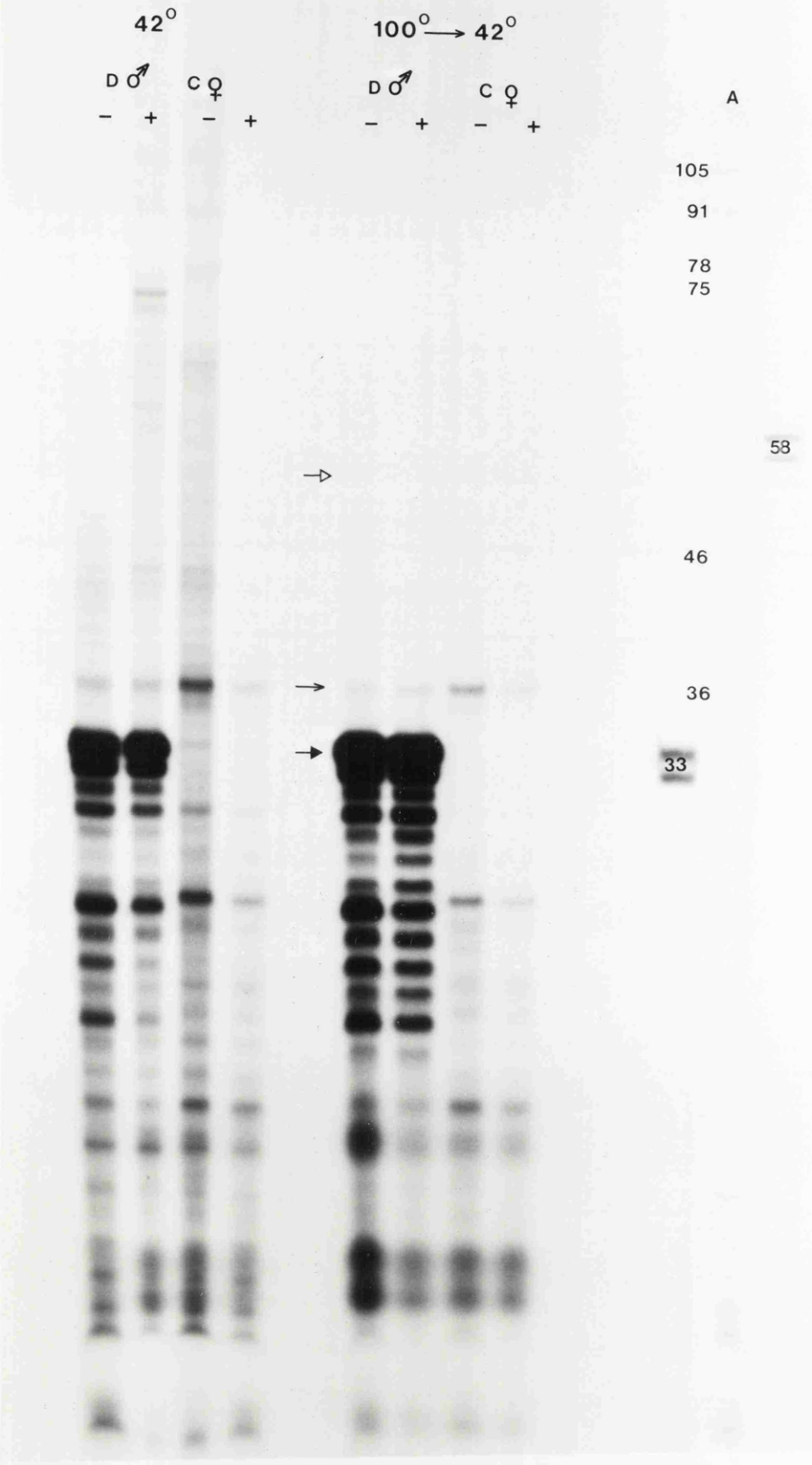
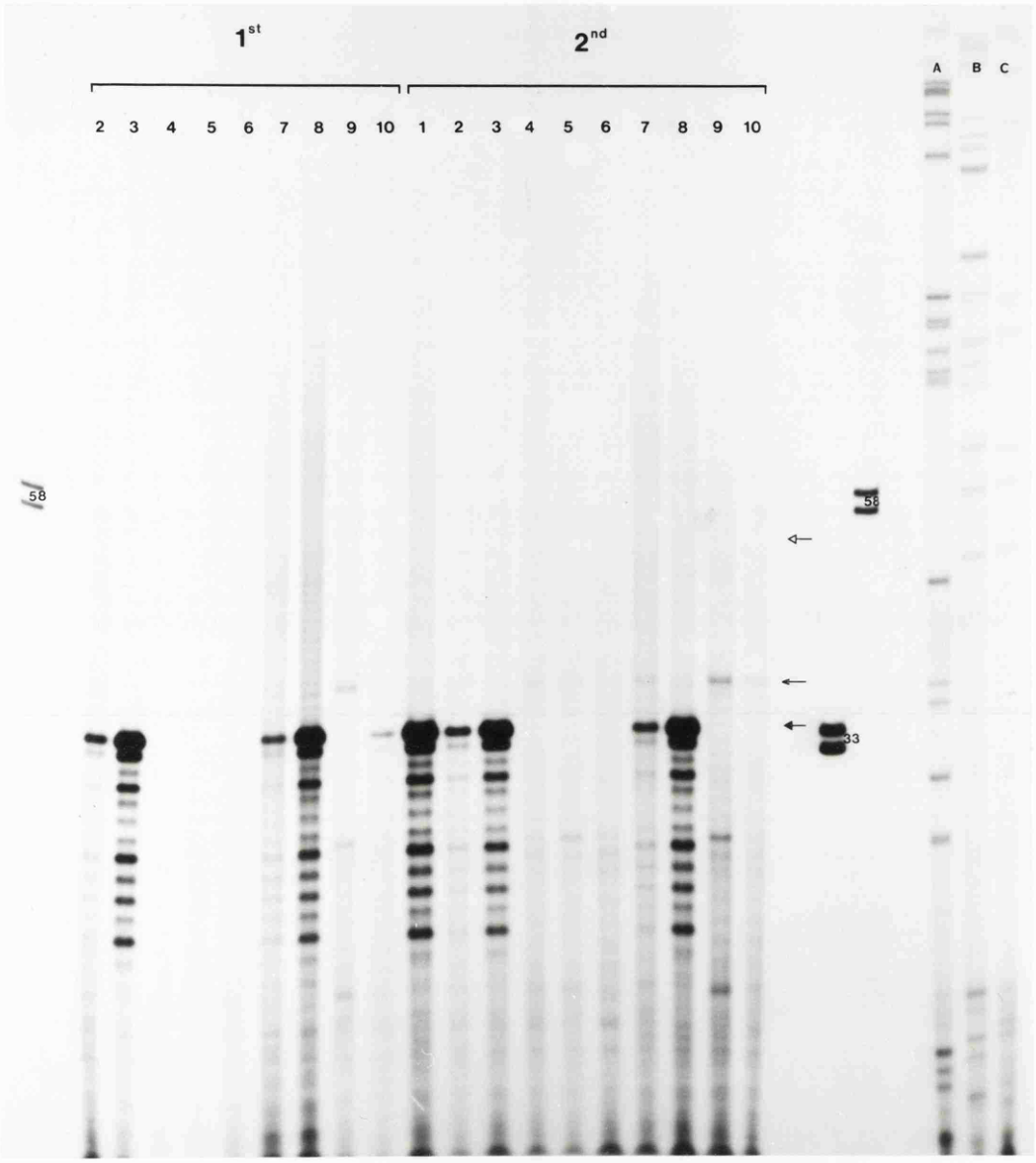

42°
100° → 42°
D ♂
C ♀
− + − +
D ♂
C ♀
− + − +
A
105
91
78
75
58
46
36
33

**Fig.63** - Extension products primed by REI/REII on SMG mRNA from both testosterone experiments (1st and 2nd)

| Lane | Sample | Lane | Marker |
|---|---|---|---|
| 1 | ♂ DSN | A | pBR322 |
| 2 | ♂ DSC | B | pBR322 |
| 3 | ♂ DSC/T | C | pBR322 |
| 4 | ♂ CSN | | |
| 5 | ♂ CSC | additional markers: | |
| 6 | ♂ CSC/T | 33bp and 58bp | |
| 7 | ♀ DSN | | |
| 8 | ♀ DST | | |
| 9 | ♀ CSN | | |
| 10 | ♀ CST | | |

⇨ termination point for *Ren*1
→ unknown extension product
➞ *Ren*2 extension product


1st
2nd
2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
A B C
58
58
33

give a product of 51 bases in length, but such a product is not detected in either C57BL/10 or DBA/2 tracks, even after long exposure of the auto-radiograph. Instead, a product of 37 bases in length is synthesised from both mRNAs. If this were *Ren*-1 specific it would suggest a sequencing error in the *Ren*-1 gene sequence. Such an error is extremely unlikely since the sequence of the relevant region has been determined several times and on both strands. It may be possible to confirm the origin of this second extension product by preparative synthesis and isolation followed by 'Maxam and Gilbert' sequencing, as carried out for the MuRen extension products (Chapter 7), but given the amount of product, this would be extremely difficult. The primer extensions using REI/REII were carried out on each of the SMG mRNA samples from the second hormone experiment described in section 8.1 and the products are shown in Figure 63. The abundance of *Ren*-2 cDNAs correlated exactly with the *in-vitro* translation and hybridisation data. The 37 base termination product does not show testosterone

```
                PRIMER REI/REII*
ACTTTCCCCGCATAAGTGTTTCTCCGGAAG
    ACCCTGCATAAGTGTTTCTCCGGAAG

Ren1  Ren2
(30)  (26)
```

**Fig.64** - Alternative primer for distinguishing *Ren*1 and *Ren*2 mRNAs

dependence and may not therefore be *Ren*-1 related. Perhaps, for some unknown reason, the choice of primer was not ideal. To circumvent the problem, alternative primers have been chosen and are presently in use. One of the most promising of these is shown in Figure 64. In essence, it is similar to REI/REII except that it has been chosen to (a) have a shorter distance between the predicted *Ren*-1 and *Ren*-2 termination points and (b) terminate by the addition of ddATP. This is available as $^{32}$PαddATP which should allow labelling of cDNA products only when they terminate, resulting in no premature termination products being visible. A correspondingly clean background should be obtained, thereby allowing *Ren*-1 specific products to be more easily detected.

# CHAPTER 9

# THE SUB-LINGUAL GLAND

## 9.1 Discovery of a major translation product

During the early cDNA cloning work carried out in the laboratory, mRNA was prepared from each of the three major salivary glands and translated *in vitro*. The result was shown in Figure 14 (chapter 3) and reference was made to the profile of the translation products of the mRNA isolated from the sublingual gland. Because of the overwhelming abundance of the 16kd polypeptide, a situation quantitatively comparable to globin synthesis in reticulocytes, it was decided to study the product more closely, in parallel to the renin work.

Unfortunately there is very little literature concerning the sublingual gland and its products, but the one major product known to be made in the gland is a high molecular weight glycoprotein or 'mucin' (Roukema *et al*, 1976). Other products believed to be synthesised in the gland include lysozyme and IgA (Barka T.,1980).

Sublingual and submaxillary mucins have been purified and partially characterised by Roukema *et al*(1976) and it was possible that the 16kd peptide was related to such a mucin. The polypeptide subunits of mucins, however, are of the order of 50-70kd in size (Prof.A.Allen, personal communication) and this 16kd peptide would therefore have to form an oligomer.

Close observation of the autoradiograph shown in Figure 14 (chapter 3) revealed that the 16kd species may not be a single polypeptide since the band has a slight 'dumb-bell' shape. Attempts to separate these species using 10-15% poly- acrylamide-SDS gels and various lengths of electrophoresis failed, but analysis on a 17.5% gel (Fig.6[illegible]; lane 1) revealed the presence of at least three distinct polypeptides of approximately equal abundance between 16 and 17kd in size. They must therefore differ in size by only 4 or 5 amino acids.

9.2 Characterisation of the *in vitro* products of sublingual mRNA

The characterisation of sublingual mucin by Roukema *et al* included the analysis of its amino-acid composition, which proved to be very unusual and potentially useful. The amino-acid content of sublingual mucin is listed in Table 10, which reveals that approximately 60% of the protein consists of only three amino-acids: threonine(21.2%), serine(20.4%) and alanine (17.6%).

**Table 10** - Amino acid composition of sublingual mucin

| amino acid | sublingual mucin (mol/100 mol) |
|---|---|
| Ala | 17.6±1.56 |
| Asp | 3.6±0.62 |
| Cys | 0.6±0.41 |
| Glu | 5.1±0.71 |
| Gly | 9.7±0.68 |
| Ile | 1.7±0.40 |
| Leu | 2.8±0.68 |
| Met | 1.6±0.71 |
| Phe | 1.4±0.36 |
| Pro | 5.7±1.62 |
| Ser | 20.4±1.12 |
| Thr | 21.2±1.56 |
| Tyr | 0.8±0.25 |
| Val | 3.7±0.38 |
| Arg | 2.0±0.24 |
| His | 0.5±0.21 |
| Lys | 1.6±0.40 |

There are very low levels of both methionine and cysteine and it should therefore be possible to label the *in-vitro* translation products with both $^{3}H$ and $^{35}S$, the tritium label being in any one of the three abundant amino-acids and either methionine or cysteine providing the second label. Tritiated serine and tritiated alanine were both available commercially, as were $^{35}S$ labelled methionine and cysteine. Three problems exist in the design of such an experiment: firstly, the tritiated amino-acids are only available at a comparatively low specific activity. Secondly, the

reticulocyte lysates contain enormous 'pools' of certain amino-acids including alanine and serine (typically 238$\mu$M and 91$\mu$M, respectively) and these pools vary between lysate preparations. Thirdly, however large or small the amino-acid pools are, if they are not determined accurately for each experiment, the specific activity of the amino-acids cannot be calculated.

The presence of large quantities of unlabelled amino-acids was overcome by using an amino-acid depleted lysate provided by R.Roberts (Amersham International). Reticulocyte lysate may be depleted of amino-acids by passage over Sephadex G50 or a similar column, but activity can be significantly reduced in the process. The different specific activities of the tritiated and $^{35}S$-labelled amino-acids was circumvented by testing various ratios of labelled amino-acids until the number of $^{3}H$ counts incorporated was approximately equal to the number of $^{35}S$ counts incorporated. Finally, the problem of variable specific activity was avoided by translating an mRNA whose product was of known amino-acid sequence, which could then be used as a standard in calculating the test samples.

The fractionated lysate was optimised for $K^{+}$ and $Mg^{++}$ and found to require the addition of $K^{+}$ to a final concentration of 125 mM. The addition of $Mg^{++}$ did not increase translational activity, and above a final concentration of 0.5 mM had a detrimental effect. After *in-vitro* translation in the presence of two labelled amino-acids, the products were separated by polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose paper by the method of Towbin *et al*(1979). The transfer was carried out for 4 hours at 60V(0.2A). The blot was air dried and placed on film. After location of the translation products using autoradiography, they were cut out from the blot and counted using a dual channel counter (0-12,12-156). The efficiency of counting was calculated by counting a known amount of $^{3}H$ and $^{35}S$ in the two channels in the presence of a strip of nitrocellulose paper. The actual dpms for each nuclide were then

calculated using the following equations:

$E_1$ = Efficiency of lower energy radionuclide in lower energy region

$E_2$ = Efficiency of lower energy radionuclide in higher energy region

$E_3$ = Efficiency of higher energy radionuclide in lower energy region

$E_4$ = Efficiency of higher energy radionuclide in higher energy region

A = Total cpm in the lower energy region

B = Total cpm in the higher energy region

X = dpm of the lower energy radionuclide

Y = dpm of the higher energy radionuclide

$$A = E_1X + E_3Y \qquad B = E_2X + E_4Y$$

Therefore: $$X = \frac{AE_4 - BE_3}{E_1E_4 - E_2E_3} \quad \text{and} \quad Y = \frac{BE_1 - AE_2}{E_1E_4 - E_2E_3}$$

A double-labelling experiment was carried out using $^3$H-serine and $^{35}$S-methionine. In addition to the translation of sublingual mRNA, mRNAs isolated from human placenta and bovine pituitary were translated in parallel reactions. A Western blot of the translation products was prepared as described above. Four 16-17kd sublingual species were observed and cut out from the blot using the autoradiograph as a template. The major translation products of human placental mRNA and bovine pituitary mRNA, pre-human placental lactogen and pituitary pre-prolactin respectively, were also located using the autoradiograph and excised. The nitrocellulose strips were then placed into 5mls of 'Fisofluor 3', a non-aqueous scintillant, and counted as described above. An identical experiment was carried out using $^3$H-alanine and $^{35}$S-methionine. The results of both experiments are summarised in Tables 11 and 12.

The true serine/methionine and alanine/methionine ratios for pre-HPL are 2.86 (20/7) and 1.43 (10/7) respectively (Shine *et al*,1977; Sherwood *et*

**TABLE 11** - Serine/methionine ratios for sublingual *in vitro* translation products

| SAMPLE | $^{3}H$ (SER) | $^{35}S$ (MET) | RATIO | CORRECTION RATIO |
|---|---|---|---|---|
| PHPL | 1821 | 3420 | 0.53 | (2.86) |
| BPPL | 18398 | 43451 | 0.42 | 2.3 |
| SL1 | 1868 | 3399 | 0.55 | 3.0 |
| SL2 | 3544 | 5650 | 0.63 | 3.4 |
| SL3 | 4076 | 5848 | 0.70 | 3.8 |
| SL4 | 1906 | 2999 | 0.64 | 3.5 |

**TABLE 12** - Alanine/methionine ratios for sublingual gland *in vitro* translation products

| SAMPLE | $^{3}H$ (ALA) | $^{35}S$ (MET) | RATIO | CORRECTION RATIO |
|---|---|---|---|---|
| HPL | 2166 | 4307 | 0.50 | (1.43) |
| BP | 11600 | 30528 | 0.38 | 1.1 |
| SL1 | 2272 | 4673 | 0.49 | 1.4 |
| SL2 | 4632 | 8752 | 0.53 | 1.5 |
| SL3 | 4728 | 8394 | 0.56 | 1.6 |
| SL4 | 2564 | 4502 | 0.57 | 1.6 |

*al*,1979). As described earlier, the observed ratios were obtained due to the different specific activities of the two amino-acids and therefore the real ratio divided by the observed ratio will give a correction factor enabling the true ratios of the sublingual polypeptides to be determined. To check that this extrapolation holds true, the serine/methionine and alanine/methionine ratios for bovine prolactin were calculated using human placental pre-prolactogen as a standard. The corrected serine/methionine ratio was 2.3 and the alanine/methionine ratio was 1.1. These compared well with the true ratios of 2.5 and 1.25 respectively (Sasavage *et al*,1982), the error being 8% in each case and therefore the corrected ratios for the sublingual polypeptides should be reasonably accurate.

The conclusion from these results is that the ratio of alanine or serine to methionine in the sublingual 16-17kd polypeptides is significantly different to the ratio present in sublingual mucin, and therefore means that the major *in-vitro* products of sublingual mRNA are not mucins but a novel group of polypeptides of very similar size and showing similar ratios

of serine, alanine and methionine. This is suggestive of a family of peptides being expressed simultaneously in the gland, and this possibility will be looked at in more detail below.

9.3 Synthesis of single-stranded cDNA to total sublingual mRNA

In view of the unusual *in-vitro* translation profile, single-stranded cDNA was prepared from sublingual mRNA using oligo-dT as the primer. When analysed on a denaturing acrylamide-urea gel, the products of this analysis should give an idea of the complexity of the mRNA population. Such cDNA syntheses usually result in a number of discrete species of varying abundance, and a large heterogenous population of molecules resulting from premature termination and heterogeneity in the length of polyA sequence copied during reverse transcription. Sublingual cDNA was prepared from 1μg of polyA$^+$ mRNA and after alkali denaturation, it was electrophoresed through an 8% acrylamide-urea gel at 25mA (pre-run at 25mA for 1hr). The autoradiograph of this gel is shown in Figure 65 which compares the cDNA synthesis methods of Skup *et al* (1982) and Land *et al* (1981) in the presence and absence of RNAsin. There is a single major size class of cDNA visible above the background as a discrete but very intense product of approximately 600 nucleotides. A 600 nucleotide mRNA has the potential to code for a polypeptide of 20kd, amply large enough to code for the 16-17kd species.

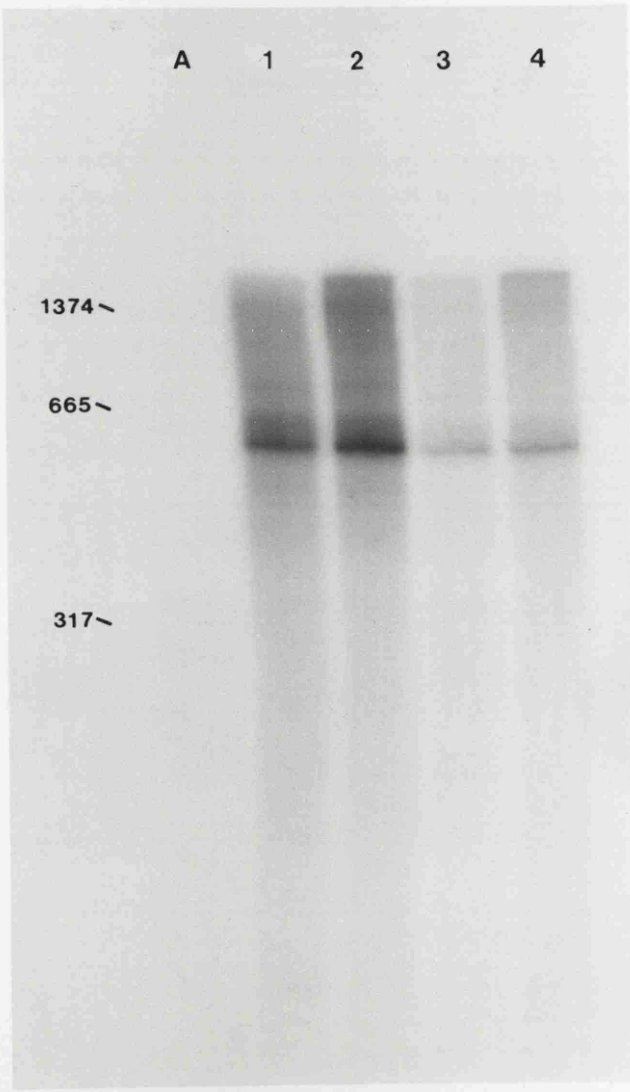
This result parallels the *in-vitro* translation insofar as it indicates that the sublingual gland contains either an exceptionally abundant mRNA or several mRNAs of very similar size.

9.4 Isolation and restriction-mapping of cDNA clones corresponding to the sublingual 16-17kd polypeptides.

The cDNA libraries made in this laboratory from DBA/2 SMG mRNA had been made from mixed salivary gland mRNA. It was therefore likely that amongst the clones were ones derived from the major sublingual mRNA(s). The filter-selection screening of salivary gland cDNA clones described in

**Fig.65** - Urea-acrylamide gel analysis of single-stranded cDNA synthesised DBA/2 sublingual gland mRNA using oligodT as a primer

| Lane | Sample |
|---|---|
| A | pBR322 x *Sau*3A (kinased) |
| 1 | method of Land *et al* (1983) |
| 2 | method of " , in presence of RNAsin |
| 3 | method of Skup *et al* (1982) |
| 4 | method of " , in presence of RNAsin |


A
1
2
3
4
1374
665
317

Chapter 4 yielded clones for every major SMG *in-vitro* translation product and mixing of SMG mRNA with sublingual mRNA prior to use in filter selection precipitated the identification of two clones, pSMG129 and pSMG147, that selected mRNA(s) translating to yield 16kd polypeptides. These clones were grown and plasmid DNA prepared, some of which was nick-translated and used to re-screen the cDNA library. Surprisingly, this yielded only one further positive clone, pSMG181. Other positive clones were found but the master colonies failed to grow and further characterisation was therefore impossible.

The three cDNA clones were restriction-mapped using the enzymes *Pst*I, *Sau*3A, *Taq*I, *Hinf*I, *Hae*III, *Msp*I, *Eco*RI, *Hha*I and *Tha*I, and the maps obtained for pSMG129 and pSMG147 showed that they were identical and therefore probably different isolates of the same clone. The restiction maps are shown in Figure 66. The clones pSMG129 and pSMG147 were found to have 375 bp inserts with identical restriction maps, whilst that of pSMG181 was 320 bp in length and overlapped with pSMG129/147.

