IN-VITRO STUDIES OF THE CONTROL OF RENIN GENE-EXPRESSION IN THE MOUSE

by

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A Thesis submitted for the Degree of Doctor of Philosophy at the University of Leicester

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I dedicate this Thesis to my mother and father and to my fiancé for their support and encouragement.

DECLARATION

I declare that all the work recorded in this thesis is original unless otherwise acknowledged in the text. None of the work has been submitted for another degree in this or any other University.

Signed..... Date.....

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ABBREVIATIONS

A	adenine
cAMP	adenosine 3',5'-cyclic monophosphate
ATP	adenosine 5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin (Pentax fraction V)
C	cytosine
CTP	cytidine 5'-triphosphate
d	2'-deoxyribo (e.g. dATP)
dd	2',3'-dideoxyribo (e.g. ddATP)
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	1,2-di(2-aminoethoxy)ethane-*NNN'N'*-tetra-acetic acid
G	guanine
GTP	guanosine 5'-triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
Hybond N	nylon membrane (Amersham)
IPTG	isopropyl β-D-thiogalactoside
Kb	kilobase(s)
Kd	kilodalton(s)
NMP	nucleoside 5'-monophosphate
NTP	nucleoside 5'-triphosphate
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
RF	replicative form
RNase	ribonuclease
RNA	ribonucleic acid
mRNA	messenger RNA
snRNA	small nuclear RNA
tRNA	transfer RNA
RNasin	ribonuclease inhibitor (Promega)
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

TTP	thymidine 5'-triphosphate
Tris.	tris(hydroxymethyl)methylamine
U	uracil
UTP	uridine 5'-triphosphate
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

Certain inbred strains of mice can be divided into two groups on the basis of their submandibular gland (SMG) renin activity. High renin producing strains have two genes *Ren-1^d* and *Ren-2^d*, whereas low producers have a single gene *Ren-1^c*. To search for the presence of regulatory elements involved in renin control, approximately 1Kb of both *Ren-1^d* and *Ren-2^d* 5' flanking sequence containing the major transcription start-site P3 were fused to a "G-free" reporter cassette, and the resulting templates transcribed in various nuclear-protein extracts. In rat liver, mouse kidney and mouse testis extracts the *Ren-2^d* promoter was 2-fold more active than the *Ren-1^d* promoter whereas in mouse liver, both promoters were transcribed equally well. Deletion-mapping data are consistent with the presence of a functional negative control element(s) within *Ren-1^d* but not *Ren-2^d*. Polymerase catalysed chain reaction (PCR) generated deletions have shown that a fragment of 62bp, containing only the P3 promoter, was sufficient for accurate *in-vitro* transcription. Disruption of the P3 TATAAAA resulted in total loss of activity. Deletion of an AP-2 consensus sequence from the *Ren-1^d* promoter had little effect on *in-vitro* transcription. Furthermore, sequences 3' to the *Ren-1^d* P3 start-site when positioned either upstream or downstream to the *Ren-1^d* promoter/G-free fusion, appeared to have a minimal effect on promoter activity suggesting that these regions are not involved in *in-vitro* regulation of the *Ren-1^d* gene. Finally, patterns of specific protein-DNA interactions observed with the mobility-shift assays, are also consistent with an overall negative control hypothesis.

CHAPTER 1

INTRODUCTION

1.1 Renin-Angiotensin System

Renin [EC 3.4.99.19] is an aspartyl protease which plays an important role in the maintenance of blood pressure (Davis, 1971; Cowely *et al.*, 1971; and MacGregor *et al.*, 1981). The enzyme is stored in granules in epithelioid cells of the afferent arterioles of the juxtaglomerular apparatus in the kidney (Figure 1.1), and is confined to the secretory granules of the granular convoluted tubules in the submandibular gland (SMG) of high producer mice (Tanaka *et al.*, 1980).

Upon release into the plasma renin cleaves angiotensinogen, a protein produced under hormonal control in the liver, to angiotensin I the N-terminal decapeptide. Angiotensin I is then quickly cleaved by angiotensin converting enzyme (ACE) to angiotensin II, by removal of two carboxy terminal amino acids (Page, 1939; Braun-Menendez et al., 1940; and Skeggs *et al.*, 1954) (Figure 1.2). Although angiotensin I has no known biological activity, angiotensin II is a potent vasopressor (Cowley *et al.*, 1971) and is directly or indirectly involved in blood pressure maintenance. First, angiotensin II has a direct vasoconstrictive action on the arterioles, resulting in increased peripheral resistance and hence elevated blood pressure. Second, it can also stimulate further secretion of its primary precursor angiotensinogen, thereby enhancing the cascade. Third, it can stimulate the secretion of aldosterone from the adrenal cortex, resulting in sodium and water retention, which leads to a greater blood volume and hence elevated blood pressure (MacGregor *et al.*, 1981).

The mechanism by which the kidney is stimulated to release renin has been intensively studied. According to the barorecepter hypothesis, the renal afferent arterioles and juxtaglomerular cells respond to changes in vascular volume and pressure (Tobian *et al.*, 1959; and Skinner *et al.*, 1964). Additional evidence that the degree of constriction of the renal

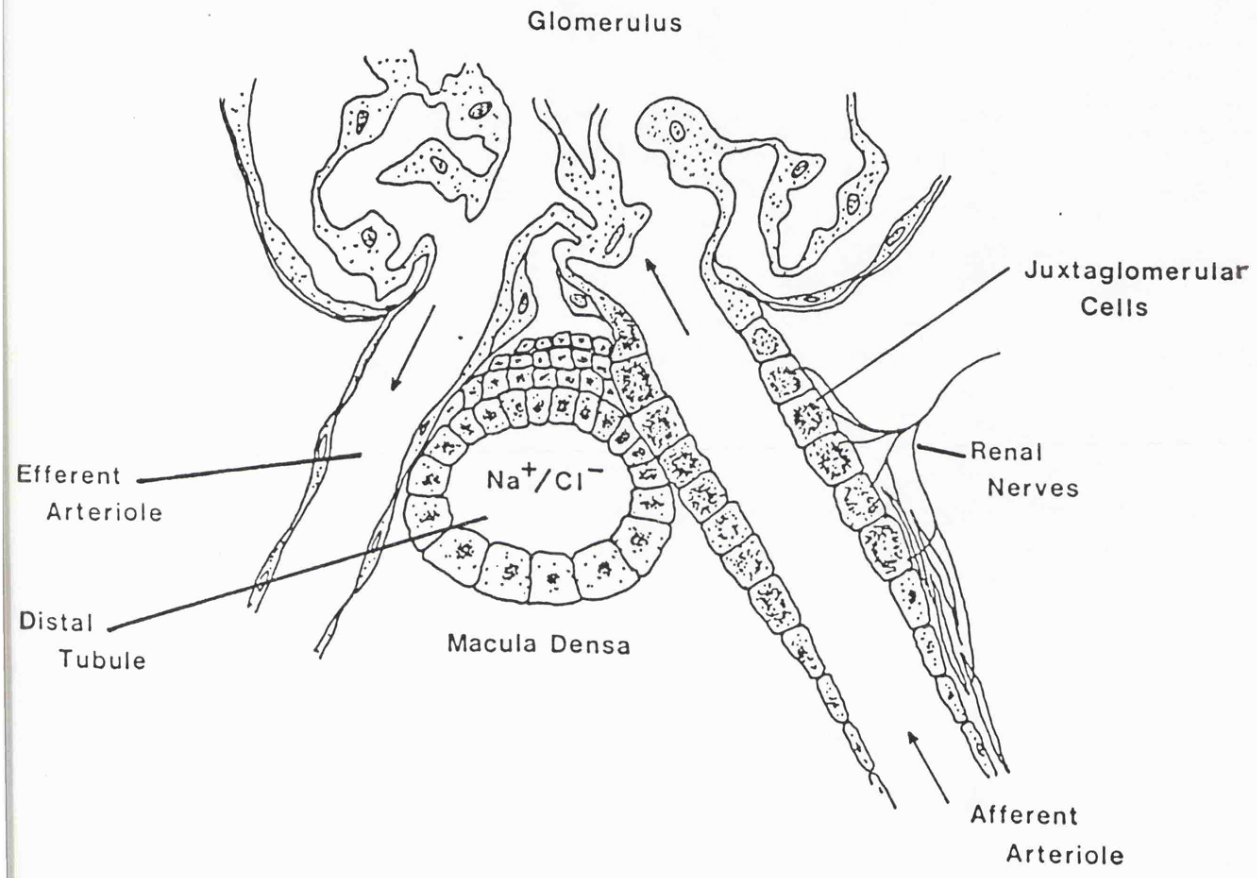


Figure 1.1

The juxtaglomerular apparatus. According to the baroreceptor hypothesis, the renal afferent arterioles and juxtaglomerular cells respond to changes in vascular volume and pressure. The macula densa is thought to respond to changes in sodium load. The juxtaglomerular cells secrete renin into the lumen of the renal afferent arteriole and into renal lymph.

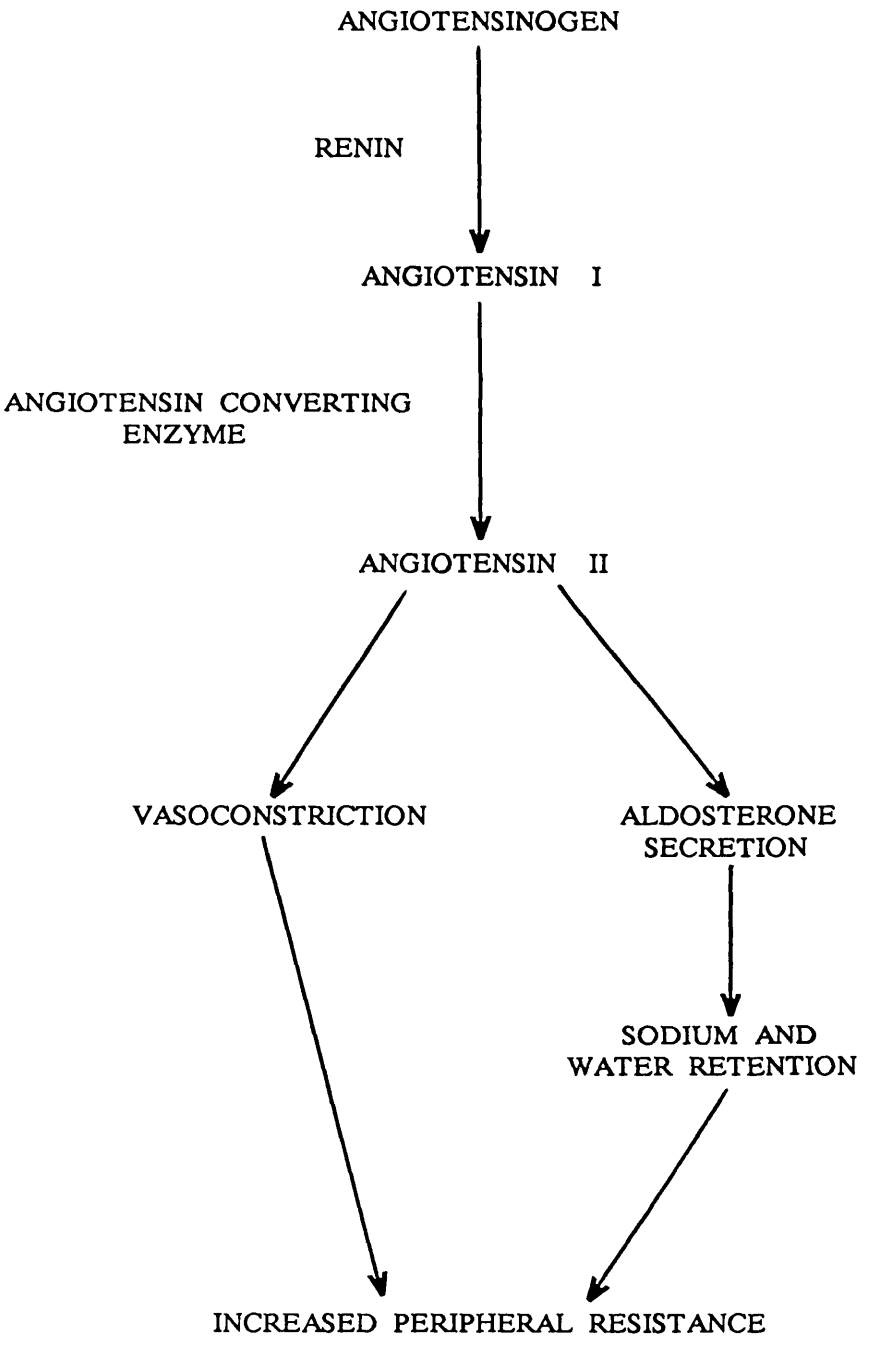


Figure 1.2

Scheme showing the overall effect of the renin-angiotensin system.

arterioles is important in renin release was provided from studies of experimental hypertension in dogs by Ayers *et al.* (1969), who reported that a decrease in renal arteriolar resistance was associated with increased renin activity. The macula densa is also thought to be involved in the renin release mechanism, and Vander and Miller (1964) and later Vander and Carlson (1969) showed that a decrease in sodium load at the macula densa leads to renin release. Renin gene-expression in the kidney is also influenced by sodium, since depletion was shown to stimulate renal renin mRNA levels 3.5- to 7.0-fold above control levels (Nakamura *et al.*, 1985; and Ingelfinger *et al.*, 1986). The renal nerves have also been shown to be important. Vander (1965) reported that electrical stimulation of the renal sympathetic nerves released renin, and Mogil *et al.* (1969) demonstrated that plasma renin activity failed to increase in response to sodium depletion in dogs with renal denervation. It is plausible that all the above mechanisms are operative in controlling renin release, but the extent of dominance may vary and be dependent upon physiological conditions.

Finally, because the renin-angiotensin system is involved in maintaining blood pressure, it is closely associated with the kallikrein-kinin system, which releases the vasodilatory peptides bradykinin and lysyl-bradykinin. The two systems are "linked" by ACE, which not only produces the vasoactive peptide angiotensin II but also cleaves and thereby inactivates bradykinin, nullifying the antagonistic response of the kallikrein-kinin system. Unlike renin, kallikrein is localised to the convoluted distal tubule of the kidney (Orstivvik and Inagami, 1982).

1.2 Renin Purification and Characterisation

Since its discovery, many workers have attempted to purify and characterise renin. The first large-scale purification of renin was that developed by Haas *et al.* (1953) who used 67Kg of hog kidney in a procedure which involved ten steps and gave a 56000-fold overall purification. The difficulty in purifying renal renin lay in its very low concentration in the kidney, and the above method was not significantly improved upon until the advent of modern affinity chromatographic techniques. Inagami and Murakami (1977) used a combination of pepstatin-affinity chromatography together with DEAE- and

CM-cellulose chromatography to produce a 133,000-fold purification of renin from hog kidney. The high purification obtained with this method paved the way for the first detailed characterisation of renin, which was found to be a 36Kd glycoprotein containing glucosamine residues. Hog renin was homogeneous and stable at neutral pH and gave a discrete band when assayed by polyacrylamide gel-electrophoresis (PAGE), SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) and isoelectric focusing.

Although much has been learned from the isolation and characterisation of hog kidney renin, many workers were turning their attention to the isolation of human kidney renin in an attempt to study its role in clinical hypertension. Galen *et al.* (1979) reported the first complete purification of human renin from renin-producing juxtaglomerula cell tumours. The use of tumours had two advantages: very high renin concentrations and simple and rapid processing. Purification involved three steps: gel-filtration, DEAE-cellulose chromatography, and preparative isoelectric focusing. Gel-filtration separated the major renin fraction of 40Kd, containing 97% of total activity, from two high molecular weight forms. The 40Kd fraction was found to be heterogeneous and contained five renin species, all of which were glycoproteins, separable by isoelectric focusing. The three major species B,C and D, when analysed by SDS-PAGE, were found to have molecular weights of 50Kd, 25Kd and 20Kd, respectively. All three species possessed renin activity, although the relationship between the 40Kd renin and the "small" renins is not clear. The discrepancy between the molecular weights found by gel-filtration (40Kd) and SDS-PAGE (50Kd) was thought to be due to glycosylation, leading to overestimation of the molecular weight in SDS-gels. In contrast to Galen *et al.* (1979), Yokosawa *et al.* (1980) reported the complete purification of human renin from non-cancerous, autopsied kidneys. Final purification on DEAE-cellulose separated renin into three peaks but, unlike the report by Galen *et al.* (1979), the major peak was found to be homogeneous. The molecular weight of the pure enzyme, determined by sedimentation equilibrium analysis, was 40Kd and affinity for concanavalin A suggested the presence of carbohydrate. McIntyre *et al.* (1983) used a potent peptide inhibitor of renin, H.77, as an affinity ligand to achieve rapid isolation of a highly purified and stable human renin preparation. The purified enzyme, when examined on

SDS-PAGE, consisted of two proteins with apparent molecular weights of 40Kd and 17Kd. The larger protein was thought to correspond to the 40Kd renin purified by Yokosawa *et al.* (1980), but the nature of the 17Kd fraction was not clear and may represent a fragment of renin formed by limited proteolysis.

Whilst there was much interest in renal renin many groups turned their attention to extra-renal sources of renin activity. One major source is the SMG of the mouse (Werle *et al.*, 1957). Purification of SMG renin was reported by Cohen *et al.* (1972), who separated the enzyme into five fractions which were chromatographically and electrophoretically distinct but were found to be antigenically related. The two major fractions (A and C) were purified further and were both found to be glycoproteins having molecular weights of 43Kd and 36-37Kd respectively. Their isoelectric points also differed, being 5.40 for A and 5.62 for C. Intravenous injection of either preparation into suitably treated animals resulted in a prolonged (over 30 minutes) elevation of mean blood pressure, demonstrating that both enzymes were biologically active *in-vivo*. Using affinity chromatography both Suzuki *et al.* (1981) and Ho *et al.* (1982) purified five forms of SMG renin. Interestingly the first group of workers found that only two fractions were active *in-vivo*, whereas the second group found that four out of the five fractions were able to elevate blood pressure in nephrectomised rats. Ho *et al.* (1982) also reported that each of the four renin fractions had molecular weights of 40Kd.

Although enzymological and molecular properties of human renin are generally analogous to renins purified from other mammalian species, the human enzyme shows a number of properties which distinguish it from other renins. Active low molecular weight renins isolated from other mammalian species have true molecular weights between 36Kd and 37Kd, whereas human renin has a molecular weight of 40Kd. Furthermore, isoelectric points of renins from other species are between 4.5 and 5.4 whereas for human renin it is 5.7. Finally, human renin does not cross-react with antibodies raised against renin from other species, whilst renins from other mammalian species cross-react with each other (Cohen *et al.*, 1972; Inagami and Murakami, 1977; Galen *et al.*, 1979; Yokosawa *et al.*, 1980; and McIntre *et al.*, 1983).

1.3 Renin Biosynthesis and Secretion

One of the first attempts to demonstrate in which form renin is stored in the SMG was that reported by Nielsen *et al.* (1979), who used renin antibody and pure specific immunoreactive Fab fragments of the antirenin molecule in their assays. The proteolytic activity of serine-, metallo- and sulphhydryl enzymes during homogenisation was inhibited, but no inactive or high molecular weight form could be detected either enzymatically or antigenically after gel-filtration. The only form of renin in the SMG which they demonstrated was the fully active 40Kd form (Cohen *et al.*, 1972; Suzuki *et al.*, 1981; and Ho *et al.*, 1982).

The first direct evidence that renin is synthesised as a high molecular weight precursor came from work by Poulsen *et al.* (1979). Two mRNA-dependent cell-free systems from wheat germ and reticulocyte lysate respectively were used, and the radioactive translation products were immunoprecipitated with antirenin or pure renin-specific Fab fragments and subsequently characterised by SDS-PAGE. Poulsen *et al.* (1979) were able to demonstrate that renin was initially synthesised as a precursor 10Kd larger than the enzymatically active 40Kd renin and that the precursor was a single chain polypeptide, a prorenin or even a preprorenin. Although the postulated 50Kd renin precursor proved later to be an overestimation (Morris and Catanzaro, 1981) it provided the first insight into renin biosynthesis.

Biosynthesis of prorenin and its conversion to renin intracellularly was first demonstrated by Morris and Catanzaro (1981). Radioactive amino acids were added to tissue actively synthesising renin and antiserum to renin was used to remove newly synthesised radioactive proteins from the mixture. These were then analysed by electrophoresis to determine molecular weight and the time course of processing. Using such a continuous labelling experiment and SMG of male mice a 44.5Kd molecule was immunoprecipitated with antiserum to renin and was converted to a 40Kd molecule in a time-dependent fashion. The 44.5Kd molecule was assumed to be prorenin. Further work showed that when SMG mRNA was translated in a cell-free system derived from rabbit reticulocyte lysate, a 46Kd molecule was immunoprecipitated with renin antiserum. This was assumed to be preprorenin since the size difference

between preprorenin and prorenin (some 1.5Kd) is consistent with the size generally found for signal sequences of secretory proteins.

Further evidence for cleavage of a signal peptide during renin biosynthesis was provided by translating mRNA in the presence of dog pancreatic microsomal membranes (Catanzaro *et al.*, 1983), which contain signal peptidases capable of cotranslationally hydrolysing the presequence from nascent polypeptide chains (Lingappa *et al.*, 1977). Using such a system Catanzaro et al. (1983) were able to show that a 45Kd preprorenin was converted to a 43Kd prorenin with removal of a 2Kd peptide. The 43Kd prorenin was subsequently rapidly converted to a 38Kd renin which was then hydrolysed slowly to give two chains of 33Kd and 5Kd held together by disulphide bonds (Figure 1.3). Both single and two chain 38Kd renins bound to pepstatin, suggesting that both were enzymatically active and have an exposed active site. The biosynthetic intermediates observed by Catanzaro *et al.* (1983) were also demonstrated by Morris *et al.* (1983) who also found that both single and two-chain 38Kd renin were enzymatically active. This was confirmed by Pratt *et al.* (1983) who have shown that the one-chain renin has a 6-fold higher specific activity than the two-chain form and that the light-chain was essential for enzymatic activity. The same group also observed heterogeneity in the isoelectric points of the various forms of renin. Each renin species appeared to have four or five isoelectric points. Prorenin was found to have four isoelectric forms with pIs similar to those of the one-chain renin. Preprorenin on the other hand showed only one isoelectric form with a pI of 5.3. Glycosylation of SMG renin was not thought to be the cause of the heterogenicity in pIs, because carbohydrate analysis of pure mouse SMG renin failed to detect significant carbohydrate moieties. However heterogeneity may be due to differences in amino acid compositions as a result of minor variations in the cleavage positions. Alternatively it may arise from other posttranslational modifications such as methylation, acetylation or phosphorylation.

After isolating renin mRNA from mouse SMG and subsequently producing recombinant cDNA clones, Panthier *et al.* (1982a) deduced the amino acid sequence for SMG renin and showed it to be synthesised as a 44,209 molecular weight precursor. N-terminal sequence analysis of renin

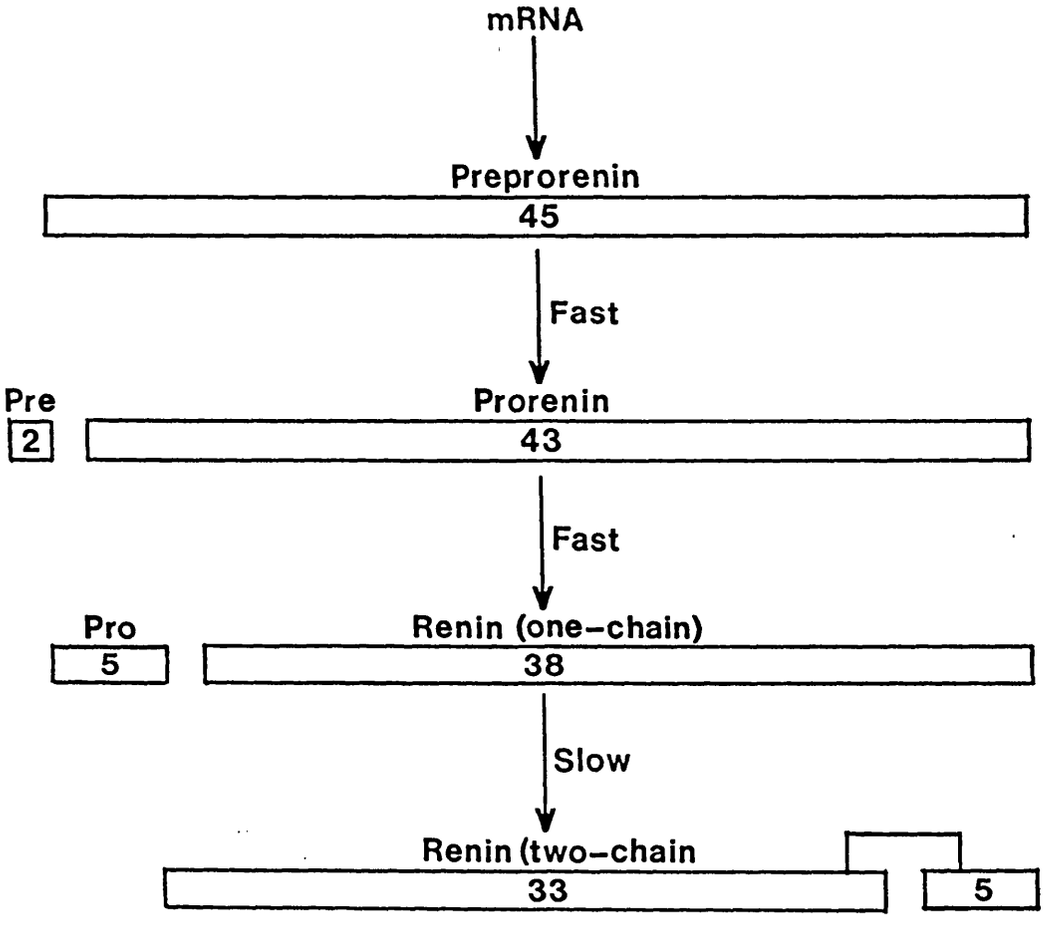


Figure 1.3

Processing of preprorenin. A 2Kd signal peptide (pre) is removed by microsomal proteases to give prorenin, which is converted rapidly to renin by removal of a 5Kd pro region. Renin of 38Kd is then hydrolysed more slowly to give two chains of 33Kd and 5Kd held together by a disulphide bond.

revealed that the precursor was converted by a proteolytic cleavage into active renin consisting of two chains of 35Kd and 3Kd. These results closely correlate with those of Catanzaro *et al.* (1983).

Pratt *et al.* (1983) went on to study the subcellular localisation of prorenin, one-chain renin and two-chain renin in mouse SMG, using subcellular fractions and electrophoretic blotting techniques. One-chain renin together with trace quantities of prorenin and two-chain renin were contained predominantly within enriched Golgi. The two-chain form (80-90%) and to a lesser extent the one-chain form (10-20%) were contained primarily within mature granules. They also observed that both one-chain and two-chain renins were secreted, but the biological relevance is unknown.

More recently further evidence for the involvement of multiple pathways in renin biosynthesis and secretion has been forthcoming. Hirose *et al.* (1985) showed that when microinjected with human renin mRNA, *Xenopus* oocytes secrete a truncated form of prorenin into the culture medium. By using pulse labelling experiments, Pratt *et al.* (1987) showed that prorenin secreted by the kidney was identical to intracellular prorenin, a truncated form was not observed. Furthermore, when the kinetics of processing and secretion were examined, newly synthesised prorenin was found to be quickly secreted, while only a small fraction was processed intracellularly to mature renin. These workers also showed that monensin, which disrupts the transport of proteins through the Glogi, reduced the secretion of prorenin and increased the amount of intracellular prorenin. The differences in secretion kinetics and the selective inhibition of prorenin secretion by monensin suggest that mature renin and prorenin are secreted independently via two pathways: a constitutive pathway from the Golgi that rapidly releases prorenin and a regulated pathway that secretes mature renin from mature granules. Further evidence for different secretory pathways of renin came from Pratt *et al.* (1988) who showed that transfected fibroblasts (mouse L929 and Chinese hamster ovary cells) synthesise prorenin only and that secretion is constitutive and is not influenced by secretagogues. Transfected pituitory tumour cells (mouse AtT-20), however, were shown to synthesise both prorenin and mature renin. Prorenin secretion was constitutive whereas mature renin was secreted by a regulated

pathway since it could be stimulated by the secretagogue 8-bromo-cAMP.

The proposed pathways of renin biosynthesis and secretion are illustrated in Figure 1.4. Translation of renin mRNA yields preprorenin. Preprorenin is internalised cotranslationally into the rough endoplasmic reticulum and hydrolysed by signal peptidase to produce prorenin. Prorenin has two major fates. It is rapidly secreted by a constitutive pathway probably directly from the Golgi. Prorenin also may be packaged into immature granules where it is further processed to form mature renin during condensation and maturation of the secretory granules. In normal human kidney the latter process is relatively slow.

1.4 Renin Gene-Expression

Renin gene expression is complex and under genetic, developmental, hormonal and tissue-specific controls. The renin gene therefore provides an interesting system in which to study these controlling mechanisms.