## 9.5 Sequence analysis of clones pSMG129(147) and pSMG181

The sequencing strategy used to determine the cDNA sequence of clones pSMG129(147) and pSMG181 is also summarised in Figure 66. The sequences of the two cDNAs agreed perfectly within the region of overlap and contained an open reading frame starting 5' of the cloned sequence and reading through to residue 259. The 3' non-coding region is 63 bp in length and contains the consensus 'AATAAA' polyadenylation signal, 17 residues from the site of polyadenylation (Fig.67). The encoded polypeptide has a molecular weight of 9.8kd and therefore the clones contain over half of the coding region. As expected from the double labelling experiment, the amino-acid sequence does not show the levels of serine, threonine and alanine expected if the protein was related to the mucin described by Roukema *et al* (1976). Indeed, if the rest of the polypeptide consisted solely of these three amino-acids then the levels would still not approach those of the mucin. The polypeptide

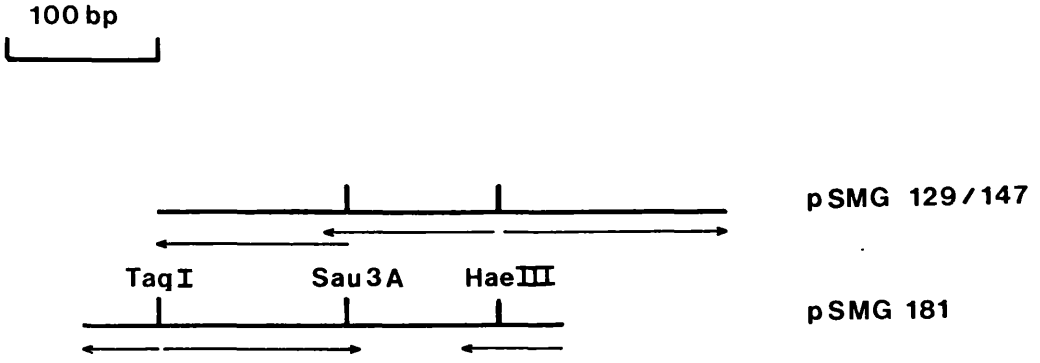



Fig.66 - Restriction map and sequencing strategy for sublingual cDNA clones 129/147 and 181

sequence shown in Figure 67 was used in a search of the Doolittle data base (Doolittle,1981), carried out by Dr.P.Stockwell (ICRF, London) and revealed no significant homology to any listed sequences. A similar search of the EMBO nucleic acid sequence data base was also negative.

```
        10         20         30         40         50         60
TCTAAGGAAGAGCACATCATTGGGGTCGAAGGCACGTATAATCCTTCTGCTTTAACTCAG
SerLysGluGluHisIleIleGlyValGluGlyThrTyrAsnProSerAlaLeuThrGln
                                          ---------
        70         80         90        100        110        120
ATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGGTGGGGTATCACGTGGGTAATTAT
IleIlePheThrThrAsnGlnProArgGlnLeuMetValGlyTyrHisValGlyAsnTyr

       130        140        150        160        170        180
CAGTACTCTAGCTACCCTGATGATCCCTGCCTTGTGCTCAAGGGAGCTTGTGTCTCTTGG
GlnTyrSerSerTyrProAspAspProCysLeuValLeuLysGlyAlaCysValSerTrp

       190        200        210        220        230        240
AGGGCAGGTGGCATAAAGAGTATCTTGTTTTTGTGGGGAAGTGAAAACAGTTCATGTGTG
ArgAlaGlyGlyIleLysSerIleLeuPheLeuTrpGlySerGluAsnSerSerCysVal
                                                  ---------
       250        260        270        280        290        300
AAATATGGCCACTCAGGCTGAAGTGGTCTGTGTCACGGTACTTCCAAACTGAACCCATGA
LysTyrGlyHisSerGly***                                         -

       310        320        330        340        350        360
ATAAATACAACATGTGTGCATG -------> POLY A
-----
```

**Fig.67** - Nucleotide sequence and encoded polypeptide of pSMG129/181

9.6 Hybrid-selection of mRNA(s) encoding the 16kd polypeptides and comparison of male and female SL mRNA translation products

The suggestion was made in section 9.2 that the polypeptides seen during analysis of sublingual mRNA translation products might be a family of peptides. To explore this possibility, pSMG129 was used as a probe for the hybrid-selection of sublingual mRNAs. Care was taken during the wash-steps to ensure that hybrids were thoroughly washed of non-hybridising mRNAs. The selected mRNAs were recovered and translated *in-vitro* using the rabbit reticulocyte lysate system, and the products were analysed by electrophoresis on a 17.5% poly- acrylamide-SDS gel. The fluorograph of the gel is shown in Figure 68a, and suggests that not only are all the mRNA(s) encoding the 16kd peptides selected by pSMG129 but those encoding 32kd

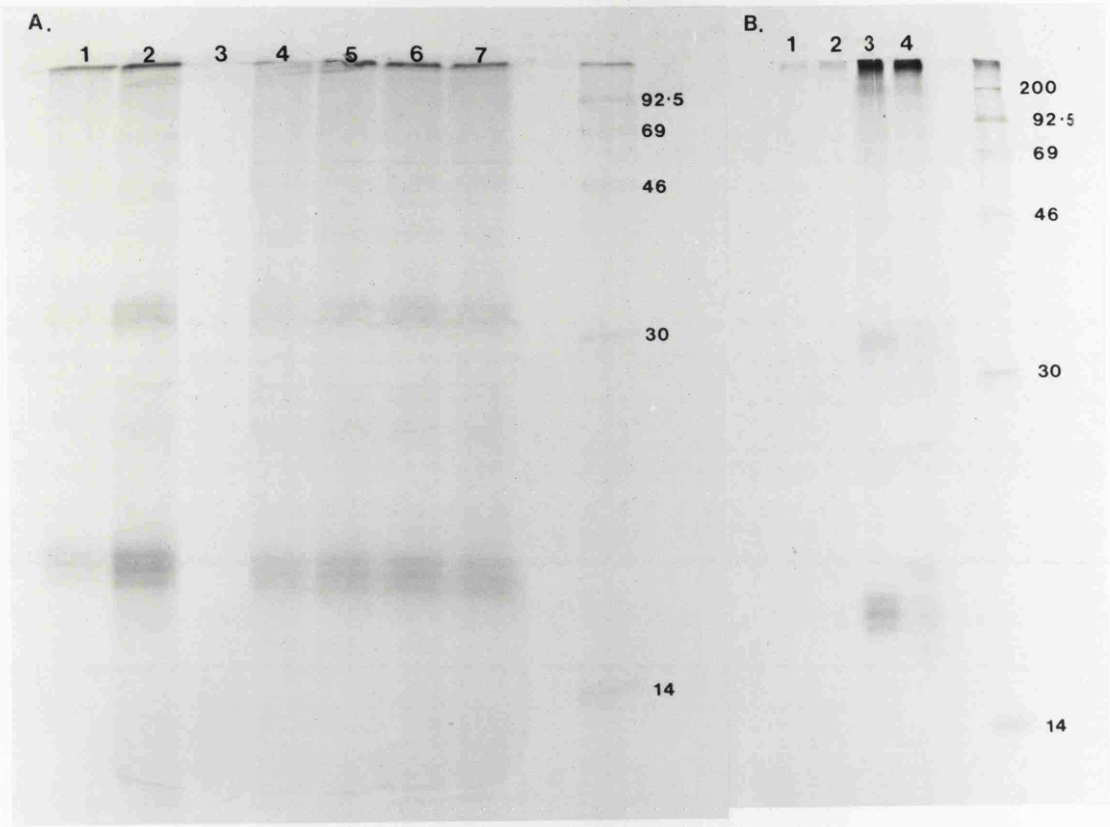
Fig.68 - (a) Translation products of DBA/2 sublingual mRNAs

(17.5% polyacrylamide gel)

| Lane | Sample |
|---|---|
| 1 | total sublingual mRNA |
| 2 | hybrid selection of sublingual mRNA (pSMG129) |
| 3 | – RNA |
| 4 | normal male |
| 5 | castrate male |
| 6 | normal female |
| 7 | testosterone treated female |
| A | $^{14}C$ M.Wt. markers |

(b) *In vitro* processing of the translation products of male DBA/2 sublingual mRNA

| Lane | Sample |
|---|---|
| 1 | – mRNA, no dog pancreas membranes (DPM) |
| 2 | – mRNA, + DPM |
| 3 | + mRNA, – DPM |
| 4 | + mRNA, + DPM |
| A | $^{14}C$ M.Wt.markers |


A.
1
2
3
4
5
6
7
92·5
69
46
30
14
B.
1
2
3
4
200
92·5
69
46
30
14

peptides are also purified. Minor species of molecular weight 45-70kd are seen not to be selected. The presence of the 32kd polypeptides adds weight to the possibility that they are related to the 16kd species.

Figure 68a also shows the translation products of sublingual mRNA preparations from normal and castrate male DBA/2 mice with those from normal and testosterone-treated female DBA/2 mice (treatment was as described in chapter 8). The translation profiles of the four RNA preparations were found to be almost identical. All the major polypeptides are present at the same level in each sample, indicating that testosterone is probably not involved in controlling the expression of the gene(s) coding for these polypeptides.

## 9.7 Processing of *in-vitro* translation products using dog pancreas membranes

The results described thus far show that the sublingual gland is the site of synthesis of a family of androgen-independent peptides of approximately 16kd in size, whose mRNAs are also of very similar size (approximately 600 bases). These peptides are distinct from what was previously believed to be the major product of the gland, a high molecular weight glycoprotein, and they may be glycosylated since the amino-acid sequence predicted from nucleotide sequencing of partial cDNAs shows two potential sites for N-linked glycosylation (Heath *et al*,1979)(see Fig.67). It would be expected that since the sublingual gland is a secretory tissue and the major mRNA(s) is so abundant, the 16kd species might be secreted from the gland and that the *in-vitro* precursors would therefore include signal sequences. As already reported for renin, these can be removed *in-vitro* by dog pancreas membranes.

Male DBA/2 sublingual mRNA was therefore translated in both the presence and absence of DPM, and the products analysed as described previously. Figure 68b reveals that the inclusion of membranes results in the appearance of at least two additional polypeptides, one of lower

molecular weight and the other (probably a doublet) of apparently higher molecular weight. Dog pancreas membranes are not very efficient at glycosylation *in-vitro* (Heath *et al*,1979) and therefore this apparent increase in molecular weight may be an artifact due to the peptide(s) running anomolously during gel electrophoresis (similar examples are quoted by Windass *et al*,(1984). An early experiment using tunicamycin failed to prevent formation of the larger polypeptide(s) suggesting that any increase in molecular weight was not due to core-glycosylation. In summary, at least two of the 16kd polypeptides are cleaved by dog pancreas membranes and are likely to be precursors of secreted proteins.

## 9.8 Preparation and screening of a cDNA library from sublingual mRNA using the vector M13mp10

Evidence was quite strong for the existence of a family of 16kd polypeptides but the two cDNA clones isolated from the existing cDNA library had identical sequences in the region of overlap. It was therefore decided to make a cDNA library from the sublingual mRNA in order to obtain a large number of clones corresponding to the 16kd species, and compare their nucleotide sequences. If there really was a family of proteins then the cDNA sequences should fall into several related groups.

To sequence a large number of clones by the method of Maxam and Gilbert (1980) would have been both time consuming and expensive. Therefore, with the advent of M13 cloning and dideoxy-sequencing, the major sublingual mRNAs seemed an ideal choice to test the usefulness of M13 as a vector for cDNA cloning.

Single-stranded cDNA was synthesised from 15$\mu$g of polyA$^+$ DBA/2 sublingual mRNA, using the method of Skup *et al* (1982). The synthesis was 16.7% efficient yielding 2.5$\mu$g of sscDNA. This sscDNA was used in a single reaction to produce dscDNA and the 0.5$\mu$g of dscDNA obtained was 78% resistant to 1 units/$\mu$l of $S_1$ nuclease at 37°C for one minute. The $S_1$ resistant cDNA was then blunt-ended using the large fragment of the enzyme

DNA polymerase (*E.coli*). The choice of enzyme for this step was a poor one as $T_4$ polymerase has a much higher 3'-exonuclease activity and would produce blunt-ended molecules with much higher efficiency.

The replicative form (RF) of the bacteriophage M13 consists of a double-stranded circular DNA molecule of approximately 7kb in length. The genome of the phage has been manipulated such that the *E.coli lac*Z gene is present in a non-essential region (Messing,1983). The *lac* promoter-operator is also present and the expression of *lac*Z can be induced by inclusion of IPTG in the growth medium. When the chromogenic substrate X-Gal is also included in the medium, expression of *lac*Z is made visible by the appearance of the cleavage product which has a blue colour. Within the coding region of *lac*Z, short sequences have been introduced that contain recognition sites for restriction endonucleases useful in molecular cloning. The introduction of such sequences does not alter the expression of *lac*Z, but use of one or more restriction sites to clone foreign DNA results in the inactivation of the *lac*Z gene. The recombinants are white due to the lack of B-galactosidase activity, and can be easily distinguished from parental phage.

One of the most important aspects of M13 is that as a mature phage its genome consists of a circular single-stranded DNA molecule and isolation of this form is relatively easy. The purified single-stranded DNA can be used as a template on which primer-directed synthesis of the complementary strand can take place. The synthesis starts very close to the cloning site by the use of a "universal" primer, and is carried out in quadruple, one reaction for each nucleotide. Each reaction proceeds in the presence of the corresponding dideoxynucleotide whose ratio to the deoxynucleotide is determined empirically to yield the maximum sequence information. Two of the drawbacks are that large inserts are relatively unstable, and using a single primer, no more than 350 bases can be confidently determined. As the size of the sublingual major mRNA is approximately 600 nucleotides, it is an ideal length for cloning, since all the sequence could be determined by either using the reverse primer or by inverting the cDNA insert.

Within the nest of cloning sites of M13mp11 is a site for the enzyme *Sma*I, which cleaves the DNA duplex to leave a blunt ended molecule. Removal of the 5' phosphate residue leaves this cleaved vector molecule unable to religate unless a DNA fragment possessing terminal phosphates is introduced.

The double-stranded, blunt-ended, sublingual cDNA made above was therefore ligated to phosphatased, *Sma*I-cut M13mp11 DNA using $T_4$ DNA ligase. The reaction was allowed to proceed overnight at 15°C, and different ratios of insert DNA to vector were used to optimise the ligation. The ligated DNA (10$\mu$l) was used to transform 200$\mu$l of competent cells made from the *E.coli* strain JM103. Approximately two-fold more blue plaques than white plaques were generated, and in this initial transformation a total of 49 white plaques and 107 blue plaques were obtained. This gave a potential cDNA library of 1250 clones. Subsequent work in this laboratory has shown that $T_4$ ligase from either P.L.Biochemicals or New England Biolabs gives up to two orders of magnitude more recombinants during blunt-ended ligation than BRL $T_4$ ligase. The methodology, therefore, has the potential for very high efficiency cloning.

Template DNA was made from the 49 recombinants by picking the plaques into 1ml of exponentially growing JM103 cells and incubating for 6 hours. After harvesting the phage the single-stranded DNA was extracted by phenol-treatment of the phage particles and ethanol-precipitation. The DNA pellets were resuspended in 20$\mu$l of TE buffer and 1$\mu$l of each template used for dot hybridisation analysis. The clone pSMG129 was nick-translated and used as a hybridisation probe to identify those templates derived from related clones.

From the 49 recombinants, 18 gave positive hybridisation signals and were T-screened to determine orientation, relatedness and template condition. This screening proved very useful and highlighted two problems. A minor one was that screening with the clone pSMG129, which has a short polyA tail, resulted in the isolation of recombinants that contained only

short stretches of polyA. The second problem was that although T-screening often allowed the orientation of clones to be determined due to the presence of a run of 'T's, attempts to sequence through such a structure was often unsuccessful, probably due to polymerase slipping on the polyA tract. This means that unless the oligo-A sequence is short, it is not possible to sequence clones with the 3'-end towards the universal priming site.

Since the methods used for cDNA synthesis place a bias towards obtaining 3' cDNA clones, and most sequence heterogeneity might be expected to manifest itself in the 3' untranslated region, a primer was designed to allow sequencing of the clones in the 5'→3' orientation with respect to the universal priming site. This would give a set of related sequences from a constant reference point for ease of comparison. The primer chosen is shown below (Fig.69) and covers nucleotides 229 to 248 in figure 67. Clones in the correct orientation were screened with the primer and the sequences

5' AGTTCATGTGTGAAATATG 3'

Fig.69 - Sublingual primer

obtained indicated that some clones extended to the polyA tail, others terminated between the priming site and the polyA tail, whilst the third group gave no sequence using the primer, suggesting that they terminated before the priming site.

The sequences determined to date are compared in Figure 70. These sequences have been determined by using the templates made above and before being 100% confident , the inserts should be recloned into the sister vector M13mp10, to determine the complimentary strand. From the sequences shown, two patterns emerge: firstly, where there are regions of 5-6 overlapping sequences, sequence differences can be seen which are present in only one of the clones. Such differences are probably sequencing errors or artifacts. The second type of sequence heterogeneity is more interesting: certain

Fig.70 - Nucleotide sequences of sublingual gland cDNA clones

The extent of the sequences cloned is indicated by the symbol *. Regions cloned but whose sequences have not yet been determined are indicated by the symbols < and > showing that they extend 5' and 3' respectively.

```
           10        20        30        40        50        60        70        80        90       100       110       120

181  TCTAAGGAAGAGCACATCATTGGGGTCGAAGGCACATATAATCCTTCTGCTTTAACTCAGATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGGTGGGGTATCACGTGGGTAATTAT
147                               *CACATATAATCCTTCTGCTTTAACTCAGATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGGTGGGGTATCACGTGGGTAATTAT
#54              *ATCAT?GCGG-CCAAGGCACATACTCTCCTTCTGCTTTAACTCAGATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGG*
#9   AATAACGAAGAGTCCATCATTGCGG-CGAAGGCACATACTCTCCTTCGGCTTTAACTCAGATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGGTGGGGTATTATGTGGGCAGTTCT
#37  AGTAACGAAGAGTCCATCATTGCGG-CGAAGGCACATACTCTCCTTCGGCTTTAACTCAGATAATCTTTACGACAAACCAGCCTCGCCAGCTAATGGTGGGGTATTATGTGGGCAGTTCT
#39                                                  *AGATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGGTGGGGTATCACGTGGGTAATTAT
#42 〈AATAACGAAGAGTCCATCAT??GGG-GCGAGGCACATACTCTCCTTCGGCTTTAACT?AGATAATCTTTACGACCAACCAGCCTCGCCAGCTAATGGTGGGGTATTATGTGGGCAGTTCT
#38                                                                                                  *TATTATGTGGGC?GTTCT

           130       140       150       160       170       180       190       200       210       220       230       240

181  CAGTACTCTAGCTACCCTGATGATCCCAGCCTTGTGCTCAAGGGAGCTTGTG*
147  CAGTACTCTAGCTACCCTGATGATCCCAGCCTTGTGCTCAAGGGAGCTTGTGTCTCTTGGAGGGCAGGTGGCATAAAGAGTATCTTGTTTTTGTGGGGAAGTGAAAACAGTTCATGTGTG

#9   GAGTACTCTAGCTTCCCTGACGATCCCAGCCATGTGCTCAAGGGAGCTTGTGTCTCTT〉
#37  GAGTACTCTAGCTTCCCTGACGATCCCAGCCATGTGCTCAAGGGAGCTTGTGTCTCTTGGAGGGCAGGTGGCATAAAGAGTATCTTGTTTTTGTGGGGAACTGAAAACAGTTCATGTGTG
#39  CAGTACTCTAGCTACCCTGATGATCCCAGCCTTGTGCTCAAGGGAGCTTGTGTCACTTGGAGGGCAGGTGGCATAAAGAGTATCTTGTTTTTGTGGGGAAGTGAAAACAGTTCATGTGTG
#42  GAGTACTCTAGCTTCCCTGACGATCCCAGCCATGTGCTCAAGGGAGCTTGTGTCTCTT*
#38  GAGTACTCTAGCTTCCCTGACGATCCCAGCCATGTGCTCAAGGGAGCATGTGTCTCTTGGAGGGCAGGTGGCATAAAGAGTATCTTGTTTTTGTGGGGAAGTGAAAACAGTTCATGTGTG
#32                                                                                                                  *GTG

           250       260       270       280       290       300       310       320

147  AAATATGGCCACTCAGGCTGAAGTGGTCTGTGTCAGGTACTTCCAAACTGAACCCATGAATAAATACAACATGTGCATG

#37  AAATATGGCCACTCAGGCTGAAGTG〉
#39  AAATATGGCCACTCAGGCTGA〉
#38  AAATATGGCCACTCAGGCTGAAGTGGTCTGTGTCAGGTACT*
#11            *TCAGGCTGAAGTGGTCTGTGTCAGGTACTTCCAAACTGAACCCATGAATAAATACAACATGTGCATG
#13                                              *AACCCATGAATAAATACAACATCTGCATG
#32  AAATATGGCCACTCAGGCTGAAGTGGTCTGTGTCAGGTACTTCCAAACTGAACCCATGAATAAATAC*
#29                                                  *AATACAACATGTGCATG
```

regions show two distinct sequences and the clones covering such regions divide themselves more evenly. Such differences are consistent with the existence of a family of closely related mRNA molecules encoding very similar polypeptides.

Further analysis of these and other clones is continuing but the data presented in this chapter establishes the existence of a major new family of tissue-specific, androgen-independent secreted proteins.

# CHAPTER 10

# HUMAN RENIN

## 10.1 Molecular cloning and identification of cDNA copies of Human kidney renin mRNA

The mouse has proved an excellent animal model for studying genetically determined diseases. Mutants and inbred mice are available that stably inherit specific defects e.g. *his/his* mice (Kacser *et al*,1973).

As mentioned in the introduction, one of the commonest medical problems existing today is hypertension, and although not necessarily caused by elevated renin levels, the enzyme is intimately involved in the maintenance of the hypertensive state. Since it is such a key element in the condition, it is a logical place to attack in order to try to control the problem. At present, medical treatment concentrates on inhibiting angiotensin converting enzyme (ACE) and although such treatment has been very successful it can result in an increase of plasma renin by a feedback mechanism. In addition, inhibition of ACE by Captopril or similar drugs can have unpleasant side-effects. Although the major source of renin is the kidney, the level of enzyme in the human kidney is extremely low, some $10^6$ fold less than the mouse submaxillary gland or $10^4$ fold less than the mouse kidney. This makes the purification of the enzyme for amino-acid sequencing and crystallography extremely difficult and to date the problem has proved insurmountable. In order to purify enough human renin to crystallise for X-ray studies (approximately 100mgs) 60,000 human kidneys would have to be obtained. The structure of the enzyme as determined by X-ray crystallography is the most powerful way of being able to design inhibitors.

The mouse renin system is not a model for human hypertension or even human renin expression, but the cloning of mouse renin cDNAs provides a tool to open the human system to further study. Thus a mouse submaxillary gland renin cDNA clone can be used as a probe to search for human renin cDNA clones. Assuming that the level of renin mRNA in the human kidney is of

similar relative abundance as the protein, the task of finding a clone, even given such a probe, would be a very difficult one. However, there exists a clinical condition termed renal arteriostenosis in which patients suffer from high blood-pressure as a result of blockage of the renal artery. The effect of this blockage is two-fold; the kidney is starved of blood and begins to die, and secondly the baroreceptors on the far side of the blockage detect a drop in blood-pressure and initiate the renin cascade to correct the problem (which really doesn't exist), leading to hypertension. Such kidneys may produce enormously elevated levels of renin and these would presumably be reflected in the levels of renin mRNA. Such material is therefore potentially an excellent source of mRNA for cDNA cloning, if it can be obtained in a fresh condition. Unfortunately, although arteriostenosis is not uncommon, removal of the kidney is now rarely undertaken. The normal remedy is simply to by-pass the kidney and allow it to die *in-situ*. Dr.Brenda Leckie (Glasgow) was able to obtain a small amount of such a kidney and kindly donated 5g of the material to our laboratory.

Total RNA was prepared from the 5g sample using the same methodology employed to extract RNA from mouse tissues. The kidney sample was in an advanced state of necrosis and not very amenable to homogenisation. 2.7mgs of RNA was obtained and of this 15$\mu$g were retained on oligo-dT cellulose. In view of the small amount of polyA$^+$ RNA recovered it was not re-chromatographed. The mRNA was translated *in-vitro* (1$\mu$g) and the products immunoprecipitated with either normal rabbit serum or anti-human kidney renin antiserum (a monoclonal antibody provided by Dr.J.Major, ICI, Pharmaceuticals Division). Although the tissue contained high levels of renin (J.Major, personal communication), no detectable immunoprecipitable *in-vitro* product was seen.

The advantages and success of using M13 as a vector for cDNA cloning prompted its use for cloning human renin cDNAs. In addition to the advantages already outlined, the technique allows the rapid screening of a large number of recombinants in a similar manner to the screening of a

genomic library made using a λ vector. Since the mouse cDNA sequence was known, direct sequencing of positive clones should quickly confirm their identity.

Single-stranded cDNA was prepared from 10μg of polyA$^+$ RNA and the yield was 1.2μg (12%). After alkali denaturation this was used in a single second-strand reaction. Synthesis of second strand was very poor and only 0.05μg of dscDNA was recovered. 80% of this was $S_1$-resistant and was made blunt-ended using $T_4$ DNA polymerase.

The dscDNA was then ligated to phosphatased, *Sma*I-cut M13mp11, and transformed into *E.coli* JM101 (JM103 was reported to contain a cryptic exonuclease). Three thousand recombinants were obtained and were plated out onto five 12.5cm lawns of JM101, prior to "Benton and Davis" -type screening (Benton and Davis,1977). Although putative positives were obtained when the filters were probed with pSMG142, the soft agar top did not give good plaque lifts and therefore the library was amplified and re-screened.

Approximately 15,000 phage from the amplified library were screened using pSMG142 at a density of 3000 plaques per 12.5cm filter. Twenty-one positives were found and purified, which on T-screening fell into 7 families. Sequencing of representative members of each family yielded two clones, #11 and #19, having homology to the mouse SMG cDNA sequence. Clone #11 contained approximately 750bp of 3'cDNA and in addition possessed a long polyA tail. The length of clone #19 was shorter (360bp) and it also had a polyA tail. The existence of two overlapping clones helps to confirm the sequence data. Both clones were in the 5'→3' orientation with respect to the universal priming site, and were therefore able to be sequenced directly. Because of the lengths of the clones, not all their sequence could be determined using the universal primer and therefore five primers were chosen to complete the task. The sequences determined using the universal and purpose-designed primers are shown in Figure 71. The figure compares the human sequence to that of the mouse SMG and indicates the positions of the primers used in the sequence determination. Mouse SMG renin cDNA and

**Fig.71** - Comparison of the nucleotide sequence of human kidney cDNA clones #11 and #19 with the mouse SMG cDNA sequence of Panthier *et al* (1982a) (revised by Soubrier *et al* (1983)

Positions of interest in either the mRNA or the protein are indicated with respect to the nucleotide sequence by the following symbols:

*1 initiation codon

*2 signal sequence cleavage point

*3 pro-segment cleavage point

*4 cleavage point between A and B chains

*5 termination codon

*6 polyadenylation signal

```
       10        20        30        40        50        60        70        80        90       100       110       120
TCTGGGCTACACAGCTCTTAGAAAGCCTTGGCTGAACCAGATGGACAGGAGGAGGATGCCTCTCTGGGCACTCTTGTTGCTCTGGAGTCCTTGCACCTTCAGTCTCCCAACGGGCACCAC
                                    *1                                                                  *2

      130       140       150       160       170       180       190       200       210       220       230       240
CTTTGAACGAATCCCACTCAAGAAAATGCCCTCTGTCCGGGAAATCCTGGAGGAGCGGGGAGTGGACATGACCAGGCTCAGTGCTGAATGGGACGTATTCACAAAGAGGTCTTCCTTGAC
                                                                                                                *3

      250       260       270       280       290       300       310       320       330       340       350       360
TGATCTTATCTCCCCCGTGGTCCTCACCAACTACCTGAATAGCCAGTACTATGGCGAGATCGGCATTGGTACCCCACCCCAGACCTTCAAAGTCATGTTTGACACGGGCTCCGCCAACCT

      370       380       390       400       410       420       430       440       450       460       470       480
CTGGGTGCCCTCCACCAAGTGCAGCCGCCTCTACCTTGCTTGTGGGATTCACAGCCTCTATGAGTCCTCTGACTCCTCCAGCTACATGGAGAATGGAGACGACTTCACCATCCACTACGG

      490       500       510       520       530       540       550       560       570       580       590       600
ATCAGGGAGAGTCAAAGGTTTCCTCAGCCAGGACTCGGTGACTGTGGGTGGAATCACTGTGACACAGACCTTTGGAGAGGTCACCGAGCTGCCCCTGATCCCTTTCATGCTGGCCCAGTT

      610       620       630       640       650       660       670       680       690       700       710       720
                                                          HUMAN cDNA #11        GCTAAAAGAGGACGTCTTCTCTTTCTACTACAACAG
TGACGGGGTTCTAGGCATGGGCTTTCCCGCTCAGGCCGTCGGCGGGGTCACCCCTGTCTTTGACCACATTCTCTCCCAGGGGGTGCTGAAGGAGAAAGTGTTCTCTGTCTACTACAACAG

      730       740       750       760       770       780       790       800       810       820       830       840
AGATTCCGAGAATTCCCAATCGCTGGGAGGACAGATTGTGCTGGGAGGCAGCGACCCCCAGCATTACGAAGGGAATTTCCACTATATCAACCTCATCAAGACTGGTGTCTGGCAGATTCA
---------GGGTCCCCACCTGCTGGGGGGCGAGGTGGTGCTAGGAGGCAGCGACCCGGAGCATTACCAAGGCGATTTTCACTATGTGAGCCTCAGCAAGACTGATTCCTGGCAGATCAC

      850       860       870       880       890       900       910       920       930       940       950       960
AATGAAGGGGGTGTCTGTGGGGTCATCCACCTTGCTCTGTGAAGACGGCTGCCTGGCATTGGTAGACACCGGTGCATCCTACATCTCAGGTTCTACCAGCTCCATAGAGAAGCTCATGGA
AATGAAGGGGGTGTCTGTGGGGTCTTCCACCCTACTGTGTGAAGAAGGCTGTGAGGTAGTGGTGGACACTGGTTCATCCTTTATCTCGGCTCCTACGAGCTCCCTGAAGTTGATCATGCA

      970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
GGCCTTGGGAGCCAAG---AAGAGGCTGTTTGATTATGTCGTGAAGTGTAACGAGGGCCCTACACTCCCCGACATCTCTTTCCACCTGGGAGGCAAAGAATACACGCTCACCAGCGCGGA
AGCCCTGGGAGCCAAGGAGAAGAGACTACATGAATATGTTGTGAGCTGTAGCCAGGTGCCCACCCTCCCCGACATCTCCTTCAACCTGGGAGGCAGGGCCTACACACTCAGCAGTACGGA

     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                       Human cDNA #19          TCCACGCCATGGATATCCCGCCACCCACTGGACCCACGTGGGCCCTGGGGGCCACCTTCATCCTAAAGTT
CTATGTATTTCAGGAATCCTACAGTAGTAAAAAGCTGTGCACAGTGGCCATCCACGCCATGGATATCCCGCCACCCACTGGACCCACCTGGGCCCTGGGGGCCACCTTCATCCTAAAGTT
CTACGTGCTACAGTATCCCAACAGGAGAGACAAGCTGTGCACAGTGGCTCTCCATGCCATGGACATCCCACCACCCACTGGGCCTGTGTGGGTCCTGGGTGCCACCTTCATCCGCAAGTT
                                *4

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
CTACACAGAGTTTGATCGGCGTAACAACCGCATTGGCTTCGCCTTGGCCCGCTGAGGCCCTCTGCCACCCAGGCAGGCCCTGCCTTA?GCCCT-GGCCCAGAGCTGGAAC-ACTCT>
CTACACAGAGTTTGATCGGCGTAACAACCGCATTGGCTTCGCCTTGGCCCGCTGAGGCCCTCTGCCACCCAGGCAGGCCCTGCCTTCAGCCCT-GGCCCAGAGCTGGAAC-ACTCTCTGA
CTATACAGAGTTTGATCGGCATAACAATCGCATTGGATTCGCCTTGGCCCGCTAAGGCCCTCTGCCACCCAG------------TAA-CCCTAGGCCAAGCCAAGCTGGCACTC-CTGG
                                          *5

     1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440

GATGCCCCTCTGCCTGGGCTTATGCCCTCAGATGGAGACATTGGATGTGGAGCTCCTGCTGGATGCGTGCCCTGACCCCTGCACCAG------CCCTTCCCTGCTTTGAGGACAAAGAGA
G---------GGCCATTTTGTCTGGCTTTGTCCCAACATAGGGACACTGGACACAGAGACCCTAACGAGTGTGTGCCCCTTCACTGCACCTCACCCTTCCCTGCTTTAAGGAAAAACCGA

     1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560

ATAAAGACTTCATGTTCACA
ATAAAGACTTCATGTT
 *6
```

human kidney renin cDNA show 81% homology in the 3' half of the coding region (residues 673-1252) and 62% homology in the 3' non-coding region. This is clearly revealed by a computer-aided comparison of the two sequences in which a $^{6}/_{6}$ or $^{6}/_{9}$ stretch of homology is represented by a diagonal line. The line of homology is seen in Figure 72 in which positions of insertion/deletion can be clearly seen as line displacements.

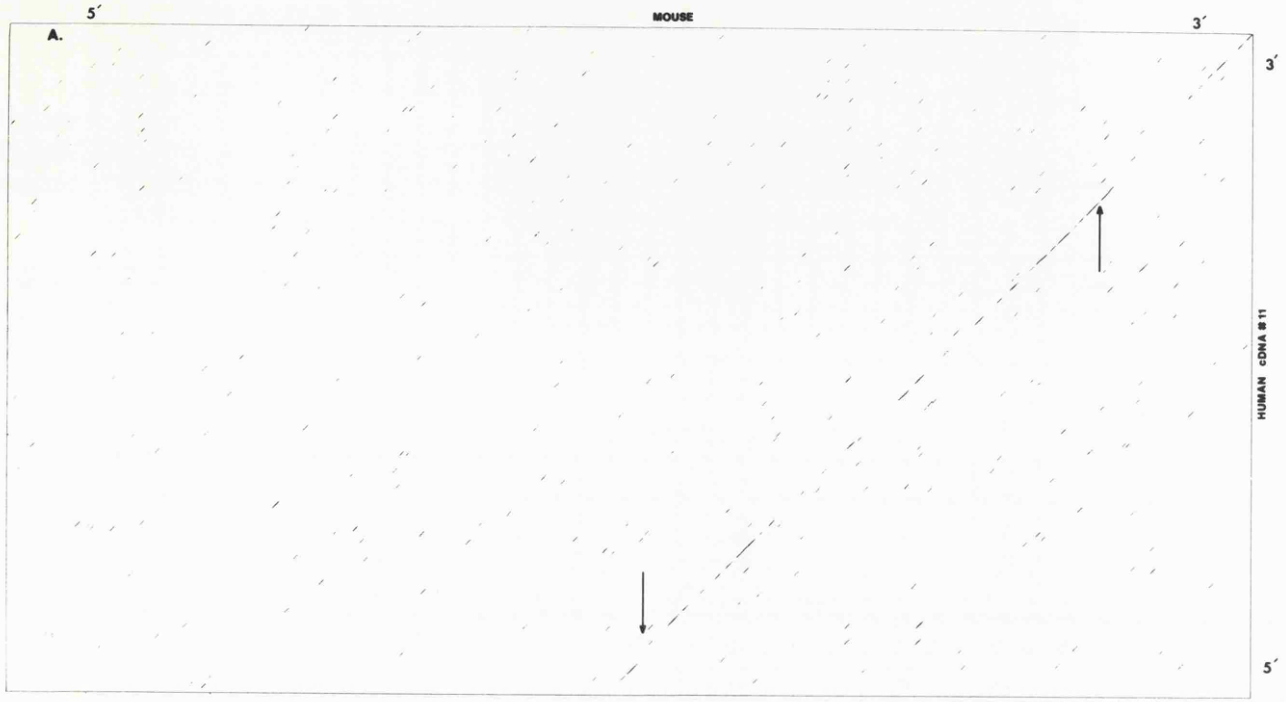
The sequence of #11 was translated into amino-acid sequence and compared (Fig.73) to the amino-acid sequences of mouse SMG renin (*Ren*-2) and the high producer *Ren*-1 gene products. One interesting difference between the human and mouse sequences is around the cleavage point between the A and B chains of the renin molecule. The Arg-Arg↓Asp cleavage point of the mouse proteins changes in the human enzyme to the sequence Ser-Ser-Lys, and although there is an alternative potential cleavage point slightly further towards the carboxyl-end of the molecule, the observation is striking amid rumours that mature human kidney renin consists of a single chain.

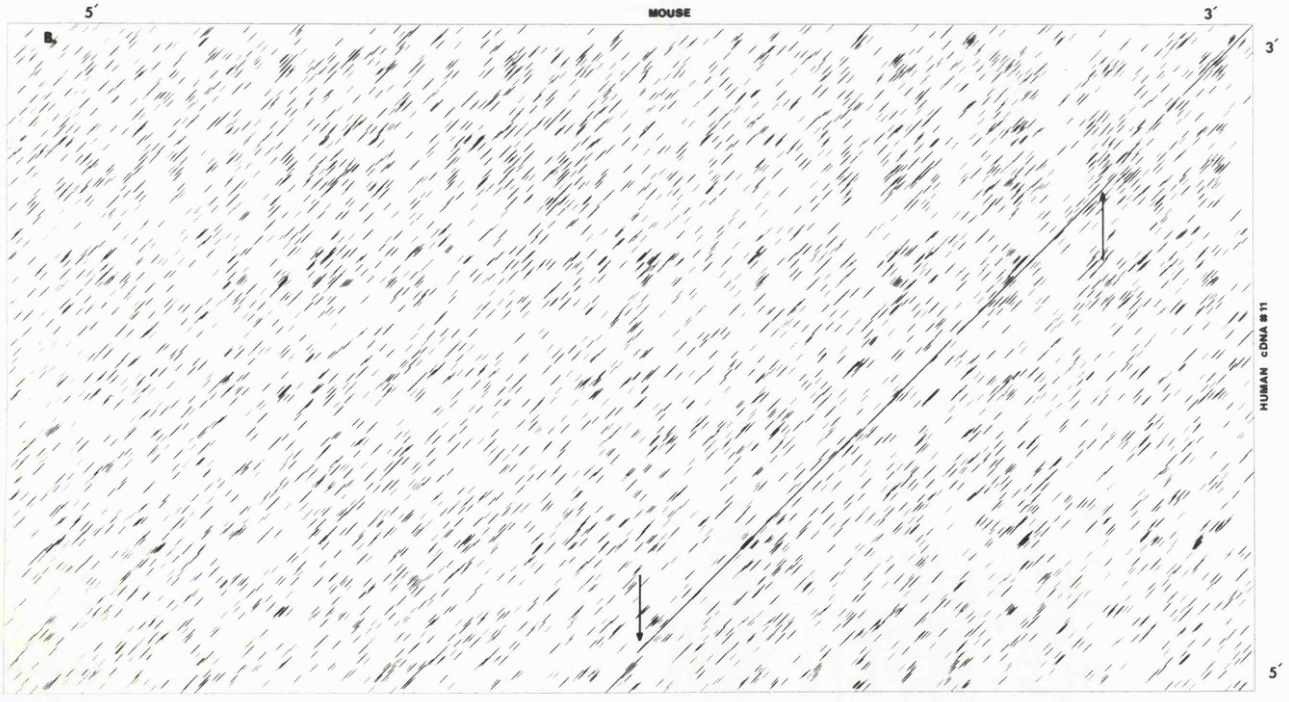
The availability of some human renin amino-acid sequence helps significantly in model-building of renin structure, and the availability of a sizable cDNA paves the way towards expression of renin for purification and crystallisation. If a full-length cDNA was isolated and expressed in *E.coli* there would be no guarantee that the product would be active since post-translational modifications are involved *in-vivo*. A full-length cDNA has recently been reported by Imai *et al* (1983) and agrees well with the sequence presented in figure 71. Speculation as to whether single-chain mouse renin is active (i.e. prior to the cleavage of the A and B chains) was answered by Pratt *et al* (1983) who demonstrated that single-chain renin was more active than the mature form! Expression of a cDNA in *E.coli* may well therefore generate an active product. Modelling studies by Blundel and colleagues place the A-B cleavage point on the surface of the molecule and indicate that the cleavage does not seriously alter the conformation of the molecule. Thus the cleavage may not be involved in activation of renin, and

**Fig.72** - Homology between human kidney renin cDNA sequence and mouse SMG renin cDNA sequence

The human cDNA sequence, clone 11, was compared by a 6/6 (a) or 6/9 (b) dot matrix analysis. The position at which there are three extra amino acids in the human sequence is seen as a line displacement and is indicated by a downward arrow. The upwards arrow shows the point at which the 3' untranslated region starts.

**Fig.73** - Comparison between the amino acid sequences of mouse and human renins **(overleaf)**

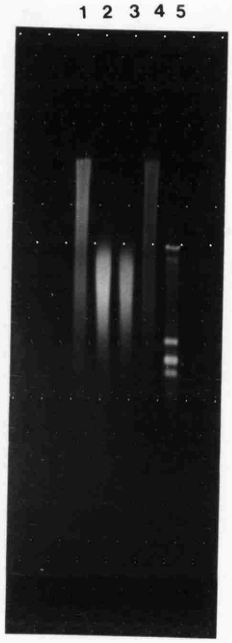

5′
MOUSE
3′
A.
3′
HUMAN cDNA #11
5′


5′
MOUSE
3′
B.
3′
HUMAN cDNA #11
5′

```
                                    220                                 230
Human Kidney Renin  L  K  E  D  V  F  S  F  Y  Y  N  R  D  S  E  N  S
Mouse SMG Renin     L  K  E  K  V  F  S  V  Y  Y  N  R  -  -  -  G  P
Mouse Kidney Renin  L  K  E  E  V  F  S  V  Y  Y  N  R  -  -  -  G  S

                                       240
Human Kidney Renin  Q  S  L  G  G  Q  I  V  L  G  G  S  D  P  Q  H  Y
Mouse SMG Renin     H  L  L  G  G  E  V  V  L  G  G  S  D  P  E  H  Y
Mouse Kidney Renin  H  L  L  G  G  E  V  V  L  G  G  S  D  P  Q  H  Y

                250                                 260
Human Kidney Renin  E  G  N  F  H  Y  I  N  L  I  K  T  G  V  W  Q  I
Mouse SMG Renin     Q  G  D  F  H  Y  V  S  L  S  K  T  D  S  W  Q  I
Mouse Kidney Renin  Q  G  N  F  H  Y  V  S  I  S  K  T  D  S  W  Q  I

                         270                                 280
Human Kidney Renin  Q  M  K  G  V  S  V  G  S  S  T  L  L  C  E  D  G
Mouse SMG Renin     T  M  K  G  V  S  V  G  S  S  T  L  L  C  E  E  G
Mouse Kidney Renin  T  M  K  G  V  S  V  G  S  S  T  L  L  C  E  E  G

                                    290                                 300
Human Kidney Renin  C  L  A  L  V  D  T  G  A  S  Y  I  S  G  S  T  S
Mouse SMG Renin     C  E  V  V  V  D  T  G  S  S  F  I  S  A  P  T  S
Mouse Kidney Renin  C  A  V  V  V  D  T  G  S  S  F  I  S  A  P  T  S

                                       310
Human Kidney Renin  S  I  E  K  L  M  E  A  L  G  A  K  -  K  R  L  F
Mouse SMG Renin     S  L  K  L  I  M  Q  A  L  G  A  K  E  K  R  L  H
Mouse Kidney Renin  S  L  K  L  I  M  Q  A  L  G  A  K  E  K  R  I  D

                         320                                 330
Human Kidney Renin  D  Y  V  V  K  C  N  E  G  P  T  L  P  D  I  S  F
Mouse SMG Renin     E  Y  V  V  S  C  S  Q  V  P  T  L  P  D  I  S  F
Mouse Kidney Renin  E  Y  V  V  N  C  S  Q  V  P  T  L  P  D  I  S  F

                                    340                                 350
Human Kidney Renin  H  L  G  G  K  E  Y  T  L  T  S  A  D  Y  V  F  Q
Mouse SMG Renin     N  L  G  G  R  A  Y  T  L  S  S  T  D  Y  V  L  Q
Mouse Kidney Renin  D  L  G  G  R  A  Y  T  L  S  S  T  D  Y  V  L  Q

                                       360
Human Kidney Renin  E  S  Y  S  S  K  K  L  C  T  L  A  I  H  A  M  D
Mouse SMG Renin     Y  P  N  R  R  D  K  L  C  T  V  A  L  H  A  M  D
Mouse Kidney Renin  Y  P  Y  R  R  D  K  L  C  T  L  A  L  H  A  M  D

                         370                                 380
Human Kidney Renin  I  P  P  P  T  G  P  T  W  A  L  G  A  T  F  I  R
Mouse SMG Renin     I  P  P  P  T  G  P  V  W  V  L  G  A  T  F  I  R
Mouse Kidney Renin  I  P  P  P  T  G  P  V  W  V  L  G  A  T  F  I  R

                                    390                                 400
Human Kidney Renin  K  F  Y  T  E  F  D  R  R  N  N  R  I  G  F  A  L
Mouse SMG Renin     K  F  Y  T  E  F  D  R  H  N  N  R  I  G  F  A  L
Mouse Kidney Renin  K  F  Y  T  E  F  D  R  H  N  N  R  I  G  F  A  L

Human Kidney Renin  A  R
Mouse SMG Renin     A  R
Mouse Kidney Renin  A  R
```

**Fig.74** - Self-ligation of sized human genomic DNA

Lane
1&4 Sized DNA after O/N ligation
2&3 Sized DNA
5 λ x *Eco*RI


1 2 3 4 5

single-chain product could be very useful in the design of inhibitors.

10.2 Preparation of a human genomic library in the λ vector DB287

Given the potential problems associated with expression of a human renin cDNA in *E.coli*, an alternative pathway towards expression could be valuable. One such pathway would be to introduce the entire gene or a derivative of it such as a mini-gene into a suitable mammalian cell-line. Prior to studying the expression of a human renin gene(s) the detailed structure of the gene(s) must be determined.

As a start to this goal a genomic library was constructed in the λ vector DB287. Human DNA isolated from the placenta of a male foetus was provided by Dr.A.Jeffreys (Dept.of Genetics) and 300μg was partially cut by the restriction enzyme *Sau*3A, as previously described for the mouse genomic library. The partially cut fragments were sized by centrifugation through two NaCl gradients and checked for ability to self-ligate (Fig.74). Three micrograms of the sized DNA was ligated to 14μg of λ arms prepared from the vector DNA by digestion with *Bam*HI and *Sal*I, and pelleting through a potassium acetate gradient (see materials and methods). The ligated DNA was then packaged using the "Packagene" kit (available from P&S Biochemicals, Ltd., Liverpool). The library was titred and found to contain 9.8 x $10^5$ recombinant phage which are presently being screened and should allow the structure of the human gene to be determined and reveal if there is one or more human renin genes.It would be of interest to study the renin genes of patients suffering from high-renin hypertension, in which the increase in blood-pressure is a consequence of elevated plasma renin activity. Is this caused by an altered renin gene?

# CHAPTER 11

## SUMMARY AND DISCUSSION

At the onset of the work described in this thesis the salivary glands were known both to contain and synthesise many enzymes, growth factors and structural proteins, several of which were of widespread interest. With the exception of α-amylase, however, these products had been studied exclusively by protein chemists, histologists, physiologists and biochemists. It seemed appropriate to apply the enormous potential of molecular biological techniques to these tissues, both to define new areas of study and to give a new dimension to the study of some of the potentially more interesting products.

Initially, messenger RNA was isolated from the three major salivary glands and translated *in vitro*. The profile of products observed was an important guide-line for the directions taken.