1.4.1 Genetic and Developmental Control.

Inbred strains of mice can be divided into two groups on the basis of their SMG renin activity. The initial discovery of high (e.g. DBA/2, Swiss mice, AKR, SWR) and low (e.g. C57BL/6, BALB/c) producer mice was made by Bing *et al.* (1967), who also demonstrated with bioassay techniques that the activity in the SMG was under genetic control, since crosses between high and low producer strains resulted in F1 hybrids with intermediate levels of renin activity. SMG renin activity is also under developmental control, since the difference between high and low producer strains is not expressed until approximately four weeks of age and prior to that time activity is low in both strains (Wilson *et al.*, 1981). Further evidence for the genetic control of renin came from Wilson *et al.* (1977), who proposed that the difference in renin activity between high and low producers was due to a single genetic locus *Rnr* located on chromosome 1 and closely linked to the *Pep 3* gene (previously called *Dip 1*) (Wilson *et al.*, 1978). Wilson *et al.* (1982) utilized inbred strain-specific differences in renin stability at 60°C to determine the relationship

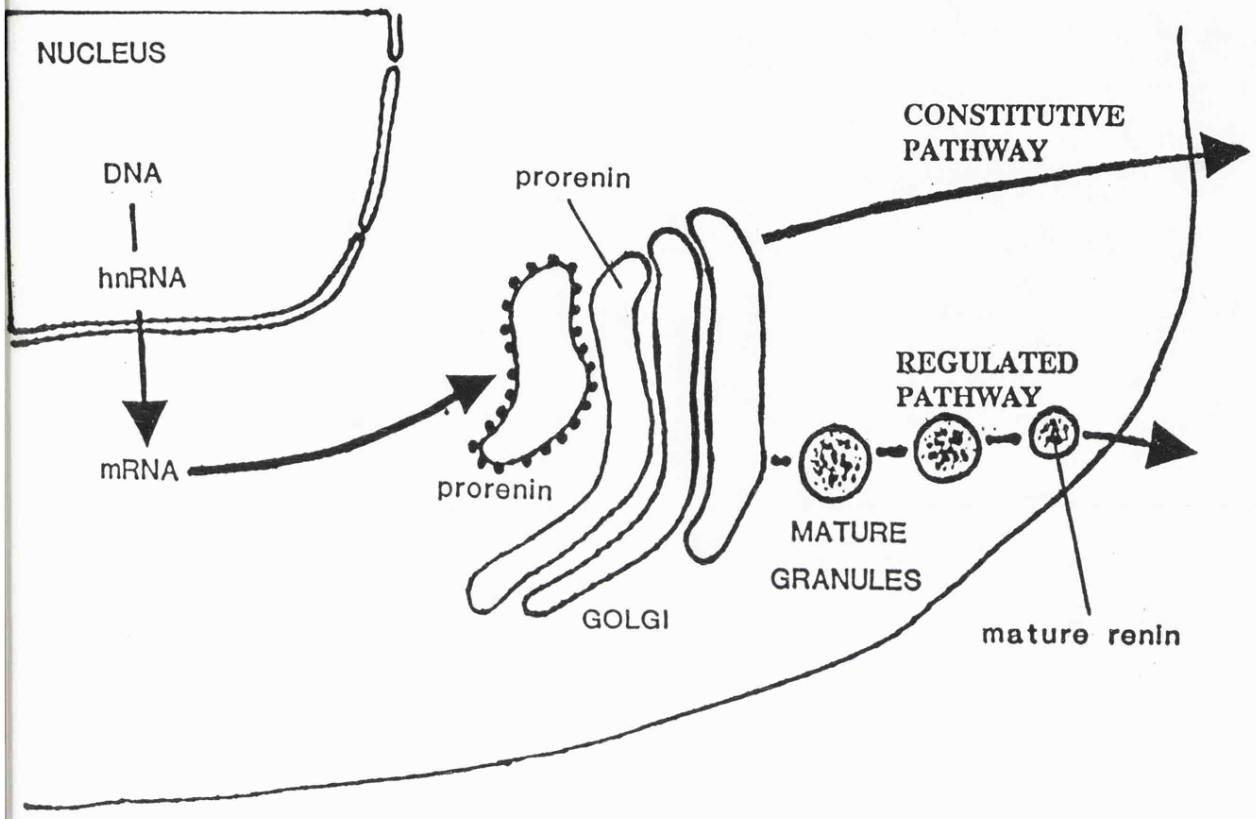


Figure 1.4

Proposed intracellular localisation of the various forms of renin during biosynthesis and secretion.

between *Rnr* and the renin structural gene. They showed that SMG renin from low producer strains (*Rnr*^{b} allele) was thermostable whereas SMG renin from high producer strains (*Rnr*^{s} allele) was thermolabile. Futhermore they observed that kidney renin from either strain was thermostable. On the basis of these observations these workers proposed three models to explain SMG renin expression (Table 1.1).

Model I assumes that there is one structural gene *Rnr* for renin and that both qualitative and quantitative differences among strains can be explained by allelic variation at this genetic locus. In Model II renin in the SMG is controlled by two separate but closely linked genes. *Ren–r* symbolises the amount of renin activity whereas *Ren–s* symbolises the renin structural gene. Finally Model III proposes that renin activity in the mouse SMG is determined by two different structural genes. *Ren–1* codes for the thermolabile isoenzyme Renin-1, and *Ren–2* codes for the thermostable isoenzyme Renin-2.

Wilson *et al.* (1982) found Model III appealing because it provided a simple explanation for their observation that an alloantibody which inhibited SWR (high producer) SMG renin, did not affect SWR kidney renin or C57BL/6 (low producer) SMG or kidney renin. They suggested that *Ren–1* was expressed only in the SMG of mice that carry the *s* allele (Table 1.1) at this locus whereas *Ren–2* was expressed in both tissues. It should be noted that the nomenclature has now been reversed so that the low renin producing strains have one gene *Ren–1*^{c} (thermostable phenotype) whereas high renin producing strains have two genes *Ren–1*^{d} and *Ren–2*^{d} (thermolabile phenotype). Hereafter the new nomenclature will be used.

Evidence in support of Model III has been obtained by several workers. By choosing hybridisation conditions which would identify only those sequences closely homologous to SMG renin mRNA, and by using Southern-blot hybridisation, Panthier *et al.* (1982b) showed that low producer mice (C57BL/6, Balb/c) have one renin structural gene *Ren–1*^{c} (*Ren–A* or *Rn–1*) per haploid genome, whilst high producer mice (AKR, Swiss mice) have two renin genes *Ren–1*^{d} and *Ren–2*^{d} (*Ren–B* or *Rn–2*). Piccini *et al.* (1982) isolated a cDNA

Table 1.1 Three models to explain the genetic control of renin

	HIGH PRODUCER MICE	LOW PRODUCER MICE
MODEL I 1 Structural Gene: 2 Alleles	*Ren*^{s} ↓ Renin-S	*Ren*^{b} ↓ Renin-B
MODEL II 1 Structural Gene: 2 Alleles 1 Regulatory Gene: 2 Alleles	[*Ren-s*^{s},*Ren-r*^{s}] ↓ Renin-S	[*Ren-s*^{b},*Ren-r*^{b}] ↓ Renin-B
MODEL III 2 Structural Genes: Null Allele	*Ren-1*^{s} *Ren-2* ↓ ↓ Renin-1 Renin-2	*Ren-1*^{n} *Ren-2* ↓ Renin-2

recombinant clone for SMG renin, and used it in Southern-blot analysis to study the organisation of homologous DNA sequences in both high and low producer strains. Restriction digest patterns of DNA from high renin strains were found to be distinct from patterns observed for low renin strains, suggesting a structural gene duplication at the chromosome 1 locus in high renin strains. Via genomic cloning Mullins *et al.* (1982) showed that there are two similar but distinct renin genes in DBA/2 mouse DNA, and that the homology between *Ren-1*^{d} and *Ren-2*^{d} regions of the genome extends over at least 10Kb and stretches well beyond the renin genes. Finally Panthier and Rougeon (1983) reported the molecular cloning of cDNA copies of Swiss mouse kidney renin mRNA, and after comparison of these sequences with the sequence of Swiss mouse SMG mRNA (Panthier *et al.*, 1982b) demonstrated that Swiss mice express the two non-allelic genes *Ren-1*^{d} and *Ren-2*^{d}.

Occurrence of a gene duplication event at the chromosome 1 locus in high producer mice was first reported by Piccini *et al.* (1982). They went on to suggest that the mechanism of such an event may be the result of an unequal meiotic crossover, between misaligned chromosomes, through homologous recombination between short direct repeats of DNA sequences located on both sides of the ancestral gene. Dickinson *et al.* (1984) observed that two renin genes were only present in wild-derived mouse species closely related to the (modern) inbred strains: more distantly related species, including the rat, all appeared to carry only one gene. On the basis of the limited phylogenetic distribution of the two gene animals, these workers suggested that the renin gene duplication event in mice probably occured prior to their divergence 2.75-5.50 million years ago.

The observation that only high producer mice have the *Ren-2*^{d} gene, suggests that either two mouse populations were established at the time of the duplication of the ancestral renin gene (one possessing two renin genes and the other one renin gene), or the duplication of the ancestral renin gene was followed by a deletion event resulting in the loss of the *Ren-2*^{d} gene in low producer mice (Figure 1.5).

Burt *et al.* (in preparation) compared the restriction site maps and

nucleotide sequence of *Ren–1^d*, *Ren–2^d* and *Ren–1^c* genes. They observed that divergence between *Ren–1^d* and *Ren–1^c* was less than that between *Ren–1^c* and *Ren–2^d*, suggesting that the renin gene duplication event was followed by deletion of the *Ren–2^d* gene in low producer mice. Gene duplication was calculated to have occured 13 million years ago followed by the deletion event approximately 6.7 million years ago, and is consistent with the data reported by Dickinson *et al.* (1984).

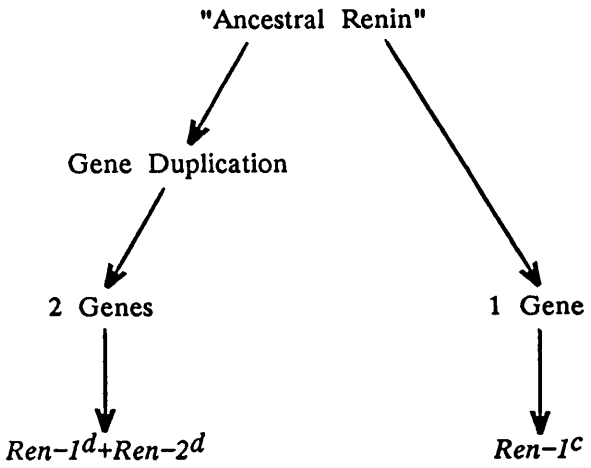
Finally, by using pulse field gel–electrophoresis to study the physical arrangement of the two renin genes in DBA/2 mice, Abel and Gross (1988) were able to construct a restriction site map of the *Ren* loci spanning roughly 120Kb. The results indicated that the genes are transcribed in the same relative direction, that *Ren–2^d* lies upstream relative to *Ren–1^d*, and that the respective coding sequences are separated by approximately 20Kb.

1.4.2 Hormonal Control

Both androgen (testosterone) and thyroid hormone (thyroxine) have been shown to regulate SMG renin gene–expression in both high and low producer mice. Renal renin on the other hand is not affected, suggesting possible tissue–specific control.

In the SMG testosterone has a major regulating influence and is responsible for the high renin synthesis in the male SMG that occurs after puberty (Oliver and Gross, 1967). Furthermore testosterone, when given to female mice, can increase renin mRNA levels to male values (Catanzaro *et al.*, 1985). However Wilson *et al.* (1981) showed that the difference in renin activity between female mice of high and low producer strains was not due to enhanced sensitivity of high producers to the small amounts of endogenous androgen present in the normal female mouse, since removal of all sources of androgen formation by ovarectomy and adrenalectomy did not prevent the normal increase in SMG renin seen after four weeks of age in female high producer mice. Catanzaro *et al.* (1985) reported that the time course of the effect of testosterone was similar to that generally observed for the action of steroid hormones and suggested that the

MODEL I



MODEL II

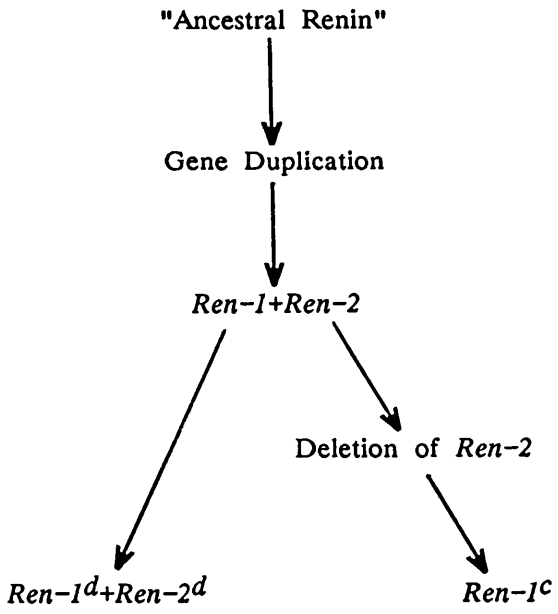


Figure 1.5

Models for the evolution of mouse renin genes. (See text for details).

hormone was acting directly on its target gene rather than a gene coding for other proteins which in turn regulate renin genes.

Wilson *et al.* (1982) injected hypophysectomised or intact female mice with dihydroxytestosterone (DHT) and thyroxine. Thyroxine and DHT administered together produced renin specific activity levels in hypophysectomised mice identical to those of intact mice treated with DHT or DHT plus thyroxine. Furthermore thyroxine was found to act independently of androgen, inducing SMG renin in *Tfm/Y* mice that are resistant to DHT because they lack functional androgen receptors. Female mice treated with propylthiouracil to ablate chemical thyroid hormone production showed a substantial decrease in mRNA and renin, suggesting the importance of thyroid hormone in regulation of renin synthesis in the female SMG. The response resembled that observed when testosterone was used where direct action was suggested. In male mice ablation of the thyroid or administration of thyroxine had little effect, showing the overiding importance of testosterone in males.

Hormonal control of SMG renin appears complex and it is of current interest to determine whether the increase in renin mRNA and renin in response to either testosterone or thyroxine is a result of enhanced transcription, stabilisation of nuclear precursor mRNA or other causes.

1.4.3 Tissue-Specific Control

The most well characterised tissue-specific control of renin has been in the SMG of the mouse. Panthier *et al.* (1982b) demonstrated that induced levels of SMG renin in high producer mice were 100x higher than in low producer mice. Similar results were also observed by Wilson *et al.* (1982) who found that maximum SMG renin activity in low producer mice given thyroxine and/or DHT were 100x lower than in identically treated high producer mice. Field *et al.* (1984) observed that on a per cell basis, the accumulation of *Ren-1*^{d} transcripts in the kidney and *Ren-2*^{d} transcripts in the SMG were roughly equivalent. Field and Gross (1985) found that *Ren-1*^{d} and *Ren-2*^{d} mRNAs accumulated in the kidney of two gene strains to approximately equal

Table 1.2 Differential expression on a per cell basis of *Ren-1*^{c}, *Ren-1*^{d}, and *Ren-2*^{d} in mouse SMG and kidney (see Section 1.4.3. for references).

		LOW PRODUCER MICE	HIGH PRODUCER MICE	
		Ren-1^{c}	*Ren-1*^{d}	*Ren-2*^{d}
SMG	♂	10	10	10^{3}
	♀	1	1	200
Kidney	♂	10^{4}	10^{4}	10^{4}
	♀	10^{4}	10^{4}	10^{4}

levels. Tronik *et al.* (1987) reported that a *Ren-2^d* transgene present in a *Ren-1^c* background was expressed in transgenic mice identically to that observed in wild-type animals in which *Ren-1^d* and *Ren-2^d* are closely linked on chromosome 1. In the kidney *Ren-1^c* and *Ren-2^d* mRNAs were present at a comparable level whereas in the SMG *Ren-2^d* mRNA was at least 100-fold more abundent than *Ren-1^c* mRNA. Correct expression of the *Ren-2^d* transgene in a *Ren-1^c* background suggests that the enhanced expression of the former gene is due to the presence of *cis*-acting regulatory elements in the *Ren-2^d* transgene. Furthermore, using RNase-protection analysis Miller *et al.* (1989) found equal levels of *Ren-1^d* and *Ren-2^d* mRNAs in kidney and approximately 200-fold excess of *Ren-2^d* over *Ren-1^d* mRNAs in SMG. These workers also observed that *Ren-1^c* was expressed at slightly higher levels than *Ren-1^d* in SMG. The general patterns of *Ren-1^c*, *Ren-1^d* and *Ren-2^d* gene-expression in SMG and kidney of high and low producer mice from these and other observations are summarised in Table 1.2.

Mouse renin mRNAs have also been detected in other extrarenal sites including liver, testis, adrenal, heart, brain, and pituitary (Dzau *et al.*, 1984; Dzau *et al.*, 1987; Dzau and Re, 1987; and Lillycrop, unpublished data). Furthermore Miller *et al.* (1989) demonstrated transcriptional activity from *Ren-1^d* in testis, liver, brain, and heart in high producer (DBA/2) mice whereas *Ren-2^d* was clearly expressed only in testis. Similar analysis of *Ren-1^c* transcripts in low producer (C57BL/6) mice showed expression in testis, brain and heart, but unlike *Ren-1^d*, *Ren-1^c* transcripts were not detected in liver.

Finally tissue-specific renin gene-expression has also been demonstrated in the rat, where renin mRNA has been detected in varying amounts in kidney, liver, testis, brain, adrenal, aorta, heart, lung, and spleen (Dzau *et al.*, 1987; Ariel *et al.*, 1988; and Samani *et al.*, 1988).

1.5 Renin Gene Structure

There has been extensive structural analysis of the mouse renin genes in this and other laboratories. A structural comparison of the two genes in

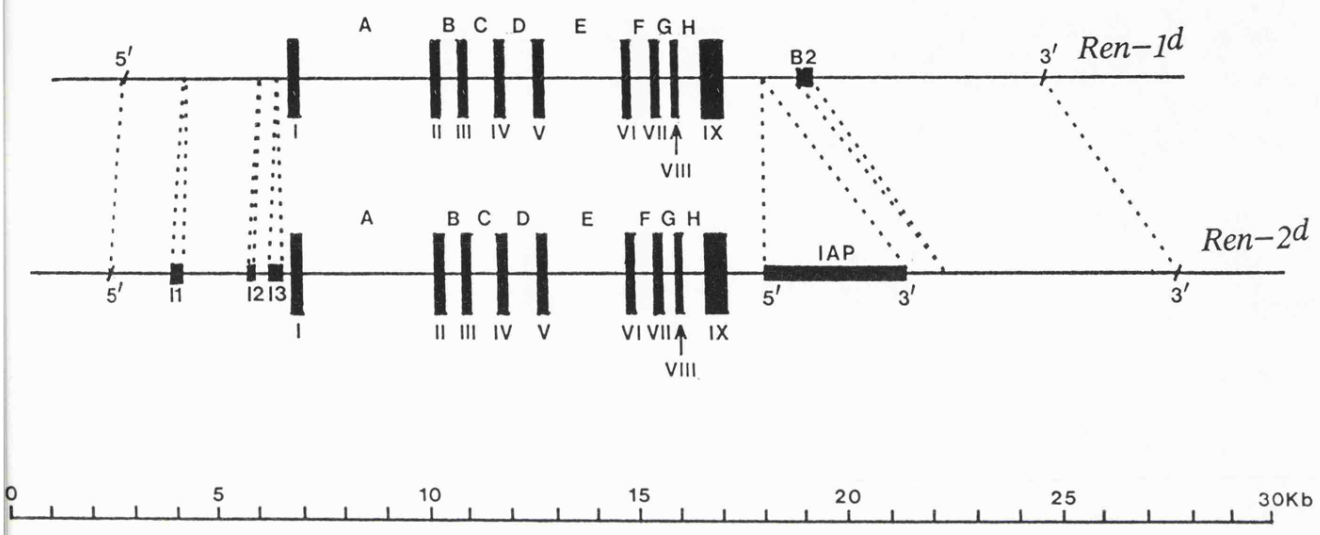


Figure 1.6

Comparison of mouse *Ren–1*^{d} and *Ren–2*^{d} gene structure. Exons are labelled I–IX and introns are labelled A–H. B2, *Ren–1*^{d} B2 *Alu* equivalent mobile element; I1 and I2, 329bp and 158bp insertions respectively; I3, *Ren–2*^{d} B2 *Alu* equivalent mobile element; IAP, intracisternal A particle (3Kb element). The limits of homology are defined by the slash symbols (/).

high producer strains, by restriction endonuclease mapping and DNA sequence analysis, reveals extensive homology between *Ren–1^d* and *Ren–2^d* (Figure 1.6). Each gene spans 9.6Kb and contains nine exons and eight introns of highly variable size. The first exon, encoding the signal peptide of preprorenin, is separated from the eight following exons by a 3Kb intron (intron A). These eight exons are organised into two clusters of four separated by a 2Kb intron (intron E). DNA stretches encoding the aspartyl residues of the active site are located at homologous positions in both clusters. The close homology suggests that the two genes have arisen by a duplication and fusion of an ancestral gene containing five exons (Holm *et al.*, 1984). Comparison of the patterns of restriction endonuclease sites together with heteroduplex analysis has revealed one insertion in the *Ren–1^d* gene (with respect to the *Ren–2^d* gene), and four insertions in the *Ren–2^d* gene (with respect to the *Ren–1^d* gene).

1.5.1 *Ren–1^d* Insertion

The *Ren–1^d* insertion is situated 3' to exon IX (Figure 1.6) and DNA sequence comparisons have shown that it is 228bp long and has 83% homology to the consensus sequence of mouse B2 (*Alu* equivalent) mobile elements (Burt *et al.*, in preparation; and Krayev *et al.*, 1982). B2 elements belong to a major family (including B1) of short interspersed repeats which are present in more than 10^5 copies scattered throughout the mouse genome. B2 contains regions of homology to the RNA polymerase III split promoter and to 4.5s snRNA I, and they contain regions which resemble junctions between exons and introns. B1 and B2 are thought to represent a class of transposon–like elements in eukaryotic genomes that may be involved in genome reorganisation and DNA replication. It has also been suggested that these elements are involved in the control of gene–expression at the level of RNA splicing (Krayev *et al.*, 1982) since they have homology to snRNAs involved in this process.

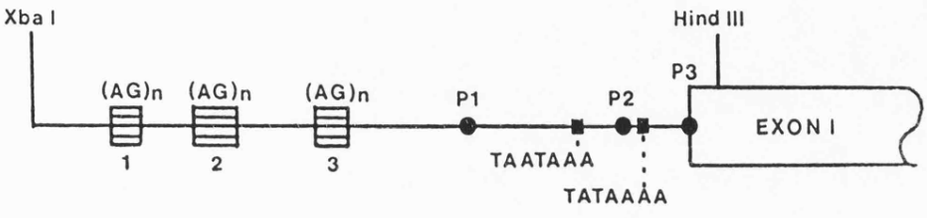
1.5.2 *Ren–2^d* Insertions

The four insertions within the *Ren–2^d* gene include a "3Kb element" 3' to exon IX and three insertions 5' to exon I referred to as I1, I2

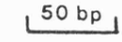
and I3 (Figure 1.6). I3 (214bp) has been identified as a mouse B2 (*Alu* equivalent) mobile element (Field *et al.*, 1984). I1 and I2 (329bp and 158bp respectively) lack homology with any known insertion sequence, and Burt *et al.* (in preparation) proposed that the difference between *Ren-2^d* and *Ren-1^d* may be due to deletion of sequence from the *Ren-1^d* gene. The 3Kb element has been found to be highly homologous to sequences in intracisternal A particle (IAP) genomes (Burt *et al.*, 1984). These are non-infectious retrovirus-like structures found generally in early mouse embryos and mouse tumours. It has been demonstrated that the integration/transposition event of these genomes can alter the expression of cellular genes. For example the murine leukaemia viruses (MuLVs) have been associated with coat colour (Copeland *et al.*, 1983). However Field *et al.* (1984) thought it unlikely that this sequence was of importance with respect to the differential expression of *Ren-2^d*, as a related species *Mus hortulanus* exhibiting evidence of renin gene duplication and similar tissue-specific expression, lacks such an insertion (Dickinson *et al.*, 1984).

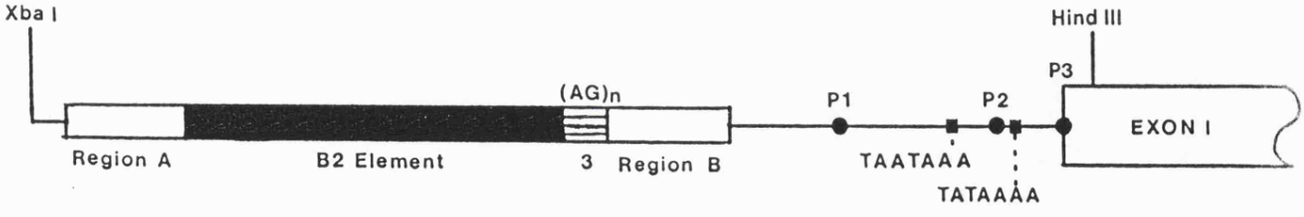
1.5.3 Detailed Comparison of the *Ren-1^d* and *Ren-2^d* 5' Flanking Sequences

A schematic representation of the 5' promoter region of *Ren-1^d* and *Ren-2^d* is given in Figure 1.7 and the DNA sequence is given in Figure 1.8. Using nuclease S1 mapping and primer-extension analysis, Field *et al.* (1984) identified three transcription start-sites designated P1, P2 and P3 at positions -125, -37 and +1 respectively. Utilisation of these sites shows tissue-specificity: in the kidney only P3 is used whereas in the SMG all three sites are functional, although P3 is the major transcription start-site. Since P3 is the major transcription start-site for *Ren-1^c*, *Ren-1^d* and *Ren-2^d* in SMG and kidney, the tissue-specific difference in renin mRNA levels is probably due to relative utilisation of this transcription initiation site, and is not due to utilisation of different promoters (Field *et al.*, 1984). Transcription initiation from P2 and P3 occurs 25 to 26bp downstream of their respective TATA boxes, suggesting that these TATA boxes are functional *in-vivo*. A TATA box consensus has not been identified for P1. Furthermore unlike other eukaryotic promoters (Benoist *et al.*,