Three submaxillary gland products were potential candidates for study. Epidermal growth factor and nerve growth factor were known to be important hormones in the growth and development of cells, and the molecular cloning of EGF in particular seemed a realistic possibility. In retrospect, the assumption that because EGF was a small molecule it was likely that its precursor was also comparatively small was a naive one and the subsequent inability to immunoprecipitate the *in vitro* precursor may have had several explanations: antibody made to the mature protein would be extremely unlikely to be capable of recognising a precursor molecule 40-45 times its size. If, however, it had been able to do so, then the gel systems used would not have been able to resolve such a large polypeptide. Lastly, from the work of Gray *et al*(1983) EGF is probably not as major a product of the SMG as had been previously supposed. Nerve growth factor was known to have a precursor of 22kd and with this in mind, it was not surprising that antibody to the mature protein did not recognise this species (data not shown).

Therefore there were no suitable "handles" to use as tools to isolate cDNA clones.

The SMG product that offered the greatest opportunity of success was renin. Again, there appeared to be no obvious reason why such a protein should be made in large quantities in this tissue, but added to the fact that there was a genetic basis to the level at which it was produced and that as an enzyme it was medically important strongly favoured it as an SMG product worthy of study. Since it had been shown by Poulsen *et al* (1979b) that antibody to the mature protein was capable of recognising precursor forms, the handle needed for further exploitation was available. Using a combination of SMG renin antiserum and mRNA/cDNA hybridisation, cDNA clones were isolated encoding DBA/2 mouse SMG renin. Before sequencing of these clones was undertaken, Panthier *et al* (1982a) published the sequence of two cDNA clones which together encoded the entire renin precursor molecule. From a comparison of restriction maps, their data confirmed indirectly that our clones were indeed derived from renin mRNA. Subsequent events (particularly their sequence corrections) have shown that although the renin sequence had been published, it would have been valuable to have determined the nucleotide sequence of our own clones at that time, but since the amino acid sequence had been discovered, it appeared that there was little new to be gained by it.

The predicted amino acid sequence of the renin precursor proposed a signal sequence of 18 amino acids (≈ 2kd) and although renin was known to be a secreted product of the SMG, the existence of a signal sequence had not been shown directly. The success of the hybrid-selection technique prompted the isolation of a highly enriched renin mRNA preparation which was translated *in vitro* in both the presence and the absence of dog pancreas membranes. A comparison of the translation products revealed that the addition of DPM to the reaction resulted in the removal of ≈2 kd from the renin precursor, providing direct evidence for the hypothesised signal sequence and linking *in-vitro* tranlation data with results obtained from

the incubation of tissue slices *in-vitro*.

A comparison of the relative abundances of high and low producer renins by *in vitro* translation of their respective SMG mRNAs revealed that the high versus low phenotype was a result of a difference in translatable renin mRNA levels and not simply a variation in the specific activity of the enzymes. In addition, the two precursors were indistinguishable in size showing that there was no gross difference between the proteins. The difference in renin levels between male and female mice was also due to the levels of translatable mRNA indicating that renin expression is controlled at either the level of transcription or the stability of the mRNA.

Southern blotting of genomic DBA/2 DNA and probing with the renin cDNA clone pSMG142 led to the discovery of two distinct renin genes in the high producer strains. This opened the way to a possible explanation of high and low phenotypes, although it was clear that more was involved than gene dosage. Although no data are presented, attempts were made to determine the number of renin genes in the low producer. The southern blots revealed only one band per track in contrast to those of DBA/2 but the result was not unequivocal. The publication by Piccini *et al* (1982) that a DNA polymorphism was associated with high SMG renin production confirmed that the high producer mouse had two renin genes and showed that there was only a single gene in the low producer. The hypothesis of two renin genes in the high producer mouse was confirmed by the isolation of genomic clones from a DBA/2 genomic DNA library. These clones could be divided into two groups on the basis of their restriction maps which showed a significant difference at their 3' ends. It was this difference that accounted for the DNA polymorphism observed by Piccini, and showed that the interpretation of the Southern blotting data presented in Chapter 6 was correct. The *Ren*-2 gene, specific to the high producer, was found to possess an extra 3 kb of DNA downstream of the structural gene. The sequencing of this region of DNA has revealed significant homology to a type 1A retrovirus possessing several characteristic features including LTR sequences. The length of this stretch

of DNA would suggest that it represents a defective provirus, disabled by the deletion of approximately 4 kb of DNA. Many retroviruses have been shown to possess enhancer sequences capable of increasing the rate of transcription, and although not proven, it is possible that the high producer renin phenotype is a result of enhancer activity of this kind. In this respect, it would act over an unusually long distance and the fact that it is 3' of the gene is of particular interest. From $S_1$ mapping and primer extension studies, the most active promoter was found to be the one closest to the 3 kb element. In addition, P2 (the second closest) is more active than the most 5' potential promoter P1, but even P1 appears to be more active in the high producer mouse than the low producer. It would be a useful experiment to mutate P3 such that P2 becomes the closest promoter to the 3kb element and/or to introduce a potential promoter between P3 and the ATG initiation codon. Such experiments would tell if there was 3' regulatory activity acting on the gene.

The 3kb element is unlikely to be responsible for androgen inducibility since the *Ren*1 gene is, in real terms, more inducible than *Ren*2. Similarly its involvement in tissue-specificity is questionable since the *Ren*1 gene shows tissue-specificity, being expressed in significant quantities only in the SMG and kidney. Two observations can therefore be made: firstly, that if this element is responsible for elevated expression of the *Ren*2 gene then its enhancing activity is sensitive to regulation by normal tissue-specific and androgen controls. Secondly, the question must be asked - why is *Ren*2 not active in the kidney? Does the 3' element adversely affect kidney-specific expression and if so how, or are there other *Ren*2 features yet to be recognised which determine the lack of expression in the kidney. It would be informative to ask such questions by intoducing hybrid genes into either primary culture cells or into the mouse germ-line. The possibility of placing genes into the germ-line provides a unique opportunity of answering questions of tissue specificity and developmental control, and although at present few experiments have been carried out in this area those that have

demonstrate that such genes can exhibit normal tissue-specific and developmental patterns. If the 3kb element was placed near *Ren*1 (or a low producer *Ren*1-type gene) in the appropriate position would that gene then be active in the kidney? The parallel experiment of determining the effect of removing the 3kb element from *Ren*2 may well show any involvement of the element in tissue specific expression (with respect to the kidney) and clarify its role, if any, in androgen induction. If the element is shown not to be involved in tissue specificity then a direct comparison between the nucleotide sequences of *Ren*1,*Ren*2, and low-producer gene, preferably over a significant distance 5', may prompt similar hybrid experiments in which mutations have been introduced in order to alter the tissue specificity. Recent evidence points to the hormonal control of certain genes being dependent on defined stretches of DNA (in the case of mouse mammary tumor virus a maximum of 100bp (Buetti and Diggelmann,1983)) but it is not clear how the binding of a receptor complex to such a region of DNA alters the expression of a particular gene. In the case of androgens, however no such stretches of DNA have been defined to date.

The SMG provides a unique opportunity to compare genes which are expressed in the same tissue either under androgen control or independent of androgen. Since discrete DNA sequences are being implicated in tissue specificity (Gillies *et al*,1983; Banerji *et al*,1983) a close comparison of such genes may help in an understanding of this phenomenon. On a wider footing the SMG would be an interesting developmental tissue to study, since testosterone can elicit marked changes in morphology in a reversible manner. These changes start to take place within a very short timescale, and it may be possible, perhaps via a tissue culture model, to look at the very early stages of androgen induction. How does testosterone produce major changes in cellular structure in addition to affecting the expression of a specific gene?

The renin system generally still has some important gaps which recent progress has provided a way of filling. The identities of the enzymes which

actually cleave the renin precursor (prorenin) to form the 38kd single chain molecule and the 35+5kd two chain form are still unknown. Their exact site of expression and their control are also therefore unknown and these facts are essential for a complete understanding of the physiology of the renin-angiotensin system. The availability of cloned cDNAs means that it should be possible to synthesis and purify various renin precursor molecules and use them as tools to assay for cleavage enzymes. It would also be of interest to find out if maturation can take place in the bloodstream, and whether,in light of the work of Pratt *et al* (1983), renin is secreted as a highly active single-chain enzyme and then cleaved later as a regulatory mechanism to quickly decrease overall renin activity.

cDNA probes are currently being used to determine whether renin is expressed in tissues other than the SMG and the kidney. There is evidence for renin activity in many tissues but the arguements about its expression may be settled by hybridisation histochemistry and thereby give a clearer picture of renin physiology.

The primer-extension and $S_1$ mapping studies have highlighted more than one candidate for the renin promoter. It would appear that P2 and P3 are both influenced by androgen and the 3 kb element but I have not observed any variation in the relative amounts of P2- and P3-initiated transcription. The presence of two species of renin mRNA of such similar size would not have been detected on the Northern blot, but I would have expected, perhaps, to have been able to detect transcripts from P1 after a long exposure. It is difficult at present to be confident of P1 as a promoter, and to regard it as a firm candidate would require $S_1$ mapping using a restriction fragment from the low producer gene and/or primer extension from a more 5' start point. These studies are presently underway.

The mRNA hybridisation experiment described in chapter 8 shows that renin mRNA levels correlate with the androgen level and although the puzzling data of the first experiment is unresolved, the data of the larger scale experiment have been independently verified (Catanzaro *et al*, in

press). The interesting observation that the female kidney has higher levels of renin mRNA than the male in both high and low producer mice, would seem to invalidate the possible explanation that this was due to suppression of kidney levels in the male due to SMG renin feedback inhibition. This observation has still to be explained.

As regards the models referred to earlier in the thesis, none are totally satisfactory. Of the models proposed by Wilson *et al*(1982b), the suggestion of two structural genes being present, one of which (the more active one) is a null allele in the low producer, is the closest approximation to the facts, but no account is made of the activity of the genes in the kidney. The model of Panthier *et al* (1982b) is a good working model but suggests levels of activity for which there is no evidence ie. *Ren*-1 activity in the SMG and a total absence of *Ren*-2 activity in the kidney. In addition, it does not take account of the variation in kidney renin mRNA levels between males and female. Therefore, although such models are a useful guideline, their inferences must not be taken too strictly.

There are many areas of renin physiology and molecular biology still to be explored but during the last 3-4 years significant progress has been made in many directions.

The sublingual gland provided a good contrast to the renin project: instead of a well defined system, it offers a tissue which is new territory to both biochemists and molecular biologists. One of the most important findings is that mucin is probably not, as previously supposed, the major product of the gland. Instead, a family of small polypeptides of 16-17 kd are synthesised. There appears to be no significant difference in the levels of production between males and females and in many respects this family can be regarded as the gland's equivalent of the '36' kd polypeptides of the SMG (Windass *et al*,1984). The amino acid sequence defines a previously unrecognised protein and the primary objective is to complete the sequence by a selective cloning experiment. Using a combination of the cDNA synthesis method of Gubler and Hoffman (1983), size-selection and M13

cloning and sequencing, it should be possible to generate a more extensive group of sequences for comparison. Not only will the complete amino acid sequence be determined, but a better idea of the number of members of the family should be obtained. Such analysis would tell if this family results from the transcription of many genes or the differential splicing of a small number. From examples like the major urinary proteins (MUPs) and the 36 kd SMG polypeptide, it would be tempting to suggest that there was a large family of closely nested genes but confirmation of this requires the isolation of genomic clones. As to their function, once again we have a major protein with no known activity but the availability of clones makes possible the design of physiological experiments along the lines of the NGF and EGF studies. Whatever the function, as for the MUPs, their discovery means that there is another interesting and potentially informative group of proteins to be studied.

The cloning of the human kidney renin cDNAs highlights the indirect use of animal systems for human applications. Documented differences between mouse and human renin must have some explanation in the differences found by comparing their sequences. This has been an exciting sideline to the main project and, as suggested in chapter 10, there may well be clinically serious variation in the expression of the human renin gene(s) which would make the human system more worthwhile to study. However, the mouse genes are proving to be a very good system for experimental investigation, possessing features from many areas of current interest.

The expression of several well-characterised genes is affected by various steroid hormones, whose presence usually induces the synthesis of the corresponding product. One of the best studied systems is the ovalbumin gene which is controlled by several steroid hormones, including oestrogen and progesterone (Palmiter *et al*, 1981). Although purified progesterone receptors have been shown to bind to specific sequences *in vitro* (Mulvihill *et al*, 1982) the mechanism by which such hormone-receptor complexes regulate gene expression is unknown. Still less is known about the mechanism of androgen induction and whilst androgen inducible genes have been isolated and characterised (Page and Parker, 1983), studies of the receptor are made difficult by its instability.

One of the most informative areas has been the study of Mouse Mammary Tumour Virus (MMTV) which is known to be hormonally regulated (Varmus *et al*, 1979). The entire viral genome has been cloned and shown to retain its response to glucocorticoid hormones when introduced into tissue culture cells (Buetti and Diggelmann, 1981). The purification of the glucocorticoid receptor from liver cells led to the demonstration that it bound specifically to the MMTV promoter region (Pfahl, 1982; Payvar *et al*, 1981), implying a direct interaction with DNA. Chandler *et al* (1983) found that a 340bp MMTV DNA fragment inserted upstream of the HSV thymidine kinase (tk) gene was sufficient,in the presence of dexamethasone, to increase the number of $tk^+$ transfectants. The region within this fragment which was responsible for the interaction was termed the glucocorticoid responsive element (GRE) and could confer glucocorticoid responsiveness in either orientation. The precise nature of the GRE has been further defined initially by Buetti and Digglemann (1983) as being present between -105 and -204 nucleotides upstream from the initiation site of viral transcription and by Schneidereit *et al* (1983) who identified a similar region and from nuclease protection studies proposed several receptor-binding sequences

sharing the hexanucleotide sequence TGTTCT. More recently Scheidereit and Beato (1984) have shown the presence of four binding sites within a GRE between -72 and -192 nucleotides upstream of the viral initiation site, which interact directly with the receptor. The G residue within the TGTTCT sequence has been shown to be critical for the interaction and if methylated prevents receptor binding.

In summary, although the regulation of many genes has been shown to involve steroid hormones, to date there is little known as to the mechanism of action of the hormone-receptor complex, even though in certain cases they are known to bind specifically to regions close to the promoter for the regulated gene. The actual mechanism whereby such binding effects regulation is at present unclear.

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# The Biosynthetic Pathway of Renin in Mouse Submandibular Gland*

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**The present study set out to demonstrate the biosynthesis of a prorenin and its processing in the cell, using the richest known natural source of renin, the mouse submandibular gland. Cell-free translation of total poly(A$^+$) RNA or mRNA selected using a renin cDNA yielded $M_r$ = 45,000 preprorenin which in the presence of dog pancreatic microsomal membranes, was converted to $M_r$ = 43,000 prorenin. The latter was seen during *in vitro* labeling of tissue with [$^{35}$S]methionine. Prorenin was synthesized first and converted rapidly to $M_r$ = 38,000 single chain renin. Renin was then hydrolyzed slowly to give two chains of $M_r$ = 33,000 and 5,000 held together by disulfide bonds. The $M_r$ = 38,000 and 33,000 species had similar peptide maps. Western blotting of fractions from a pepstatin affinity column identified the separation of prorenin from renin. The results suggested that both single and two-chain renin have an exposed active site. Testosterone stimulated synthesis of prorenin during *in vitro* labeling of female tissue. Thus, mouse renin is synthesized as a preprorenin ($M_r$ = 45,000) which is converted to a prorenin ($M_r$ = 43,000) and then to renin ($M_r$ = 38,000) by rapid processing within the cell, after which renin is cut slowly to give a two-chain form.**

Renin (EC 3.4.99.19) plays a pivotal role in the regulation of blood pressure via its highly specific enzymatic cleavage of the plasma protein angiotensinogen to form angiotensin I. Further hydrolysis of this decapeptide by the ectoenzyme dipeptidyl carboxypeptidase yields the physiologically active octapeptide angiotensin II. The discovery in 1971 of an inactive form of human renin that could be activated by partial hydrolysis led Lumbers (1) and Morris and Lumbers (2) to postulate the existence of a renin precursor, prorenin. However, rigorous testing of this hypothesis has been impeded because renin and, presumably, prorenin are present only in low concentrations in the tissues of most species (3-5). Only recently has a prorenin-like molecule been isolated, from hog kidney, requiring a three million-fold purification (6). Other attempts to identify prorenin have utilized the submandibular gland of the adult male mouse, the richest source of renin known (2% of protein) (7-9). Although early experiments by Nielsen and co-workers (10) failed to find prorenin in extracts of MSG[1], Poulsen and co-workers (11) did show that the primary translation product of MSG mRNA gave a renin-immunoreactive protein that was $M_r$ = 10,000 larger than renin. Recently, Panthier *et al.* (12) deduced a complete amino acid sequence encoded by MSG renin cDNA. This sequence possessed possible target sites for enzymatic cleavage which could give rise to molecules intermediate in size between the primary translation product and the isolated form.

In the present paper we examine the biosynthesis of renin in the MSG and present direct evidence for a "pro" form.

## EXPERIMENTAL PROCEDURES

*Materials*—Phosphorylase *b*, bovine serum albumin, ovalbumin, α-chymotrypsinogen, ribonuclease A, basic pancreatic trypsin inhibitor, creatine phosphate and creatine phosphokinase were obtained from Sigma. L-[$^{35}$S]Methionine (≥1000 Ci/mmol), dog pancreatic microsomal membranes, Econofluor, and Protosol were obtained from New England Nuclear. Rabbit reticulocyte lysate was prepared by the method of Pelham and Jackson (13). L-[$^{35}$S]Methionine for cell-free translation experiments was from Amersham, United Kingdom. Testosterone propionate (Testoviron) was from Schering AG, Berlin. Oligo(dT)-cellulose (Type 3) was from Collaborative Research Inc., Waltham, MA.

*Preparation of Renin and Antirenin*—Renin was isolated from the submandibular glands of adult male Quackenbush mice by two methods (14, 15). Pure renin eluted from CM52-cellulose as two major peaks, equal in proportion, with specific activities of 1.0-1.1 × $10^8$ pmol of angiotensin I/h/mg of protein determined as described previously (9). Two-dimensional gel electrophoresis suggested that these were similar in size ($M_r$ = 33,000) but differed in pI (5.4 and 5.6). Each also had a 5-kDa subunit which will be described later. Antiserum was raised in Castle Hill White rabbits. It was judged to be monospecific by one- and two-dimensional electrophoresis and Ouchterlony double diffusion analysis (16); 5-20 μl were required to precipitate 1 μg of renin in a total volume of 200 μl. $^{125}$I-labeled renin was prepared using a lactoperoxidase-glucose oxidase radioiodination kit, and specific activities obtained were 2-5 × $10^6$ cpm/μg.

*In Vitro Incorporation Experiments*—Pieces of tissue from testosterone-induced, castrated male mice were incubated with [$^{35}$S]methionine using the method of Berger and Shooter (17). Specific details of individual experiments are given in the figure legends. Renin and renin-like proteins were obtained by immunoprecipitation with renin antiserum, where the optimal amount was determined by titration with $^{125}$I-labeled renin as described (17). After incubation overnight at 4 °C, the immunoprecipitate was sedimented using an Eppendorf microfuge and washed three times in 0.025 M sodium phosphate buffer, pH 7.6, containing 0.1 M NaCl and 0.25% Nonidet P-40. The yield was 90-96% based on recovery of $^{125}$I-labeled renin. The effect of testosterone on synthesis in tissue from female mice was examined by adding the steroid to tissue medium after 1 h of incubation *in vitro*.

*Electrophoresis*—SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (18). In some experiments, samples were run simultaneously on duplicate gels so that one gel could be dried (using a Bio-Rad Model 224 Gel Slab Dryer) and autoradi-

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[1] The abbreviations used are: MSG, mouse submandibular gland; SDS, sodium dodecyl sulfate.

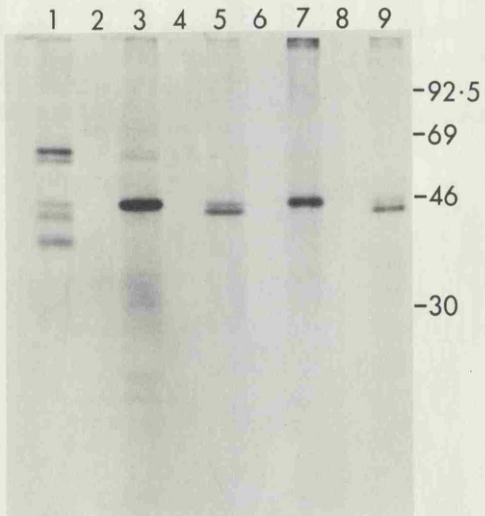


FIG. 1. **Cell-free translation of MSG mRNA.** *Lane 1*, total poly(A$^+$) RNA (0.02 $\mu$g); *lane 2*, no mRNA (control); *lane 3*, translation of mRNA selected by hybridization with renin cDNA; *lane 4*, same as *lane 5*, but without mRNA; *lane 5*, hybrid selected translation in the presence of dog pancreatic microsomal membranes; *lane 6*, pre-immune control of *lane 7*; *lane 7*, immunoprecipitation of *lane 3* with antirenin before electrophoresis; *lane 8*, pre-immune control of *lane 9*; *lane 9*, immunoprecipitation of *lane 5* before electrophoresis. Molecular weight markers in ascending order of size were carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase *b* (shown as $M_r \times 10^{-3}$).

ographed (with Kodak X-Omat S film) at −70 °C, and the stained bands in the other gel could be excised and their content of radioactivity determined after elution of gel slices at 37 °C overnight in 10 ml of Econofluor containing 3% Protosol and liquid scintillation counting using a Beckman LS 3150P counter. Autoradiographs were scanned with a Camag Electrophoresis Scanner. Two-dimensional electrophoresis was performed as described by O'Farrell (19) except that the solution of acrylamide for the first dimension (isoelectric focusing) was made freshly each time. Peptide mapping on SDS gels was carried out by the method of Cleveland *et al.* (20). Reduction and *S*-carboxymethylation of pure renin were performed as described previously (10). Western blotting was performed by the method of Burnette (21).

*Precipitation with Trichloroacetic Acid*—Portions (2 $\mu$l) of submandibular gland supernatants and redissolved immunoprecipitates were spotted on Whatman No. 3MM filter paper. The filters were washed once with 10% trichloroacetic acid, boiled for 10 min in fresh 10% trichloroacetic acid, washed with three changes of 10% trichloroacetic acid at 22 °C, with two changes of ethanol, and with one of ether, then dried at 100 °C. Radioactivity was solubilized by incubating each filter with 0.5 ml of Protosol at 60 °C for 30 min. After neutralization with 50 $\mu$l of glacial acetic acid, 10 ml of Econofluor were added and radioactivity was determined.

*Isolation of mRNA*—RNA was prepared by a modification of the method of Noyes *et al.* (22), and poly(A$^+$) RNA was purified by chromatography on oligo(dT)-cellulose. Hybrid selection of renin mRNA was carried out by the method of Wood *et al.*[2] using a partial renin cDNA clone, pSMG213, which had been isolated from an adult male DBA/2 mouse submandibular gland cDNA library (23).

*Translation of mRNA*—Total and hybrid selected mRNA was translated in a reticulocyte lysate system containing 20 $\mu$M each amino acid (except methionine), 1 mM ATP, 0.2 mM GTP, 80 mM KCl, 2 mM magnesium acetate, 10 mM Tris-HCl, pH 7.6, 2 mM glucose, 7 mg/ml of creatine phosphate, 50 $\mu$g/ml of calf liver tRNA, 1 mCi/ml of [$^{35}$S]methionine (specific activity, 1160 Ci/mmol) and 50 $\mu$g/ml of creatine phosphokinase. Incubation was at 30 °C for 90 minutes. For *in vitro* processing experiments, the reaction mixture was supplemented with 4 $A_{260}$ units/ml of dog pancreatic microsomes. Translation products were immunoprecipitated by a modification of the method of Dobberstein *et al.* (24) and electrophoresed on a 10% polyacrylamide gel, which was dried and autoradiographed at −70 °C.

[2] D. Wood, A. Blanchetot, and A. J. Jeffreys, manuscript in preparation.