Ren–1^d





Ren–2^d

Figure 1.7

Detailed comparison of *Ren–1^d* and *Ren–2^d* promoter structure. P1, P2, P3, start–sites for mRNA transcription; TATAAAA, P3 TATA box; TAATAAA, P2 TATA box; (AG)n, polypurine tracts; Region A and Region B, rearranged sequence flanking the B2 *Alu* equivalent mobile element (see text).

```
          -700        -690        -680        -670        -660
Ren-1d TCTAGAGTCATTGGGCTCAGcCACCCTTCCCACACCCCCATGCCTGCCAC
Ren-2d TCTAGAGTCATTGGGCTCAG.CACCCTTCCCACACCCCCATGCCTGCCAC
         -1050       -1040       -1030       -1020       -1010

          -650        -640        -630        -620
Ren-1d CACTCTGCTCTGCGACCAG.GTtcTGCTTATCCTATACCT.....
Ren-2d CACTCTGCTCTGCGACCAGtGTctTGCTTATCCTATACCTcacctaagct
          -1000       -990        -980        -970        -960


Ren-1d .................................................
Ren-2d acatgccataaatcaatgcttgttattcaaggcatcatctcctttggggg
          -950        -940        -930        -920        -910


Ren-1d .................................................
Ren-2d gcacttctcaaaaagtcctctcctgtccctagatatctgagactcctcag
          -900        -890        -880        -870        -860

                                                      -610
Ren-1d ..........................................ACcTaAcTTgg
Ren-2d tggccttccatttagaagggtgttgtctctgaagtaaaaACtTcAgTTcc
          -850        -840        -830        -820        -810

          -600        -590        -580        -570        -560
Ren-1d TCTCACAGGCTAgaATTTATCAGgaCTGcCCTGCCATGGGCCTTGTTGGg
Ren-2d TCTCACAGGCTAagATTTATCAGagCTG.CCTGCCATGGGCCTTGTTGG.
          -800        -790        -780        -770        -760

          -550        -540        -530        -520        -510
Ren-1d CAcCTGGCaTGGGgaAATAAaGGaGGTGGgCGAGAcCTGTGTCtCacCAG
Ren-2d CA.CTGGCgTGGG..AATAA.GGgGGTGG.CGAGA.CTGTGTC.CcgCAG
             -750        -740        -730        -720

          -500        -490        -480        -470        -460
Ren-1d AGCTCAGAgtGACTGGatgGGCAGACAGCAGGgGAAGgCACTGGGTGGTC
Ren-2d AGCTCAGA..GACTGGgcaGGCAGACAGCAGGaGAAGaCACTGGGTGGTC
          -710        -700        -690        -680        -670

          -450        -440        -430        -420        -410
Ren-1d TGGCAGCTGGAAACGCTGGGAGGCCTTCTTGgGGGAaATTAGATGAaGCT
Ren-2d TGGCAGCTGGAAACGCTGGGAGGCCTTCTTGcGGGAgATTAGATGAgGCT
          -660        -650        -640        -630        -620
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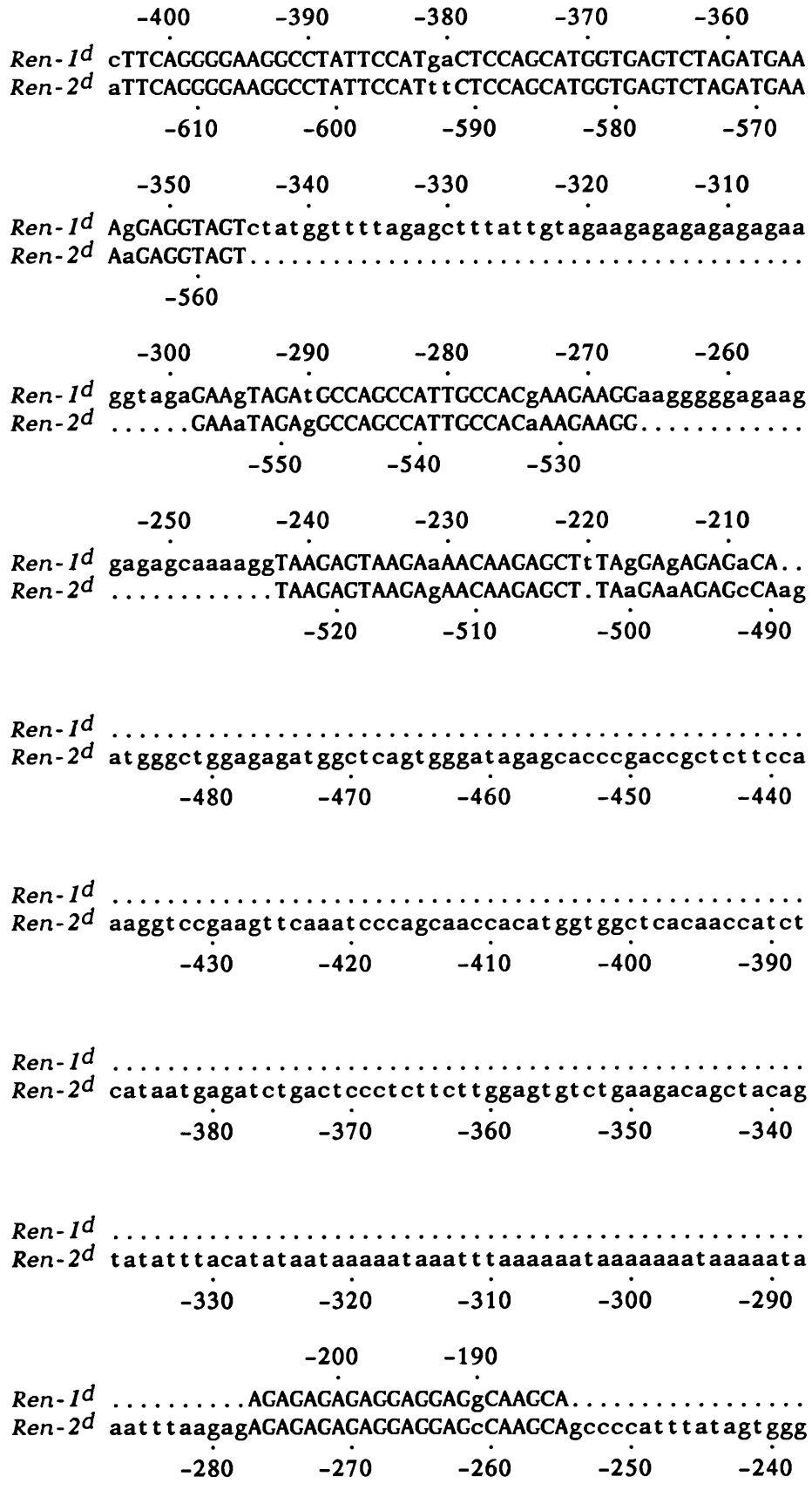

-400 -390 -380 -370 -360
Ren-1d cTTCAGGGGAAGGCCTATTCCATgaCTCCAGCATGGTGAGTCTAGATGAA
Ren-2d aTTCAGGGGAAGGCCTATTCCATttCTCCAGCATGGTGAGTCTAGATGAA
-610 -600 -590 -580 -570
-350 -340 -330 -320 -310
Ren-1d AgGAGGTAGTctatggttttagagctttattgtagaagagagagagagaa
Ren-2d AaGAGGTAGT.....
-560
-300 -290 -280 -270 -260
Ren-1d ggtagaGAAgTAGAtGCCAGCCATTGCCACgAAGAAGGaaggggggagaag
Ren-2d ......GAAaTAGAgGCCAGCCATTGCCACaAAGAAGG.....
-550 -540 -530
-250 -240 -230 -220 -210
Ren-1d gagagcaaaaggTAAGAGTAAGAaAACAAGAGCTtTAgGAgAGAGaCA..
Ren-2d .....TAAGAGTAAGAgAACAAGAGCT.TAaGAaAGAGcCAag
-520 -510 -500 -490
Ren-1d .....
Ren-2d atgggctggagagatggctcagtgggatagagcacccgaccgctcttcca
-480 -470 -460 -450 -440
Ren-1d .....
Ren-2d aaggtccgaagttcaaatcccagcaaccacatggtggctcacaaccatct
-430 -420 -410 -400 -390
Ren-1d .....
Ren-2d cataatgagatctgactccctcttcttggagtgtctgaagacagctacag
-380 -370 -360 -350 -340
Ren-1d .....
Ren-2d tatatttacatataataaaaataaatttaaaaaataaaaaaataaaaata
-330 -320 -310 -300 -290
-200 -190
Ren-1d .....AGAGAGAGAGGAGGAGgCAAGCA.....
Ren-2d aatttaagagAGAGAGAGAGGAGGAGcCAAGCAgccccatttatagtggg
-280 -270 -260 -250 -240

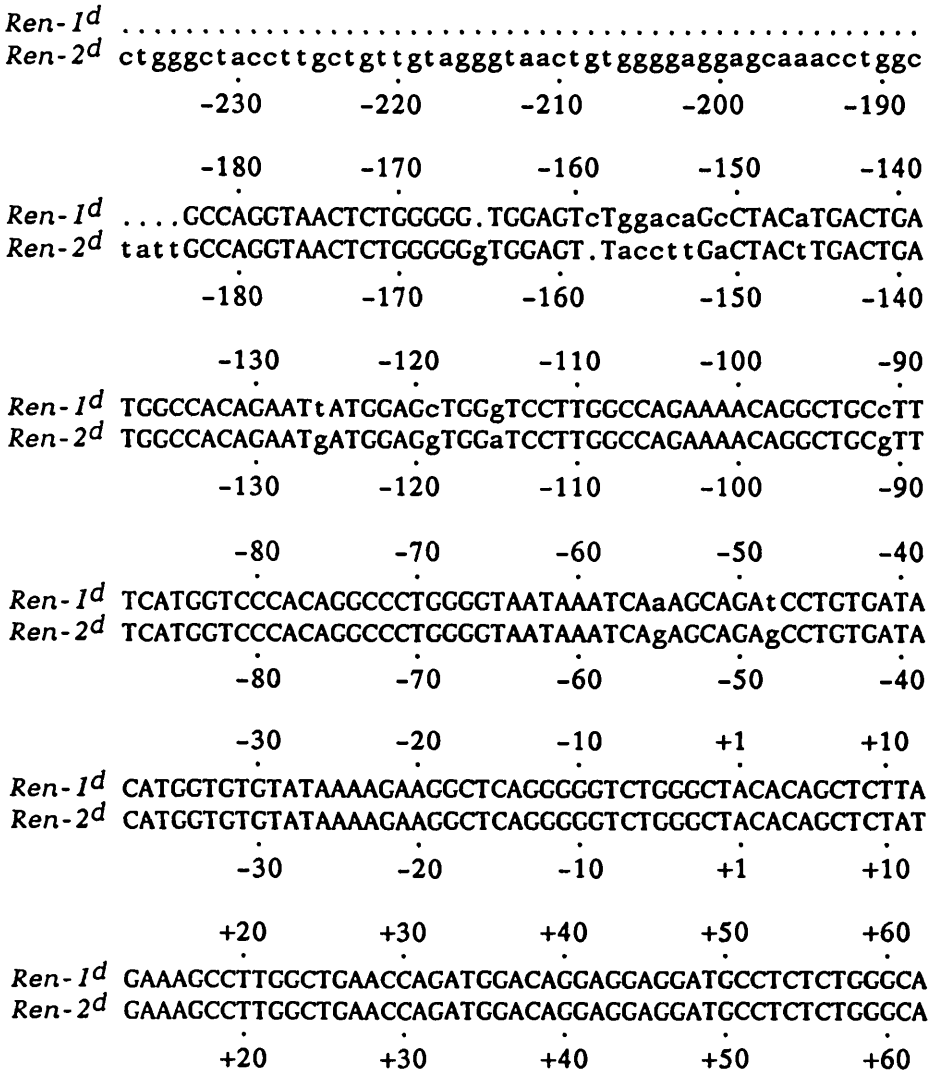


Figure 1.8

Comparison of the nucleotide sequence between *Ren-1*^{d} and *Ren-2*^{d} from −704 and −1054 respectively to +62. Nucleotide +1 corresponds to the P3 transcription start-site.

1980; Bienz and Pelham, 1986; and Cereghini *et al.*, 1987) a CCAAT box, usually found upstream of TATA boxes, is absent. In contrast, in the human renin gene a CCAAT box is present at position -92 (Soubrier *et al.*, 1986).

The greatest perturbation between *Ren-1*^{d} and *Ren-2*^{d} promoter regions is due to an insertion of a B2 mobile element in *Ren-2*^{d}. Furthermore, the insertion event appears to have been responsible for a number of rearrangements either side of the element. These include a 5' region, in which stretches of homology with *Ren-1*^{d} are interspersed with novel sequences (Region A in Figure 1.7), and a 3' region, which has no homology with *Ren-1*^{d} (Region B). Polypurine tracts that are predominantly (AG)n are also present within *Ren-1*^{d} at positions -321 to -303, -274 to -250, and -207 to -190. In the *Ren-2*^{d} gene the two most 5' polypurine tracts are absent, probably as a result of the B2 insertion event, and the third tract which has been retained is now located further upstream with respect to the P3 start-site (Field *et al.*, 1984). The functional significance of these tracts is still unknown.

Soubrier *et al.* (1986) compared the promoter region (500bp upstream from the major transcription start-site) of the human renin gene with that of the mouse *Ren-1*^{d} and *Ren-2*^{d} genes, but suprisingly they were unable to find any regions of extensive homology. However, four discrete regions did present a high degree of homology: these include the P2 and P3 TATA boxes of the mouse renin genes, a 12bp (GGGTCTGGGCTA) sequence at nucleotide -361 in the human and -7 in the mouse renin genes, and a 10bp (AGCTGGAAAC) sequence at nucleotide -346 in the human and -446 and -667 in the *Ren-1*^{d} and *Ren-2*^{d} mouse renin genes respectively. The reason for the different locations in the mouse renin genes is thought to be due to the insertion of the B2 element in *Ren-2*^{d}. This decanucleotide encompasses the sequence TGGAAA which is the consensus core sequence found in viral enhancer elements (Weiher *et al.*, 1983), but again its functional significance with respect to renin is unknown. The apparent lack of homology between the human and mouse renin gene promoters was later shown to be due to a large insertion of a transposable-like element in mouse *Ren-1*^{d} and *Ren-2*^{d} genes but

absent from the human renin gene (Tronik *et al.*, 1988) (Figure 1.9). Since the insertion is present only in those genes which are expressed in the SMG, these workers proposed that the elements necessary for SMG expression are included within this element. Finally when Williams *et al.* (1985) compared the 5' regions of five androgen responsive genes (rat seminal vesicle F and S genes, and rat prostate genes C1, C2 and C3) with both mouse renin genes, no obvious sequence motifs relating to androgen responsive elements (AREs) were identified. AREs may display tissue-specific and/or species-specific complexity, or secondary structure rather than simple sequence motifs may be important. Nevertheless it seems likely that other approaches will be needed to define the androgen control locus.

1.6 Experimental Approach

The observation that a single gene is under different control mechanisms in different tissues is not an exceptional situation (Schibler *et al.*, 1980; and Young *et al.*, 1981). Catanzaro *et al.* (1985) examined the transcriptional activity of the mouse renin genes in the kidney and SMG, by measurement of renin mRNA in comparison with renin activity. They found that under conditions known to alter renin activity there was a proportional change, irrespective of the stimulus, between the concentration of renin mRNA and renin activity. Karen and Morris (1986) also demonstrated a proportional increase in renin mRNA and renin in the SMG of the female mouse in response to administration of thyroid hormone. Since the concentration of mRNA is primarily determined by the transcriptional activity of its gene rather than the rate of degradation, transport or nuclear processing (Derman *et al.*, 1981), it seems more than likely that the renin genes are controlled principally at the level of transcription. One may suggest two general concepts for the differential expression of the renin genes. *Ren-1*^{c}, *Ren-1*^{d} and *Ren-2*^{d} may lie within different domains of chromatin and the tissue-specific expression of the genes is due to their availability to the transcriptional machinery of a particular cell-type. However this seems unlikely, since recent experiments with transgenic mice carrying an exogenous *Ren-2*^{d} gene, showed that the transgene was expressed normally although it was randomly integrated within the recipient genome (Tronik *et al.*, 1987). On the other hand the interaction of tissue-specific cellular factors with

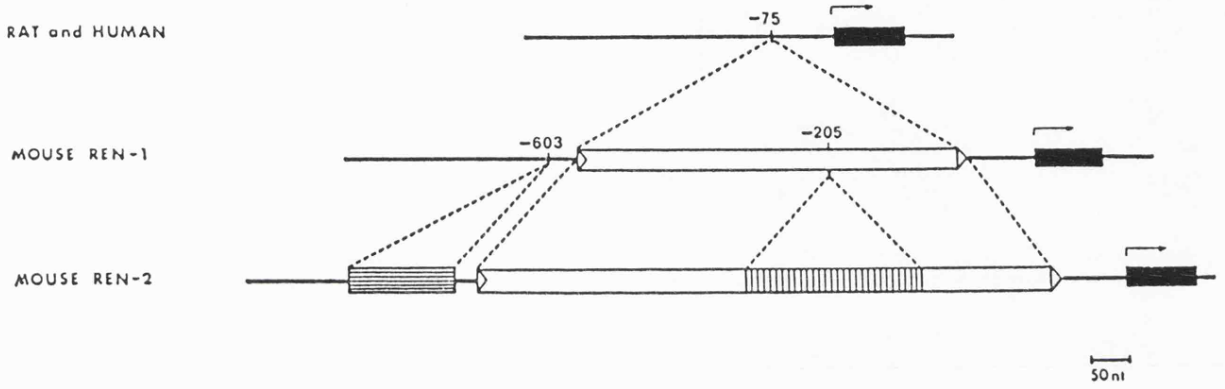


Figure 1.9

Comparative organisation of rat, human and mouse renin promoters. The blackened boxes represent exon I with the arrows indicating the direction of transcription. The large insertion in the mouse genes is represented by the open boxes with triangles for the direct repeats at its borders. Vertically striped box, B2 *Alu* equivalent mobile element; horizontally striped box, 158bp insertion (I2).

sequences lying within or near the genes, may be responsible for their differential expression. Three major approaches one may consider when investigating those regions involved in gene control are transgenic mouse technology, transient expression assays in various cell-lines and *in-vitro* transcription assays.

With transgenic mouse technology the transgene is integrated into the host's genome so that every cell contains one or more copies of the foreign DNA. Although this assay system would be ideal for studying tissue-specific gene-expression, the method can be cumbersome and time-consuming when large numbers of constructs need to be screened. Furthermore the extent of expression of the transgene is dependent on the site of integration into the host's genome. Dominant control regions (DCR), characterised by DNase I hypersensitive sites, conferring erythroid-specific and T cell-specific integration-independent and copynumber-dependent expression of a β-globin mini-gene (Grosveld *et al.* 1987) and a human CD2 mini-gene (Greaves *et al.* 1989) respectively have been reported. Whether DCRs are associated with other genes has yet to be determined but such regions will prove useful when studying tissue-specific gene-expression in transgenic mice.

Alternatively, transient expression assays of fused genes in cultured cells is another useful method used to dissect gene promoters. The best cell-line for such a primary screening would be one which expressed detectable renin mRNA and which could be induced to secrete the protein product; ideally this would be a permanent cell-line to facilitate comparison of different experimental results. However the lack of such a cell-line has been a continuing problem. The juxtaglomerular cells of the kidney which produce renin are rare and are not as fast growing as fibroblasts which eventually take-over a primary culture. Methods have been published which allow for enrichment of juxtaglomerular cells (Kurtz *et al.*, 1986) but the problem of successfully immortalising these cells remains unsolved. Recently B. Leckie (MRC Blood Pressure Unit, Western General Hospital, Glasgow) kindly donated a culture of cells derived from a Wilm's tumour, a rare malignant tumour of the kidney. Although this particular cell-line secreted large amounts of human renin into the tissue culture fluid, the cells were too slow growing to be of any use. After five weeks of culture they had failed to achieve more

than 5–10% confluency of a 25cm² flask. With a non renin-expressing cell-line, although it would still be possible to study general regulatory elements associated with the promoter, tissue-specific gene-expression could not be investigated.

The third approach is to perform the initial screening for promoter elements in an *in-vitro* assay system which, although it may only approximate the conditions *in-vivo*, might provide clues about those regions of the renin gene worth investigating by full transgenic analysis. Also *in-vitro* transcription systems are relatively quick to set up and have yielded much information about promoter elements and their associated proteins (see Chapter 3). Gorski *et al.* (1986) and more recently Monaci *et al.* (1988) reported tissue-specific *in-vitro* transcription, from the mouse albumin and human α1-antitrypsin promoters, respectively, using nuclear-protein extracts prepared from various rat tissues. For these reasons an entirely *in-vitro* approach, involving the preparation of nuclear-protein extracts from various mouse tissues, was used for investigating renin gene-expression in the mouse. Information gained using this approach would then form the basis for further investigations in an *in-vivo* assay system.

CHAPTER 2

MATERIALS AND METHODS

2.1 General Buffers and Stock Solutions

2.1.1 LB (Luria Bertani) Agar

Difco Bacto tryptone	10g/litre
Difco Bacto yeast extract	5g/litre
NaCl	5g/litre
Difco Bacto agar	15g/litre

pH was adjusted to 7.5 with NaOH before the addition of agar.

2.1.2 LB Broth

Difco Bacto tryptone	10g/litre
Difco Bacto yeast extract	5g/litre
NaCl	5g/litre

pH was adjusted to 7.5 with NaOH

2.1.3 2xTY Broth

Difco Bacto tryptone	16g/litre
Difco Bacto yeast extract	10g/litre
NaCl	5g/litre

pH unaltered

2.1.4 H Plate Agar

Difco Bacto tryptone	10g/litre
NaCl	8g/litre
Difco Bacto agar	12g/litre

pH unaltered

2.1.5 H Top Agar

Difco Bacto tryptone	1g/litre
NaCl	0.8g/litre
Difco Bacto agar	8g/litre
pH unaltered	

2.1.6 TE Buffer [pH 8.0]

Tris.HCl [pH 8.0]	10mM
EDTA [pH 8.0]	1mM

2.1.7 Tris.acetate Electrophoresis (TAE) Buffer

Tris.acetate	40mM
EDTA [pH 8.0]	1mM

10x Stock Solution:

Tris.base	48.4g/litre
Glacial acetic acid	11.42ml/litre
0.5M EDTA [pH 8.0]	20ml/litre

2.1.8 Tris.borate Electrophoresis (TBE) Buffer

Tris.borate	89mM
Boric acid	89mM
EDTA [pH 8.0]	2mM

10x Stock Solution:

Tris.base	108g/litre
Boric acid	55g/litre
0.5M EDTA [pH 8.0]	40ml/litre

2.1.9 20x SSC

NaCl	3M
Trisodium citrate	0.3M

2.1.10 100x Denhardt's Solution

BSA	2% (w/v)
Ficoll type 400	2% (w/v)
Polyvinyl pyrollidone	2% (w/v)

2.1.11 Ampicillin

A 25mg/ml solution of the sodium salt of ampicillin in distilled deionised H_2O was prepared, sterilised by filtration and stored in aliquots at -20°C

2.1.12 Tetracycline

A 12.5mg/ml solution of tetracycline hydrochloride in 50% (v/v) ethanol was prepared, sterilised by filtration and stored in aliquots at -20°C in the dark.

2.1.13 OLB Buffer

Solution O:

Tris.HCl [pH 8.0]	1.25M
$MgCl_2$	0.125M

Solution A:

Solution O	1000μl
2-mercaptoethanol	18μl
0.1M dATP	5μl
0.1M dTTP	5μl
0.1M dGTP	5μl

Solution B:

2M HEPES [pH 6.6] (titrated with 4M NaOH)

Solution C:

Prepare a solution of 90 A_{260} units/ml hexadeoxynucleotides (Pharmacia).

OLB Buffer:

Mix solutions A, B and C in the ratio 10:25:15 (50μl). This is sufficient for 10 labelling reactions.

2.2 Oligonucleotide Sequences

	5'	3'
ADS1	GCTACACAACTCTTAAAAAGCTT	
ADS19	GGATCTGCTTTGATTTATTACTGTGGGACCATGAAAGGCA	
ADS21	TGTGGGACCATGAAAGGCAGCTGGCCAAGGACCCAGCTCC	
ADS32	GAATTCCCAGAAAACAGGCTGCCTTT	
ADS33	GAATTCCATGGTCCCACAGGCCCTGG	
ADS34	GAATTCGGTAATAAATCAAAGCAGAT	
ADS35	GAATTCCCTGTGATACATGGTGTGTA	
ADS36	GAATTCTAAAAGAAGGCTCAGGGGGT	
ADS37	AAGCTTGGGCTGCAGGTCGACTCTAG	

2.3 Bacterial Strains

E. coli JM101 : Δ(*lacpro*),*thi*,*supE*,F'*traD36*,*proAB*,*lacIq*,*lacZ*Δ*M15*.

E. coli HB101 : F$^-$,*hsdS20*(r_B^-,m_B^-),*recA13*,*ara*$-$*14*,*proA2lacY1*,*galK2*, *rpsL20*(Sm^r),*xyl*$-$*5*,*mtl*$-$*1*,*supE44*,λ^-.

E. coli JM83 : Δ(*lacpro*),*ara*,*strA*,*thi*,(φ*80lacZ*Δ*M15*).

E. coli TG1 : *K12*,Δ(*lacpro*),*supE*,*thi*,*hsdD5*/*F'traD36*,*proAB*, *lacIq*, *lacZ*Δ*M15*.

E. coli RZ1032: *HfrKL16 PO145[lysA(61–62)],dut1,ung,thi1,relA1, Zbd–279::Tn10,supE44.*

2.4 Maintenance of Bacterial Stocks

Bacterial strains were stored at -70°C as frozen cultures, which were prepared by mixing 0.85ml of overnight culture with 0.15ml sterile glycerol.

2.5 Siliconising Glassware

Glassware was siliconised by coating it directly with a 2% (v/v) solution of diethyldichlorosilane in 1,1,1-trichloroethane, followed by baking at 180°C.

2.6 Preparation of Phenol

8-Hydroxyquinoline was added to commercially distilled liquified phenol to a final concentration of 0.1%. The phenol was then extracted several times with an equal volume of 1M Tris.HCl [pH 8.0], followed by 0.1M Tris.HCl [pH 8.0] and 0.2% (v/v) 2-mercaptoethanol, until the pH of the aqueous phase was >7.6. The phenol solution was stored at -20°C under equilibration buffer.

2.7 Preparation of Chloroform:Isoamyl Alcohol

A mixture of chloroform and isoamyl alcohol (24:1 (v/v)) was used to remove proteins from preparations of nucleic acids. The chloroform denatures proteins, while isoamyl alcohol reduces foaming during the extraction and facilitates the separation of the aqueous and organic phases. The mixture was stored at room temperature.

It should be noted that hereafter, phenol/chloroform refers to a mixture of one volume of phenol to one volume of chloroform:isoamyl alcohol. In other words a mixture of phenol,chloroform and isoamyl alcohol in the ratio 25:24:1 (v/v) respectively. Furthermore, chloroform refers to a mixture of chloroform and isoamyl alcohol in the ratio 24:1 (v/v) respectively.

2.8 Preparation of Dialysis Tubing

Tubing was cut into convenient lengths (10–20cm) and boiled for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1mM EDTA [pH 8.0]. After rinsing thoroughly in distilled deionised H_2O, the tubing was boiled for a further 10 minutes in 1mM EDTA [pH 8.0] and was stored in the same solution at 4°C upon cooling, making sure that the tubing was always submerged. Before use the tubing was rinsed thoroughly in distilled deionised H_2O.

2.9 Preparation of Sheared Salmon Sperm DNA

A 10mg/ml stock solution of salmon sperm DNA, in distilled deionised H_2O, was prepared and sheared by passing through a narrow bore hypodermic syringe needle (size 25Gx⅝) at least 20 times. Aliquots were stored at −20°C.

2.10 Restriction Endonuclease Digestions

Restriction endonuclease digestions were carried out according to the manufacturers' instructions, in a reaction volume made to 10–200μl with distilled deionised H_2O.

2.11 Ligation Reactions

Ligation reactions contained 50mM Tris.HCl [pH 7.4], 10mM $MgCl_2$, 10mM DTT, 1mM spermidine, 1mM ATP, 0.1mg/ml BSA (DNase free), 100ng of DNA (with an insert to vector ratio between 2:1 to 5:1 respectively) and 2 units of T4 DNA ligase, made to 20μl with distilled deionised H_2O. After vortexing briefly, the mixture was incubated at 4°C for 12–16 hours and was then used to transform a suitable host strain.

2.12 Phosphatasing DNA

To prevent vector DNA molecules from religating without an insert fragment, the 5' phosphate groups were removed. Each reaction contained 50mM Tris.HCl [pH 9.0], 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine, 10μg of DNA and 10 units of calf intestinal phosphatase (CIP), made to

50μl with distilled deionised H_2O. After vortexing, the mixture was incubated at 37°C for 15 minutes and then at 56°C for 15 minutes. A further 10 units of CIP were added and the incubations at both temperatures were repeated. The phosphatased DNA was purified by direct extraction.

2.13 Blunt-Ending of DNA Molecules

Blunt-ending of DNA molecules was achieved by either filling-in recessed 3' ends or trimming back projecting 3' ends.

2.13.1 With Nuclease S1

Nuclease S1 degrades single-stranded DNA to yield 5' phosphoryl mono- or oligonucleotides. Double-stranded DNA, double-stranded RNA and DNA·RNA hybrids are relatively resistant to the enzyme. Each reaction contained 50mM sodium acetate [pH 4.5], 0.2M NaCl, 1mM $ZnSO_4$, 0.5% (v/v) glycerol, 10-50μg of DNA and 5 units of nuclease S1, made to 200μl with distilled deionised H_2O. After vortexing the mixture was incubated at 4°C for 30 minutes and the DNA was then purified by direct extraction.

2.13.2 With Klenow

Filling-in recessed 3' ends of double-stranded DNA was achieved by utilising the 5' to 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. Each reaction contained 50mM Tris.HCl [pH 7.2], 10mM $MgSO_4$, 0.1mM DTT, 50μg/ml BSA (DNase free), 100μM dATP, 100μM dTTP, 100μM dGTP, 100μM dCTP, 10-30μg of DNA and 30 units of Klenow made, to 20μl with distilled deionised H_2O. After vortexing the mixture was incubated at room temperature for 60 minutes and the DNA was then purified by direct extraction.

2.13.3 With T4 DNA Polymerase

Like the Klenow fragment, T4 DNA polymerase possesses a 5' to 3' polymerase activity and a 3' to 5' exonuclease activity. However the exonuclease activity of T4 DNA polymerase is 200x more active than

that for Klenow. Because the polymerase activity is more active than the exonuclease activity, an exchange reaction was exploited to generate blunt-ends. If only one dNTP is present in the reaction mixture, the 3' to 5' exonuclease activity will degrade DNA from the 3'-OH end, until a base is exposed that is complementary to the dNTP. At this position an exchange reaction occurs.

Each reaction contained 33mM Tris.acetate [pH7.9], 66mM potassium acetate, 1mM magnesium acetate, 0.5mM DTT, 0.1mg/ml BSA (DNase free), 100μM appropriate dNTP, 10-50μg of DNA and 50 units of T4 DNA polymerase, made to 100μl with distilled deionised H_2O. After vortexing, the mixture was incubated at 37°C for 60 minutes and the DNA was purified by direct extraction.

2.14 Radio-Labelling of DNA Molecules

2.14.1 End-Labelling

Labelling double-stranded DNA molecules by filling-in recessed 3' ends was achieved using the Klenow fragment of *E. coli* DNA polymerase I. Each reaction contained 50mM Tris. HCl [pH 7.2], 10mM $MgSO_4$, 0.1mM DTT, 50μg/ml BSA (DNase free), 100μM dTTP, 100μM dGTP, 100μM dCTP, 10μCi of 3000Ci/mmol [α^{32}P]dATP, 10-30ng of DNA and 10 units of Klenow, made to 20μl with distilled deionised H_2O. After vortexing, the mixture was incubated at room temperature for 30 minutes and the DNA was purified by direct extraction.

2.14.2 Oligonucleotide-Labelling Using Hexadeoxynucleotide Primers

Each reaction contained 1x OLB buffer (see Section 2.2.11), 0.5mg/ml BSA (DNase free), 25ng of DNA, 25μCi of 3000Ci/mmol [α^{32}P]dCTP and 10 units of Klenow, made to 25μl with distilled deionised H_2O. Note that the H_2O and DNA solution were premixed and boiled for 5 minutes, prior to the addition of the other reagents and the whole mixture was then incubated at room temperature for 12-16 hours. The reaction was stopped by the addition of 100μl of stop buffer (20mM Tris.HCl [pH 7.5], 20mM NaCl, 2mM EDTA [pH 8.0], 0.25% (w/v)

SDS and 1μM dCTP in distilled deionised H_2O) and the whole mixture was used in colony hybridisation studies (see Section 2.20).

2.15 Electrophoretic Techniques

2.15.1 Agarose Gels

Gels of between 0.4–2.0% (w/v) were prepared, by adding the appropriate amount of Miles HSB agarose to 1x TAE buffer. After dissolving with warming, ethidium bromide was added to final concentration of 0.5μg/ml, and the solution was poured into a gel former and allowed to set around a perspex comb. The comb was removed and the gel was transferred to a gel–tank containing 1x TAE buffer and 0.5μg/ml ethidium bromide. Sufficient buffer was added so that the gel was submerged by 2–3mm. Prior to loading, 6x gel–loading buffer (15% (w/v) Ficoll type 400, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF in distilled deionised H_2O) was added to each sample to a final concentration of 1x and electrophoresis was carried out between 5–20V/cm. DNA bands were visualised on a UV transilluminator.

2.15.2 Polyacrylamide Gels

The various recipes for polyacrylamide gels are given in Table 2.1. After mixing in 30μl TEMED (*N*,*N*,*N*',*N*'–tetramethylenediamine), the solution was poured immediately between two gel plates separated by 0.4mm (for sequencing and *in–vitro* transcription assays) or 0.8mm (for mobility–shift assays) spacers. The appropriate comb was inserted into place and the gel allowed to polymerise at room temperature for 20 minutes. After attachment of the plates in a vertical position in a gel–tank, both the upper and lower reservoirs were filled with 1x TBE buffer (for sequencing and *in–vitro* transcription assays) or 0.5x TBE buffer (for mobility–shift assays) and the sample–wells were flushed out with the same buffer. Samples were loaded in appropriate buffer and electrophoresis was carried out until the bromophenol blue dye was 2–3cm from the bottom. The two glass plates were then separated so that the gel adhered to one plate (aided by siliconising the opposing plate), and the gel, still attached to the plate, was

Table 2.1 Recipes for DNA sequencing (A), *in-vitro* transcription (B) and mobility-shift (C) polyacrylamide gels

	A	B	C
Urea	42g	42g	-
40% (w/v) Acrylamide	15ml	10ml	-
30% (w/v) Acrylamide	-	-	13.3ml
10x TBE buffer [pH 8.0]	10ml	10ml	5ml
H_2O to:	100ml	100ml	100ml
10% (w/v) Ammonium persulphate	600μl	600μl	-
3% (w/v) Ammonium persulphate	-	-	2.1ml
TEMED	30μl	30μl	30μl

immersed in fixing solution (10% (v/v) ethanol and 10% (v/v) glacial acetic acid in distilled deionised H_2O) for 15 minutes. After blotting onto Whatman 3MM filter paper, the gel was covered in Saran-Wrap and dried in a Bio-Rad 483 slab drier at 80°C for approximately 2 hours. DNA bands were visualised by autoradiography, using Fuji RX100 X-ray film at -70°C with a screen (for [α^{32}P]) or at room temperature without a screen (for [α^{35}S]).

2.16 DNA Purification

2.16.1 By Direct Extraction

The volume of the DNA sample was measured, and an equal volume of phenol added and mixed by vortexing, until an emulsion formed. The mixture was centrifuged for 3 minutes at room temperature and the upper aqueous phase was transferred to a fresh tube. The extraction was repeated a further two times with phenol/chloroform and chloroform respectively. Sufficient 2.5M sodium acetate [pH 5.2] was then added, so that the final concentration was 0.25M. After adding exactly 2 volumes of ethanol and vortexing, the mixture was incubated in a dry-ice/ethanol bath for 30-60 minutes and was then centrifuged at 12000g for 15 minutes at room temperature. The supernatant was removed by aspiration and discarded. The DNA pellet was dried for 2 minutes in a vacuum desiccator and was dissolved in the required volume of TE buffer [pH 8.0].

2.16.2 From Low Melting Point (LMP) Agarose Gels

The DNA sample was first run on a LMP agarose gel containing 0.5μg/ml ethidium bromide. The DNA was visualised using a long wavelength UV lamp, and the band of interest was cut out using a sharp scalpel blade and transferred to an Eppendorf tube. The volume of the gel-slice was estimated and approximately 5 volumes of TE buffer [pH 8.0] was added. The mixture was incubated in a 65°C water bath for 10 minutes, to dissolve the gel-slice, and then reincubated at the same temperature for a further 10 minutes. After making sure that the gel-slice was fully dissolved, the DNA was purified by direct extraction.

2.17 Preparation of Competent Cells

10ml of LB broth was inoculated with a single bacterial colony and incubated with vigorous shaking at 37°C for 1.5–2.0 hours until the absorbace at 550nm was 0.5. The culture was chilled on ice for 10 minutes and then centrifuged in a Labfuge 6000 at 4000rpm for 5 minutes at 4°C. The supernatant was discarded, and the cells resuspended in half the original culture volume (5ml) of an ice-cold sterile solution of 50mM $CaCl_2$ and 10mM Tris.HCl [pH 8.0]. The cells were incubated on ice for 15 minutes and then centrifuged at 4000rpm for 5 minutes at 4°C. The supernatant was discarded, and the cells resuspended in 1/20 the original culture volume (0.5ml) of an ice-cold sterile solution of 50mM $CaCl_2$ and 10mM Tris.HCl [pH 8.0]. 200μl aliquots of the cell suspension were dispensed into prechilled Eppendorf tubes and stored at 4°C for 12–24 hours, during which time the cells became competent.

2.18 Transformation

2.18.1 Of Plasmid DNA

40ng of DNA in 50μl of TE buffer [pH 8.0], was added to 200μl of competent cells and the mixture incubated on ice for 30 minutes. The cells were then transferred to a 42°C water bath for exactly 2 minutes, after which 1ml of LB broth was added and the mixture incubated at 37°C for 60 minutes. (This period allowed the bacteria to recover and begin to express antibiotic resistance). 100μl aliquots of the cell suspension, were spread onto LB agar plates containing 40μg/ml Xgal, 47.6μg/ml IPTG and 50μg/ml of appropriate antibiotic. The plates were inverted and incubated at 37°C for 12–16 hours. Recombinant clones produced white colonies against a blue colony background.

2.18.2 Of M13 RF DNA

40ng of DNA in 50μl of TE buffer [pH 8.0], was added to 200μl of competent cells and the mixture incubated on ice for 30 minutes. The cells were then transferred to a 42°C water bath for exactly 2 minutes and then placed on ice once more. Meanwhile, to 5ml sterile culture

tubes was added 40μl of 23.8mg/ml IPTG, 40μl of 20mg/ml Xgal and 200μl of an overnight culture of either *E. coli* JM101 or TG1 cells. 50μl of heat-shocked cells was then added to each tube, followed by 3.5ml of molten H top agar at 55°C. The mixture was vortexed briefly and poured immediately onto H plates. After setting the plates were inverted and incubated at 37°C for 12-16 hours. Recombinant clones produced white plaques against a blue plaque background.

2.19 Preparative DNA Methods

2.19.1 Small-Scale Preparation of Plasmid DNA

10 ml of LB broth, containing 50μg/ml of appropriate antibiotic, was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking. 1.5ml of culture was transferred to an Eppendorf tube and centrifuged at room temperature for 1 minute. The supernatant was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended by vortexing, in 100μl of an ice-cold solution of 50mM glucose, 10mM EDTA [pH 8.0] and 25mM Tris.HCl [pH 8.0], and the mixture incubated at room temperature for 5 minutes. 200μl of a freshly prepared solution of 0.2M NaOH and 1% (w/v) SDS was then added and mixed, by inverting the tube rapidly 2-3 times and the mixture incubated on ice for 5 minutes. 150μl of an ice-cold solution of potassium acetate [≈pH 4.8] was now added, and the mixture vortexed gently for 10 seconds and then incubated on ice for 5 minutes. (A potassium acetate stock solution was prepared by adding 11.5ml of glacial acetic acid to 60ml of 5M potassium acetate, and making to 100ml with distilled deionised H_2O). The lysate was centrifuged for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. An equal volume of phenol/chloroform was added, mixed by vortexing, and the mixture centrifuged for 3 minutes at room temperature. The upper aqueous phase was transferred to a fresh tube and exactly 2 volumes of ethanol was added, mixed by vortexing, and incubated in a dry-ice/ethanol bath for 10 minutes. The mixture was centrifuged for 15 minutes and the supernatant was removed by aspiration. The DNA pellet was washed with 1ml of ice-cold 70% (v/v) ethanol, and recentrifuged for 5 minutes at room temperature. The supernatant was