## RESULTS

Translation of mRNA yielded a predominant renin-immunoreactive protein of $M_r$ = 45,000. Prior selection of renin mRNA, using its corresponding cDNA, and *in vitro* processing of the translation product with dog pancreatic membranes resulted in a decrease in molecular weight of 2,000 (Fig. 1), consistent with the presence of a signal peptide on the translation product of the renin mRNA. The processed translation product as well as its precursor could be specifically immunoprecipitated with renin antiserum.

In continuous labeling experiments, two major species of labeled protein were precipitated by renin antiserum. A $M_r$ = 43,000 band was the first to appear and remained constant

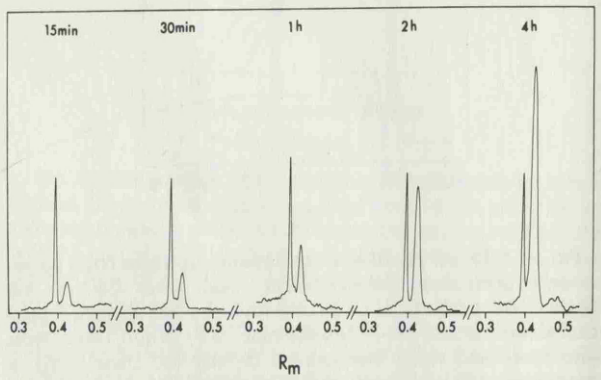


FIG. 2. **SDS gel profiles of immunoprecipitates from continuous labeling experiments.** Submandibular gland pieces (540 mg wet weight) were incubated in 1.0 ml of methionine-free medium supplemented with 1.0 mCi of L-[$^{35}$S]methionine. At the indicated times, 90–100 mg of tissue (wet weight) were removed and homogenized in 10 volumes of buffer. After centrifugation at 100,000 × *g* for 1 h at 4 °C, 0.5 ml of supernatant was mixed with 0.5 ml of renin antiserum and 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.2. After incubation overnight at 4 °C, the precipitate was collected by centrifugation, washed, dissolved in 100 $\mu$l of SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, containing 10% (v/v) glycerol, 5% (v/v) $\beta$-mercaptoethanol, and 1.2% sodium dodecyl sulfate), and then placed in a boiling water bath for 1 min. 20 $\mu$l were applied to a 12.5% discontinuous gel for electrophoresis. The gel was then fixed, dried, and exposed to x-ray film for 2 days. The autoradiograph was then scanned. Only the portion of each lane that contained bands of radioactivity is shown.

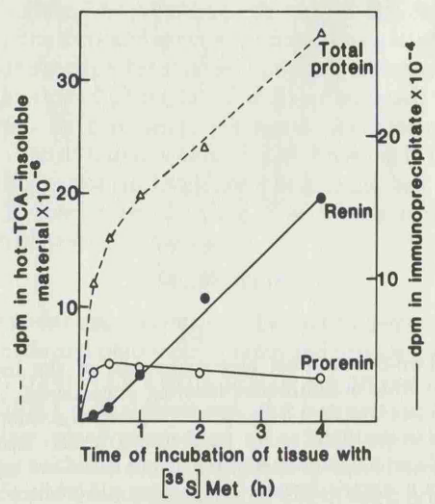


FIG. 3. **Kinetics of labeling of the $M_r$ = 43,000 species (prorenin) and $M_r$ = 38,000 renin.** Data were obtained from the experiment shown in Fig. 2 by excising stained bands from the gel and counting the amount of radioactivity in each. Radioactivity of total protein in a 0.5-ml sample is shown for comparison on a different scale. Data apply to 50 mg of tissue samples. *TCA*, trichloroacetic acid.

for all time points (Fig. 2). Another band of $M_r$ = 38,000, although initially faint, increased progressively in intensity for the duration of the labeling period (4 h), by which time radioactivity began to appear in the region of $M_r$ = 33,000. Similar results were obtained in replicate experiments. Radioactive bands were excised and counted. Fig. 3 shows the time course of incorporation of radioactivity into total protein and into the $M_r$ = 43,000 and 38,000 renin-immunoreactive material. Whereas radioactivity was rapidly incorporated into the $M_r$ = 43,000 form it was only after 30 min that radioactivity was detected in the $M_r$ = 38,000 form. After this initial lag, incorporation into the $M_r$ = 38,000 species proceeded at a constant rate, accounting for 1% of total protein synthesis.

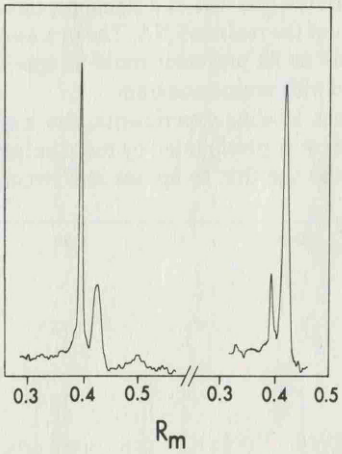


FIG. 4. **SDS gel profiles of immunoprecipitates from pulse-chase experiments.** Submandibular gland pieces (380 mg wet weight) were incubated in 1.0 ml of methionine-free medium supplemented with 1.0 mCi of L-[$^{35}$S]methionine. After 15 min, tissue pieces were rinsed and either homogenized directly (no chase) (*left*) or transferred to 25 ml of fresh medium containing 75 μg of unlabeled L-methionine/ml and no additional label. At 15 min (*right*) and longer periods, 60–70 mg of tissue (wet weight) were rinsed and homogenized and 1/5 of washed immunoprecipitates from 0.5 ml of supernatant was electrophoresed. The gels were fixed, dried, and exposed to an x-ray film for 2 days. The autoradiograph containing bands of radioactivity is shown. Chase periods of 30 and 60 min showed essentially the same labeling pattern as the 15-min chase (data not shown).

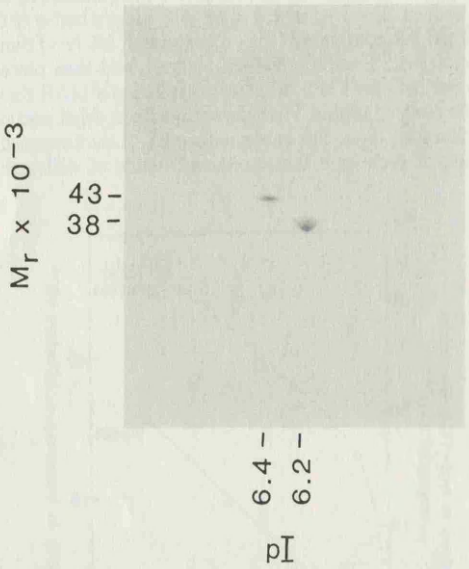


FIG. 5. **Two-dimensional electrophoresis of the immunoprecipitate from a continuous labeling experiment.** The immunoprecipitate from the 4-h sample was prepared for electrophoresis as described in the legend to Fig. 1 and then 20 μl were mixed with an equal volume of isoelectric focusing sample buffer and applied to a cylindrical isoelectric focusing gel. A linear pH gradient ranging from pH 4.7 to 7.1 was measured using a surface electrode (Activon, Sydney, Australia) applied directly on the gel at measured distances. This gel was then electrophoresed in the second dimension on a 12.5% discontinuous SDS slab gel. The gel was fixed, dried, and autoradiographed for 5 days. As well as the two major spots shown above, radioactivity was also detected in two spots of $M_r$ = 33,000 with isoelectric points of 5.4 and 5.6.

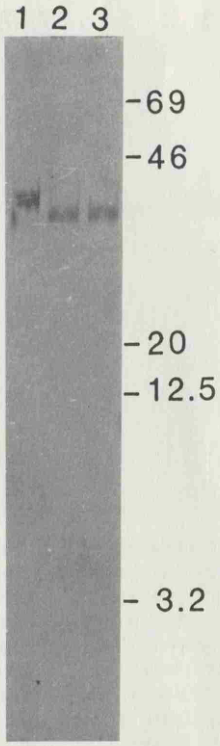


FIG. 6. **SDS gel electrophoresis of pure renin.** A highly purified preparation of MSG renin was analyzed by electrophoresis on a 15% SDS-polyacrylamide gel. The stacking gel was omitted to allow pre-electrophoresis in order to remove ammonium persulfate from the gel. After 1 h of pre-electrophoresis in lower gel buffer, this was replaced with Tris glycine running buffer and the samples were loaded. Samples were prepared exactly as described by Nielsen *et al.* (10). Each lane contained 10 μg of renin. *Lane 1*, renin dissolved in 50 μl of sample buffer and incubated at 37 °C for 20 min, then placed in a boiling water bath for 2 min; *lane 2*, as *lane 1* plus 10 μl of 80 mM dithiothrietol; *lane 3*, as *lane 2* but incubated for 1 h at 37 °C with 5 μl of 0.5 *M* α-iodoacetamide. Molecular weight markers in ascending order of size were insulin B chain, ribonuclease A, soybean trypsin inhibitor, ovalbumin, and bovine serum albumin.

In pulse-chase experiments, a similar pattern of labeling was observed (Fig. 4). During the 15-min pulse, radioactivity accumulated in a $M_r$ = 43,000 renin-immunoreactive band. After incubation for 15 min in medium containing an excess of unlabeled methionine, the radioactivity in the $M_r$ = 43,000 species decreased markedly with a concomitant increase in the $M_r$ = 38,000 form, thus indicating that conversion had occurred.

Two-dimensional electrophoresis of the 4-h sample from a continuous labeling experiment separated four labeled renin-immunoreactive species (Fig. 5). The isoelectric points of the $M_r$ = 43,000 and 38,000 forms were 6.4 and 6.2, respectively. However, the $M_r$ = 33,000 component was resolved into two forms with isoelectric points of 5.6 and 5.4. The electrophoretic properties of these corresponded to those of the two major forms of pure renin isolated from the MSG. On the stained gel, the two $M_r$ = 33,000 forms accounted for 25% of the total renin each, while the $M_r$ = 43,000 and 38,000 forms were 5 and 45%, respectively.

Electrophoretic analysis of pure renin showed that it is

composed of two subunits held together by disulfide bridges (Fig. 6). Before reduction, pure renin migrated at $M_r$ = 38,000. After reduction of disulfide bonds with dithiothreitol, bands of $M_r$ = 33,000 and 5,000 appeared. Carboxymethylation caused a slight decrease in the electrophoretic mobility of the major band, possibly by changing its SDS-binding capacity.

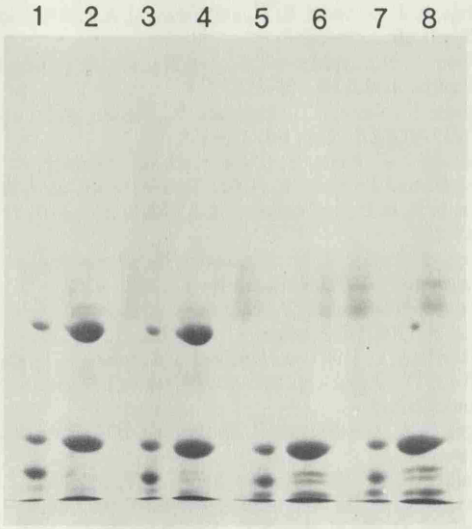


FIG. 7. **Peptide mapping of $M_r$ = 38,000 and 33,000 renin.** 50 μg of protein eluting from a pepstatin column in 0.1 M Tris-HCl, pH 7.4, 0.5 M NaCl (15) were reduced by boiling in SDS sample buffer containing 5% (v/v) β-mercaptoethanol for 1 min. After electrophoresis in a 12.5% SDS-polyacrylamide gel, the $M_r$ = 38,000 and 33,000 bands were excised and prepared for peptide mapping on a 15% SDS-polyacrylamide gel containing 1 mM EDTA. *Lanes 1, 3, 5,* and *7*, $M_r$ = 38,000 renin incubated with 0.1, 1, 5, and 10 μg, respectively, of V8 protease from *Staphylococcus aureus* (Miles); *lanes 2, 4, 6,* and *8*, $M_r$ 33,000 renin incubated with each respective concentration of V8 protease.

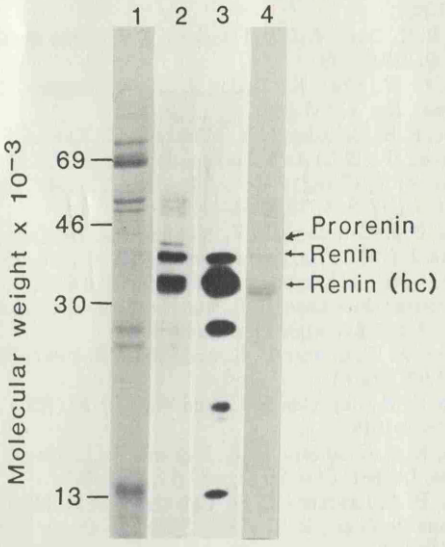


FIG. 8. **Western blotting of fractions from a pepstatin affinity column.** Pepstatin affinity chromatography was performed as described (15) using an extract of male MSG. The electrophoretic pattern of stained protein in fractions B and C of Ref. 15 is shown in *lanes 1* and *4*. Alongside each of these lanes are the corresonding autoradiographs from Western blotting (*lanes 2* and *3*). The renin fraction (*lane 3*) contained single chain $M_r$ = 38,000 renin, $M_r$ = 33,000 renin heavy chain (*hc*), together with bands of $M_r$ = 14,000, 20,000, and 28,000, revealed by long exposure and which are most likely fragments generated by proteolytic hydrolysis of renin in the crude extract of MSG. The absence of a $M_r$ = 43,000 band from the renin fraction and its presence in the early fraction indicate substantial concealment of the active site of prorenin. Renin activity in fraction B was < 1% of that in fraction C.

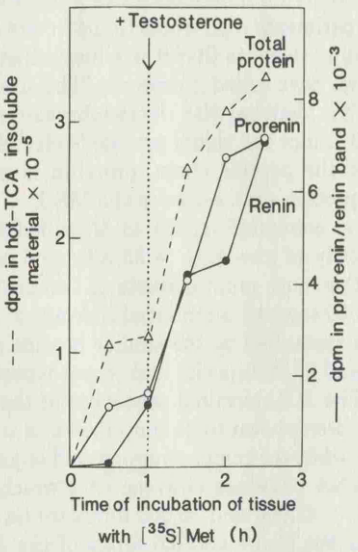


FIG. 9. **Stimulation of synthesis in female tissue by testosterone.** Pieces of submandibular gland from female mice (280 mg wet weight) were incubated in methionine-free medium supplemented with 0.5 mCi of L-[$^{35}$S]methionine. After incubation for 1 h, testosterone (20 μl of a 1-mg/ml solution in sesame oil) was added and incubation continued for a further 1.5 h. At the indicated times, 50–60 mg of tissue (wet weight) were removed, rinsed, homogenized, and immunoprecipitated as described in the legend to Fig. 2 except that 0.2 ml of supernatant was incubated with 0.2 ml of antiserum and the immunoprecipitate dissolved in 50 μl of SDS sample buffer. Conditions for electrophoresis and autoradiography were the same as described in the legend to Fig. 2. The kinetic analysis was constructed by integrating the area under scanned peaks and converting these to disintegrations/min. Data are expressed for 2-mg tissue samples. *TCA*, trichloroacetic acid.

Peptide mapping showed that the $M_r$ = 33,000 main chain of pure renin possessed peptide fragments in common with the $M_r$ = 38,000 form (Fig. 7).

Western blotting of fractions after pepstatin affinity chromatography demonstrated that the renin fraction contained only the single and two-chain forms of renin (Fig. 8). Prorenin eluted earlier and trypsin treatment (6) doubled renin activity in this fraction. The presence of $M_r$ = 33,000 and 38,000 renin bands on the blots and basal renin activity may have reflected activation shortly after elution of prorenin from the column.

Testosterone (0.02 mg/ml of medium) increased total protein synthesis and renin-immunoreactive material when added to female tissue *in vitro* (Fig. 9). Labeling in the initial 1-h control period, although lower in amount, had a pattern similar to that observed in Fig. 3 with male tissue where prorenin plateaued within 1 h.

## DISCUSSION

The present experiments are the first to demonstrate the biosynthesis and processing of renin and precursors.

Direct evidence for a signal peptide was provided by translating mRNA in the presence of dog pancreatic microsomal membranes which removed a $M_r$ = 2,000 peptide. This is consistent with the signal hypothesis of Blobel and Dobberstein (25) and the size predicted by cDNA sequence analysis (12) and complements other studies (11, 26–29) in which a $M_r$ = 45,000 primary translation product has been demonstrated. Prior selection of renin mRNA by hybridization with its cDNA obviated the possibility that some proteolytic enzyme, translated from the total MSG mRNA, could be activated by the microsomal membranes and then act on the primary renin translation product.

A $M_r$ = 43,000 renin immunoreactive protein was detected in labeling experiments with whole tissue *in vitro*. The kinetics of its labeling suggests that it is a biosynthetic precursor of renin and we have called it prorenin. The size of prorenin suggests that it is identical with the membrane-directed translation product. Since the signal peptide is cleaved before the completion of the peptide chain, prorenin is probably the largest renin protein synthesized in the MSG.

Prorenin was converted rapidly to $M_r$ = 38,000 renin and then more slowly to give a $M_r$ = 33,000 band on SDS gels. The finding that pure renin consists of two chains of $M_r$ = 33,000 and 5,000 suggests an internal cleavage of $M_r$ = 38,000 renin. This is supported by the similar peptide maps of the $M_r$ = 38,000 and 33,000 species and recent reports by others (12, 30–32). The $NH_2$-terminal sequences of the two chains have recently been shown to be contained in a single mRNA molecule (12), while the complete amino acid sequence derived from this mRNA possesses cleavage sites which would give rise to the $M_r$ = 43,000 and 38,000 forms we have observed. In considering the pI 5.4 and 5.6 forms of the $M_r$ = 33,000 species, it is noteworthy that amino acid sequencing of the heavy chain (31) did not detect the COOH-terminal Arg-Arg predicted from cDNA (12, 32).

The function of prorenin is not known, though analogy with other proenzymes would suggest that it is inactive. Unlike pepsin whose inactive precursor pepsinogen is both the storage and secretory form (33), the short life span of prorenin obviates a regulatory role. Similarly, the function of $M_r$ = 38,000 single chain renin is not clear. The presence of $M_r$ = 38,000 and 33,000 species in almost equal quantities in immunoprecipitates indicates that conversion to the two-chain form occurs only slowly in the cell. The 4-h time lag in continuous labeling experiments between the appearance of $M_r$ = 38,000 renin and the $M_r$ = 33,000 heavy chain suggests that processing may occur at some site distal to the site of synthesis and renders unlikely the possibility that conversion to two-chain renin occurs only during extraction. Both single and two-chain $M_r$ = 38,000 renins bound to pepstatin, suggesting that both are enzymatically active. Prorenin eluted earlier, suggesting a concealed active site and low or no activity. Another aspartyl protease, cathepsin D, also possesses fully active single and two-chain forms as well as a pro form that is processed in much the same way as described here for prorenin (34).

The ability of testosterone to stimulate synthesis in female tissue *in vitro*, although modest compared to the increase in renin activity seen after injection intraperitoneally (35), suggests that (dihydro)testosterone causes transcription of renin mRNA.

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# Molecular cloning of two distinct renin genes from the DBA/2 mouse

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**We report the molecular cloning of cDNA copies of DBA/2 mouse submaxillary gland (SMG) renin mRNA, which were used to probe Southern transfers of mouse genomic DNA. The results suggested either that there is a single renin gene containing a large intron in that part of the gene corresponding to the probe, or that there are two distinct renin genes. We have shown that the latter is the case by cloning and isolating two similar but distinct renin genes from DBA/2 mouse DNA. Restriction maps of the regions containing the two renin genes are presented, together with nucleotide sequence data locating a complete exon coding for amino acids 268–315 of mouse SMG renin.**

*Key words:* renin/submaxillary gland/mouse/genomic cloning

## Introduction

Renin is an endopeptidase (EC 3.4.99.19) that specifically cleaves angiotensinogen to produce angiotensin I (Cowley *et al.*, 1971). The latter is subsequently cleaved to produce the vasoactive octapeptide angiotensin II, which is involved in the maintenance of normal blood pressure. Although the primary site of renin synthesis is the kidney, renin can also be produced in large amounts by the submaxillary gland (SMG) of the mouse. SMG renin production is under genetic control and is both androgen- and thyroxine-dependent (Wilson *et al.*, 1977, 1981, 1982). Kidney and SMG renins are immunologically and physicochemically similar (Michelakis *et al.*, 1974; Malling and Poulsen, 1977; Poulsen and Nielsen, 1981), but are secreted under different stimuli. The structure of SMG renin has been determined (Panthier *et al.*, 1982; Misono and Inagami, 1982), and the kidney and SMG renin mRNAs are similar in size and have close sequence homology (Rougeon *et al.*, 1981). Although SMG renin is active in the bloodstream (Bing *et al.*, 1980), the release of large quantities from the gland leads to no significant increase in blood pressure (Bing and Poulsen, 1979). The relationship between the kidney and SMG renins and the physiological function of the submaxillary enzyme are unknown.

Inbred mouse strains can be divided into two groups having significantly different levels of SMG renin. This genetic variation is due to a single gene, *Rnr*, located near the *Pep-3* locus on chromosome 1 (Wilson *et al.*, 1978). The SMG renins of high and low producers differ in their thermostability and immunological properties, suggesting that two distinct proteins are involved and that the *Rnr* locus is either identical with or closely linked to a gene that influences the structure of the renin molecule (Wilson and Taylor, 1982). Recently, Piccini *et al.* (1982) reported that restriction endonuclease digests of the DNA of high and low renin strains are characteristically distinct and suggestive of a *Rnr* gene duplication in high renin strains.

We report the presence in the DBA/2 mouse, a high renin-producer, of two renin genes that show substantial homology in both coding and non-coding regions.

## Results

### *Isolation and identification of renin cDNA clones*

The cell-free translation product of SMG renin mRNA was identified by immunoprecipitation with mouse SMG renin antiserum (kindly supplied by K. Poulsen). Figure 1 shows the precipitation of the renin precursor, a 44-K polypeptide (Panthier *et al.*, 1982).

An SMG cDNA library was constructed in the vector pAT153 as reported (Skup *et al.*, 1982). Recombinant plasmids were screened for their ability to hybridise to renin

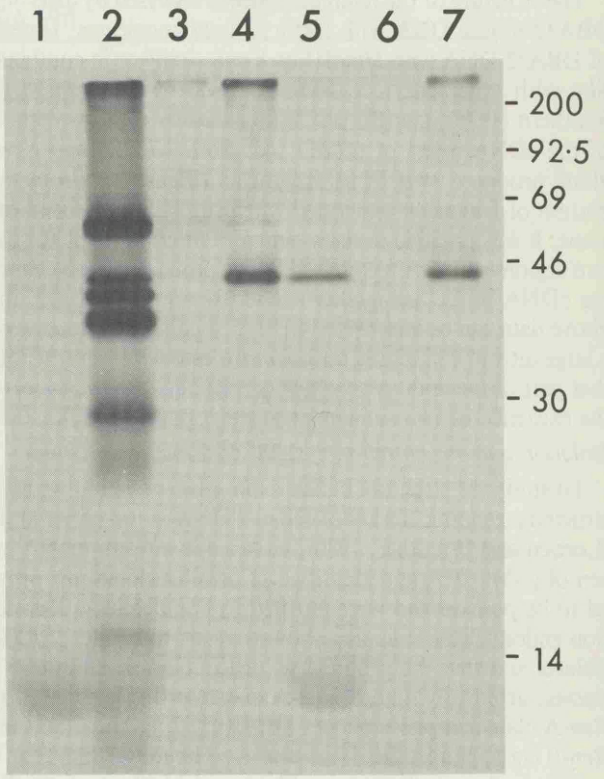


**Fig. 1.** Immunoprecipitation of the renin precursor from the translation products of total SMG mRNA and mRNA selected by hybridization to a renin cDNA clone. **Lane 1**, control translation with no added mRNA; **lane 2**, translation products of total male DBA/2 SMG mRNA; **lanes 3** and **4**, immunoprecipitation of the translation products of total mRNA (**lane 2**) with normal rabbit serum and renin antiserum, respectively. **Lane 5**, translation products of mRNA selected by hybridization to pSMG 142; **lanes 6** and **7**, immunoprecipitation of the translation products of pSMG 142 selected mRNA (**lane 5**), with normal rabbit serum and renin antiserum respectively. **Lanes 1, 2,** and **5** show 3 μl of sample. **Lanes 3, 4, 6,** and **7** show the immunoprecipitate from 10 μl of translation mix. The sizes of mol. wt. markers are given in kilodaltons.