removed as before, and the pellet dried for 2 minutes in a vacuum desiccator and finally, resuspended in 50μl TE buffer [pH 8.0] containing 20μg/ml DNase free pancreatic RNase A. 10μl aliqots were used for restriction endonuclease digestions.

2.19.2 Small-Scale Preparation of M13 RF DNA

1.5ml of a 1/100 dilution of an overnight culture of either *E. coli* JM101 or TG1 cells in 2xTY broth, was dispensed into a 25ml sterile universal and inoculated with a single plaque. After incubation at 37°C overnight with vigorous shaking, the mixture was transferred to an Eppendorf tube and centrifuged for 5 minutes at room temperature. The supernatant was removed by aspiration, leaving the bacterial pellet as dry as possible. The remainder of the protocol was as outlined in Section 2.19.1.

2.19.3 Large-Scale Preparation of Plasmid DNA

4x250ml of LB broth, containing 50μg/ml of appropriate antibiotic, were inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking. The cells were harvested by centrifugation, at 5000rpm in a Sorvall GSA rotor for 30 minutes at 4°C and after discarding the supernatant, the pellets were resuspended in 10ml of 50mM glucose, 25mM Tris.HCl [pH8.0], 10mM EDTA [pH 8.0] and 5mg/ml lysosyme (added freshly). 5ml aliquots of the suspension, were transferred to each of two polypropylene tubes and 2x10ml of 0.2M NaOH and 1% (w/v) SDS were added. The tops of the tubes were sealed with Nescofilm, and the contents mixed gently, by inverting the tubes several times, after which they were incubated on ice for 10 minutes. 7.5ml of potassium acetate [≈pH 4.8] was added to each tube and the contents mixed as before. After incubating on ice for 10 minutes, the lysate was centrifuged at 20000rpm in a Sorvall SS34 rotor for 20 minutes at 4°C. (During this step, the chromosomal DNA and bacterial debris formed a tight pellet on the bottom of the tube). 10ml aliquots of the supernatant were transferred to each of four 30ml Corex tubes, and to each was added exactly 2 volumes of ice-cold ethanol and the mixture was incubated at -20°C for 60 minutes. The DNA was harvested by centrifugation at 15000rpm in a

Sorvall SS34 rotor for 30 minutes at 0°C. The supernatants were discarded, and the DNA pellets were dried for 2 minutes in a vacuum desiccator and resuspended in a total volume of 8ml of TE buffer [pH 8.0].

For every 1ml of DNA solution, exactly 1g of solid ultrapure (optical grade) caesium chloride (BRL) was added and mixed gently. When all the salt had dissolved, 0.8ml of 10mg/ml ethidium bromide was added for every 10ml of caesium chloride solution and mixed well. The solution was then transferred to a tube suitable for centrifugation in a Sorvall T1270 rotor, and the remainder of the tube was filled with light paraffin oil and sealed. Centrifugation was carried out at 45000rpm for 40 hours at 20°C.

After centrifugation, two bands were visible. The upper band represented linear chromosomal DNA and the lower band was supercoiled covalently closed circular plasmid DNA. The lower plasmid band was collected, by inserting a hypodermic syringe needle (size 19Gx1½) into the wall of the tube just below the band of interest, and after making an air-hole at the top of the tube, the band was drawn-out with a syringe. The plasmid DNA solution was transferred to an Eppendorf tube and the ethidium bromide was removed as follows. An equal volume of butan-1-ol, saturated with H_2O, was added to the plasmid solution and mixed by vortexing. After centrifugation for 3 minutes at room temperature, the lower aqueous phase was transferred to a fresh tube and the extraction was repeated 4-6 times until all of the ethidium bromide disappeared. Finally, the aqueous phase was dialysed against 3x1000ml of TE buffer [pH 8.0] for 4.5 hours and the DNA concentration was measured by absorbance at 260nm.

2.19.4 Large-Scale Preparation of M13 RF DNA

4x250ml of a 1/100 dilution of an overnight culture of either *E.coli* JM101 or TG1 cells in 2xTY broth, were inoculated with a single plaque and incubated at 37°C overnight with vigorous shaking. The remainder of the protocol was as outlined in Section 2.19.3.

2.19.5 Preparation of Single-Stranded DNA for M13 Sequencing

1.5ml of a 1/100 dilution of an overnight culture of *E. coli* JM101 cells in 2xTY broth, was dispensed into a 25ml universal and inoculated with a single plaque. After incubation at 37°C overnight with vigorous shaking, the mixture was transferred to an Eppendorf tube and centrifuged for 5 minutes at room temperature. 1ml of the supernatant was then transferred to a fresh tube, being particularly careful not to transfer cells. (The supernatant could be stored for 24 hours at this stage). After adding 200μl of 20% (w/v) PEG 6000 in 2.5M NaCl, the mixture was incubated at room temperature for 15 minutes and then centrifuged for 5 minutes at the same temperature. The supernatant was discarded, and the phage pellet was recentrifuged for a further 2 minutes at room temperature. Using a drawn-out Pasteur pipette, all remaining traces of PEG were removed and the mouth of the tube was wiped with tissue. The phage pellet was then resuspended in 100μl of TE buffer [pH 8.0]. An equal volume of phenol was added, and the mixture vortexed for 15 seconds and then incubated at room temperature for 15 minutes. The mixture was vortexed for a further 15 seconds and then centrifuged for 3 minutes at room temperature. The upper aqueous phase was transferred to a fresh tube and an equal volume of phenol/chloroform added. After vortexing, the mixture was centrifuged for 3 minutes at room temperature and the upper aqueous phase transferred to a fresh tube. An equal volume of chloroform was added, and the mixture was vortexed and centrifuged as before, and the upper aqueous phase was again transferred to a fresh tube. Sufficient 2.5M sodium acetate [pH 5.2] was then added, so that the final concentration was 0.25M. Exactly 2 volumes of ethanol were now added and mixed by vortexing, and the mixture was incubated in a dry-ice/ethanol bath for 30-60 minutes. After centrifugation for 15 minutes at room temperature, the supernatant was removed by aspiration and the DNA pellet was dried for 2 minutes in a vacuum desiccator. The pellet was resuspended in 50μl of TE buffer [pH 8.0] and 5μl aliquots were used in sequencing reactions (see Section 2.21.1).

2.19.6 Preparation of Plasmid DNA for Sequencing

10ml of LB broth, containing 50μg/ml of appropriate antibiotic, was

inoculated with a single bacterial colony and incubated at 37°C for 20-24 hours with vigorous shaking. 1.5ml of culture was transferred to an Eppendorf tube and centrifuged for 1 minute. The supernatant was removed by aspiration, and an additional 1.5ml of culture was added and centrifuged as before. The supernatant was again removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended by vortexing in 100μl of an ice-cold solution of 50mM glucose, 10mM EDTA [pH 8.0] and 25mM Tris.HCl [pH 8.0]. After incubating for 5 minutes at room temperature, 200μl of a freshly prepared solution of 0.2M NaOH and 1% (w/v) SDS was added and mixed by inverting the tube 2-3 times. The mixture was incubated on ice for 5 minutes, and 150μl of an ice-cold solution of potassium acetate [≈pH 4.8] was then added and the tube vortexed gently for 10 seconds. Again, after incubating on ice for 5 minutes, the lysate was centrifuged for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. Following recentrifugation for 5 minutes at 4°C, the supernatant was transferred to a fresh tube being careful not to carry over any precipitate. RNase A was added to a final concentration of 50μg/ml and the lysate was incubated at 37°C for 30 minutes. An equal volume of phenol/chloroform was added, mixed by vortexing and the mixture was centrifuged for 3 minutes at room temperature. The upper aqueous phase was transferred to a fresh tube and the extraction was repeated with an equal volume of chloroform. 1-volume of isopropanol (propan-2-ol) was then added to the aqueous phase, mixed by vortexing and incubated at room temperature for 30-60 minutes. After centrifugation for 15 minutes at room temperature, the supernatant was removed by aspiration. The DNA pellet was rinsed with 1ml 70% (v/v) ice-cold ethanol and recentrifuged for 5 minutes at room temperature. The supernatant was again removed by aspiration and the pellet was dried for 2 minutes in a vacuum desiccator. After dissolving the DNA pellet in 16.8μl distilled deionised H_2O, 3.2μl of 5M NaCl was added and mixed, followed by 20μl of 13% (w/v) PEG 8000 and the mixture was incubated at 4°C for 1-2 hours. Following centrifugation for 10 minutes at 4°C, the supernatant was discarded and the DNA pellet was rinsed with 1ml of 70% (v/v) ice-cold ethanol, and the mixture was recentrifuged for 5 minutes at room temperature. The supernatant was discarded, and the pellet was dried for 2 minutes in a vacuum

desiccator and finally, was resuspended in 10μl distilled deionised H_2O. The DNA concentration was quantitated by measuring the absorbance at 260nm, and 2-3μg of DNA was used in sequencing reactions (see Section 2.21.2).

2.19.7 Preparation of Single-Stranded DNA for Site-Directed Mutagenesis

100ml of a 1/100 dilution of an overnight culture of *E. coli* RZ1032 cells in LB broth containing 12.5μg/ml tetracycline, was inoculated with a single plaque and incubated at 37°C for 4 hours. The cells were pelleted by centrifugation (4x25ml in 30ml Corex tubes) at 8000rpm in a Sorvall SS34 rotor for 30 minutes at 4°C, and the supernatants were transferred to fresh tubes. 0.2 volumes of 20% (w/v) PEG 6000 in 2.5M NaCl, was added to the supernatants and the mixtures were incubated at 4°C for 60 minutes. After centrifugation at 8000rpm in a Sorvall SS34 rotor for 20 minutes at 4°C, the supernatants were discarded and the phage pellets were recentrifuged as before for 5 minutes at 4°C. All remaining traces of PEG were removed with a drawn-out Pasteur pipette, and the pellets were resuspended in a total of 500μl TE buffer [pH 8.0] and transferred to an Eppendorf tube. The remaining bacterial cells were removed from the suspension by centrifugation for 5 minutes at 4°C, after which the supernatant was transferred to a fresh tube. 200μl of 20% (w/v) PEG 6000 in 2.5M NaCl, was added and the mixture incubated at room temperature for 15 minutes. (Alternatively, the mixture could be stored overnight at 4°C). After centrifugation for 5 minutes at room temperature, the supernatant was discarded and the phage pellet was recentrifuged for 2 minutes. All remaining traces of PEG were removed with a drawn-out Pasteur pipette, and any drops on the mouth of the tube were wiped off with a tissue. The pellet was then resuspended in 500μl of TE buffer [pH 8.0]. An equal volume of phenol was added, mixed by vortexing for 60 seconds, and then the mixture was incubated at room temperature for 15 minutes. After vortexing for a further 30 seconds, the mixture was centrifuged for 3 minutes at room temperature. The upper aqueous phase was transferred to a fresh tube, and the phenol extraction was repeated. An equal volume of phenol/chloroform was then added to the aqueous phase and mixed by

vortexing. After centrifugation for 3 minutes at room temperature, the upper aqueous phase was transferred to a fresh tube and the phenol/chloroform extraction was repeated. An equal volume of chloroform was then added to the aqueous phase and mixed by vortexing. After centrifugation for 3 minutes at room temperature, the upper aqueous phase was transferred to a fresh tube and the chloroform extraction was repeated. Sufficient 2.5M sodium acetate [pH 5.2] was added to the aqueous phase, so that the final concentration was 0.25M. Exactly 2 volumes of ethanol was then added and mixed by vortexing, and the mixture was incubated in a dry-ice/ethanol bath for 30-60 minutes. After centrifugation for 15 minutes at room temperature, the supernatant was removed by aspiration and the DNA pellet was dried for 2 minutes in a vacuum desiccator. Finally, the pellet was resuspended in 50μl TE buffer [pH 8.0] and the DNA concentration was quantitated by measuring the absorbace at 260nm.

2.20 Colony Hybridisation

82mm gridded Hybond-N membranes, were carefully placed onto each of two identical selection plates. Both plates were marked with a needle hole through the membrane, so that both templates could be aligned. Using sterile toothpicks, single colonies were streaked onto corresponding grid squares on both the experimental and reference plates, and these were then incubated at 37°C for 12-16 hours. The reference plate was stored at 4°C. The membrane, from the experimental plate, was placed colony-side up on a pad of absorbant filter paper soaked in denaturing solution (1.5M NaCl and 0.5M NaOH) and left for 7 minutes. The membrane was then transferred, colony-side up, onto a pad of absorbant filter paper soaked in neutralising solution (1.5M NaCl, 0.5M Tris.HCl [pH 7.2] and 1mM EDTA [pH 8.0]) and left for 3 minutes. This step was repeated, with a fresh pad soaked in neutralising solution. The membrane was then transferred, colony-side up, onto a pad of absorbant filter paper soaked in 2x SSC and left for 3 minutes. Finally, the membrane was transferred to dry filter paper and allowed to air dry, colony-side up, and was then wrapped in Saran-Wrap and placed, colony-side down, on a UV transilluminator for exactly 30 seconds, to crosslink the denatured DNA to the membrane.

25ml of prehybridisation solution (3x SSC, 5x Denhardt's, 0.1% (w/v) SDS and 6% (w/v) PEG 6000 in distilled deionised H_2O) was added to the membrane, followed by 0.5ml of a 10mg/ml solution of denatured (by boiling for 5 minutes) sheared salmon sperm DNA. Prehybridisation was carried out for at least 1 hour in a 65°C shaking water bath. The prehybridisation solution was then replaced with 25ml of hybridisation solution (3x SSC, 2x Denhardt's, 0.1% (w/v) SDS and 6% (w/v) PEG 6000 in distilled deionised H_2O), followed by the addition of denatured sheared salmon sperm DNA, containing 125μl of oligonucleotide-labelled probe DNA solution (see Section 2.14.2). Hybridisation was carried out for 12-16 hours in a 65°C shaking water bath.

The membrane was washed, in solutions of increasing stringency, for 30 minutes in a 65°C water bath until no more radioactivity was eluted from the membrane. The first wash solution contained 2x SSC and 0.1% (w/v) SDS; the second contained 0.5x SSC and 0.1% (w/v) SDS; and the third contained 0.1x SSC and 0.1% (w/v) SDS. The membrane was then air dried, as before, wrapped in Saran-Wrap and autoradiographed with screens at -70°C. Positive colonies were picked from the reference plate and used for further analysis.

2.21 DNA Sequencing

2.21.1 Of Single-Stranded M13 DNA Templates

A primer mix (2.5ng of primer (17mer), 20mM Tris.HCl [pH 7.5] and 10mM $MgCl_2$, made to 5μl with distilled deionised H_2O), was added to 5μl of single-stranded template DNA (see Section 2.19.5) and incubated in a 65°C oven for 1 hour. The mixture was then allowed to cool, for 30 minutes, to room temperature and was subsequently called the annealing mix. Meanwhile, a labelling mix (4.5 units of Klenow and 8μCi of 400Ci/mmol [α^{35}S]dATPαS, made to 9μl with distilled deionised H_2O) was prepared. Each set of sequencing reactions (A,T,G and C) contained 2μl of annealing mix, 2μl of respective A*,T*,G* and C* mixes (Table 2.2) and 2μl labelling mix, and the resulting concoction was incubated at 37°C for 20 minutes. 0.25mM dNTP chase solutions were added to their respective A,T,G and C mixes, which were then incubated at 37°C for a further 20