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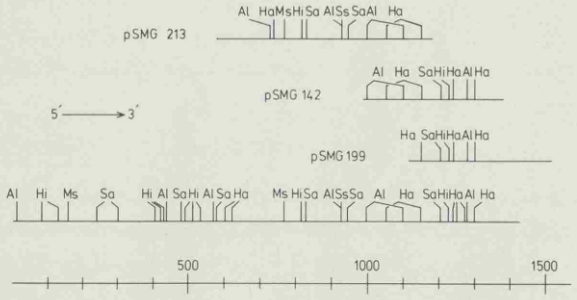


**Fig. 2.** Restriction maps of renin cDNA clones pSMG 213, pSMG 142, and pSMG 199. The maps are drawn relative to the restriction map of a full length cDNA, which was derived from a computer search of the DNA sequence determined by Panthier *et al.* (1982). The scale is drawn in base pairs from the 5′ end of the sequence mentioned above. Symbols for restriction enzymes are as follows: Al = *Alu*I; Hi = *Hinf*I; Ms = *Msp*I; Sa = *Sau*3A; Ha = *Hæ*III; Ss = *Sst*I.

mRNA, assayed by *in vitro* translation of the selected mRNAs and immunoprecipitation of their products (Figure 1). Clone pSMG 142, identified as a renin clone, was then used to re-screen the complete cDNA library. Figure 2 shows the restriction maps of three renin cDNA clones, pSMG 199, pSMG 142, and pSMG 213, indicating their position with respect to the renin cDNA sequence of Panthier *et al.* (1982).

*Southern analysis of genomic DNA*

The structure of the renin gene(s) was analysed by digesting DBA/2 mouse DNA with restriction endonucleases. Digests of DBA/2 DNA with *Hind*III or *Kpn*I, singly or in combination with other enzymes, were analysed by the method of Southern (1975), using a probe prepared by nick-translation of the cDNA insert of pSMG 142. Both *Hind*III and *Kpn*I alone produced two DNA fragments (Figure 3). From the pattern of fragments generated when cut with the second enzyme, it was possible to draw simple restriction maps for the two regions of DNA recognised by the probe (Figure 3). Since the cDNA probe has neither a *Hind*III nor a *Kpn*I site, the above data can be interpreted to suggest either the presence of a large intron, containing both *Hind*III and *Kpn*I sites, within that part of the renin gene which corresponds to the probe, or the existence of two similar but distinct renin genes.

*Isolation and structural analysis of genomic renin clones*

To study the structure of the renin gene (or genes) we constructed a genomic library of DBA/2 DNA in the vector λL47 (Loenen and Brammar, 1980), screened it with the cDNA insert of pSMG 213, and identified 11 possible clones. Six proved to be positive and were purified to homogeneity. Restriction endonuclease mapping showed all six to be independent isolates and that the genomic sequences cloned fell into two classes, arbitrarily named *Ren*-A and *Ren*-B (Figure 4). The *Ren*-A class comprises phages *DBA*Rn1, 2, 6, and 7 and the *Ren*-B class, phages *DBA*Rn5 and 11. The *Ren*-A and *Ren*-B regions in these isolates cover 21 kb and 14 kb, respectively. The *Ren*-A phage DNAs overlap in a region that corresponds to the promoter-distal section of the *Ren*-A gene, since it hybridizes to the pSMG 213 cDNA clone derived from the 3′ end of the SMG renin mRNA. Comparison of the restriction maps derived for the two genomic regions, *Ren*-A and *Ren*-B, indicates their substantial homology and the position of an insertion or deletion event.

This conclusion was confirmed by the analysis of heteroduplexes formed between the DNAs of λ*Ren*-A and λ*Ren*-B phages (Figure 5). Heteroduplexes of λ*DBA*Rn1/λ*DBA*Rn11,

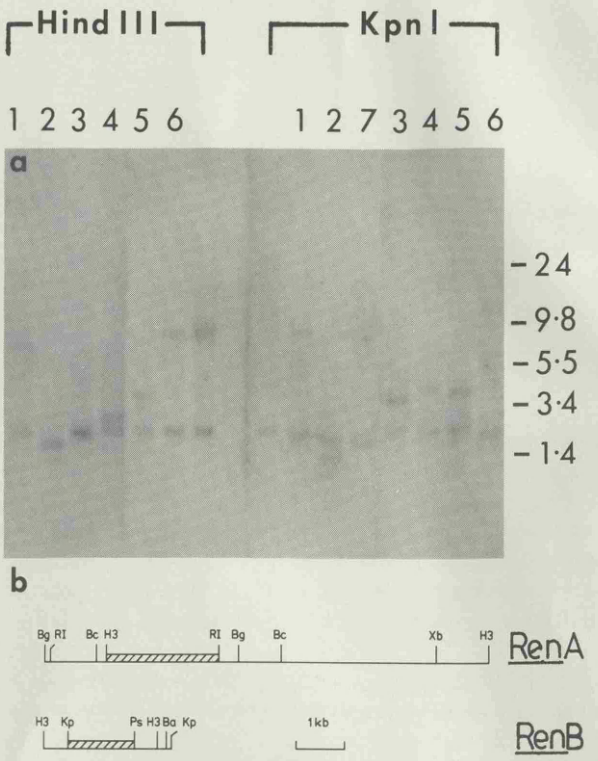


**Fig. 3.** Analysis of DBA/2 mouse DNA by Southern transfer and hybridization to the renin cDNA clone pSMG 142. **(a)** Restriction digests of liver DNA were run on an 0.8% agarose gel, transferred as described in Materials and methods, and probed with $^{32}$P-labelled pSMG 142. DNA was cleaved with either *Hind*III or *Kpn*I and then, where indicated, recut with a second enzyme. Restriction enzymes are numbered as follows: 1 = *Xba*I; 2 = *Pst*I; 3 = *Eco*RI; 4 = *Bgl*II; 5 = *Bcl*I; 6 = *Bam*HI; 7 = *Hind*III. **(b)** Restriction maps of the two regions of DNA hybridizing to the probe pSMG 142 as determined from the Southern transfer data above **(a)**. The regions of genome homologous to the probe must be within the hatched areas. The sizes of mol. wt. markers are given in kilobase pairs. The two homologous regions are designated *Ren*-A and *Ren*-B to show their correspondence with the maps derived from renin genomic clones (see Figure 4).

λ*DBA*Rn2/λ*DBA*Rn11, and λ*DBA*Rn6/λ*DBA*Rn5 all showed extensive duplex formation and three deletion loops (Figure 5). The central loop contained ~3 kb of DNA in each case and the two terminal loops of varying size represent the non-overlapping regions at the opposite ends of the cloned DNA. Heteroduplexes between λ*DBA*Rn7 and λ*DBA*Rn11 also showed a central deletion loop of ~3 kb of DNA, but one of the terminal loops was of the substitution type, showing that the DNA sequence at one end of the cloned DNA in these two recombinants was non-homologous. The third type of heteroduplex molecule was seen when DNA of λ*DBA*Rn1 was paired with that of λ*DBA*Rn5; the entire central region appeared as a substitution loop. This is the behaviour expected if the cloned sequences were in opposing orientations relative to the flanking regions of the phage genome. The structures of the *Ren*-A and *Ren*-B regions of the DBA/2 mouse genome, as deduced from restriction mapping and heteroduplex analysis are summarised in Figure 5.

*Orientation of the renin genes*

The appropriate positions of the renin-coding sequences and their orientations with respect to the restriction maps were determined by investigating the homology of different restriction fragments with cDNA probes made from pSMG 199 and pSMG 213. The former recombinant contains se-

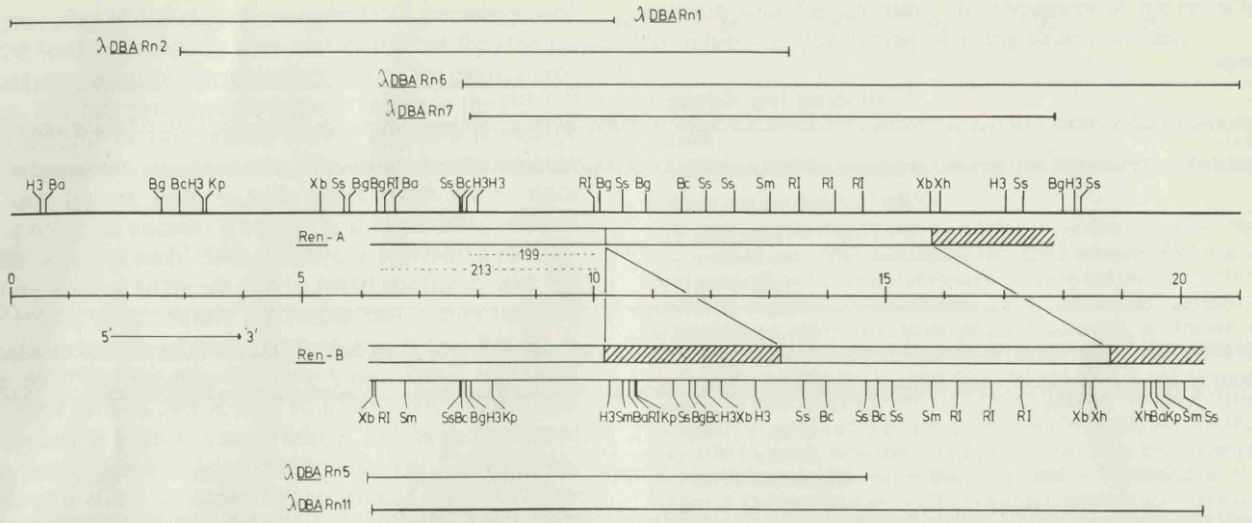


**Fig. 4.** Physical maps of the regions containing the renin genes. Restriction maps were derived from digests of six recombinant phage DNAs. The extents of the regions cloned in each phage are shown at the top and bottom of the figure. The double lines between the restriction maps summarise the data from heteroduplex mapping, showing homologous (——) and non-homologous (▨) regions. The accuracy in the placement of the ends of the homologous regions by heteroduplex mapping is ± 200 bp, so that it is likely that the *Xba*I and *Xho*I targets at co-ordinate 16 kb in the *Ren*-A map and 18.5 kb in *Ren*-B are within the homologous sequences. The dotted lines show the DNA fragments that hybridize to the pSMG 199 and pSMG 213 cDNA probes and allow the orientation of the coding sequences to be deduced. The central scale is graduated in kilobase pairs. Symbols for restriction enzymes are as follows: Ba = *Bam*HI, Bc = *Bcl*I; Bg = *Bgl*II; H3 = *Hind*III; Kp = *Kpn*I; RI = *Eco*RI; Ss = *Sst*I; Xb = *Xba*I; Xh = *Xho*I.

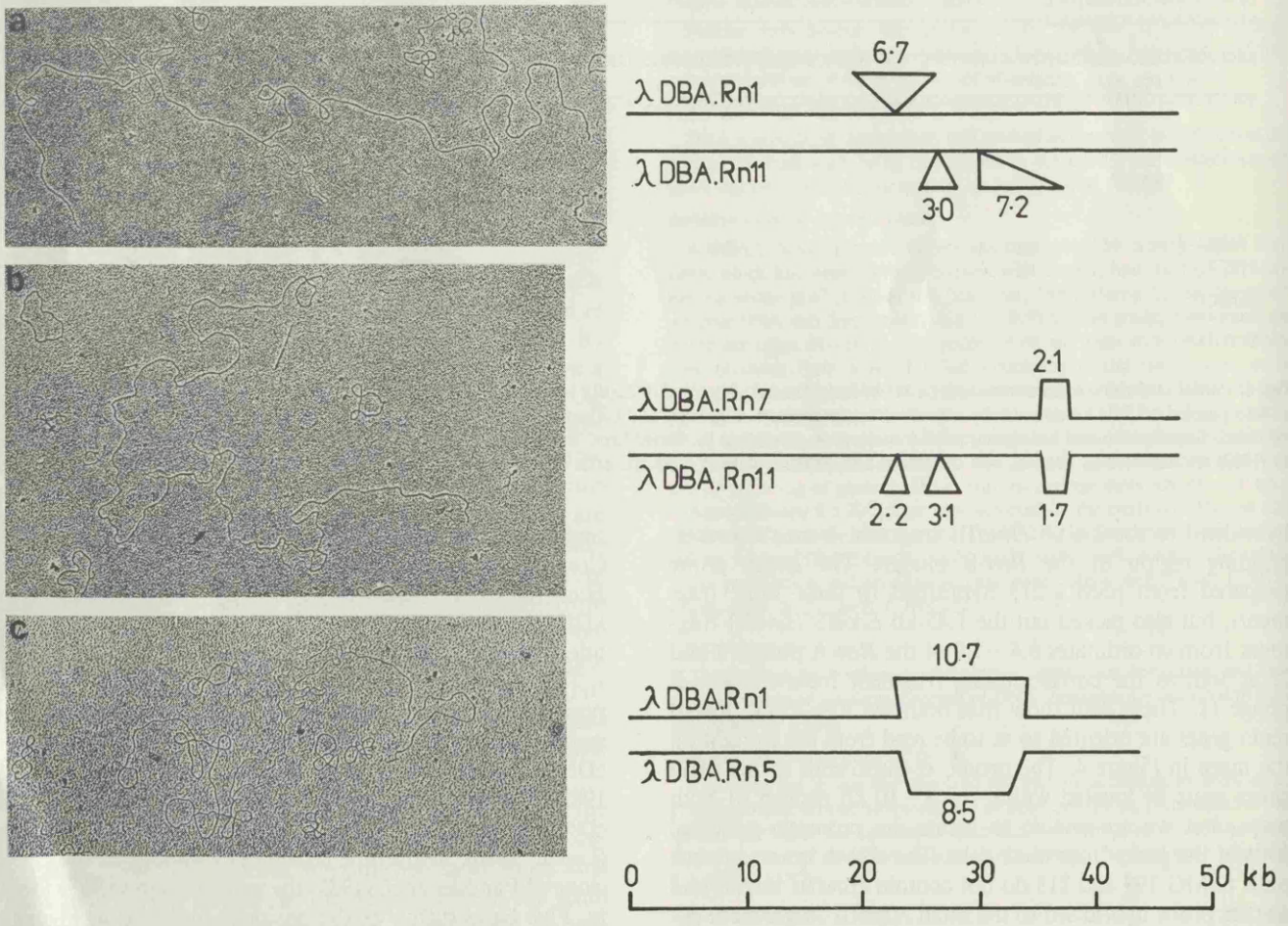


**Fig. 5.** Heteroduplex mapping of the genomes of recombinant phages carrying mouse renin genes. Heteroduplex DNA molecules formed between DNAs from (**a**) λ*DBA*Rn1 and λ*DBA*Rn11, (**b**) λ*DBA*Rn7 and *DBA*Rn11, and (**c**) λ*DBA*Rn1 and λ*DBA*Rn5 are shown. The line drawings give the sizes and positions of the deletion and substitution loops derived from measurements of ~20 molecules.

uences corresponding to ~375 nucleotides at the 3′ end of ıe SMG renin mRNA, while pSMG 213 extends another 450 ucleotides towards the 5′ end. The pSMG 199 probe hybrid-

ised with the 2.2-kb *Hind*III/*Eco*RI fragment from coordinates 8 – 10 kb of the *Ren*-A series of phages, but showed no detectable homology with flanking fragments. It also

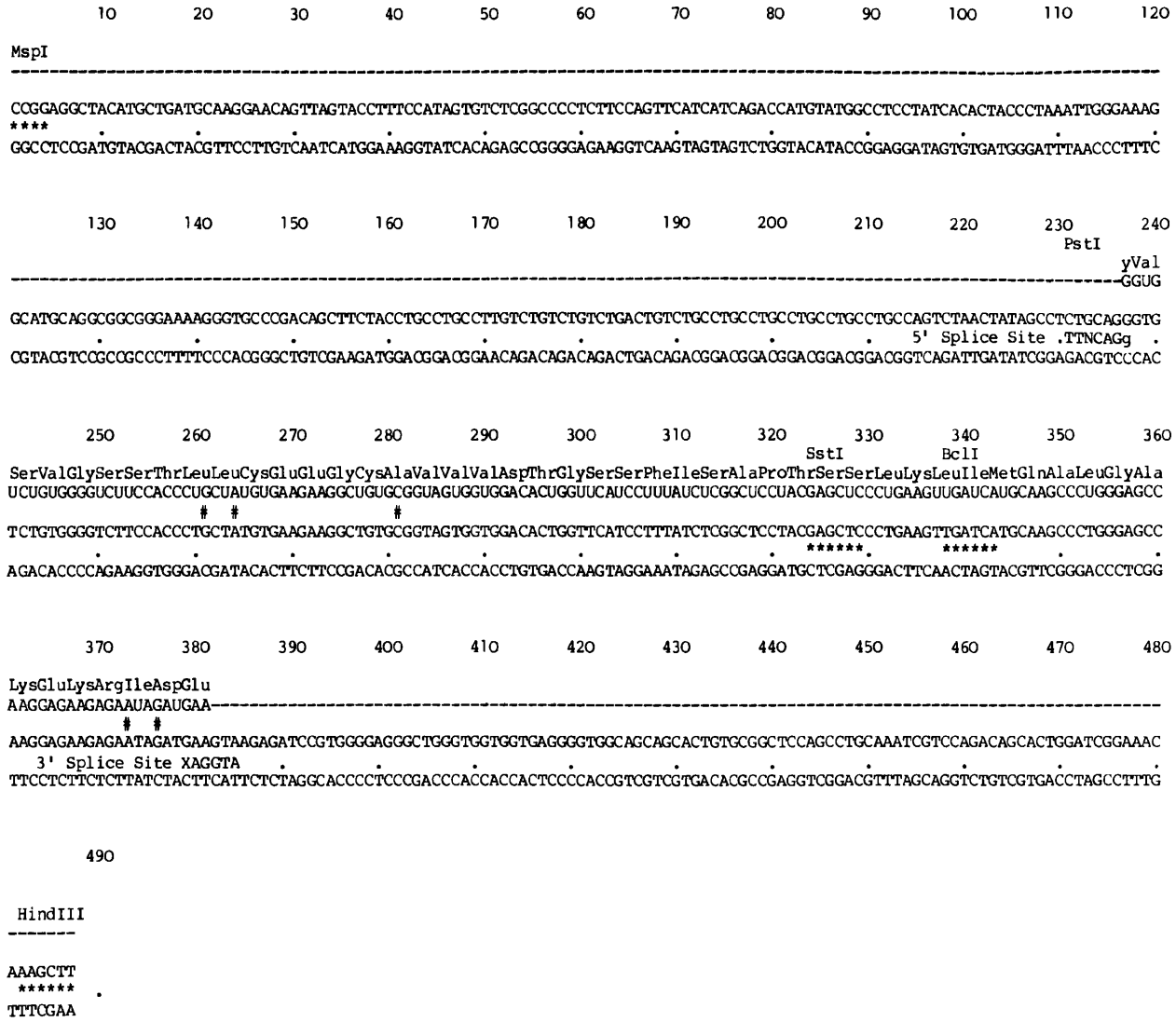


**Fig. 6.** Partial nucleotide sequence analysis of λ*DBA*Rn1. The 1.45-kb *Hind*III/*Eco*RI fragment of λ*DBA*Rn1 between co-ordinates 6.5 and 7.9 was subcloned into plasmid pAT 153, and partially sequenced by the method of Maxam and Gilbert (1980). Those restriction endonuclease sites used for sequencing are indicated. Sequences spliced out during mRNA maturation are shown by dotted lines. Bases in the coding region that differ from the sequence of Panthier *et al.* (1982) are indicated by hatches, and the amino acid sequence derived from the exon is indicated above the mRNA sequence.

hybridised to the 2.6-kb *Hind*III fragment from the corresponding region of the *Ren*-B phages. The larger probe prepared from pSMG 213 hybridised to these same fragments, but also picked out the 1.45-kb *Eco*RI/*Hind*III fragment from co-ordinates 6.4 – 7.9 of the *Ren*-A phages 1 and 2, as well as the corresponding fragment from the *Ren*-B phage 11. These data show that both the *Ren*-A and *Ren*-B renin genes are oriented so as to be read from left to right on the maps in Figure 4. The promoter-distal ends of the renin genes must be located within the 8 – 10 kb regions of both maps, but we are unable to locate the promoter-proximal ends of the genes from these data. The cDNA inserts in plasmids pSMG 199 and 213 do not contain *Hind*III targets and neither probe hybridised to the small *Hind*III fragment at co-ordinate 8 of the *Ren*-A region, suggesting that this region of the genomic map must constitute an intron.

*Partial sequence analysis of Ren-A DNA*

The presence of neighbouring *Sst*I and *Bcl*I sites at co-ordinate 7.8 kb from the left end of the *Ren*-A genomic map was consistent with this region's correspondence to the 3′ end of the renin mRNA sequence, as these restriction sites are similarly placed in the cDNA clone of Panthier *et al.* (1982). Confirmation of this was obtained by subcloning the 1.45-kb *Hind*III-*Eco*RI fragment covering this region, from λ*DBA*Rn1 into plasmid pAT153, followed by partial nucleotide sequence analysis of this subclone.

The sequence (Figure 6) confirmed the orientation and position of the *Ren*-A gene and revealed an exon closely corresponding to the published sequence of the SMG renin cDNA, between nucleotides 841 and 985 (Panthier *et al.*, 1982). This exon is flanked by two sequences not found in the cDNA and is bounded by consensus intron/exon junctions (Lewin, 1980). Within the region of homology to the cDNA clone of Panthier *et al.* (1982) the sequence shows 96% identity. Five bases differ, at the positions indicated in Figure 6, though two of these alterations lead to no change of amino acid in the protein coded.

## Discussion

Molecular cloning of DNA copies of the mouse mRNA for SMG renin has provided a probe for the analysis of renin genes and for the isolation of genomic clones. Restriction en-

zyme mapping of the cloned genomic DNA correlates well with the Southern mapping data and establishes the absence of detectable sequence rearrangement during the cloning procedures. The mapping data prove the existence in the DBA/2 mouse genome of two regions having extensive homology with the renin-coding sequence. We cannot be certain that these two regions both include intact renin genes, since without complete nucleotide sequences the possibility remains that one sequence could be incomplete or represent a pseudogene. For ease of reference and to avoid confusion with existing genetic nomenclature for mouse renin genes we have called the two renin-related sequences *Ren*-A and *Ren*-B.

The homology between the *Ren*-A and *Ren*-B regions of the genome extends over at least 10 kb of DNA and stretches well beyond the renin genes. Within the large duplicated region there has been a deletion or insertion of 3 kb of DNA, downstream of the renin-coding region. The region of homology could be >10 kb, since the six recombinant phages studied have not defined the left end of the homologous region.

Although the extended homology between *Ren*-A and *Ren*-B would allow the inclusion of multiple copies of the renin gene in each chromosomal region, the Southern mapping of the DBA/2 chromosomal DNA makes this possibility very unlikely. A double digest of chromosomal DNA with *Hind*III and *Pst*I yields only 1.9-kb fragments hybridising with the cDNA probe. After digestion with *Kpn*I plus *Pst*I, the only fragment from the *Ren*-B region that is revealed by the probe is 1.4 kb long. Since the renin mRNA sequence is ~1.5 kb long, and the cDNA insert in the pSMG 142 probe is ~400 bp, it is very unlikely that the 1.4-kb fragment contains two copies of the region homologous to the probe. The observations are strongly suggestive of there being only one renin gene in each of the *Ren*-A and *Ren*-B regions.

The renin level in the SMG of the mouse is governed by a regulatory locus, *Rnr*, mapped to chromosome 1 (Wilson *et al.*, 1978). Piccini *et al.* (1982) have recently shown, by Southern transfer analysis with a renin cDNA probe, that a specific DNA polymorphism is intimately associated with the allele at the *Rnr* locus and have suggested that high-producer strains carry a duplication of the renin gene. Our data show that one high renin-producing strain, DBA/2, does have two renin genes, though it remains to be established that they are both active. The genomic mapping of Piccini *et al.* (1982) shows that *Kpn*I generates fragments of 20 kb and 2 kb from DBA/2 DNA, but produces only the larger fragment from the DNA of C57BL/6J, a low renin strain. Our data show that the 2-kb *Kpn*I fragment derives from the *Ren*-B region and that the *Ren*-A renin gene is contained within a *Kpn*I fragment of >18 kb. Taken together, these findings suggest that the low renin strain C57BL/6J contains the *Ren*-A renin gene but does not carry the *Ren*-B copy.