Table 2.2 Recipes for $A^{\bullet}$, $T^{\bullet}$, $G^{\bullet}$ and $C^{\bullet}$ mixes (volumes in μl)

	$A^{\bullet}$	$T^{\bullet}$	$G^{\bullet}$	$C^{\bullet}$
0.5mM dTTP	500	25	500	500
0.5mM dGTP	500	500	25	500
0.5mM dCTP	500	500	500	25
10mM ddATP	1			
10mM ddTTP		50		
10mM ddGTP			16	
10mM ddCTP				8
TE buffer [pH 8.0]	1000	1000	1000	1000

minutes. After adding 2μl of formamide dye (98% (v/v) deionised formamide, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF and 10mM EDTA [pH 8.0]), the mixtures were incubated in a boiling water bath for 3 minutes and 2μl of each reaction were loaded onto a 6% (w/v) polyacrylamide (acrylamide:bisacrylamide ratio of 38:2), 7M urea sequencing gel. Electrophoresis was carried out at 1400V, until the bromophenol blue dye was 2-3cm from the bottom of the gel.

2.21.2 Of Plasmid DNA Templates

2-3μg of plasmid DNA (see Section 2.19.6), in 8μl of distilled deionised H_2O, were denatured by adding 2μl of 2M NaOH and incubating at room temperature for 10 minutes. 3μl of 3M sodium acetate [pH 4.8], 7μl of distilled deionised H_2O and 60μl of ice-cold ethanol, were then added and mixed, and incubated in a dry-ice/ethanol bath for 30 minutes. After centrifugation for 15 minutes at room temperature, the supernatant was removed by aspiration and the DNA pellet rinsed with 1ml of 70% (v/v) ice-cold ethanol. Following recentrifugation for 10 minutes at room temperature, the supernatant was again removed by aspiration, and the pellet was dried for 2 minutes in a vacuum desiccator and resuspended in 10μl distilled deionised H_2O. 2μl of annealing buffer (280mM Tris.HCl [pH 7.5], 100mM $MgCl_2$ and 350mM NaCl) and 2μl of primer (5ng/μl of a 17mer), were added and the mixture (now called the annealing mix) incubated at 37°C for 20 minutes. The annealing mix was then incubated at room temperature for at least 15 minutes, followed by the addition of 2μl of labelling mix (2μM dGTP, 2μM dCTP and 2μM dTTP), 1μl of 0.3M DTT, 1μl of 400Ci/mmol [α^{35}S]dATPαS and 2μl of 1.5 units/μl T7 DNA polymerase. After incubating at room temperature for 5 minutes, the labelling reaction was terminated, by transferring 4.5μl of the mixture into each of four prewarmed tubes containing 2.5μl of A,T,G and C mixes (15μM ddATP, 15μM ddTTP, 15μM ddGTP and 15μM ddCTP respectively, together with 150μM dATP, 150μM dTTP, 150μM dGTP, 150μM dCTP, 10mM $MgCl_2$, 40mM Tris.HCl [pH 7.5] and 50mM NaCl) respectively, and the concoction was incubated at 37°C for 5 minutes. After adding 5μl of stop solution (95% (v/v) deionised formamide, 0.05% (w/v)

bromophenol blue, 0.05% (w/v) xylene cyanol FF and 20mM EDTA [pH 8.0]) to each tube, the mixtures were heated to 75-80°C in a water bath for 3 minutes and 2μl of each reaction mix were loaded onto a 6% (w/v) polyacrylamide (acrylamide:bisacrylamide ratio of 38:2), 7M urea sequencing gel. Electrophoresis was carried out at 1400V, until the bromophenol blue dye was 2-3cm from the bottom of the gel.

2.22 Deletion-Mapping

2.22.1 With Nuclease *Bal*31

A reaction mixture containing 20mM Tris.HCl [pH 8.0], 12mM $MgCl_2$, 12mM CaCl, 0.6M NaCl, 2mM EDTA [pH 8.0], 10μg of linearised DNA and 4.2 units of nuclease *Bal*31, made to 100μl with distilled deionised H_2O, was incubated at 30°C for 30 minutes. At regular intervals the reaction was stopped, by transferring 9μl of the mixture to an Eppendorf tube containing 1μl of 0.2M EGTA [pH 8.0] and the sample was then stored on ice. To each sample was added 90μl of distilled deionised H_2O, and the DNA was purified by direct extraction and resuspended in 50μl of TE buffer [pH 8.0]. Although nuclease *Bal*31 degrades both strands of DNA at approximately the same rate, as a precaution the DNA was blunt-ended using the Klenow fragment of *E coli* DNA polymerase I. The linear DNA fragments in each sample, were then recircularised using T4 DNA ligase and the reaction mixtures were used to transform *E. coli* JM101 cells respectively. After preparing plasmid DNA, transformants were initially screened by restriction endonuclease digestion, followed by plasmid sequencing.

2.22.2 By Polymerase Catalysed Chain Reaction (PCR)

A full description of the way in which PCRs work is given in Section 4.4. Each reaction contained 67mM Tris.HCl [pH 8.8], 16.6mM ammonium sulphate, 6.7mM $MgCl_2$, 10mM 2-mercaptoethanol, 6.7μM EDTA [pH 8.0], 1.5mM dATP, 1.5mM dTTP, 1.5mM dGTP, 1.5mM dCTP, 170μg/ml BSA (DNase free), 10μM each primer, 2ng DNA and 2 units of *Taq*I DNA polymerase, made to 20μl with distilled

deionised H_2O. After overlaying the mixture with liquid paraffin, to prevent evaporation, vacuum grease was wiped around the base of the tube to allow good contact with the heating block. PCR was carried out for 30 cycles and the standby temperature was 37°C. Each cycle consisted of three steps: denaturing at 95°C for 1.3 minutes; annealing at 55°C for 1.5 minutes; and extension at 70°C for 10 minutes. 180μl of distilled deionised H_2O was added to the reaction mixture and the supernatant was carefully transferred to a fresh tube being careful not to transfer any liquid paraffin. The DNA was then purified by direct extraction.

2.23 Site-Directed Mutagenesis

The mutagenesis protocol used was one developed by Kunkel (1985) (see Section 3.3.1). Following the preparation of single-stranded template DNA (see Section 2.19.7), two reaction steps were involved in the procedure: in the first, the mutant oligonucleotide was annealed to the DNA; and in the second, synthesis and ligation of the mutant complementary strand was achieved.

Each annealing reaction contained 50mM Tris.HCl [pH 7.2], 10mM $MgSO_4$, 0.1mM DTT, 50μg/ml BSA (DNase free), 2pmol DNA and 1-2pmol mutant oligonucleotide, made to 20μl with distilled deionised H_2O. After vortexing, the mixture was incubated at 70°C for 3 minutes and then at 37°C for 30 minutes. Synthesis and ligation of the mutant strand was achieved by adding to the annealing mix 50mM Tris.HCl [pH 7.4], 10mM $MgCl_2$, 10mM DTT, 1mM spermidine, 1mM ATP, 0.1mg/ml BSA (DNase free), 2mM dATP, 2mM dTTP, 2mM dGTP, 2mM dCTP, 12 units of Klenow and 12units of T4 DNA ligase, made to 50μl with distilled deionised H_2O. After vortexing, the mixture was incubated at 16°C for 12-16 hours. The mixture was then used to transform *E. coli* JM101 cells and after preparing single-stranded DNA, the transformants were screened by M13 sequencing.

2.24 Protein Estimation

The Bradford method of protein estimation (Bradford, 1976) was used throughout this work.

2.24.1 Protein Preparation

BSA (Pentax fraction V) was dissolved in appropriate buffer to a final concentration of 1mg/ml.

2.24.2 Preparation of Protein Reagent

100mg of Coomassie Brilliant Blue G-250 was dissolved in 50ml of 95% (v/v) ethanol. To this solution 100ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1000ml with distilled deionised H_2O. The final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol, and 8.5% (v/v) orthophosphoric acid.

2.24.3 Protein Assay

Protein solution containing 10-100μg protein in a volume up to 0.1ml, was pipetted into 10x15mm test tubes. The volume in each test tube was adjusted to 0.1ml with appropriate buffer. 5ml of protein reagent was added to the test tube and the contents mixed by vortexing. The absorbance at 595nm was measured after 2 minutes against a reagent blank prepared from 0.1ml of buffer and 5ml of protein reagent. The weight of protein was plotted against the corresponding absorbance, resulting in a standard curve used to determine protein concentration in unknown samples.

2.25 Preparation of Nuclear-Protein Extracts

Two methods for preparing nuclear-protein extracts have been used. Those prepared by the Schibler method (Schibler, personal communication; Gorski *et al.*, 1986) were used in *in-vitro* transcription assays, whereas those prepared by the Dignam method (Dignam *et al.*, 1983) were used in the mobility-shift assay. As much as possible work was carried out in the cold room at 4°C.

2.25.1 Schibler Method

Tissues were dissected and kept on ice in a cold beaker containing

30ml of homogenisation buffer (10mM HEPES [pH7.6], 25mM KCl, 0.5mM spermidine, 0.15mM spermine, 1mM EDTA(K^+) [pH 8.0], 2M sucrose, 10% (v/v) glycerol, 0.5mM DTT and 0.5mM PMSF). Following homogenisation in a motorised glass-teflon homogeniser, where >90% of the cells were lysed, the homogenate was diluted to 100ml with homogenisation buffer and layered in 4x25ml aliquots over 4x10ml cushions of the same buffer. After centrifugation at 25000rpm in a Sorvall AH629 rotor for 60 minutes at 0°C, the nuclear pellet was cleaned as follows. Using a spatula the solid disc of cell debris, which floated at the top of the tube, was removed and the supernatant was carefully removed by aspiration with a 10ml plastic pipette. The tube was inverted and its wall rinsed with distilled deionised H_2O from a 60ml syringe with a long bent needle. After wiping off excess liquid with a tissue, the nuclei were resuspended in 10ml of nuclear-lysis buffer (10mM HEPES [pH 7.6], 10% (v/v) glycerol, 0.1M KCl, 3mM $MgCl_2$, 0.1mM EDTA(K^+), 1mM DTT and 0.1mM PMSF) and homogenised in an all glass Kontes homogeniser (15ml) using pestle B. A 1 in 50 dilution of the homogenate in 0.5% (w/v) SDS was made and the absorbance at 260nm measured against a blank of 1 in 50 dilution of nuclear-lysis buffer. The DNA concentration was calculated (1mg/ml gives an absorbance at 260nm of 20) and the nuclear suspension was diluted with nuclear-lysis buffer to give a DNA concentration of 0.5mg/ml. 3M KCl was then added to give a final concentration of 0.55M (taking into consideration that the lysis buffer already contained 0.1M KCl), and the suspension was incubated on ice for 30 minutes with occasional mixing. (Chromatin comes out of solution at this stage). Following centrifugation at 26000rpm in a Sorvall AH629 rotor for 30 minutes at 0°C, the supernatant was immediately transferred to a prechilled measuring cylinder, being careful not to carry-over any pelleted chromatin. The volume was measured and 0.3g of solid $(NH_4)_2SO_4$/ml of supernatant was added, allowed to dissolve and incubated on ice for 20-60 minutes. After centrifugation at 26000rpm in a Sorvall AH629 rotor for 20 minutes at 0°C, the supernatant was removed by aspiration and discarded. The protein pellet was resuspended in 500-1000μl of dialysis buffer (25mM HEPES [pH 7.6], 10% (v/v) glycerol, 40mM KCl, 0.1mM EDTA(K^+) [pH 8.0] and 1mM DTT) to give a protein concentration of 10-20mg/ml, and dialysed at 4°C for 2x2 hours each

time against 500-1000 volumes of dialysis buffer. The dialysate was transferred to an Eppendorf tube and the white precipitate, produced during dialysis, was pelleted by centrifugation for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. Protein estimation was by the Bradford method and the nuclear-protein extract was frozen in aliquots in liquid nitrogen, at a protein concentration of 10-20mg/ml.

2.25.2 Dignam Method

Tissues were dissected and kept on ice in 10ml of buffer A (10mM HEPES [pH 7.9], 1.5mM $MgCl_2$, 10mM KCl and 0.5mM DTT) containing 0.6M sucrose. Following homogenisation in a manual glass teflon homogeniser, the homogenate was layered in 4x2.5ml aliquots over 4x10ml cushions of the same buffer containing 2M sucrose, and centrifuged at 5000rpm in a Sorvall HB4 rotor for 30 minutes at 4°C. The supernatant was discarded leaving the nuclear pellet as clean as possible, which was then resuspended in 3ml of buffer C (20mM HEPES [pH 7.9], 25% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA [pH 8.0], 0.5mM PMSF and 0.5mM DTT) per 10^9 cells (on the basis that there are approximately $3x10^8$ cells/g of tissue). The suspension was stirred gently on ice for 30 minutes and then centrifuged at 16500rpm in a Sorvall SS34 rotor for 30 minutes at 4°C. The supernatant was collected and dialysed against 300 volumes of buffer D (20mM HEPES [pH 7.9], 20% (v/v) glycerol, 0.1M KCl, 0.2mM EDTA [pH 8.0], 0.5mM PMSF and 0.5mM DTT) for 5 hours. The dialysate was centrifuged in a Sorvall SS34 rotor at 16500rpm for 20 minutes at 4°C, to pellet precipitated material formed during dialysis, and the supernatant (nuclear-protein extract) was transferred to a fresh tube. Protein estimation was by the Bradford method and the nuclear-protein extract was frozen in aliquots in liquid nitrogen, at a protein concentration of 0.7-1.0mg/ml.

2.26 *In-Vitro* Transcription Assay

Each reaction contained 10mM HEPES [pH 7.6], 25.5mM KCl, 6mM $MgCl_2$, 0.63mM ATP, 0.63mM CTP, 35μM UTP, 0.1mM 3'-o-methyl GTP, 3% (v/v) glycerol, 40 units RNasin, 20μCi of 800Ci/mmol

[α^{32}P]UTP, 5-10mg/ml nuclear-protein extract (45% of total reaction volume) and 342fmol template DNA, made to 20μl with distilled deionised H_2O. The nuclear-protein extract and DNA were preincubated on ice for 10 minutes, prior to mixing with the other reagents and the total mixture was then incubated at 30°C for 20 minutes. Transcription was terminated by the addition of 286μl of stop buffer (20mM Tris.HCl [pH 7.5], 0.25M NaCl, 1% (w/v) SDS, 5mM EDTA [pH 8.0], 70μg/ml yeast tRNA and 140μg/ml proteinase K) and the mixture was incubated at 37°C for 30 minutes. An equal volume of phenol/chloroform was added and after vortexing, the mixture was centrifuged for 3 minutes at room temperature. The upper aqueous phase was transferred to a fresh tube and sufficient 3M sodium acetate [pH 4.8] was added, so that the final concentration was 0.3M. Exactly 2.5 volumes of ice-cold ethanol was then added, mixed by vortexing, and incubated in a dry-ice/ethanol bath for 30 minutes. After centrifugation for 15 minutes at room temperature, the supernatant was discarded and the nucleic acid pellet dried for 2 minutes in a vacuum desiccator. The pellet was resuspended in 6μl of formamide dye (80% (v/v) deionised formamide, 0.01% (w/v) bromophenol blue and 0.01% (w/v) xylene cyanol FF, in 1x TBE) and then heated in a boiling water bath for 3 minutes. 5μl were loaded onto a 4% (w/v) polyacrylamide (acrylamide:bisacrylamide ratio of 38:2), 7M urea sequencing gel and electrophoresis was carried out at 1400V, until the bromophenol blue dye was 2-3cm from the bottom of the gel.

2.27 Mobility-Shift Assay

Each reaction contained 10mM Tris.HCl [pH 7.5], 5% (v/v) glycerol, 50mM NaCl, 1mM EDTA [pH 8.0], 1mM DTT, 2μg of the DNA copolymer duplex poly(dI-dC)·poly(dI-dC), 4fmol end labelled probe DNA and 8μg of nuclear protein extract, made to 30μl with distilled deionised H_2O. In addition competing reactions contained 200fmol and 400fmol of either specific or non-specific cold DNA. After mixing gently with a sealed capillary tube, the mixture was incubated at room temperature for 30 minutes. 6x gel-loading buffer (15% (w/v) Ficoll type 400, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF, in distilled deionised H_2O) was added to a final concentration of 1x. 30μl aliquots of each sample were then loaded onto a low ionic strength (45mM Tris.borate), non-denaturing, 4% (w/v) polyacrylamide gel

(acrylamide:bisacrylamide ratio of 29:1) and electrophoresis was carried out at 11V/cm for 3-5 hours.

CHAPTER 3

RESULTS

Comparison of the Relative Activities of the *Ren-1^d* and *Ren-2^d* Promoters in Initiating Accurate *In-Vitro* Transcription

3.1 G-free Cassette

A major advance for investigating eukaryotic transcriptional control, has been the development of soluble cell-free systems that mediate accurate *in-vitro* transcription initiation on purified genes by RNA polymerase II (Weil *et al.*, 1979; Manley *et al.*, 1980; and Dignam *et al.*, 1983). Using such systems the requirement for several protein factors in addition to RNA polymerase has been revealed (Matsui *et al.*, 1980; Tsai *et al.*, 1981; Samuels *et al.*, 1982; Dynan and Tjian, 1983; Heintz and Roeder, 1984; and Parker and Topol, 1984a, 1984b), but a major drawback in investigating these proteins has been the lack of a fast quantitative assay.

Although RNA polymerase II is able to initiate and elongate transcripts accurately *in-vitro*, transcription termination does not occur properly (reviewed by Manley 1983). For this reason, in most *in-vitro* transcription assays a truncated template is used in order to generate a discrete run-off transcript. Alternatively fairly time-consuming methods, such as nuclease S1 mapping and primer-extension, must be employed to detect the accurately initiated transcripts from supercoiled templates. In order to overcome these problems, Sawadogo and Roeder (1985a) designed the plasmid p(C_2AT), a pUC13-derivative containing a DNA fragment whose random sequence has the average composition $(C_2,A,T)_n.(G_2,T,A)_n$. One such plasmid, p$(C_2AT)_{19}$, contains a synthetic run of 380 A, C and T residues on the messenger strand (Figure 3.1). When a promoter fragment that has no guanine residues downstream of the cap-site is cloned directly upstream of this "G-free cassette", an *in-vitro* transcription template results that, in the absence of GTP, generates a specific-length transcript whose 5' and 3' boundaries are determined by the cap-site and the first guanine residue flanking the

Sst I

5'...GAATTCGAGCTCCATACCCTTCCTCCATCTATACCACCCTACTCTCCTTT

CCTCATTATTCCTCCTATTATCTTCTCCTCTTCTCTCCTTCTTCTATATT

TCCCAAATCTATCATCATTCACTCTCATCCCCTCTTCCTTCACTCCCATT

CTATTCTACTCCTTTCCCTTTCCATATCCCCTCCACCCCCCTTCCTCCCC

TCTTTCAATCTTATCCCCAATCATAAAATTATCTCAATTATATTCTCCTT

CCATACCCCCTATCATCCTCATCCCTATCACCCCCTACTCACCCAATACT

CCCTACTCATCTCATATATCCTTATCCTCTCCTCACCTCTCCCTCCTCTA

TCTCCCCCCCTCACACTCATTTCTCATTCCACTCCCGGGGATCC...3'

Sma I

Figure 3.1

Nucleotide sequence of the "G-free" cassette.

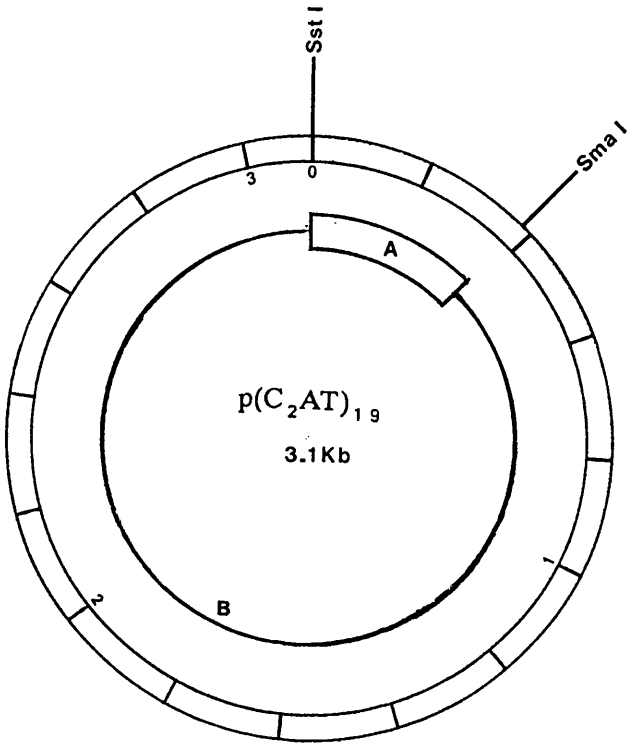
cassette, respectively. Apart from their use in rapid transcription assays in which radiolabelled transcripts are precipitated with trichloroacetic acid and the amount of label incorporated is directly determined (Sawadogo and Roeder, 1985a), such constructs have several additional advantages. First, the efficiency of circular and linear templates in promoting accurate transcription initiation can be directly compared. Second, non-specific transcripts such as those resulting from initiation at pseudo-promotors are not successfully elongated. Third, symmetrical transcription which may possibly interfere with transcription from the promoter of interest, is largely abolished through use of the G-free cassette. All of these properties help to increase the ratio of specific to non-specific transcripts, thereby enhancing the sensitivity of the assay.

Gorski *et al.* (1986) have used G-free cassette templates in conjunction with their own method for preparing transcriptionally active nuclear-protein extracts from rat liver, brain and spleen, to investigate the tissue-specific *in-vitro* transcription from the mouse albumin promoter (-650 to +22). They reported that in these soluble extracts the promoter of the albumin gene behaved, at least qualitatively, as it does *in-vivo*. It promoted efficient transcription in the presence of nuclear-proteins from liver, was only slightly active in brain extract and was almost silent in spleen extract. In addition they went on to show that albumin sequences between -170 and -55 were required for this liver-specific *in-vitro* transcription, since deletion of this region resulted in almost a 100-fold reduction in transcription. Furthermore, insertion of this sequence in either orientation upstream of the parotid-specific *Amy-1* promoter, which was poorly transcribed in liver extract, increased the activity of this promoter to a level comparable to that observed for the albumin promoter.

Similar *in-vitro* confirmation of tissue-specific *in-vivo* gene-expression has been reported for the human α1-antitrypsin promoter (Monaci *et al.*, 1988). Using nuclear-protein extracts, prepared as described by Gorski *et al.* (1986) and Lichtsteiner *et al.* (1987), from rat liver, rat spleen and a human carcinoma cell-line (HeLa) together with human α1-antitrypsin promoter driven G-free cassette templates, these workers have identified a region from -137 to -2 which directed liver-specific *in-vitro* transcription. Furthermore by site-directed mutagenesis, two *cis*-acting

Figure 3.2

Restriction site maps of I, $p(C_2AT)_{19}$ and II, $pML(C_2AT)_{19}$. I: A, G-free cassette; B, pUC13. II: A, adenovirus-2 major-late promoter (-404 to +10); B, G-free cassette; C, pUC13. Hereafter an arrow represents the direction of transcription.


Sst I
Sma I
0
1
2
3
A
B
p(C2AT)19
3.1Kb

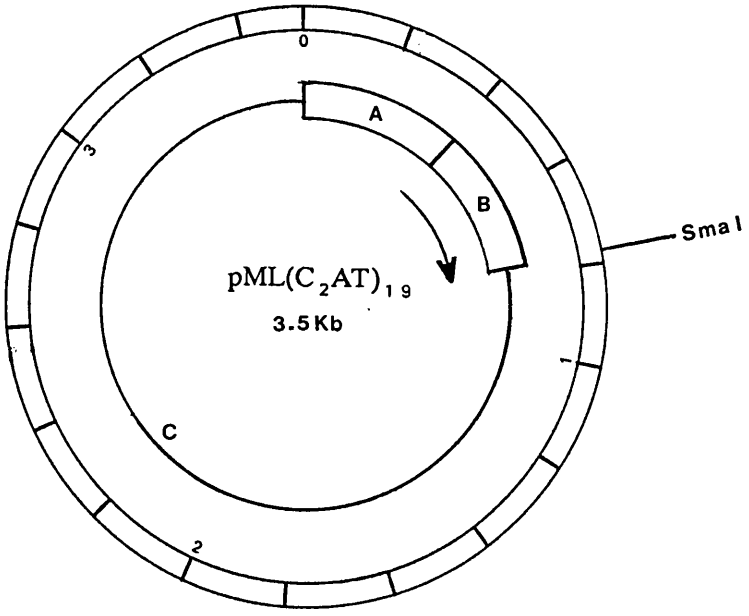
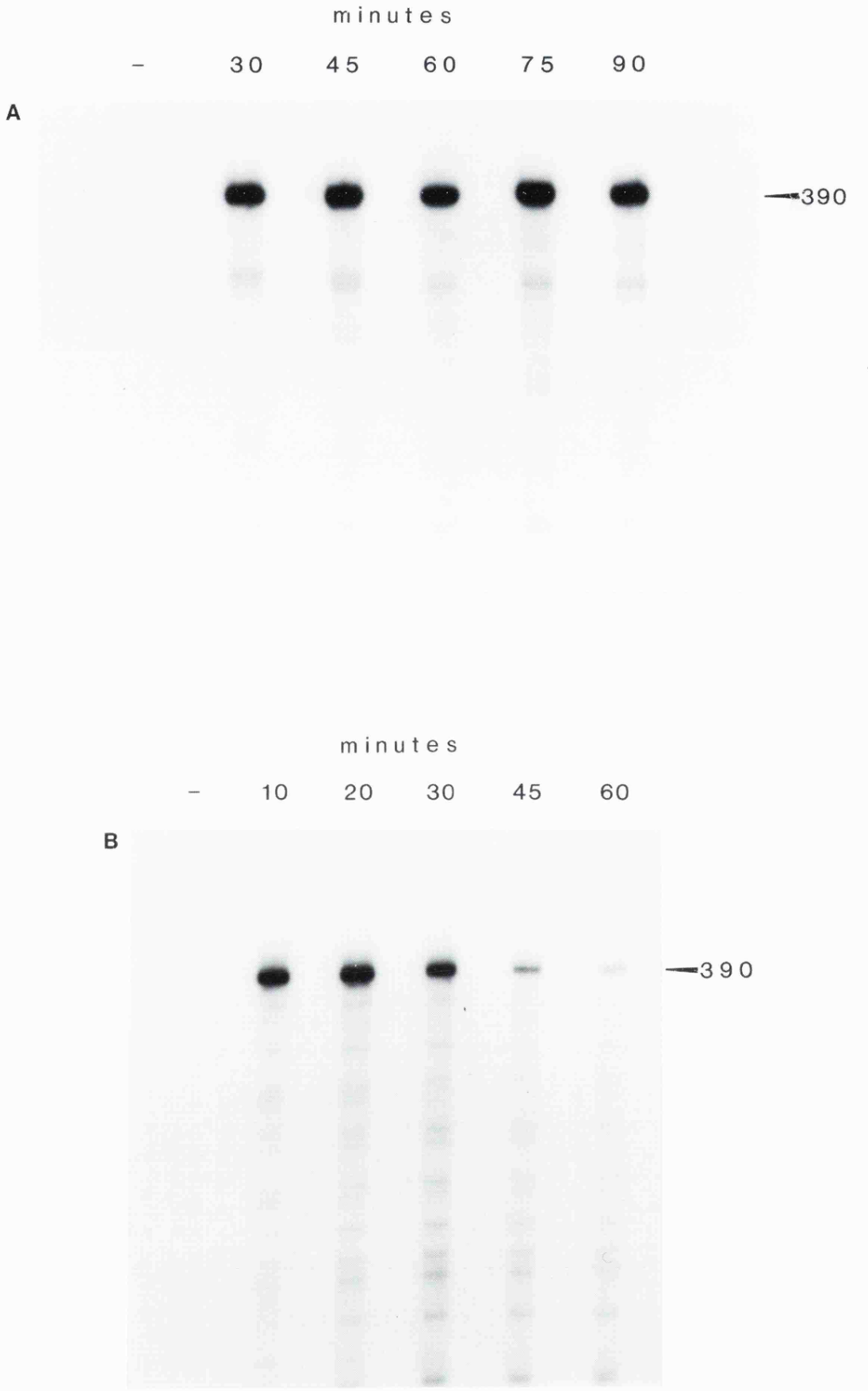
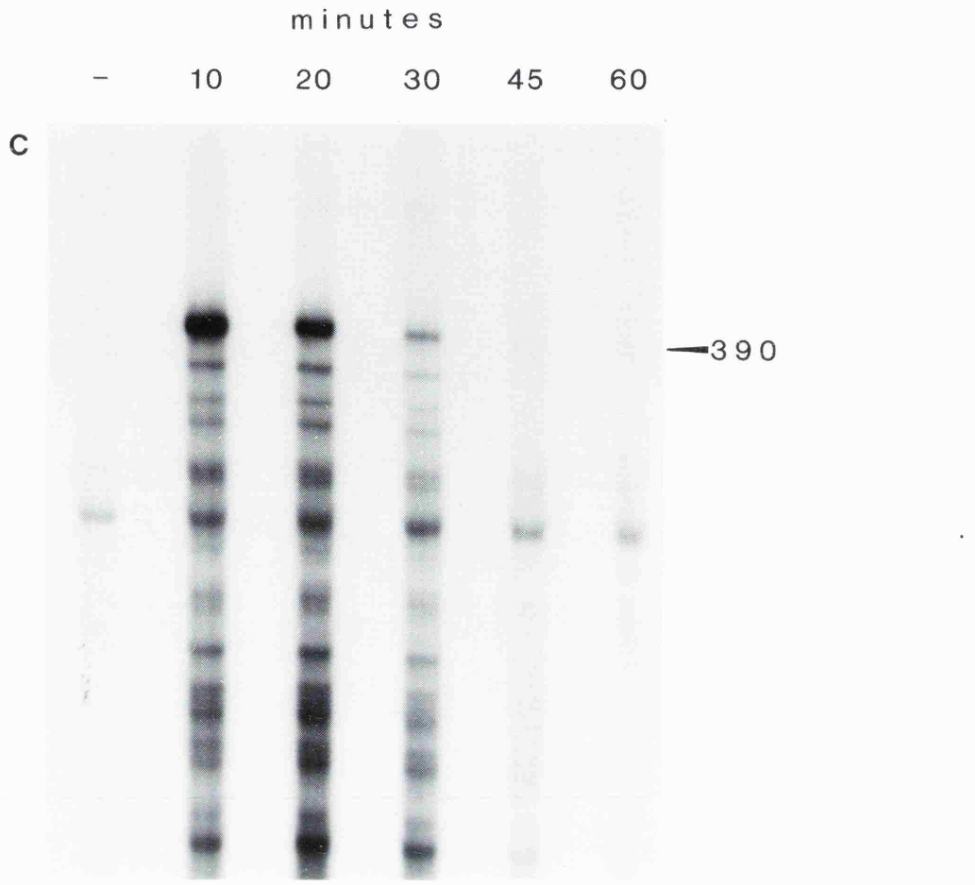
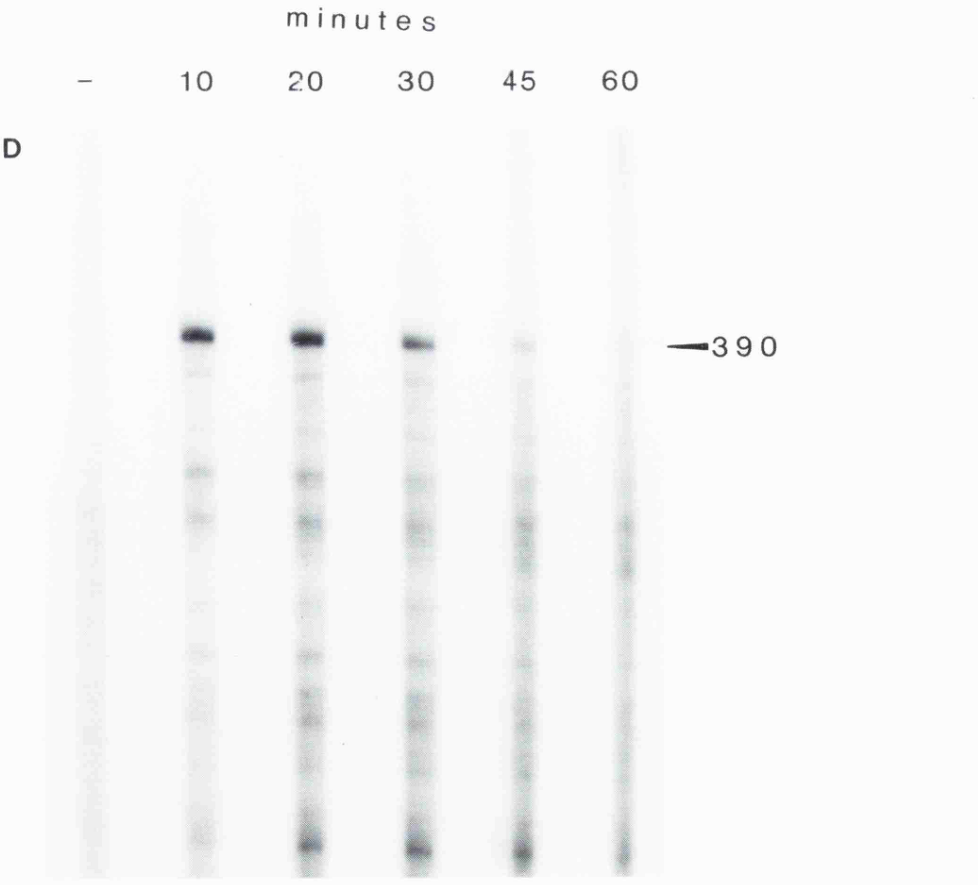

0
1
2
3
A
B
C
Sma I
pML(C2AT)19
3.5Kb

Figure 3.3

Time course for transcription of $pML(C_2AT)_{19}$ with various nuclear-protein extracts. A, rat liver; B, mouse kidney; C, mouse liver; D, mouse testis. Hereafter the symbol - represents transcription of $p(C_2AT)_{19}$ as a negative control. Figures on the right represent transcript length in bases.


minutes
–
30
45
60
75
90
A
390
minutes
–
10
20
30
45
60
B
390


minutes
–
10
20
30
45
60
C
390


minutes
–
10
20
30
45
60
D
390

elements, A and B, have been identified within this region.

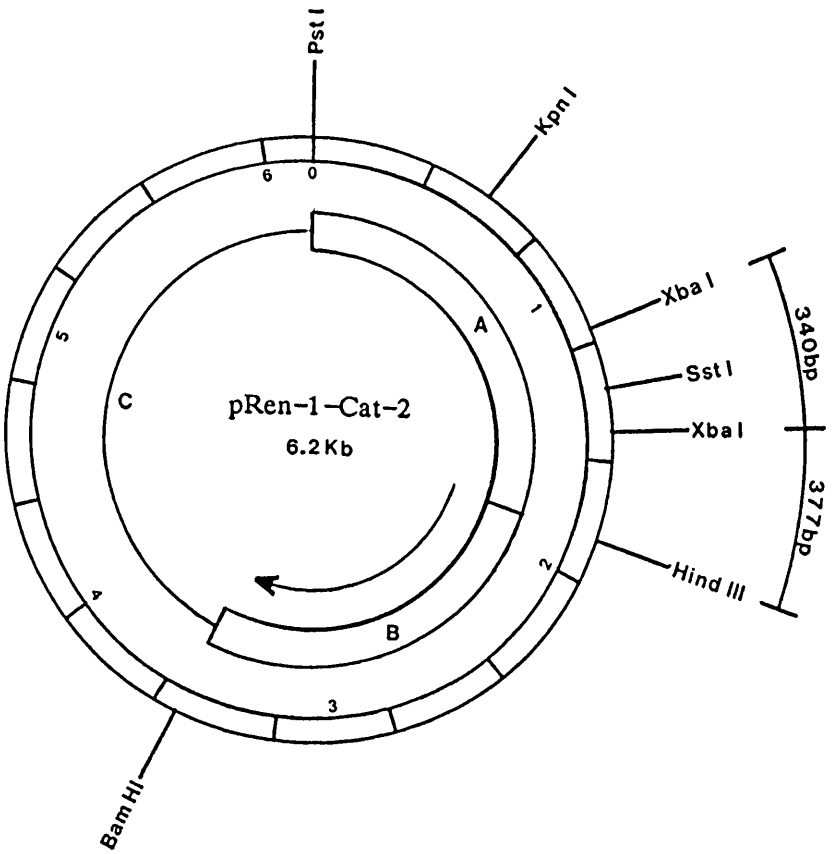
3.2 Establishing the *In–Vitro* Transcription Assay

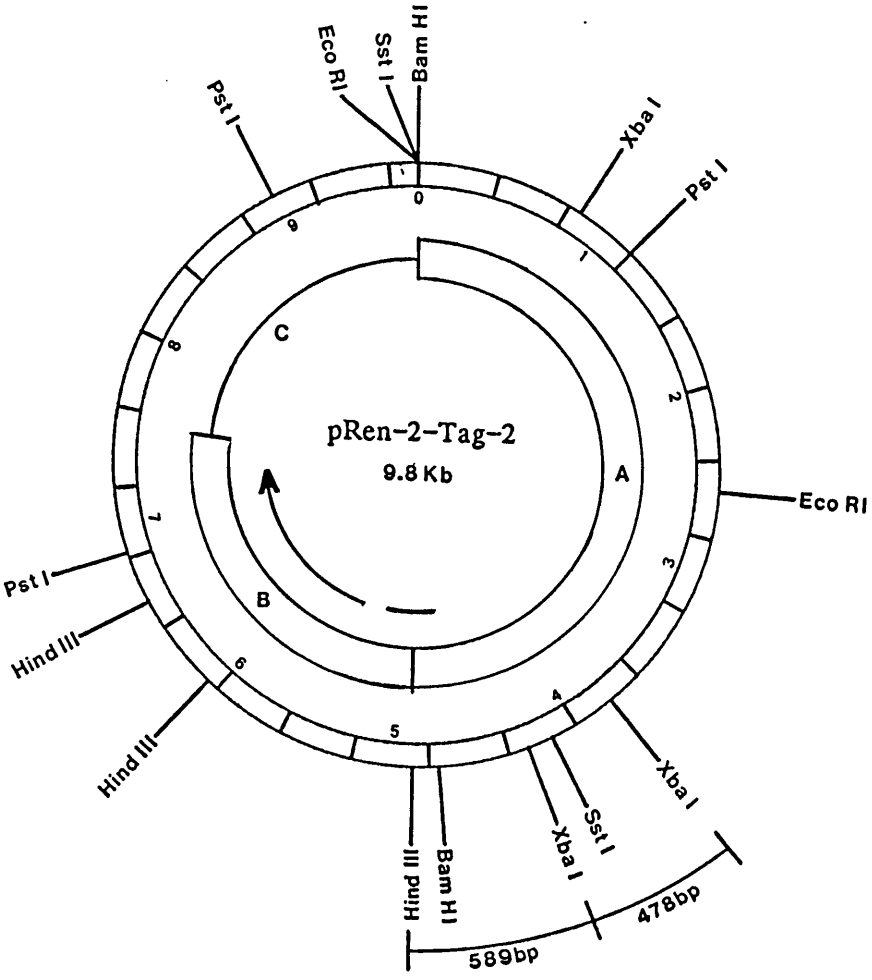
It appears from the above examples that the combination of promoter-driven G-free cassette and nuclear-protein extracts for *in–vitro* transcription would be a suitable assay for investigating tissue-specific mouse renin gene-expression. In order to establish the *in–vitro* transcription assay two templates were used, namely $p(C_2AT)_{19}$ and $pML(C_2AT)_{19}$ (which were a generous gift from M. Sawadogo and R.G. Roeder, The Rockefeller University, N.Y.) (Figures 3.2), together with nuclear-protein extracts prepared from adult Wistar male rat liver, and adult DBA/2 male mouse kidney, liver, testis and SMG. As described earlier, $p(C_2AT)_{19}$ consists of the 380bp G-free cassette; there is no promoter situated directly upstream, and hence this plasmid serves as a negative control template. $pML(C_2AT)_{19}$ has in addition the adenovirus-2 major-late (Ad-2 ML) promoter (-404 to +10 relative to the cap-site) situated 5' to the G-free cassette and under favourable conditions will generate a transcript of 390 nucleotides (Sawadogo and Roeder, 1985b). The Ad-2 ML promoter is a strong viral promoter and will generate specific-length transcripts from most transcriptionally active extracts (Gorski *et al.*, 1986; and Monaci *et al.*, 1988) and was used here as a positive control. Unless otherwise stated all plasmids used hereafter in the *in–vitro* transcription assay were covalently closed circular templates.

A time course for transcription of $pML(C_2AT)_{19}$ DNA with various nuclear-protein extracts is shown in Figure 3.3. *In–vitro* transcriptions were carried out as outlined in Section 2.26, except that 800ng of template were used for each incubation and the transcription reactions were stopped at the stated time points. Without a promoter $p(C_2AT)_{19}$ did not produce any full-length transcripts, whereas with $pML(C_2AT)_{19}$ the vast majority of transcripts were full-length 390 nucleotide RNA molecules. Although the rate of transcription with rat liver nuclear-protein extract was found to be many times greater than for any of the mouse extracts (data not shown), all were able to initiate transcription accurately by RNA polymerase II from the Ad-2 ML promoter. Furthermore, with rat liver extract there appeared to be little

Figure 3.4

Restriction site maps of I, pRen-1-Cat-2 and II, pRen-2-Tag-2. I: A, *Ren-1^d* 5' sequence; B, chloramphenicol acetyltransferase (CAT); C, pUC8. II: A, *Ren-2^d* 5' sequence; B, SV40 T-antigen; C, pAT153.


Pst I
Kpn I
Xba I
Sst I
Xba I
Hind III
Bam HI
340bp
377bp
pRen-1-Cat-2
6.2Kb
A
B
C
0
1
2
3
4
5
6


Eco RI
Sst I
Bam HI
Pst I
Xba I
Pst I
Eco RI
Xba I
Sst I
Xba I
Bam HI
Hind III
Hind III
Pst I
478bp
589bp
pRen-2-Tag-2
9.8Kb
A
B
C
0
1
2
3
4
5
6
7
8
9

if any degradation of the RNA produced, even after a 90 minutes incubation period, suggesting the absence of RNase activity with this extract preparation. However, with mouse kidney, liver and testis extracts marked degradative activity was present (compare Figure 3.3A with B, C and D), and a peak level of transcript was observed at 20 minutes. Attempts to prepare transcriptionally active mouse SMG nuclear-protein extracts have thus far been unsuccessful (data not shown). This is thought to be due to the highly degradative nature of this organ. Indeed Gorski *et al.* (1986) have reported the inability to produce transcriptionally competent nuclear-protein extract from rat parotid gland and they attributed their failure to endogenous DNase(s) rather than RNase(s) or protease(s).

Nuclear-protein extracts used for this and subsequent experiments were made from fresh tissue preparations; extracts were stored at -70°C and aliquots were thawed for individual assays. Extracts prepared from frozen tissue were found to be inactive and stored extracts which had been thawed more than once were also found to be inactive (data not shown).

3.3 Construction of pRen-$1^d(C_2AT)_{19}$ and pRen-$2^d(C_2AT)_{19}$

The 5' flanking sequences of *Ren-1^d* (1872bp) and *Ren-2^d* (4881bp) were obtained on the plasmids pRen-1-Cat-2 and pRen-2-Tag-2 respectively (which were a generous gift from C.C.J. Miller and A.T. Carter, ICI Joint Laboratory, Leicester University) (Figure 3.4). In both plasmids a *Hin*dIII restriction site has been introduced 14bp downstream of the major transcription start-site P3 (Field *et al.*, 1984) by replacement of a cytosine residue with a thymine residue at position +19. An absolute requirement for G-free cassette templates is the absence of guanine residues downstream of the cap-site of the promoter of interest. Both *Ren-1^d* and *Ren-2^d* promoters have two guanine residues at positions +6 and +13 respectively, between P3 and the *Hin*dIII restriction site (Figure 3.5). In order to prepare the renin promoters for the G-free cassette, these guanine residues have been replaced with adenine residues via site-directed mutagenesis and subsequently, pRen-$1^d(C_2AT)_{19}$ and pRen-$2^d(C_2AT)_{19}$ constructed by the following strategy.

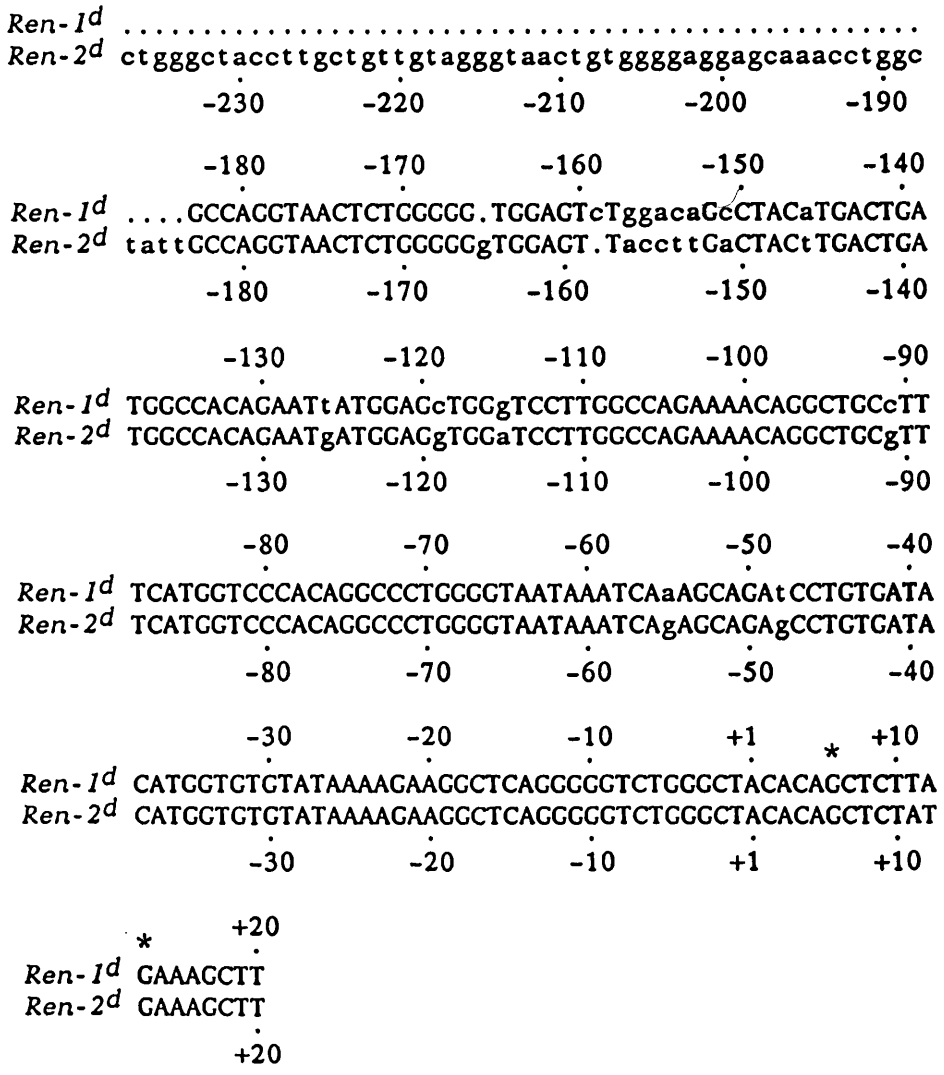


Figure 3.5

Nucleotide sequence of *Ren-1*^{d} and *Ren-2*^{d} showing the positions at which guanine residues were replaced with adenine residues (*) by site-directed mutagenesis.

3.3.1 Site-Directed Mutagenesis of the *Ren-1^d* and *Ren-2^d* Promoters

The *Xba*I-*Hin*dIII restriction fragments of both *Ren-1^d* (377bp) and *Ren-2^d* (589bp) (Figure 3.4) were purified, via LMP agarose gel-electrophoresis, and ligated into the polylinker of *Xba*I-*Hin*dIII digested M13mp19 respectively. The mixtures were used to transform *E. coli* TG1 cells and after screening RF DNA from white plaques for insert, by restriction endonuclease digestion, single-stranded template DNA for mutagenesis was prepared.

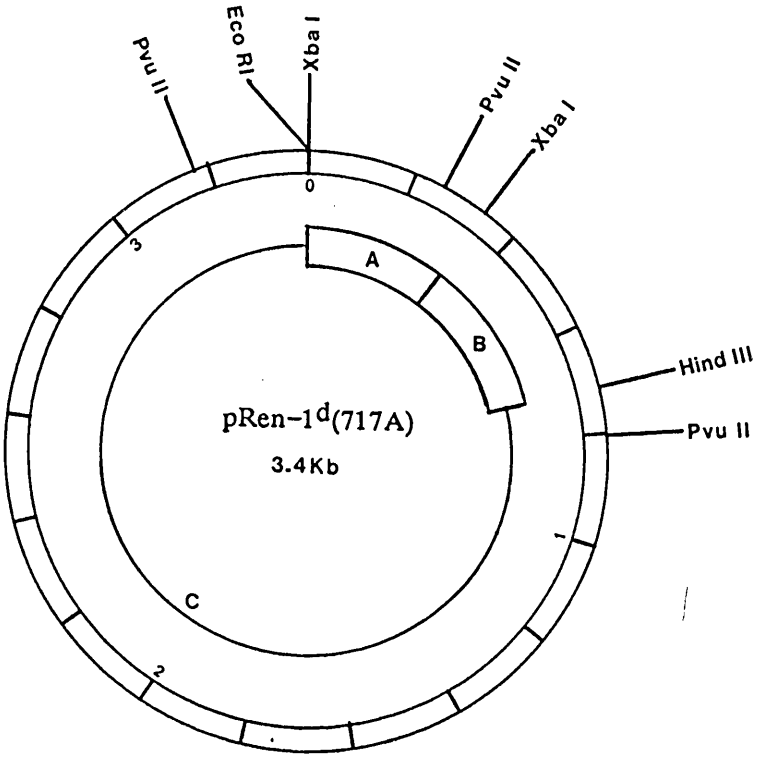
The mutagenesis protocol used was one developed by Kunkel (1985). Single-stranded template DNA was prepared from M13 phage grown in *E. coli* RZ1032, a *dut*$^-$ *ung*$^-$ strain. This strain is deficient in the enzyme dUTPase, the product of the *dut* gene, resulting in an increased intracellular pool of dUTP, which competes with dTTP for incorporation into DNA (Tye and Lehman, 1977). Uracil thus incorporated is not removed, due to the deficiency in the product of the *ung* gene, uracil glycosylase (Warner *et al.*, 1981). The M13 DNA template purified from the phage thus contains 20-30 uracil residues per genome (Sagher and Strauss, 1983). After annealing of mutant oligonucleotide ADS1 to the single-stranded templates followed by synthesis and ligation (in the absence of dUTP) of the second strand, the mixtures were used to transform a normal *dut*$^+$ *ung*$^+$ host, *E. coli* JM101 respectively. During phage replication the dUTP containing template was degraded by the hosts uracil glycosylase, whereas the mutant template remains intact. Single-stranded template was prepared from several of the transformants and M13 sequencing revealed that 80% contained the desired substitution (data not shown).

3.3.2 Preparation of pRen-1^d(717A) and pRen-2^d(1067A)

After large-scale purification of RF DNA by caesium chloride/ ethidium bromide density-gratient centrifugation, the mutated *Ren-1^d* (377bp) and *Ren-2^d* (589bp) fragments (hereafter called 377A and 589A respectively) were cut out of M13mp19 by an *Xba*I-*Hin*dIII digestion. After purification, via LMP agarose gel-electrophoresis, they were ligated into the polylinker of *Xba*I-*Hin*dIII digested pUC18

Figure 3.6

Restriction site maps of pRen-1^{d}(717A) and pRen-2^{d}(1067A). A, fragments 340 and 478 respectively; B, fragments 377A and 589A respectively; C, pUC18.


Pvu II
Eco RI
Xba I
Pvu II
Xba I
Hind III
Pvu II
pRen-1d(717A)
3.4Kb
A
B
C
0
1
2
3

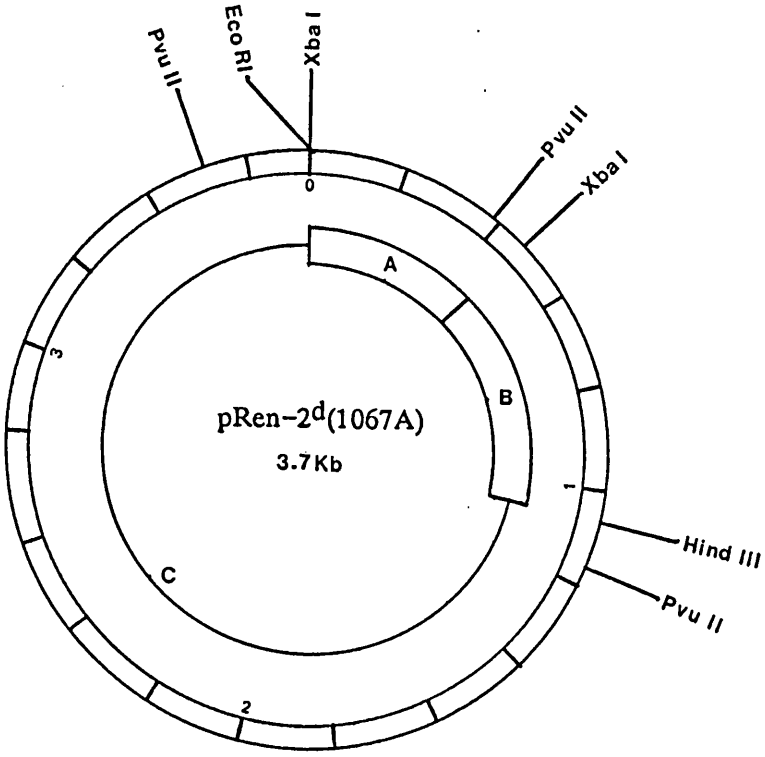
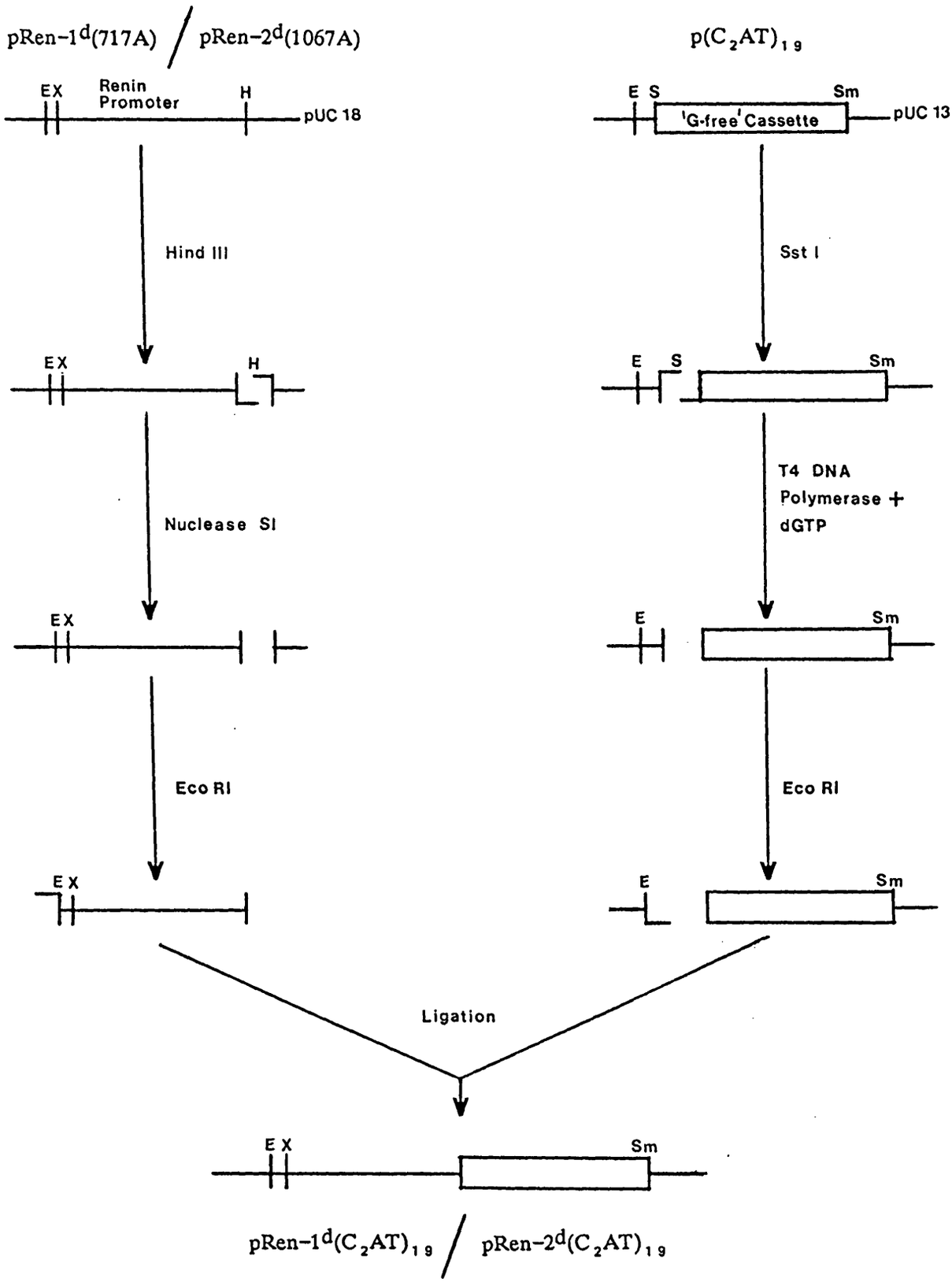

Pvu II
Eco RI
Xba I
Pvu II
Xba I
Hind III
Pvu II
pRen-2d(1067A)
3.7Kb
A
B
C
0
1
2
3

Figure 3.7

Scheme showing the construction of pRen-1$^{d}(C_2AT)_{19}$ and pRen-2$^{d}(C_2AT)_{19}$. Restriction sites are as follows: E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; Sm, *Sma*I; X, *Xba*I.


pRen-1d(717A) / pRen-2d(1067A)
EX
Renin Promoter
H
pUC 18
Hind III
EX
H
Nuclease SI
EX
Eco RI
EX
p(C2AT)19
E S
Sm
'G-free' Cassette
pUC 13
Sst I
E S
Sm
T4 DNA Polymerase + dGTP
E
Sm
Eco RI
E
Sm
Ligation
EX
Sm
pRen-1d(C2AT)19 / pRen-2d(C2AT)19

to give the plasmids pRen-1^{d}(377A) and pRen-2^{d}(589A) respectively.

The *Xba*I-*Xba*I restriction fragments of both *Ren-1^{d}* (340bp) and *Ren-2^{d}* (478bp) (Figure 3.4) (hereafter called 340 and 478 respectively) were purified, via LMP agarose gel-electrophoresis, and ligated into the *Xba*I site of pRen-1^{d}(377A) and pRen-2^{d}(589A) respectively. The orientation of fragments 340 (with respect to 377A) and 478 (with respect to 589A) were determined via restriction endonuclease digestion, due to the presence within fragments 340 and 478 of an asymmetrical *Pvu*II restriction site. The sense orientation of fragments 430 and 478 with respect to 377 and 589 gave the plasmids pRen-1^{d}(717A) and pRen-2^{d}(1067A) (Figure 3.6) respectively.

3.3.3 Fusion of the *Ren-1^{d}* and *Ren-2^{d}* Promoters to the G-free Cassette Respectively

A schematic representation of the construction of pRen-1^{d}(C_2AT)$_{19}$ and pRen-2^{d}(C_2AT)$_{19}$ is given in Figure 3.7. The plasmids pRen-1^{d}(717A) and pRen-2^{d}(1067A) were cut with the restriction endonuclease *Hin*dIII, and after purification by direct extraction, were treated with nuclease S1 to generate blunt ends. Following a second round of purification by direct extraction, both plasmids were cut with the restriction endonuclease *Eco*RI and the insert fragments (containing the renin promoters) were then purified via LMP agarose gel-electrophoresis.