Renin mRNA can be detected in both the SMG and the kidney of low producer strains, including C57BL/6J (Rougeon *et al.*, 1981; J.J. Mullins, unpublished data). Since such strains only appear to contain the *Ren*-A copy of the renin gene, it is evident that this gene must be expressed in both tissues.

As discussed by Piccini *et al.* (1982), the presence of an extra copy of the renin gene in high producers cannot account for the 100-fold elevation in SMG renin levels by mere gene dosage. In order to understand the molecular basis for the variation in renin levels it will be necessary to correlate the expression with the structures and sequences of the renin loci from high and low renin-producing strains of mice.

## Materials and methods

*Animals*

Mice (DBA/2) were supplied by Bantam and Kingman Ltd., Hull, UK.

*Isolation and translation of mRNA*

10 week-old male DBA/2 mice were killed by cervical dislocation. SMGs were removed and placed immediately into liquid nitrogen. RNA was prepared essentially by the method of Noyes *et al.* (1979) and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography. mRNA was translated in a nuclease-treated reticulocyte lysate prepared according to Pelham and Jackson (1976), containing the following: 20 μM each amino acid (minus methionine), 1 mM ATP, 0.2 mM GTP, 80 mM KCl, 2 mM magnesium acetate, 10 mM Tris-HCl pH 7.6, 2 mM glucose, 7 mg/ml creatine phosphate, 50 μg/ml calf liver tRNA, 1 mCi/ml [$^{35}$S]methionine (specific activity 1160 Ci/mmol, Amersham International), and 50 μg/ml creatine phosphokinase. Immunoprecipitation was carried out by a modification of the method of Dobberstein *et al.* (1979). Translation products and immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and visualised by fluorography.

*Construction of recombinant plasmids*

Recombinant plasmids containing cDNA copies of SMG poly(A)$^+$ RNAs were made as described by Skup *et al.* (1982). Colony hybridization was performed according to Young *et al.* (1977) and plasmid DNAs were prepared by equilibrium centrifugation in the presence of ethidium bromide (Windass and Brammar, 1979).

*Positive hybridization-translation assays*

Positive hybridization was performed by the method of A. Jeffreys (unpublished data) and the selected RNAs assayed by translation and immunoprecipitation of the products (see above).

*Preparation and Southern blotting of mouse DNA*

DNA was isolated, transferred, and probed as described by Jeffreys *et al.* (1980), the final wash being carried out in 0.5 x SSC. Nick-translation of DNA was performed by the method of Jeffreys *et al.* (1980).

*Isolation of renin genomic clones*

A DBA/2 mouse genomic library was constructed by cloning mouse liver DNA which had been partially digested with *Sau*3A, into the high capacity lambda vector λL47 (Loenen and Brammar, 1980). Partial *Sau*3A digests of genomic DNA were fractionated on a 10–40% sucrose gradient and fractions in the size range 10–18 kb were pooled. Left and right arms of λL47 DNA were prepared from a *Bam*HI/*Sal*I double-digest and fractionated on a 10–40% sucrose gradient. Fractions containing the 24 and 10 kb left and right arms were pooled. λL47 arms were annealed and ligated to genomic DNA and the ligation mixture was packaged *in vitro* into phage particles as described by Jeffreys *et al.* (1981). The yield of recombinant phages was 2 x 10$^6$ p.f.u./μg of genomic DNA, with an average input size of ~10 kb.

Approximately 8 x 10$^5$ phage were screened by the method of Benton and Davis (1977) using the cDNA probe, pSMG 213. Initial screening was carried out on the host DB102 (*met*B, *sup*E, *sup*F, *hsd*R, *trp*R, *ton*A) while subsequent purification and propagation was done with a *rec*BC host, ED8910 (*sup*E, *sup*F, *hsd*S$_k$, *rec*B21, *rec*C22).

*General techniques*

The methods for manipulation of phages, preparation, restriction, and ligation of DNAs and gel electrophoresis of DNA fragments are described in Burt and Brammar (1982).

*Southern blotting of genomic clones*

Gels were cut to size and prepared for transfer according to Wahl *et al.* (1979) and then blotted by the method of Southern (1975). Hybridization was carried out under the conditions described by Jeffreys *et al.* (1980), using 7 x 10$^6$ c.p.m./ml of nick-translated probe DNA. The final wash was with 1 x SSC at 65°C.

*DNA sequencing*

Fragments used for sequencing were derived from fragments of the genomic clones, subcloned into pAT 153; nucleotide sequencing was performed according to the procedure of Maxam and Gilbert (1980).

*Electron microscopy*

DNA heteroduplex molecules were prepared by the methods described by Davis *et al.* (1971), using DNAs at ~15 μg/ml in 0.1 M Tris, 0.01 M EDTA pH 8.5, and 50% formamide. DNAs were denatured for 1 min at 70°C and

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# Molecular cloning of two distinct renin genes from the DBA/2 mouse

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*Received on 7 October 1982*

**We report the molecular cloning of cDNA copies of DBA/2 mouse submaxillary gland (SMG) renin mRNA, which were used to probe Southern transfers of mouse genomic DNA. The results suggested either that there is a single renin gene containing a large intron in that part of the gene corresponding to the probe, or that there are two distinct renin genes. We have shown that the latter is the case by cloning and isolating two similar but distinct renin genes from DBA/2 mouse DNA. Restriction maps of the regions containing the two renin genes are presented, together with nucleotide sequence data locating a complete exon coding for amino acids 268 – 315 of mouse SMG renin.**

*Key words:* renin/submaxillary gland/mouse/genomic cloning

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## Results

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The cell-free translation product of SMG renin mRNA was identified by immunoprecipitation with mouse SMG renin antiserum (kindly supplied by K. Poulsen). Figure 1 shows the precipitation of the renin precursor, a 44-K polypeptide (Panthier *et al.*, 1982).

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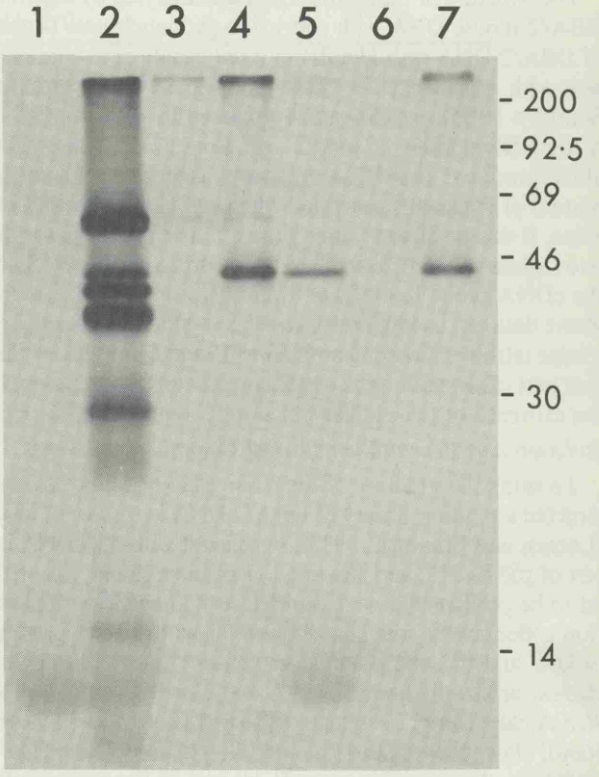


**Fig. 1.** Immunoprecipitation of the renin precursor from the translation products of total SMG mRNA and mRNA selected by hybridization to a renin cDNA clone. **Lane 1**, control translation with no added mRNA; **lane 2**, translation products of total male DBA/2 SMG mRNA; **lanes 3** and **4**, immunoprecipitation of the translation products of total mRNA (**lane 2**) with normal rabbit serum and renin antiserum, respectively. **Lane 5**, translation products of mRNA selected by hybridization to pSMG 142; **lanes 6** and **7**, immunoprecipitation of the translation products of pSMG 142 selected mRNA (**lane 5**), with normal rabbit serum and renin antiserum respectively. **Lanes 1, 2,** and **5** show 3 $\mu$l of sample. **Lanes 3, 4, 6,** and **7** show the immunoprecipitate from 10 $\mu$l of translation mix. The sizes of mol. wt. markers are given in kilodaltons.

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# Molecular cloning of cDNAs from androgen-independent mRNA species of DBA/2 mouse sub-maxillary glands

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ABSTRACT

cDNA libraries have been constructed from mRNAs isolated from mature male DBA/2 mouse submaxillary glands. Several recombinant plasmids have been assigned to particular mRNA species and their in vitro translation products by HART and hybrid selection. Clones containing copies of two abundant mRNA species that showed no sexual dimorphism were selected for detailed characterisation. Nucleotide sequences determined from one series of clones define an 850 nucleotide mRNA encoding a polypeptide of 16.5 kd having an N-terminal signal sequence, an acidic core and four glycosylation sites. A second family of clones correspond to an mRNA of 800 nucleotides, the sequence of which can be interpreted as coding for an intracellular protein of 14.7 kd. Computer searches of protein and nucleic acid sequences have not revealed the identity of either of these submaxillary gland products.

## INTRODUCTION

The mouse submaxillary gland is the site of synthesis of a wide range of physiologically important polypeptide hormones and enzymes, including nerve growth factor (NGF), epidermal growth factor (EGF), NGF esteropeptidase, EGF esteropeptidase, renin, amylase and acid phosphatase (1). The salivary glycoprotein mucin is also produced in large amounts by this gland (2). In many cases the primary structures of these products are not fully known and there is only limited information about the nature of precursor molecules and the regulation of their synthesis. This is of particular interest because the submaxillary gland shows sexual dimorphism in its production of many substances, but not all (1), and therefore offers an opportunity to study both hormonal and tissue-specific control of gene expression. In the case of at least one submaxillary gland product, renin, there is also clear evidence of direct genetic control of expression levels; mice are either genetically high ($\underline{Rnr}^s$) or low ($\underline{Rnr}^b$) submaxillary renin producers (3, 4).

In view of these physiologically, regulatory and genetically interesting

features, we have begun a programme to characterise cDNA clones derived from the major mRNAs of the male mouse submaxillary gland. This should enable us to provide detailed descriptions of the precursors of many of the gland's products. New polypeptides, which have not yet been ascribed a physiological or biochemical role, may be recognised. In addition hybridisation probes will be obtained which will permit studies on the control and genomic organisation of a set of genes with a common site of expression. Submaxillary gland renin cDNA clones have been previously described (5, 6) and used in the isolation and characterisation of mouse renin genes (6). The molecular cloning and characterisation of cDNA copies of the mRNAs for four other major androgen inducible submaxillary products - EGF, β-NGF, kallikrein and EGF binding protein - have also been recently reported (7-12). Here we describe the identification of cDNA clones corresponding to several other major submaxillary gland products and the detailed characterisation of two abundant mRNA species which, in contrast to those described to date, do not show sexual dimorphism in their expression.

## MATERIALS AND METHODS

### Animals

DBA/2 mice were supplied by Bantin and Kingman Ltd., Hull, UK. Other mice used were the gift of Dr G. Bulfield.

### Enzymes and reagents

Restriction enzymes were purchased from Bethesda Research Laboratories. All reactions were carried out according to supplier's recommended conditions. Avian myeloblastosis virus reverse transcriptase was supplied by Life Sciences Inc., Florida, USA. All radioactively labelled reagents were supplied by Amersham International, dog pancreas membranes by New England Nuclear. Formamide was purchased from Fluka.

### mRNA isolation

RNA was prepared from 11 week old male DBA/2 mice or mixed mice, by the method of Noyes *et al* (13) and poly(A)$^+$ RNA purified by oligo-dT chromatography (14).

### In vitro translation studies

Messenger RNA was translated in a nuclease-treated reticulocyte lysate, as previously described (6,15).

For *in vitro* processing, the above reaction was supplemented with 4 $OD_{260}$ units/ml of dog pancreas microsomes. Immunoprecipitation of translation products was by a modification of the method of Dobberstein *et al* (16).

Translation products and immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (17) and visualised by fluorography. Hybrid selection was performed by the method of Wood et al (18) and the selected mRNAs were translated and immunoprecipitated, as described above. Hybrid arrested translation (HART) was performed by the method of Paterson et al (19).

cDNA cloning

Recombinant plasmids containing cDNA copies of submaxillary gland poly(A)$^+$ RNAs were made as described by Skup et al (20). In addition to these, a number of clones were generated by the method of Land et al (21). The library was maintained by freezing the colonies on nitrocellulose filters according to the method of Hanahan and Meselson (22).

DNA sequencing

Nucleotide sequencing was performed according to the procedure of Maxam and Gilbert (23). Where necessary, the 5' end sequence of mRNA was determined by dideoxy primer extension using reverse transcriptase (24).

Northern blotting

RNA species were separated using a urea-agarose gel (1.5% agarose, 6M urea, 25mM citrate (pH 3.4), 15mM sodium iodo-acetate)

Prior to loading, the samples were heated in sample buffer (82.5% v/v DMSO, 9.2% glycerol in 10mM citrate (pH 3.4) for 30 minutes at 45°C. The gel was run for 1400 V. hrs overnight at 4°C in buffer (6M urea, 25mM citrate (pH 3.4), 1mM sodium iodo-acetate). To prepare the gel for blotting, it was placed in 200ml of 50mM NaOH, 5mM 2-mercaptoethanol and rocked gently for 40 minutes at room temperature, washed twice in 200ml of 25mM sodium phosphate buffer (pH6.0), 7mM iodo-acetic acid (10 minutes at room temperature), and finally washed twice in 200ml of 25mM sodium phosphate buffer (pH 6.0) (10 minutes at room temperature). The RNA was transferred to diazobenzyloxymethyl (DBM) paper prepared by the method of Alwine et al (25). Hybridisation of specific probes was carried out by the method of Thomas (26).

General Techniques

Plasmid DNAs were prepared by equilibrium centrifugation in the presence of ethidium bromide (27). Gel electrophoresis of DNA fragments on polyacrylamide gels and agarose gels was performed according to Maniatis et al (28).

## RESULTS

### In vitro translation of submaxillary gland mRNA preparations

The results of previous in vitro translation analyses of submaxillary

gland mRNA preparations (29,30) have focused attention on strain and sex-specific differences. We have also compared the in vitro translation profiles of male and female glands from mice. In addition, we have studied the processing of in vitro translation products by dog pancreas microsomes.

When rabbit reticulocyte in vitro translation products of adult (11 week old) male and female DBA/2 mouse submaxillary mRNA preparations were analysed by SDS-PAGE characteristic polypeptide patterns were seen (Figure 1a). Typically at least 15 major polypeptide species were apparent with a size-range from 6 to 90 Kdaltons (Kd). Despite the sexual dimorphism in many of the known products of submaxillary glands (1) most of the major polypeptides were clearly encoded by mRNA from the glands of both sexes. Notable exceptions are the male-specific 44 Kd species and the 26-29 Kd size class which have been previously recognised as preprorenin (31) and the esteropeptidase family (11) respectively. There is also a female-specific 30 Kd product of DBA/2 mouse submaxillary gland mRNA which has not been previously described.

As might be expected for an organ such as the submaxillary gland, which displays both exocrine and endocrine activities, many of the abundant in vitro translation products were processed when dog pancreas microsomes were included (Figure 1b). Such processing may involve both the removal of signal peptide regions and other microsome-mediated modifications (32). The dramatic change in mobility of the 36 Kd species (which by relative abundance must be the precursor of the 26 Kd processed product) is, however, difficult to explain, since signal sequences are commonly only 1-3 Kd in size. Just as some in vitro translation products were clearly processed by the microsome preparation, others, such as the abundant 14 Kd species, appeared to be unaffected.

Construction of adult male DBA/2 mouse submaxillary gland cDNA libraries

In the course of this work several cDNA libraries of 8 week old male DBA/2 mouse submaxillary gland cDNA were prepared in plasmid pAT153 (33) by conventional S1 nuclease/GC-tailing methodology. In total approximately 3600 recombinant plasmids were obtained in this manner. In addition a small library, containing about 200 recombinant plasmids, was prepared by the method of Land et al (21) which has been reported to yield more complete cDNA clones.

Identification of recombinant plasmids containing copies of abundant mRNA species

At an early stage in the screening of the first DBA/2 submaxillary gland cDNA library it became apparent that one family of plasmids contained inserts

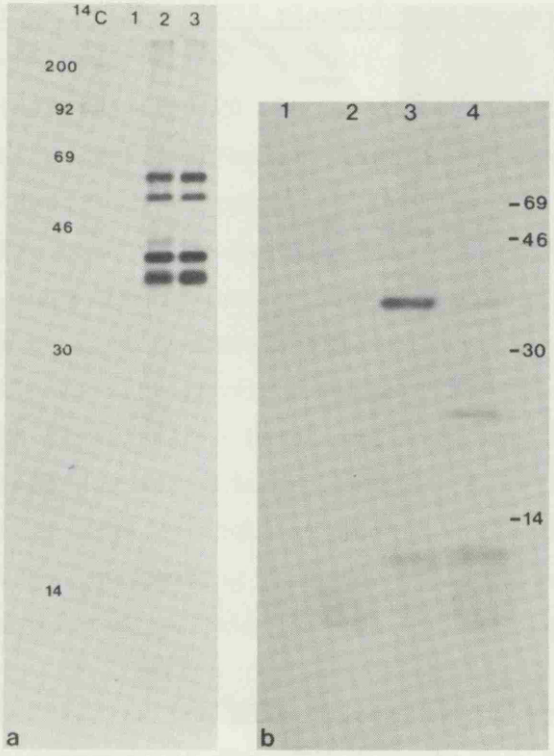



Figure 1 - Typical in vitro translation products analysed on a 12½% polyacrylamide-SDS gel.

a) Translation in the absence (1) or the presence of male (2) and female (3) submaxillary gland mRNA.

b) Processing of primary in vitro translation product by dog pancreas membranes (DPM). Lanes (1) and (2) show the products of minus RNA control translations; lanes (3) and (4) show the translation products of male submaxillary gland mRNA. Translations (1) and (3) were in the absence of DPM, (2) and (4) were in the presence of DPM.

derived from a particularly abundant mRNA. Thus the cDNA insert in plasmid pSMG3 hybridised to about 17% of the recombinant plasmids in our libraries, even under conditions of high stringency.

HART (Figure 2a) and subsequently hybrid selection (Figure 2b) analyses using pSMG3 DNA confirmed that this plasmid contained a copy of a major mRNA species and showed that the corresponding polypeptide was the one which migrated on SDS-PAGE with an apparent size of 36 Kd. Using differential hybridisation procedures, other colonies in the cDNA libraries were identified which contained copies of abundant mRNAs unrelated to pSMG3.

Plasmid DNA was isolated from several pSMG3 unrelated colonies and used in hybrid selection experiments in order to correlate each plasmid with the in vitro translation product of the mRNA species from which it had been derived. In this manner plasmids corresponding to the major polypeptides

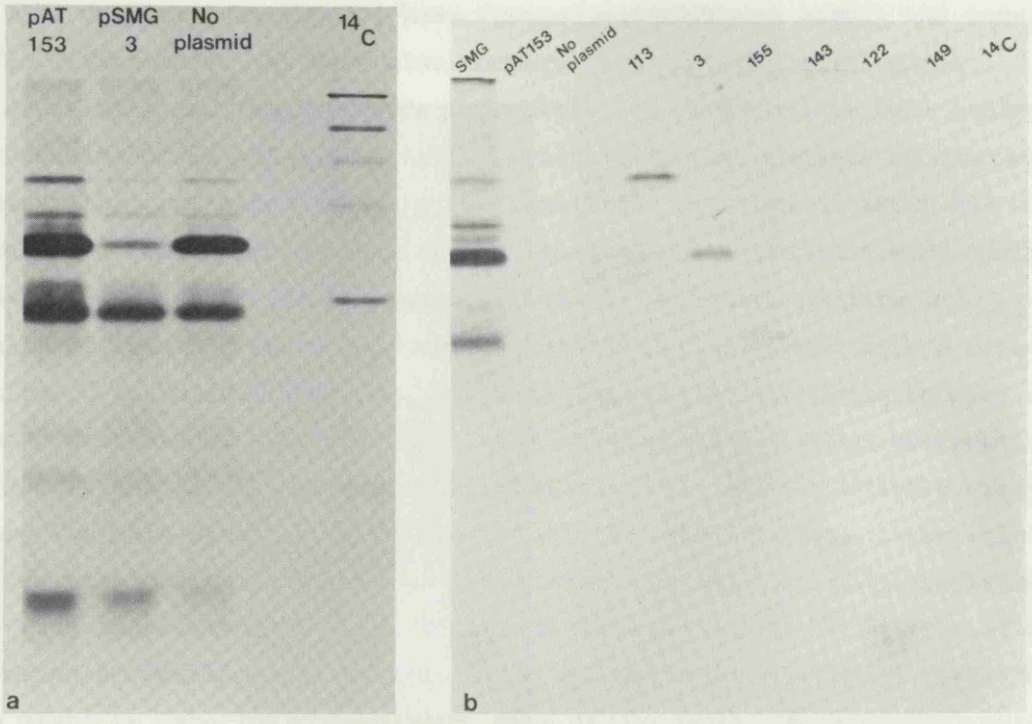



Figure 2 - Correlation of cDNA clones with the in vitro translation products of corresponding mRNA species.

a) HART analysis of adult male submaxillary gland mRNAs translated after hybridisation to pSMG 3 (lane 1), pAT153 (lane 2) or without added plasmid (lane 3).

b) Hybrid selection analyses of submaxillary gland mRNAs. Numbers above tracks correspond to the isolation numbers of the cDNA clones used in this analysis.

shown in Figure 2b were recognised. Plasmids corresponding to the 40 Kd (pSMG148) and 44 Kd (preprorenin) (pSMG142) (5) polypeptides have also been identified.

Following this preliminary correlation of plasmid isolates with in vitro translation products, plasmids pSMG3 and pSMG143 were selected for further study. This selection was based upon the features of the polypeptide products corresponding to these plasmids: they were distinct major products of the submaxillary gland and their expression did not appear to be affected by the sex of the animal from which the glands were taken. Unlike the genes for renin, EGF, NGF and the kallikreins, the expression of those encoding the 36 and 14 Kd submaxillary gland products were thus unlikely to be subject to androgen-mediated regulation. In addition, the 36 Kd product of the mRNA from which pSMG3 had been derived was processed by dog pancreas microsomes. It was therefore likely to be a major secreted product of the submaxillary gland. The 14 Kd polypeptide was not processed by microsome preparations and was thus likely to be a major intracellular submaxillary gland product.

Detailed characterisation of the pSMG3 plasmid family and the corresponding mRNA

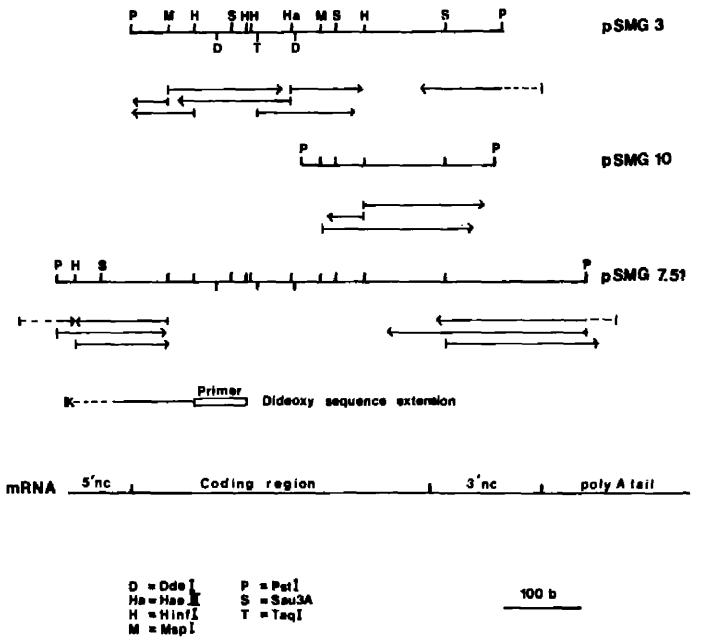
Plasmid DNA was isolated from 20 clones which showed homology to the pSMG3 insert. Detailed restriction enzyme mapping revealed that the cDNA inserts constituted a set of overlapping DNA fragments (Figure 3a). No evidence for restriction site heterogeneity was found in this analysis. It is therefore probable that the progenitor mRNA molecules had either been produced from a single gene or a closely related gene family.

Northern blot analyses also provided support for there being only one major submaxillary mRNA class with substantial homology to pSMG3. a single band of about 850 nucleotides was detected (Figure 4a). A surprising aspect of this result is that an mRNA molecule of such a size could maximally encode a polypeptide of 25-30 Kd, suggesting that the apparent molecular weight of 36 Kd observed for the product of this mRNA on SDS-PAGE gels may have been artefactually high.

To completely characterise the mRNA corresponding to the pSMG3 plasmid family, the nucleotide sequence of the cDNA inserts was determined, as indicated in Figure 3a. The total length of this sequence was 646 bases (Figure 3b) and included a 14 base oligo-A tract preceded 20 bases earlier by a consensus polyadenylation signal (AATAAA) (34) defining the 3' end of the mRNA. In order to determine whether the 5' end of the mRNA was also represented by the sequence derived from our set of clones, a 66bp HinfI DNA fragment isolated from pSMG3 was used as a primer for mRNA-dependent dideoxynucleotide sequencing with reverse transcriptase (24). The nucleotide sequence determined in this way agreed exactly with that presented in Figure 3b and moreover terminated at the same 5' nucleotide. It is thus likely that the sequence shown in Figure 3b represents a complete copy of the non-poly-A part of the submaxillary gland mRNA corresponding to plasmid pSMG3. This would imply that the mRNA has a poly-A tail of about 200 residues, i.e. the difference between the 632 base sequence in Figure 3 and the 850 base total mRNA length. A poly A tail of this size would be quite consistent with previous estimates of average tail length on newly synthesised mRNAs (35).

The most extensive continuous open reading frame starting with a translation initiation codon in the sequence we have determined for the mRNA corresponding to pSMG3, is shown in Figure 3b and is 414 bases long. It is preceded by a 69 base 5' non-coding region, containing an in phase translation termination codon, and is followed by a 14 base 3' non-coding region which contains several in phase translation termination codons. This reading frame is thus likely to describe the natural polypeptide derived from this

a




b

```
        10        20        30        40        50        60        70        80        90       100       110       120
                                  ***  5' non-coding region          MetLysPheLeuAlaLeuLeuValLeuLeuGlyValSerThrIleLeuValSer
AAGAGTCTTCCTTGTCTCCTGGTATTGGAGCTTGATCTTGTGTGTTTCCTGCTCTTCTACTTGTCACCATGAAGTTCCTGGCACTCCTTGTGTTGCTTGGTGTTTCCACTATCCTAGTCT

       130       140       150       160       170       180       190       200       210       220       230       240
  CysGlnAspProGluThrAsnSerThrGluThrSerGlyThrAlaAspSerAlaGlyGluAsnThrGlySerGluThrGlnAlaAspSerThrAspGlnAsnGlnGluValAspSerSer
CTTGCCAGGACCCGGAGACAAACAGTACTGAAACTTCTGGAACTGCTGATTCTGCTGGTGAAAATACTGGCTCTGAAACTCAGGCAGATAGTACTGATCAAAATCAAGAAGTTGATTCCT

       250       260       270       280       290       300       310       320       330       340       350       360
  AspSerValGluGluGluAsnValAsnThrAspAspThrAsnThrSerTyrGluAlaThrGluGluAspAsnLysGluGluLeuAsnAspAsnSerThrGlyAspAsnThrSerAspGln
CTGATTCAGTCGAAGAAGAAAATGTGAACACTGATGATACCAACACTAGCTATGAGGCCACTGAGGAAGACAACAAGGAAGAGTTAAATGATAACAGTACCGGGGATAATACCTCTGATC

       370       380       390       400       410       420       430       440       450       460       470       480
  AsnSerGlyValAspAsnThrGluThrGluGluSerAsnAlaLysThrLysGluGluAspMetLysThrValIleLysSerGlyValGluLysLeuLysAsnPheLeuGlnArgGly
AGAACAGTGGCGTTGACAACACTGAAACGGAAGAAGAGTCCAATGCAAAAACTAAACTTGAAGACATGAAAACCGTTATTAAAAGTGGGGTTGAAAAACTAAAGAATTTCTTACAACGTG

       490       500       510       520       530       540       550       560       570       580       590       600
  ***                                 3' non-coding region
GATGAGTTTGCATCTTCTACTACTGATCTTTCTGAAACAGACTAGCACATTCTTGATTTGATTTCCTCCAGTTATTTATCCTGACTATTCTCCCTCTCTCTCATTGCTAACTTTTATCTT

       610       620       630       640       650
TTATCAAATAAATTGTAATCATGAGCAGCATCAAAAAAAAAAAAAA
```

Figure 3 - Composite restriction map and sequence of the pSMG3 family.
a) Restriction maps of cDNA clones pSMG3, pSMG10 and pSMG751, showing sequencing strategy used and proposed mRNA structure.
b) Sequence of pSMG751, showing longest open reading frame. In phase stop codons are indicated by *** and the polyadenylation signal is boxed.

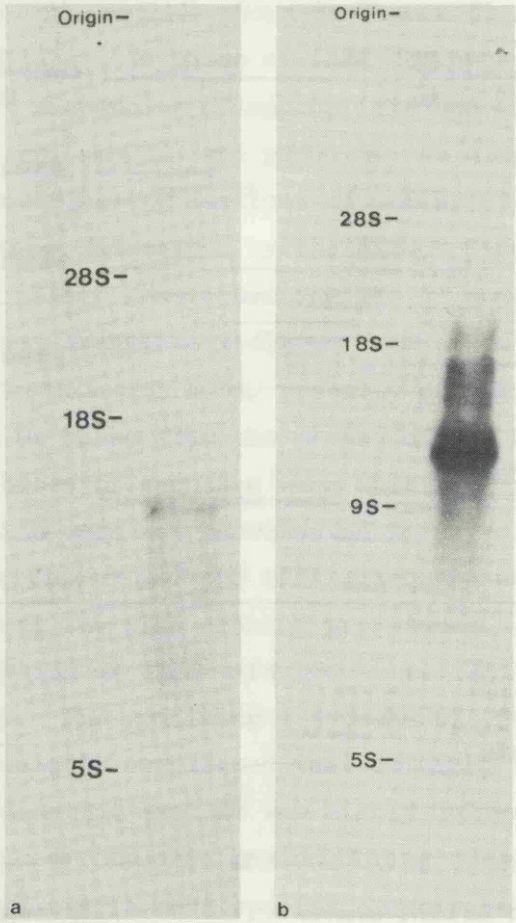



Figure 4 - Northern blots showing a) hybridisation of nick translated pSMG3 and b) hybridisation of nick translated pSMG166, to male submaxillary gland mRNA from DBA/2 mice

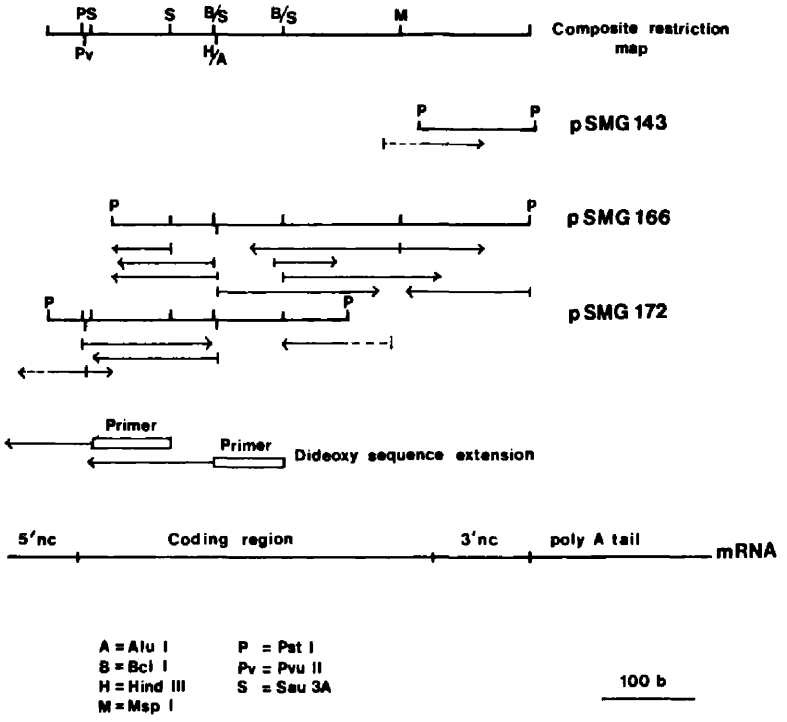
mRNA. Such a polypeptide would be 138 amino acids long with a molecular weight of 16.5 Kd, substantially different from the apparent size of 36 Kd.

Detailed characterisation of the pSMG143 plasmid family and the corresponding mRNA

Northern blot analysis of submaxillary gland mRNA using pSMG143 as a hybridisation probe suggested that the corresponding mRNA was about 800 bases long (Figure 4b). As the insert within pSMG143 was only about 200 bp in length it was used as a hybridisation probe to screen our cDNA libraries. In this way, plasmid pSMG166 with an approx 480 bp insert was isolated. Re-screening the libraries using the pSMG166 cDNA insert as a hybridisation probe resulted in the isolation of plasmids pSMG172 and pSMG173 which, as shown in Figure 5a, extended the composite restriction map of this plasmid family by approximately 70 bp. The complete composite cDNA restriction map thus obtained extended to approximately 550 bp.

Nucleotide sequence analyses, performed in the manner indicated in

a




b

```
10        20        30        40        50        60        70        80        90        100       110       120
          ***       5' non-coding region                                MetSerAlaAlaGlyIleIleGluSerGlnAspAspGluAsnVal
ATGGTXTGGTGTTCTGACTTCTCCAATATGAAGGGTCTCTCATTCACATTCAGTGCTGTGACACTCTTCTTGGTCCTATGTCTGCAGCTGGGATCATTGAAAGCCAGGATGATGAAAATG

130       140       150       160       170       180       190       200       210       220       230       240
ArgLysProLeuLeuIleGluIleAspValProSerThrAlaGlnGluAsnGlnGluIleThrValGlnValThrValGluThrGlnTyrArgGluCysMetValIleLysAlaTyrLeu
TCCGAAAGCCACTTTTGATTGAAATAGATGTTCCATCAACAGCACAGGAAAATCAGGAGATCACTGTGCAAGTTACAGTTGAAACACAATATAGAGAATGTATGGTGATCAAAGCTTACC

250       260       270       280       290       300       310       320       330       340       350       360
ValSerAsnGluProMetGluGlyAlaPheAsnTyrValGlnThrArgCysLeuCysAsnAspHisProIleArgPhePheTrpAspIleIleIleThrArgThrValThrPheAlaThr
TTGTAAGTAATGAACCAATGGAAGGTGCCTTCAACTATGTACAAACCCGCTGCCTCTGTAATGATCATCCTATTAGATTTTTCTGGGATATAATAATCACAAGAACTGTAACATTTGCAA

370       380       390       400       410       420       430       440       450       460       470       480
ValIleAspIleValArgGluLysASpIleCysProAsnAspMetAlaValValProIleThrAlaAsnArgTyrTyrThrTyrAspThrValArgMetAsn***
CAGTGATTGATATTGTTCGAGAAAAGAATATCTGTCCTAATGATATGGCAGTGGTGCCCATCACAGCAAACCGGTACTATACTTATAATACTGTGCGAATGAATTAACGGAGGCTTCTTA

490       500       510       520       530       540       550       560       570       580       590       600
                    3' non-coding region
TGCGTCTTTCAAAACCATTATCATTTTCTCCAAGTACAATTGACTGGAATAACCATTGTAGTCAGTCAGAATAAACACTTAACAAGCATAAAAAA
```

Figure 5 Composite restriction map and sequence of the pSMG143 family.
a) Restriction maps of cDNA clones pSMG143, pSMG166, pSMG172 and pSMG173 showing sequencing strategy used and the proposed mRNA structure.
b) Sequence of pSMG143 family showing longest open reading frame. In phase stop codons are indicated (***) and the polyadenylation signal is boxed.

Figure 5a, resulted in the sequence shown in Figure 5b for the mRNA corresponding to plasmid pSMG143. 534 bases of this sequence were obtained by conventional Maxam-Gilbert sequencing of appropriate DNA fragments derived from plasmids pSMG143, pSMG166, pSMG172 and pSMG173. No disparities in the sequences obtained from overlapping sections of different plasmids were found. 224 bases of sequence were determined by the direct dideoxynucleotide/reverse transcriptase mediated method for sequencing the 5' end of mRNA species, using 87 and 70 bp primer fragments isolated from plasmid pSMG173. A strong reverse transcriptase termination site, probably corresponding to the 5' end of the mRNA, was found 94 bases from the 3' end of the 87 base pair Sau3A primer fragment. 46 bases of sequence were obtained by this reverse transcriptase technique which were not represented in any of the cDNA clones and presumably corresponded to part of the mRNA which had not been effectively copied or retained during the cDNA cloning procedures. Within these 46 bases there was only one position of uncertain identity. This position is indicated by an X in Figure 5b. The remaining 178 bases of reverse transcriptase determined sequence precisely overlapped that obtained by direct sequencing of the cDNA clones, indicating that it was highly unlikely that there had been any errors introduced into this region during cDNA cloning and also that there is probably only a single mRNA species closely related to pSMG143 expressed within the adult male submaxillary gland.

The 576 bases of sequence shown in Figure 5b probably represent the virtually complete sequence of the mRNA from which plasmid pSMG143 was originally derived. As discussed above, the 5' end of the sequence closely approaches the major reverse transcriptase termination point for this mRNA. At the 3' end is the beginning of a prospective polyA tract, preceded 14 bases away by the consensus polyadenylation signal AATAAA. A 200 base polyA tract would bring the size of this mRNA close to that indicated by the Northern blot analysis.

Computer assisted analysis of the sequence shown in Figure 5b revealed only one region which could encode a polypeptide of 14,706 daltons - close to that predicted from *in vitro* translation of the mRNA. It should be noted that the translation initiation codon used for the predicted polypeptide product of this mRNA is not the first prospective one within the sequence shown in Figure 5b. Other exceptions to this principle have been described previously (10, 36). Translation initiating at the first prospective initiation codon would have resulted in a product of 5.5 Kd in molecular weight.

DISCUSSION

In this paper we have reported the construction and identification of recombinant plasmids containing cDNAs derived from the more abundant mRNAs of the adult male DBA/2 submaxillary gland. Clones corresponding to the relatively abundant mRNAs for preprorenin (5, 6) and kallikrein (11) and the three less abundant species for NGF (9, 10), EGF (7, 8) and EGF-binding protein type B (12) have previously been described. In all these cases the synthesis of the gene-product is known to be androgen-inducible. Here we have focused on major polypeptide products of the gland that do not show significant sexual dimorphism in their synthesis. Two sets of cDNA clones and their cognate messengers have been extensively characterized. The pSMG3-related clones are derived from probably the most abundant mRNA species in this gland, corresponding to about 17% of the cDNAs in the library, apparently encoding a polypeptide of 16.5 Kd that is subject to microsome-mediated modification. The pSMG143 family of clones was derived from a less abundant species of mRNA (0.5 - 1%), encoding a polypeptide of about 14 Kd that was not affected by microsomes.

Computer-assisted analysis (37) of the amino acid sequences of the putative products, determined from the nucleotide sequence of the two families of cDNA clones, has revealed features that may help in assigning a role to the proteins. The probable product of the pSMG3-related mRNA has a highly hydrophobic N-terminal sequence of amino acid residues (Figure 6a) which is characteristic of a eukaryotic signal sequence. This suggestion is consistent with the inference from the microsome-mediated processing that the corresponding protein is exported from submaxillary gland cells. Also evident in Figure 6a is the extremely acidic central tract of amino acids followed by a basic sequence towards the C-terminus. Overall, this protein would possess a high net negative charge around neutral pH, which may account for its apparently aberrant migration on SDS-PAGE. Although this translation-product has a mobility in SDS-PAGE consistent with a size of 36 Kd, its mobility in urea/polyacrylamide gels suggests a size of about 20 Kd (L.J.B.-unpublished).

A further interesting feature of the amino acid sequence of this polypeptide is the presence of four potential N-glycosylation sites, represented by the sequence Asn-X-Thr or Asn-X-Ser (32) suggesting that the protein might be highly glycosylated *in vivo*.

Thus, overall, it is likely that the polypeptide corresponding to the pSMG3 family is a major product of the mouse submaxillary gland and is secreted as an acidic, hydrophilic glycoprotein. Such properties might be expected of a protein that serves the function of a salivary lubricant. Although the

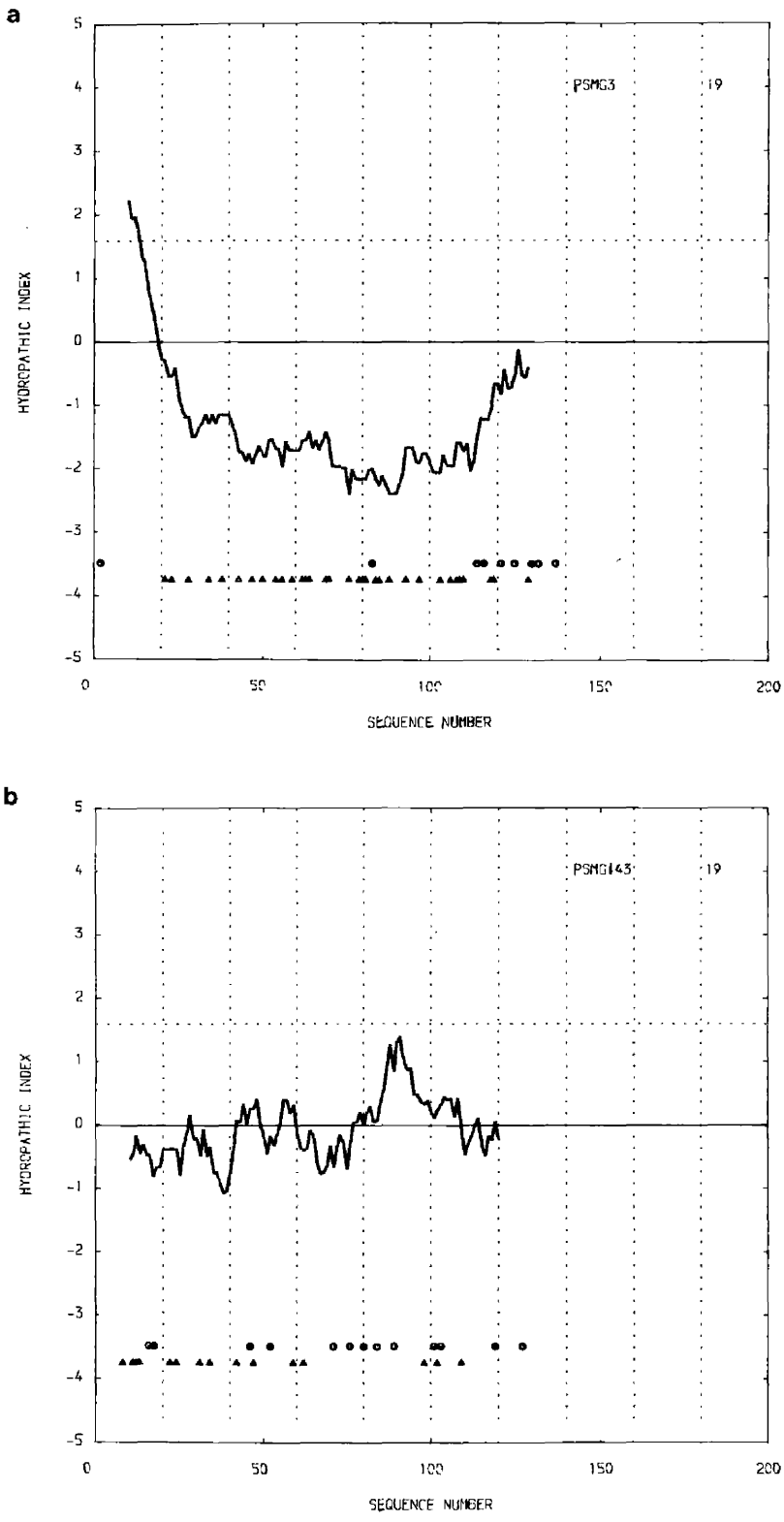



Figure 6 - Hydropathic character of the polypeptide species encoded by plasmids pSMG3 (a) and pSMG143 (b).

Analysis of the hydropathic index was carried out using the SOAP computer programme of Kyte and Doolittle (37) at a span setting of 19. The positions of acidic (Δ) and basic (o) amino acid residues within the polypeptides are indicated.

submaxillary gland is reported to synthesize mucin[2], it is apparent, particularly from the low proline content (2) that the pSMG3-protein is not closely related to presently described mucins.

Southern blots and genomic cloning of mouse DNAs, using labelled pSMG3 related DNA as the probe, have shown that the pSMG3-related mRNA is transcribed from a gene that is a member of a large gene-family in the mouse (A.Craig, unpublished observation). Analysis of RNAs from a range of different tissues by RNA/DNA hybridisation has not yet identified any sites of transcription of these genes other than the submaxillary glands.

Computer-based inspection of the prospective polypeptide product of the mRNA represented by the pSMG143 plasmid family does not reveal such striking features. This polypeptide does not have a distinctly non-polar N-terminal sequence which, together with the lack of microsome-mediated processing, indicates that it is unlikely to be either exported or integrated into membranes in the gland. In addition, this sequence lacks potential glycosylation sites and shows no unusual amino acid composition, although again it is rather acidic. It seems likely that the pSMG143-related product is a major intracellular submaxillary protein.

It is interesting to note that the polypeptide translated from the pSMG143-related mRNA was reproducibly immunoprecipitated by some batches of anti-EGF antisera. This is surprising, since no significant homology could be detected between EGF, or even pre-pro EGF (7, 8) and the prospective polypeptide product of this mRNA. The EGF preparations used in raising these antisera were of very high purity (Dr H.Gregory, personal communication) and were therefore believed to be unlikely to give rise to contaminant antibodies capable of reacting with non-EGF related products of the submaxillary gland.

A search of the Doolittle database (38) of both protein and nucleic acid sequences, kindly carried out by Dr Peter Stockwell at ICRF, Lincoln's Inn Fields, London, has not identified any sequence having significant homology with either the pSMG3 or the pSMG143 families of sequences. Antisera raised against these gene-products produced in suitably manipulated bacteria may be useful in establishing the identity and physiological role of these two major submaxillary proteins.

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## ABSTRACT

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The synthesis of mouse salivary gland proteins was studied, in particular that of renin, an enzyme normally made in the kidney and involved in the maintenance of normal blood pressure, which is produced in the submaxillary gland (SMG) in high levels. This gene expression is both androgen inducible and genetically controlled, inbred strains of mice having either high or low levels of the enzyme. It was found that the differences in renin levels reported by other workers correlated with a variation in translatable mRNA levels. The isolation and identification of cDNA clones corresponding to SMG renin facilitated the identification of two renin genes in the DBA/2 mouse (a high producer strain). Primer-extension analysis and $S_1$ mapping using a restriction fragment from one of these genes, Ren-1, revealed three potential start points for renin transcription. In the high producer all three appear to be used but in the low producer (C57BL/10) only one is significantly active (P1). Renin cDNA clones were also used to purify renin mRNA which was translated *in-vitro*. Subsequent processing of the primary translation product confirmed the presence of a leader sequence, completing the biosynthetic pathway of mouse SMG renin.

The translation profile of mRNA from the sublingual gland (SL) showed an abundant 16kd polypeptide and reverse transcription of this mRNA indicated a single major size-class of mRNA of ≈ 600 nucleotides, sufficient to encode a polypeptide of such size. *In-vitro* translation and cDNA cloning studies using M13 as a vector have shown this major product to be a previously unrecognised family of polypeptides which appear to be constitutively expressed.

The availability of renin sequence probes permitted the cloning and isolation of cDNA copies of human renal renin mRNA. The encoded polypeptide had a high degree of homology to mouse SMG and kidney renins but had three additional internal amino acids. The region corresponding to the cleavage site between the A and B chains of mouse renin is altered, giving support to reports that human renin is a single-chain molecule.