The G-free cassette plasmid p(C_2AT)$_{19}$ was cut with the restriction endonuclease *Sst*I, and after purification by direct extraction, was blunt-ended using the 3' to 5' exonuclease activity of T4 DNA polymerase in the presence of dGTP (Maniatis *et al.*, 1982). Following a second round of purification by direct extraction, p(C_2AT)$_{19}$ was cut with the restriction endonuclease *Eco*RI and the large fragment (containing the G-free cassette) was purified via LMP agarose gel-electrophoresis.

The purified insert fragments from pRen-1^{d}(717A) and pRen-2^{d}(1067A) were now ligated with the large fragment of p(C_2AT)$_{19}$ and the mixtures used to transform *E. coli* JM101 cells

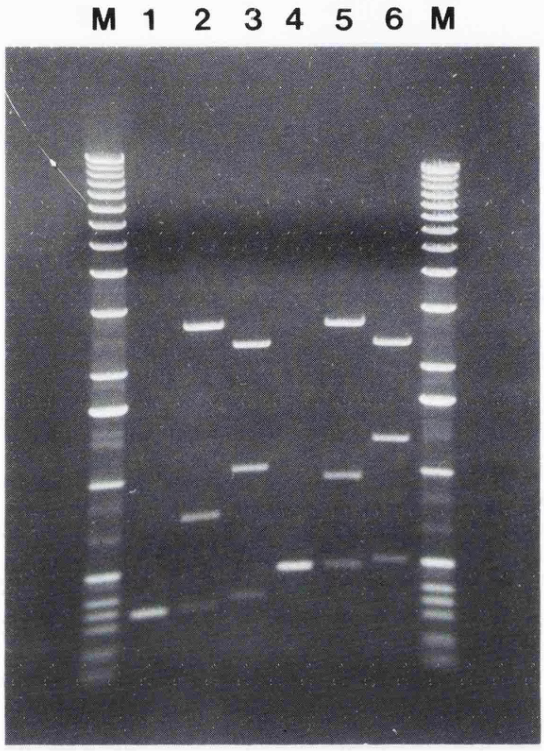


Figure 3.8

Restriction endonuclease digestion of pRen-1$^{d}(C_2AT)_{19}$ (1-3) and pRen-2$^{d}(C_2AT)_{19}$ (4-6). 1 and 4, fragments 340 and 478 respectively; 2 and 5, *Xba*I digestion; 3 and 6, *Pvu*II digestion. Hereafter M represents the 1Kb ladder (see Appendix A).

Figure 3.9

Plasmid sequencing of A, pRen-1$^{d}(C_2AT)_{19}$ and B, pRen-2$^{d}(C_2AT)_{19}$. Arrows represent the sites at which guanine residues have been replaced with adenine residues by site-directed mutagenesis.

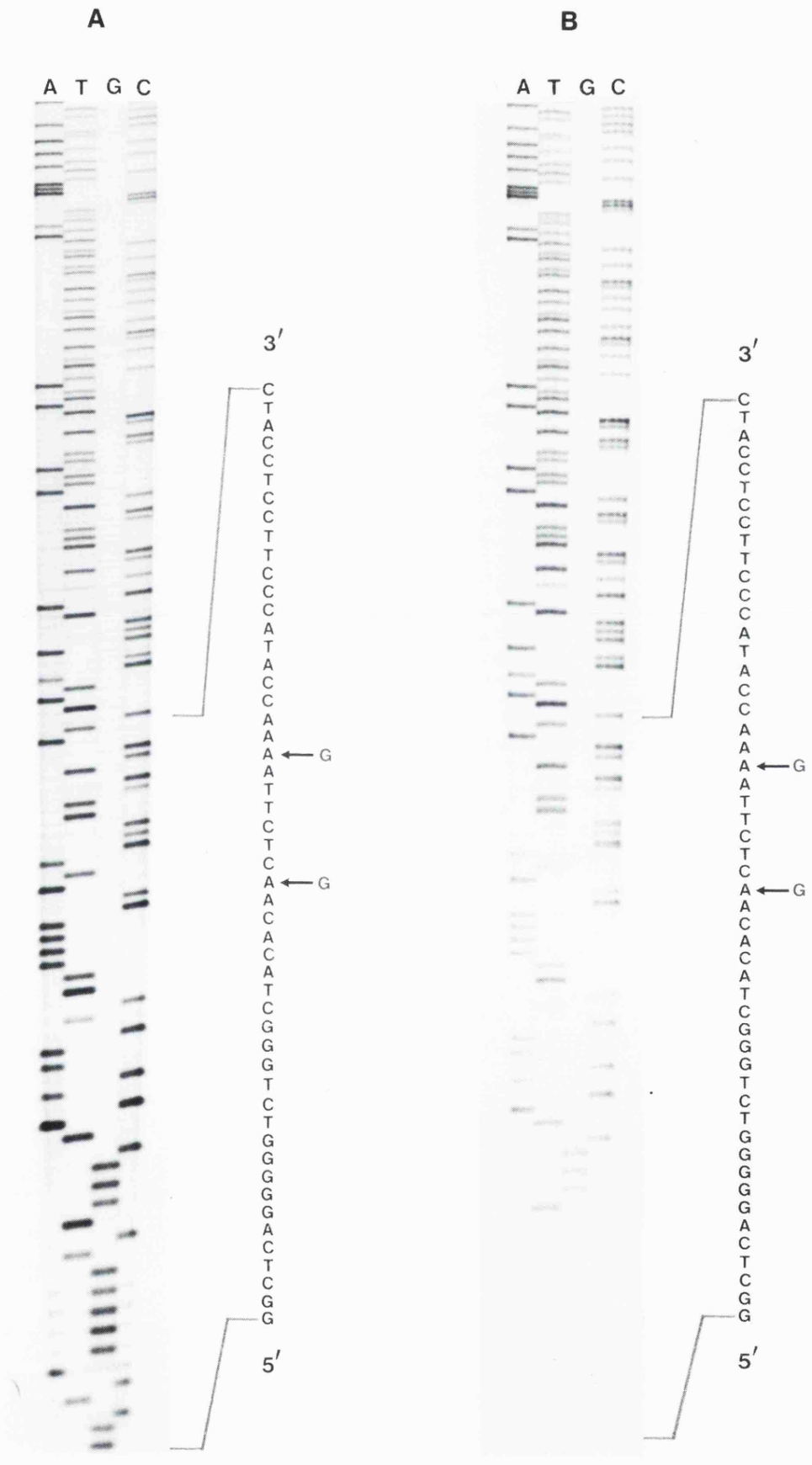
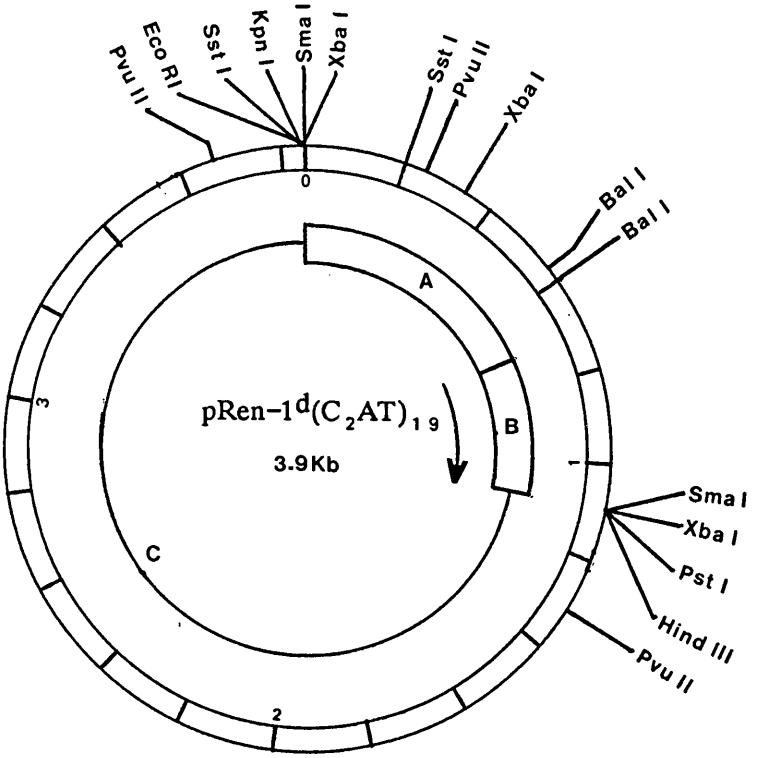

A
A T G C
3′
C T A C C T C C T T C C C C A T A C C A A A A T T C T C A A C A C A T C G G G T C T G G G G G A C T C G G
G
G
5′
B
A T G C
3′
C T A C C T C C T T C C C C A T A C C A A A A T T C T C A A C A C A T C G G G T C T G G G G G A C T C G G
G
G
5′

Figure 3.10

Restriction site maps of pRen-1$^{d}(C_2AT)_{19}$ and pRen-2$^{d}(C_2AT)_{19}$. A, *Ren-1^{d}* (717bp) and *Ren-2^{d}* (1067bp) promoters respectively; B, G-free cassette; C, pUC13.


Pvu II
Eco RI
Sst I
Kpn I
Sma I
Xba I
Sst I
Pvu II
Xba I
Bal I
Bal I
A
B
C
pRen-1d(C2AT)19
3.9Kb
Sma I
Xba I
Pst I
Hind III
Pvu II
0
1
2
3

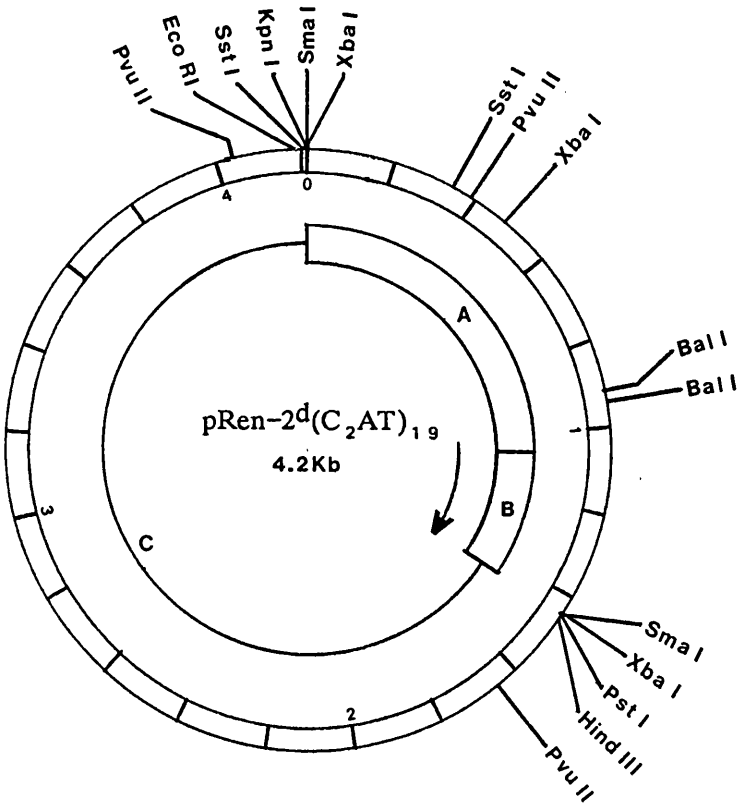
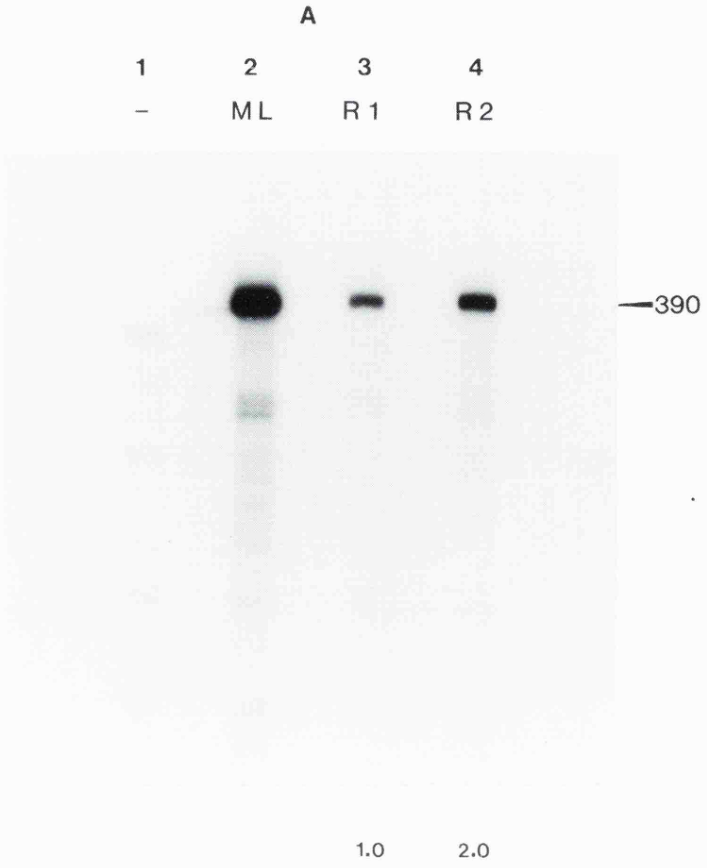

Pvu II
Eco RI
Sst I
Kpn I
Sma I
Xba I
Sst I
Pvu II
Xba I
Bal I
Bal I
A
B
C
pRen-2d(C2AT)19
4.2Kb
Sma I
Xba I
Pst I
Hind III
Pvu II
0
1
2
3
4

Figure 3.11

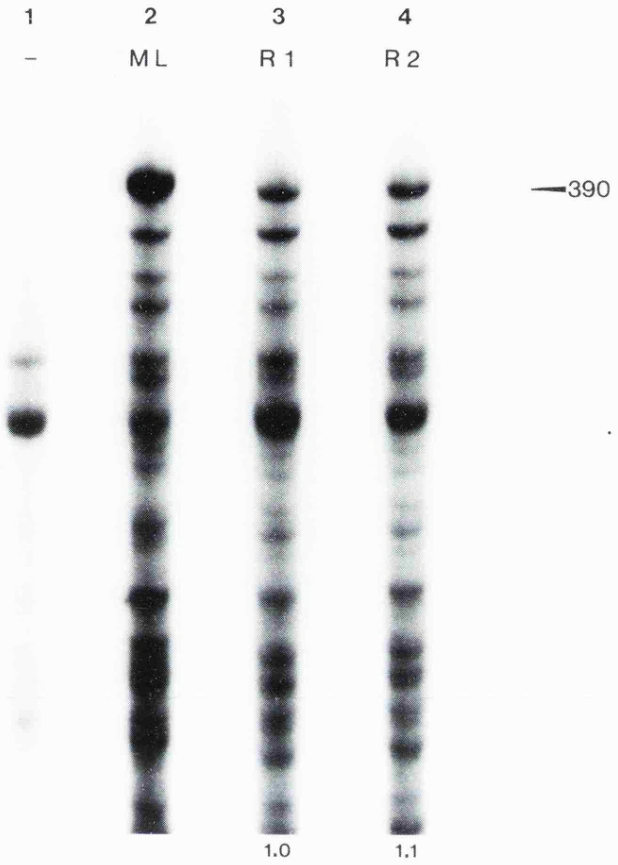
In-vitro transcription of renin promoter/G-free cassette fusion templates in A, rat liver; B, mouse kidney; C, mouse liver; and D, mouse testis nuclear-protein extracts. 1, $p(C_2AT)_{19}$; 2, $pML(C_2AT)_{19}$ (positive control); 3, $pRen\text{-}1^d(C_2AT)_{19}$; 4, $pRen\text{-}2^d(C_2AT)_{19}$. Figures on the right represent transcript length in bases. Figures on the bottom represent the relative transcription efficiency between the *Ren-1^d* and *Ren-2^d* promoters in driving the G-free cassette.

A



B

C



D

respectively. Transformants were initially screened for the presence of insert by restriction endonuclease digestion (Figure 3.8) and screened further by plasmid sequencing (Figure 3.9). Correct fusion between the renin promoters (base pairs -702 to +15 for *Ren-1^d* and base pairs -1052 to +15 for *Ren-2^d*) and the G-free cassette gave the two templates pRen-1^d(C_2AT)$_{19}$ and pRen-2^d(C_2AT)$_{19}$ respectively (Figure 3.10). Under favourable conditions these templates will generate a transcript of 390 nucleotides.

3.4 Activity of the *Ren-1^d* and *Ren-2^d* Promoters in Driving the G-free Cassette

In all incubations hereafter p(C_2AT)$_{19}$ and pML(C_2AT)$_{19}$ were used as negative and positive controls respectively, for the transcriptional competence of each extract. *In-vitro* transcriptions with pRen-1^d(C_2AT)$_{19}$ and pRen-2^d(C_2AT)$_{19}$ templates were carried out with nuclear-protein extracts prepared from adult Wistar male rat liver and adult DBA/2 male mouse kidney, liver and testis. With rat liver, mouse kidney and mouse testis extracts (Figures 3.11A, B and D respectively) the activity of pRen-2^d(C_2AT)$_{19}$ was approximately 2-fold higher than for pRen-1^d(C_2AT)$_{19}$, whereas with mouse liver (Figure 3.11C) both templates were transcribed equally well (as measured by laser densitometry). The 2-fold difference may reflect either a positive effect on the *Ren-2^d* promoter or a negative effect on the *Ren-1^d* promoter. Indeed recently the presence in *Ren-1^d* of *cis*-acting negative control element(s) has been reported by Nakamura *et al.* (1989) (see Chapters 4 and 7). Furthermore the observation of equal activity between the *Ren-1^d* and *Ren-2^d* promoters in mouse liver extract suggests that this negative control may be tissue-specific but further investigation is required.

CHAPTER 4

RESULTS

Deletion-Mapping of the *Ren-1^d* and *Ren-2^d* Promoter Fragments

4.1 Internal Control

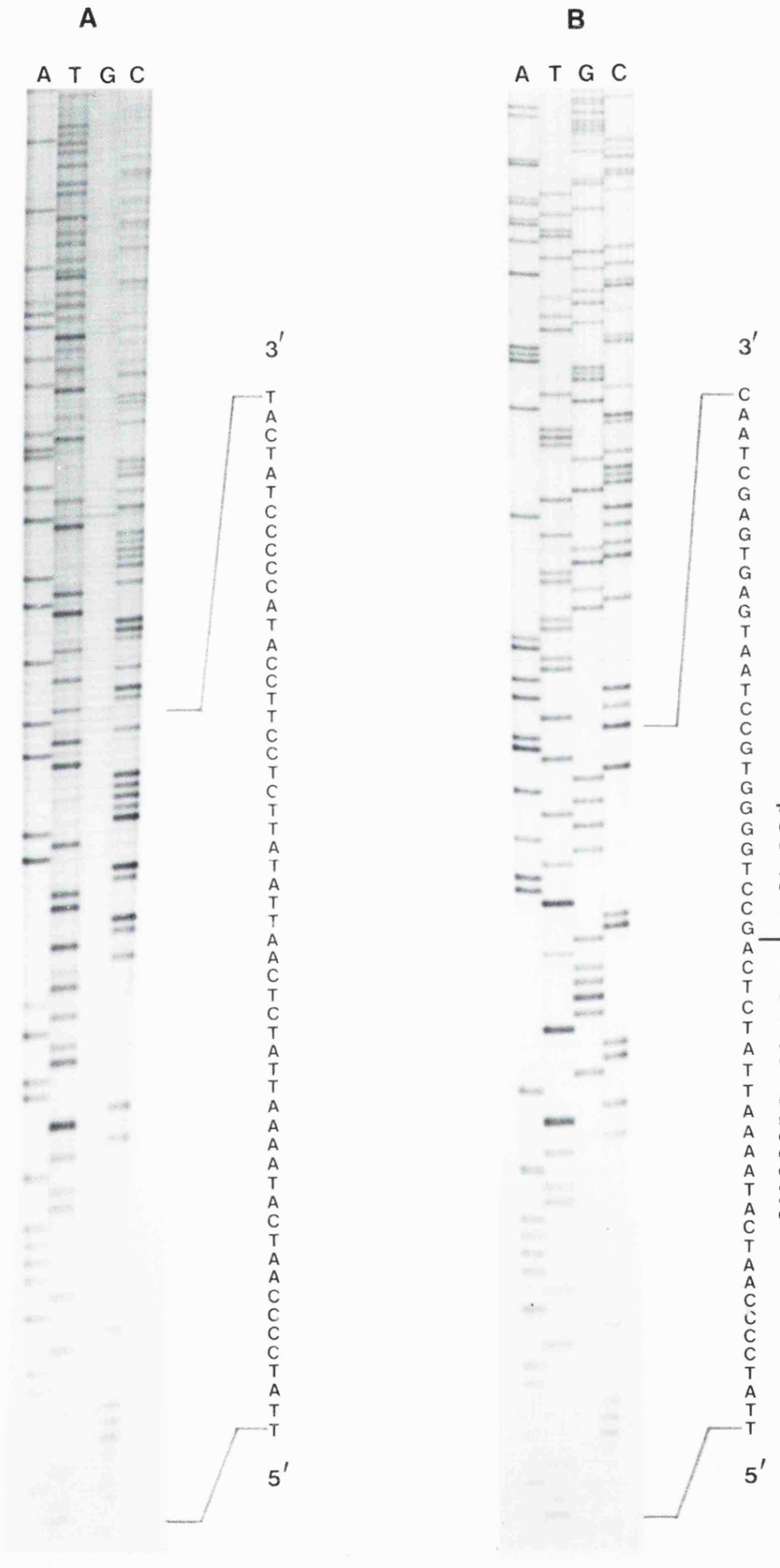
In order to investigate further the approximately 2-fold higher transcriptional activity of the *Ren-2^d* promoter over the *Ren-1^d* promoter in certain nuclear-protein extracts (see Section 3.4), deletion-mapping of both mouse promoters was embarked upon. To discriminate between real differences in transcription rate and those arising from sample gel-loading errors, an internal control template pRen-1^d(C_2AT)$_{19}$$\triangle$150 was constructed. The control template was prepared from pRen-1^d(C_2AT)$_{19}$ (see Section 3.3) and contains a shortened (by 150 dNMPs) G-free cassette fused to the *Ren-1^d* promoter; when transcribed it produces a transcript of only 240 NMPs compared to that of 390 NMPs produced by pRen-1^d(C_2AT)$_{19}$. Thus within a single track both full-length and control transcripts can be easily distinguished. When comparing activities between different test templates, the ratio of intensity between the control and test transcripts within a single track was compared with the ratios produced with other test templates.

4.1.1 Construction of pRen-1^d(C_2AT)$_{19}$$\triangle$150

pRen-1^d(C_2AT)$_{19}$ was digested with the restriction endonuclease *Pst*I and subsequently purified by direct extraction. Using the exonuclease activity of *Bal*31 a nested set of deletions was produced from the *Pst*I site and following a second round of purification by direct extraction these were blunt-ended using the activities of the Klenow fragment of *E. coli* DNA polymerase I. Following ligation each set of deleted fragments were used to transform *E. coli* JM101 cells. Transformants were initially screened by restriction endonuclease digestion and potentially suitable templates were screened further by plasmid

Figure 4.1

Plasmid sequencing of A, pRen-1$^{d}(C_2AT)_{19}$ and B, pRen-1$^{d}(C_2AT)_{19}\triangle 150$ where the G-free cassette has been shortened by 150 dNMPs.


A
A T G C
3′
TACTATCCCCCATACCTTCCTCTTATATTAACTCTATTAAAATACTAACCCCTATT
5′
B
A T G C
3′
CAATCGAGTGAGTAATCCGTGGGGTCCGACTCTATTAAAATACTAACCCCTATT
5′
pUC 13
'G-free' Cassette

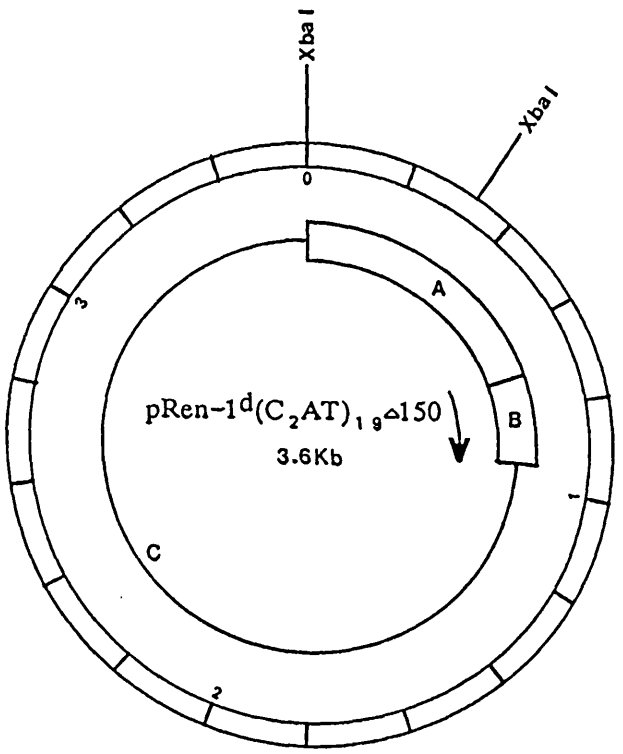


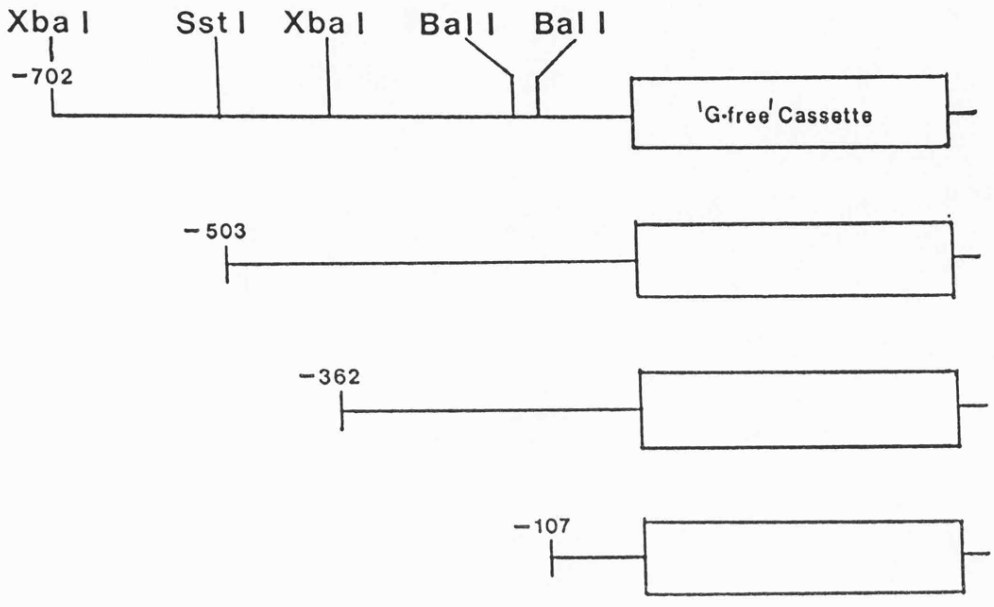
Figure 4.2

Restriction site map of pRen-$1^{d}(C_2AT)_{19}\Delta150$. A, *Ren-1^{d}* (717bp) promoter; B, shortened G-free cassette; C, pUC13.

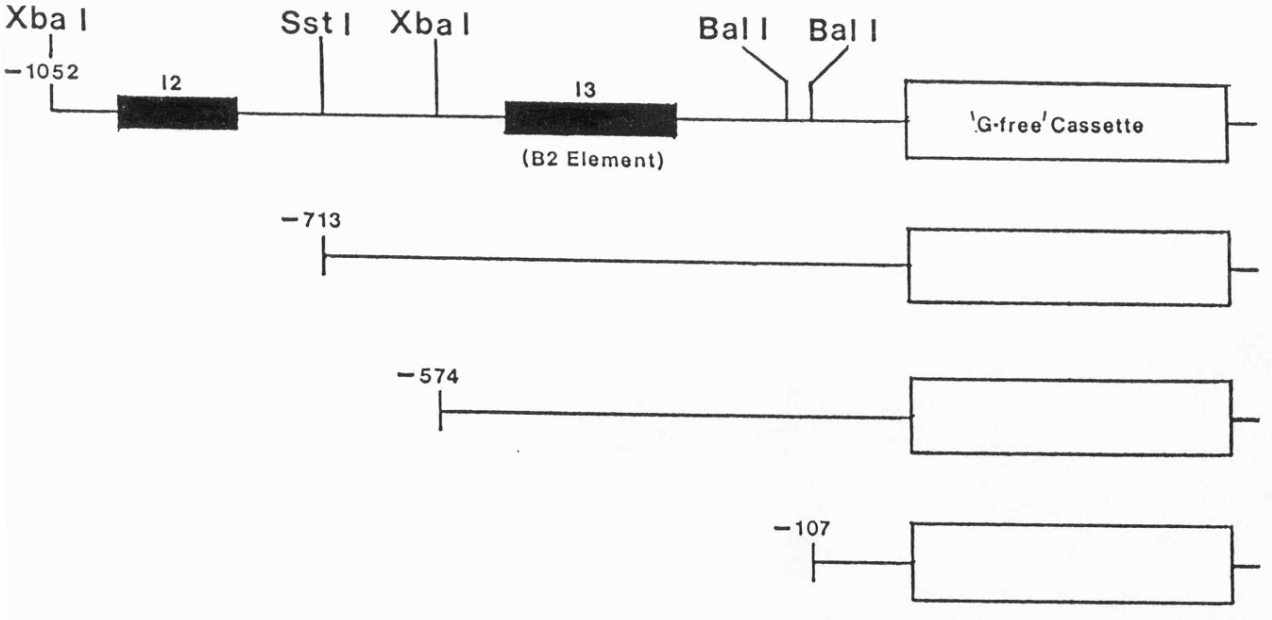
Figure 4.3

Restriction sites used for deletion-mapping of the *Ren-1*^{d} and *Ren-2*^{d} promoters.

Ren–1^d


Xba I
Sst I
Xba I
Ball
Ball
−702
'G-free' Cassette
−503
−362
−107

Ren–2^d


Xba I
Sst I
Xba I
Ball
Ball
−1052
I2
I3
(B2 Element)
'G-free' Cassette
−713
−574
−107

sequencing (Figure 4.1). One such template, pRen-1^{d}(C_2AT)$_{19}$△150 (Figure 4.2), with a shortened (by 150 dNMPs) G-free cassette was used hereafter in all incubations as an internal control template.

4.2 Gross Deletion-Mapping of the *Ren-1^{d}* and *Ren-2^{d}* Promoters by Restriction Endonuclease Digestion

Initial dissection of both *Ren-1^{d}* and *Ren-2^{d}* promoters was achieved by restriction endonuclease digestion and the sites used are illustrated in Figure 4.3. The templates pRen-1^{d}(C_2AT)$_{19}$ and pRen-2^{d}(C_2AT)$_{19}$ were used to produced deletions to the *Sst*I and *Bal*I restriction sites. However the renin promoter/G-free cassette fusions (for both pRen-1^{d}(C_2AT)$_{19}$ and pRen-2^{d}(C_2AT)$_{19}$) first had to be inverted within the pUC13 polylinker to enable deletions to the *Xba*I restriction site to be prepared (compare the templates pRen-1^{d}(C_2AT)$_{19}$ and pRen-2^{d}(C_2AT)$_{19}$, Figure 3.10, with pRen-1^{d}*(C_2AT)$_{19}$ and pRen-2^{d}*(C_2AT)$_{19}$, Figure 4.8, respectively).

4.2.1 Construction of Templates with Deletions to the *Sst*I Restriction Site

pRen-1^{d}(C_2AT)$_{19}$ and pRen-2^{d}(C_2AT)$_{19}$ were digested with the restriction endonuclease *Sst*I and the large fragments purified via LMP agarose gel-electrophoresis. Following ligation both plasmids were used to transform *E. coli* JM101 cells respectively and transformants were screened by restriction endonuclease digestion (Figure 4.4). Deletions to the *Sst*I restriction site gave the templates pRen-1^{d}△S(C_2AT)$_{19}$ and pRen-2^{d}△S(C_2AT)$_{19}$ respectively (Figure 4.5).

4.2.2 Construction of Templates with Deletions to the *Bal*I Restriction Site

The large fragments of *Eco*RI-*Bal*I digested pRen-1^{d}(C_2AT)$_{19}$ and pRen-2^{d}(C_2AT)$_{19}$ were purified, via LMP agarose gel-electrophoresis, and then blunt-ended using the 5' to 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. Following ligation both plasmids were used to transform

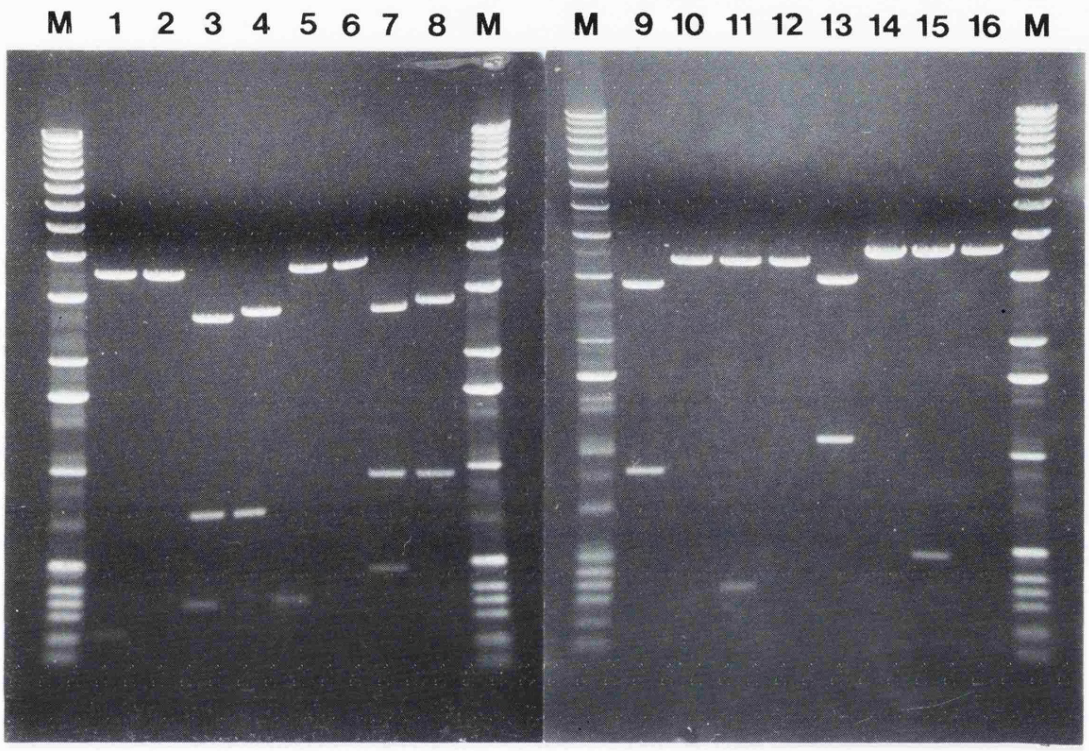
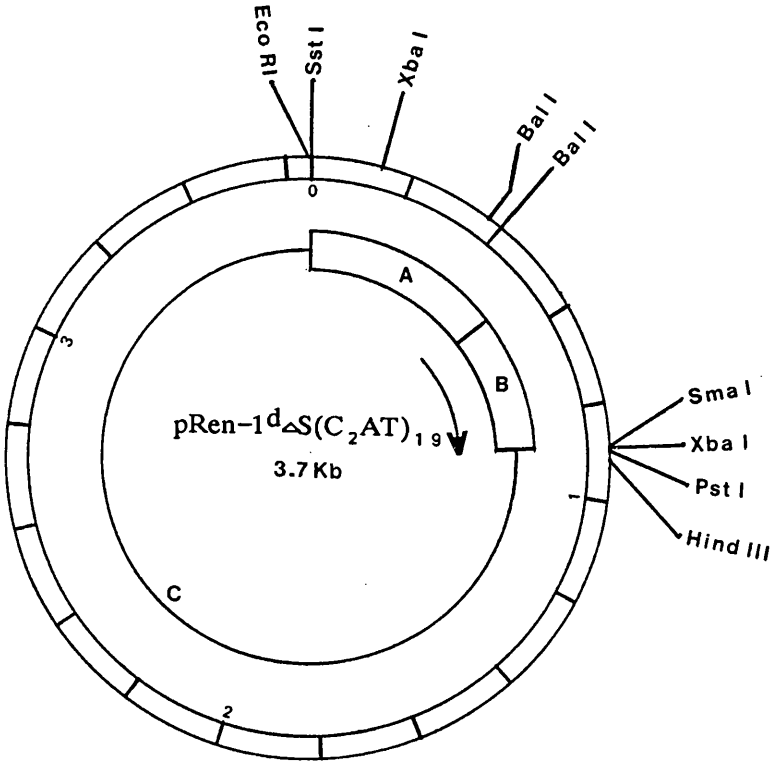


Figure 4.4

Restriction endonuclease digestion of templates with deletions to the *Sst*I and *Xba*I sites respectively. 1 and 3, pRen-1$^{d}(C_2AT)_{19}$; 5 and 7, pRen-2$^{d}(C_2AT)_{19}$; 2 and 4, pRen-1$^{d}\triangle S(C_2AT)_{19}$; 6 and 8, pRen-2$^{d}\triangle S(C_2AT)_{19}$; 9 and 11, pRen-1$^{d*}(C_2AT)_{19}$; 13 and 15, pRen-2$^{d*}(C_2AT)_{19}$; 10 and 12, pRen-1$^{d*}\triangle X(C_2AT)_{19}$; 14 and 16, pRen-2$^{d*}\triangle X(C_2AT)_{19}$. 1,2,5,6,9,10,13 and 14, *Sst*I digestion; 3,4,7,8,11,12,15 and 16, *Xba*I digestion.

Figure 4.5

Restriction site maps of pRen-1$^{d}\triangle S(C_2AT)_{19}$ and pRen-2$^{d}\triangle S(C_2AT)_{19}$. A, *Ren-1^{d}* (518bp) and *Ren-2^{d}* (728bp) promoters respectively; B, G-free cassette; C, pUC13.


Eco RI
Sst I
Xba I
Bal I
Bal I
Sma I
Xba I
Pst I
Hind III
A
B
C
pRen-1$^{d}\triangle S(C_2AT)_{19}$
3.7Kb
0
1
2
3

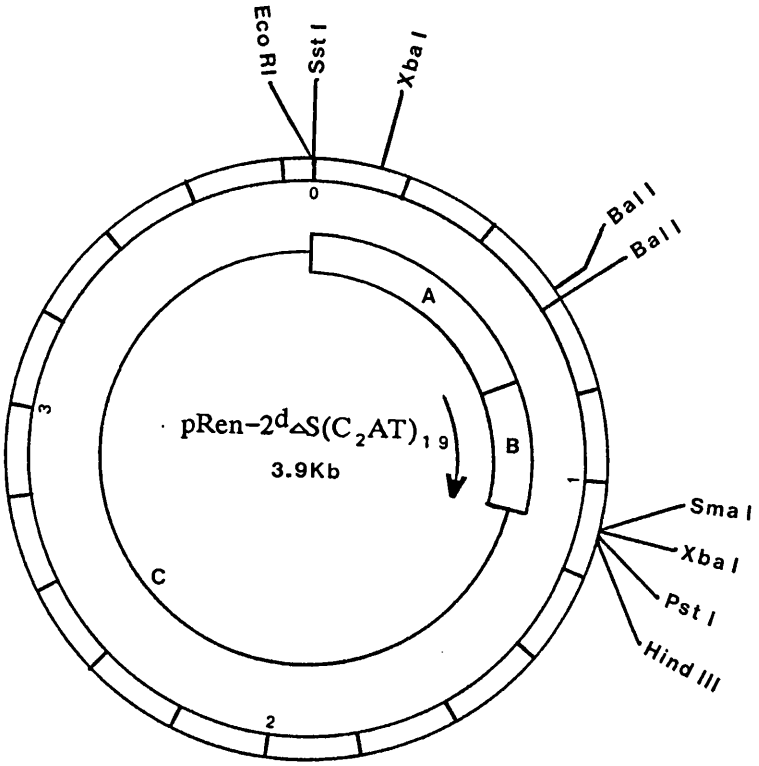
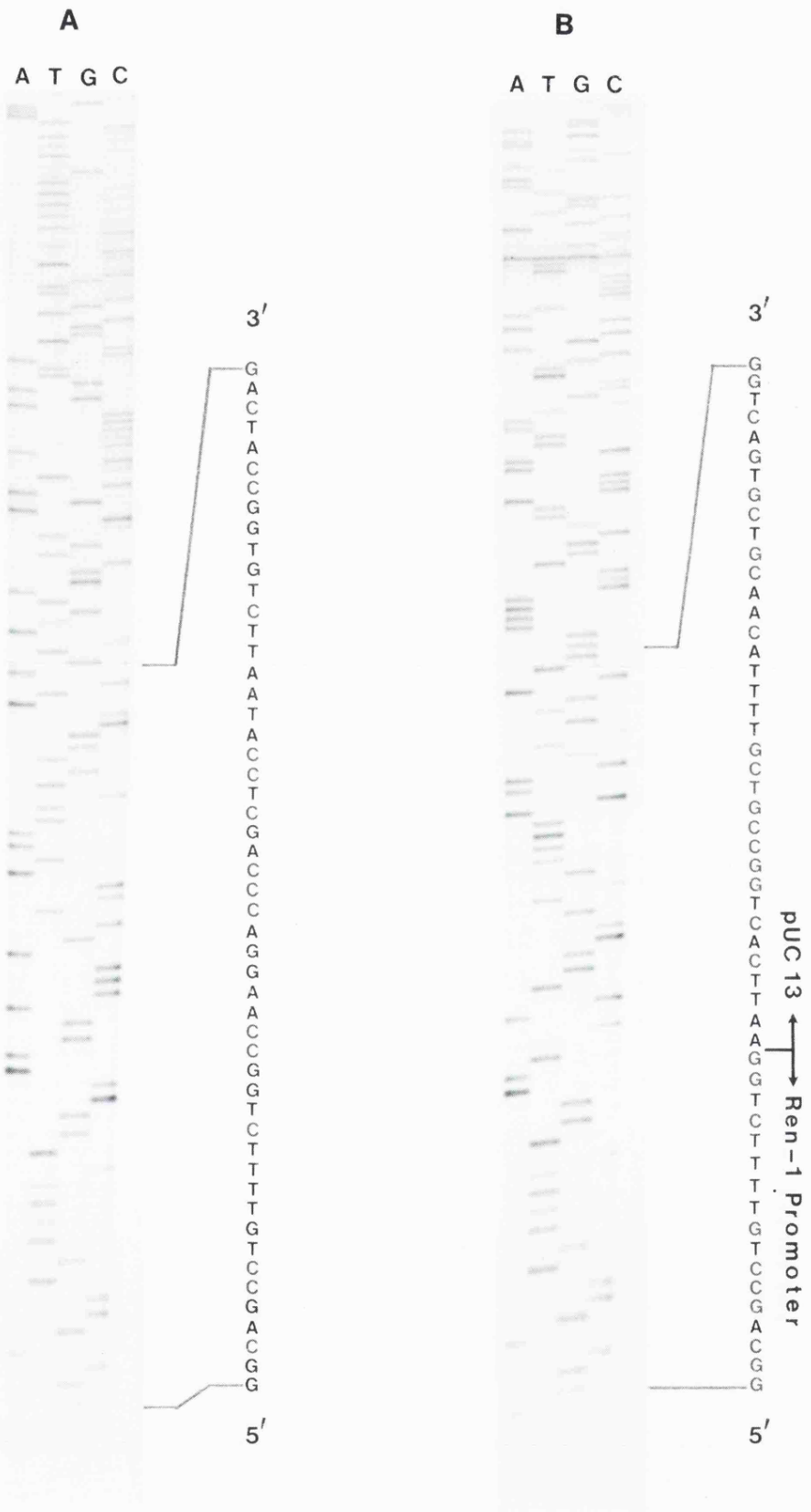

Eco RI
Sst I
Xba I
Bal I
Bal I
Sma I
Xba I
Pst I
Hind III
A
B
C
pRen-2$^{d}\triangle S(C_2AT)_{19}$
3.9Kb
0
1
2
3

Figure 4.6

Plasmid sequencing of pRen-1$^{d}\triangle B(C_2AT)_{19}$ and pRen-2$^{d}\triangle B(C_2AT)_{19}$ to confirm deletion to the *Bal*I restriction site. A, pRen-1$^{d}(C_2AT)_{19}$; B, pRen-1$^{d}\triangle B(C_2AT)_{19}$; C, pRen-2$^{d}(C_2AT)_{19}$; D, pRen-2$^{d}\triangle B(C_2AT)_{19}$.

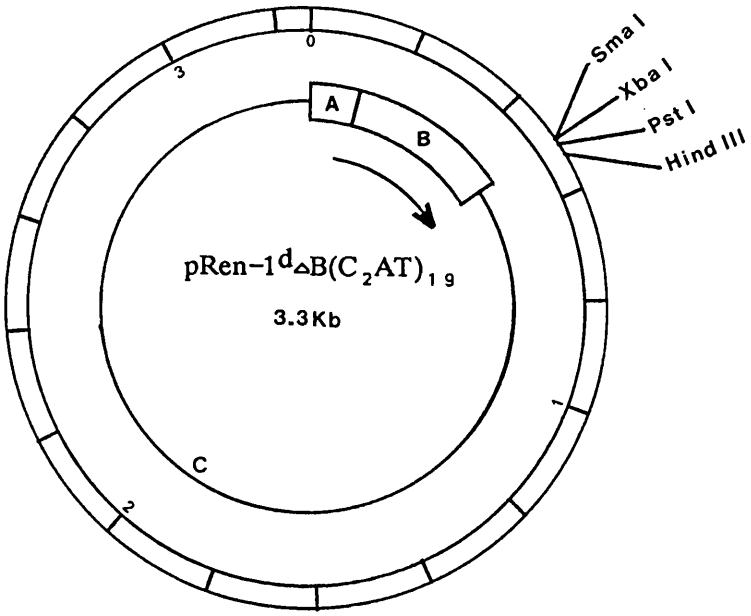

A
A T G C
3′
GACTACCGGTGTCTTAATACCTCGACCCAGGAACCGGTCTTTTGTCCGACGG
5′
B
A T G C
3′
GGTCAGTGCTGCAACATTTTGCTGCCGGTCACTTAAGGTCTTTTGTCCGACGG
5′
pUC 13
Ren-1 Promoter


C
A T G C
3′
GACTACCGGTGTCTTACTACCTCCACCTAGGAACCGGTCTTTTGTCCGACGG
5′


D
A T G C
3′
TCAGTGCTGCAACATTTTGCTGCCGGTCACTTAAGGTCTTTTGTCCGACGG
5′
pUC 13
Ren-2 Promoter

Figure 4.7

Restriction site maps of pRen-1$^{d}\triangle$B$(C_2AT)_{19}$ and pRen-2$^{d}\triangle$B$(C_2AT)_{19}$. A, *Ren-1^{d}* (122bp) and *Ren-2^{d}* (122bp) promoters respectively; B, G-free cassette; C, pUC13.


0
1
2
3
A
B
C
Sma I
Xba I
Pst I
Hind III
pRen-1$^{d}_{\Delta}$B(C$_2$AT)$_{19}$
3.3Kb

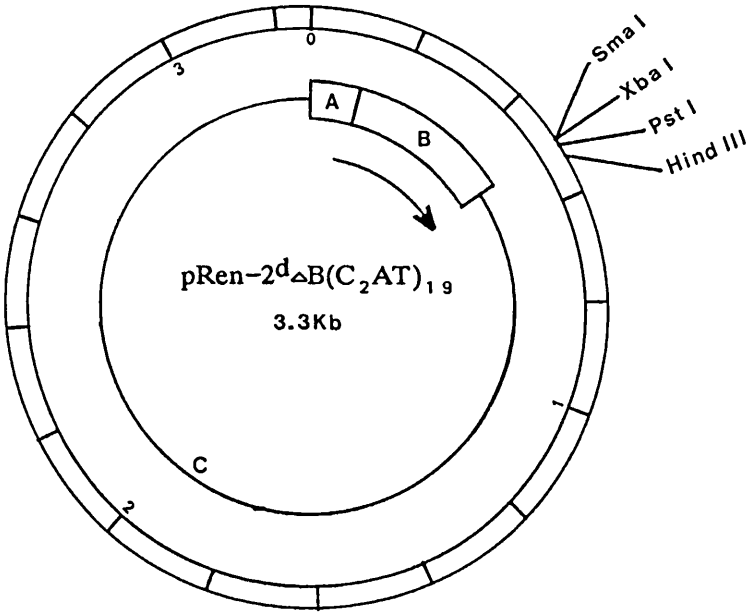
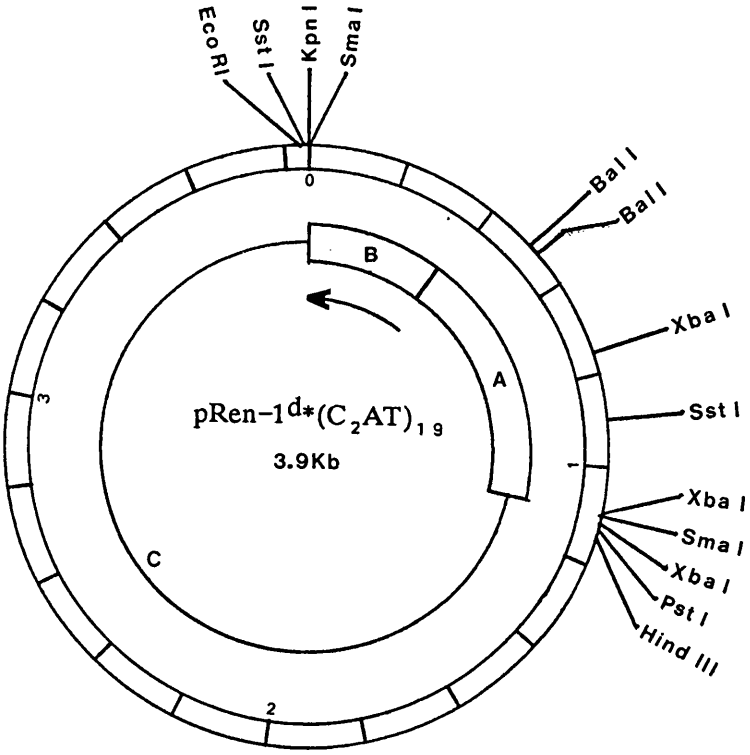

0
1
2
3
A
B
C
Sma I
Xba I
Pst I
Hind III
pRen-2$^{d}_{\Delta}$B(C$_2$AT)$_{19}$
3.3Kb

Figure 4.8

Restriction site maps of pRen-1^{d}*$(C_2AT)_{19}$ and pRen-2^{d}*$(C_2AT)_{19}$. A, *Ren-1^{d}* (717bp) and *Ren-2^{d}* (1067bp) promoters respectively; B, G-free cassette; C, pUC13.


Eco RI
Sst I
Kpn I
Sma I
Bal I
Bal I
Xba I
Sst I
Xba I
Sma I
Xba I
Pst I
Hind III
B
A
C
pRen-1d*(C2AT)19
3.9Kb
0
1
2
3

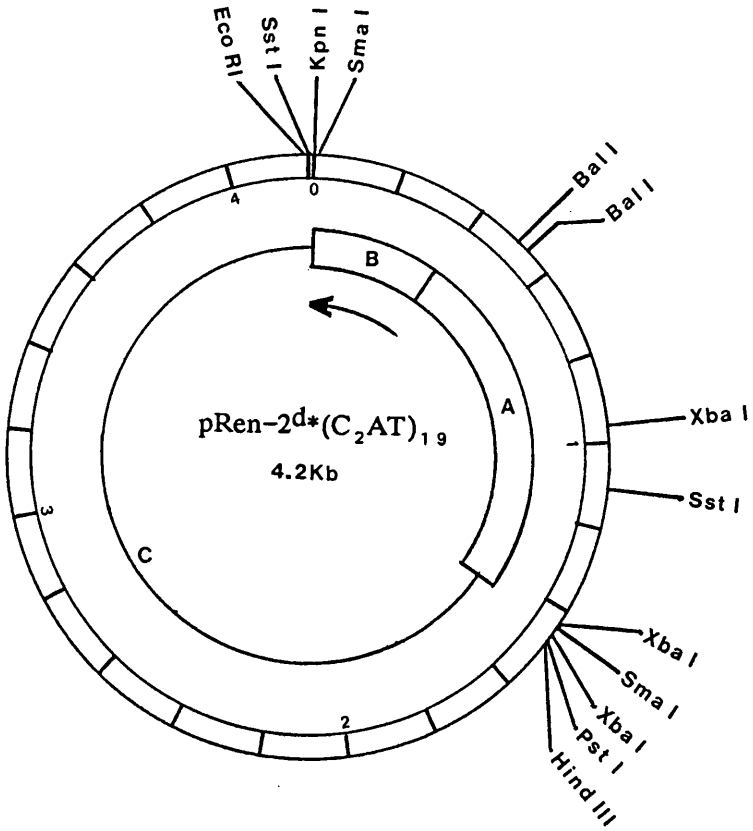
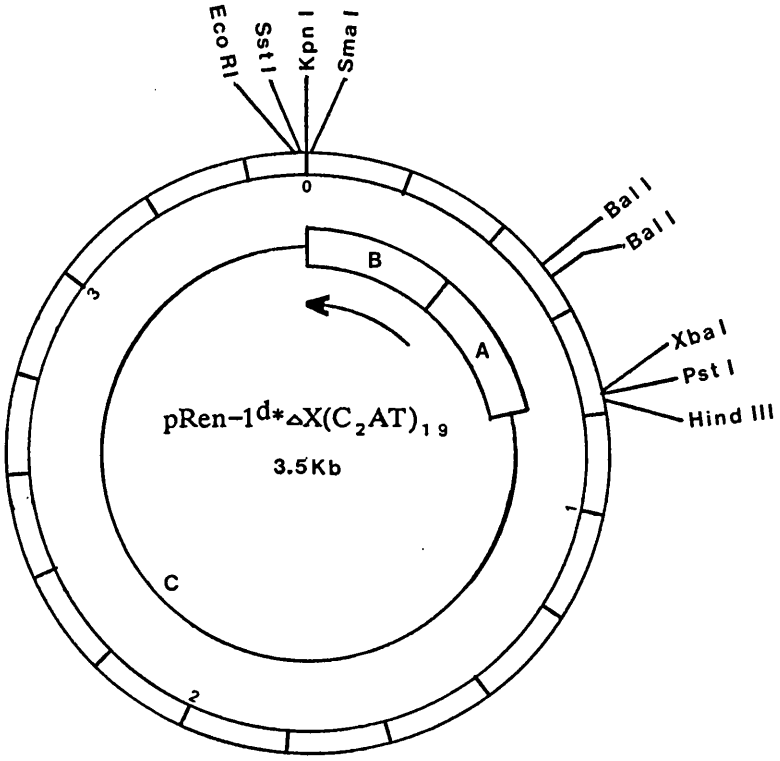
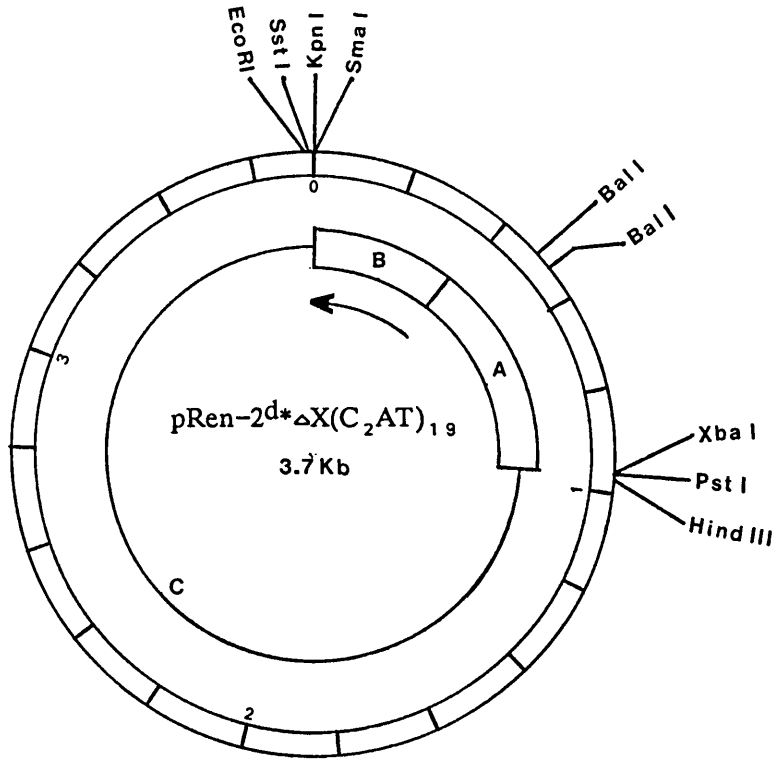

Eco RI
Sst I
Kpn I
Sma I
Bal I
Bal I
Xba I
Sst I
Xba I
Sma I
Xba I
Pst I
Hind III
B
A
C
pRen-2d*(C2AT)19
4.2Kb
0
1
2
3
4

Figure 4.9

Restriction site maps of pRen-$1^{d*}\triangle X(C_2AT)_{19}$ and pRen-$2^{d*}\triangle X(C_2AT)_{19}$. A, *Ren-1^d* (377bp) and *Ren-2^d* (589bp) promoters respectively; B, G-free cassette; C, pUC13.


Eco RI
Sst I
Kpn I
Sma I
Bal I
Bal I
Xba I
Pst I
Hind III
0
1
2
3
A
B
C
pRen-1d*ΔX(C2AT)19
3.5Kb


Eco RI
Sst I
Kpn I
Sma I
Bal I
Bal I
Xba I
Pst I
Hind III
0
1
2
3
A
B
C
pRen-2d*ΔX(C2AT)19
3.7Kb

E. coli JM101 cells respectively and transformants were initially screened by restriction endonuclease digestion and potentially suitable templates were screened further by plasmid sequencing (Figures 4.6). Deletions to the furthest *Bal*I restriction site gave the templates pRen-$1^{d}\triangle B(C_2AT)_{19}$ and pRen-$2^{d}\triangle B(C_2AT)_{19}$ respectively (Figure 4.7).

4.2.3 Construction of Templates with Deletions to the *Xba*I Restriction Site

pRen-$1^{d}(C_2AT)_{19}$ and pRen-$2^{d}(C_2AT)_{19}$ were digested with the restriction endonuclease *Sma*I and the mixtures were then religated and used to transform *E. coli* JM101 cells respectively. Transformants were initially screened for the presence of insert by colony hybridisation, using oligonucleotide-labelled *Ren-1^{d}* and *Ren-2^{d}* *Sma*I insert fragments respectively as probes. Plasmid DNA was then prepared from positive colonies and screened further by restriction endonuclease digestion (Figure 4.4). The presence of an inverted *Sma*I fragment in pRen-$1^{d}(C_2AT)_{19}$ and pRen-$2^{d}(C_2AT)_{19}$ gave the templates pRen-$1^{d*}(C_2AT)_{19}$ and pRen-$2^{d*}(C_2AT)_{19}$ respectively (Figure 4.8).

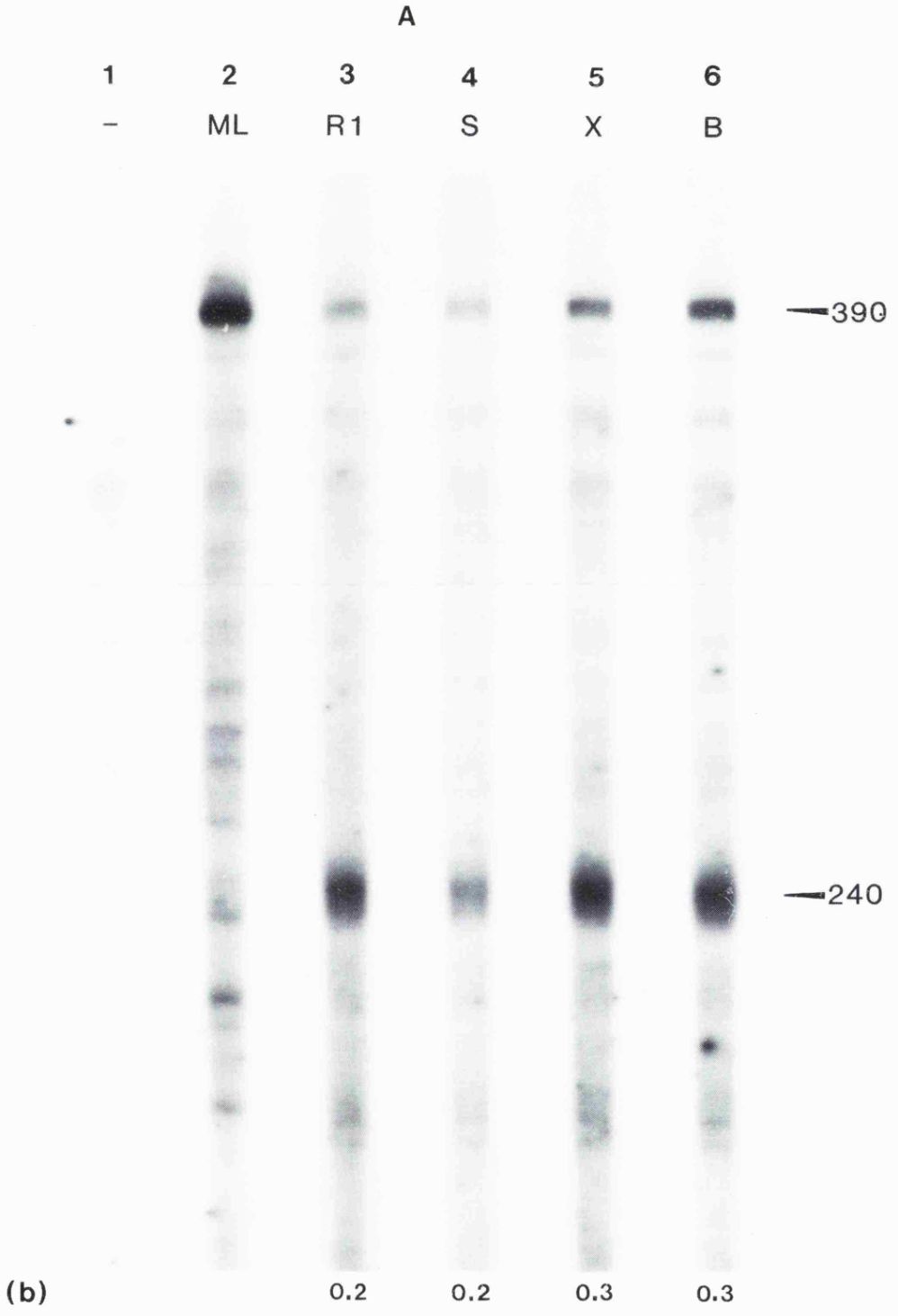
pRen-$1^{d*}(C_2AT)_{19}$ and pRen-$2^{d*}(C_2AT)_{19}$ DNAs were digested with the restriction endonuclease *Xba*I and the large fragments purified via LMP agarose gel-electrophoresis. Following ligation both plasmids were used to transform *E. coli* JM101 cells respectively and the transformants were screened by restriction endonuclease digestion (Figure 4.4). Deletions to the *Xba*I restriction site gave the templates pRen-$1^{d*}\triangle X(C_2AT)_{19}$ and pRen-$2^{d*}\triangle X(C_2AT)_{19}$ respectively (Figure 4.9).

4.3 Effect of *Sst*I, *Xba*I and *Bal*I Deletions on the Activity of Both *Ren-1^{d}* and *Ren-2^{d}* Promoter-Driven/G-free Cassettes

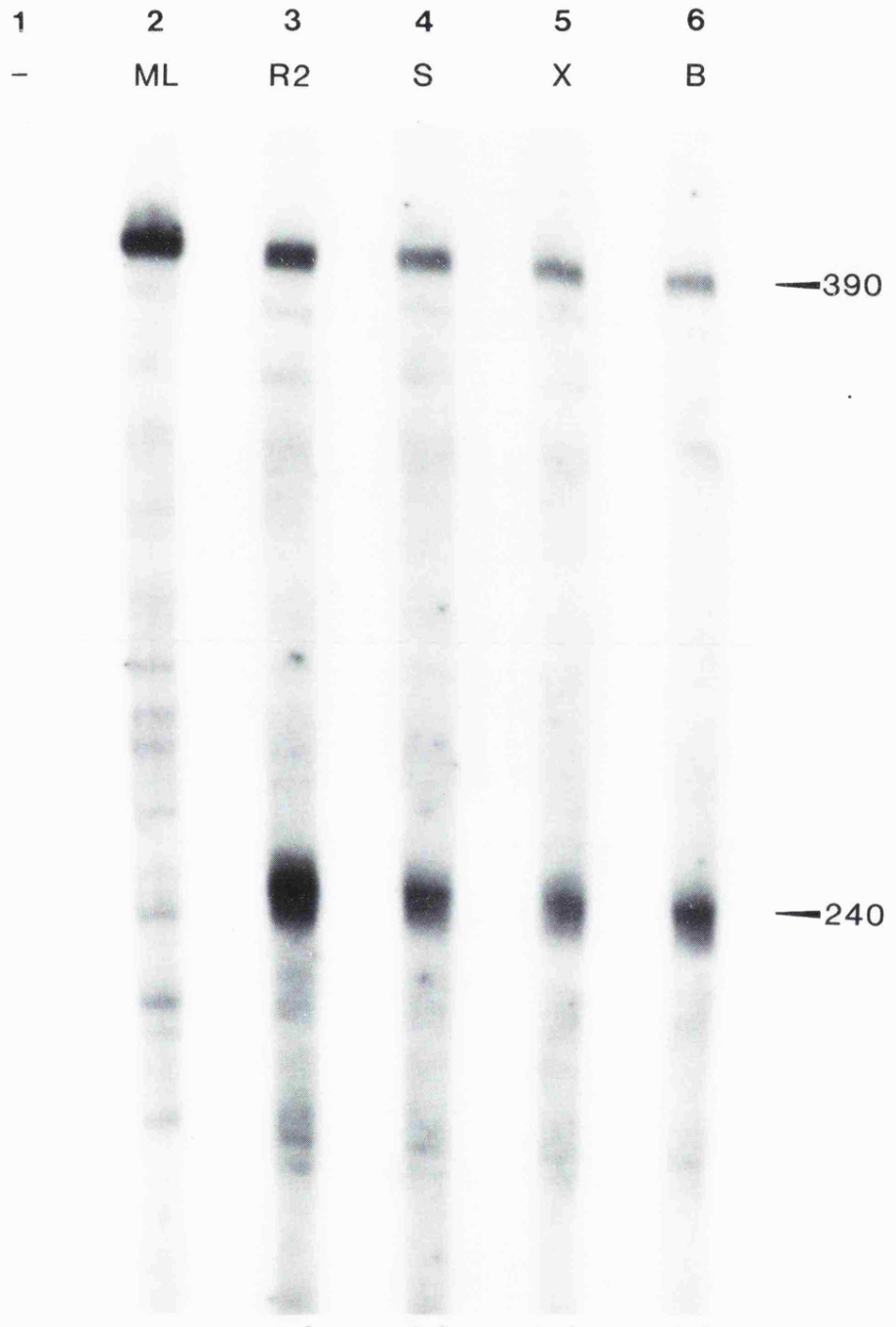
In-vitro transcriptions were carried out with nuclear-protein extract prepared from adult DBA/2 male mouse kidney. Initial comparisons of the ratio of intensity, between the control and test transcripts, showed only minor differences in activity between templates having deletions

Figure 4.10

Effect of deletion-mutants on the activity of A, *Ren-1^d* and B, *Ren-2^d* promoters in mouse kidney nuclear-protein extract. A1, p$(C_2AT)_{19}$; A2, pML$(C_2AT)_{19}$; A3, pRen-1$^d(C_2AT)_{19}$; A4, pRen-1$^d\triangle S(C_2AT)_{19}$; A5, pRen-1$^{d*}\triangle X(C_2AT)_{19}$; A6, pRen-1$^d\triangle B(C_2AT)_{19}$; B1, p$(C_2AT)_{19}$; B2, pML$(C_2AT)_{19}$; B3, pRen-2$^d(C_2AT)_{19}$; B4, pRen-2$^d\triangle S(C_2AT)_{19}$; B5, pRen-2$^{d*}\triangle X(C_2AT)_{19}$; B6, pRen-2$^d\triangle B(C_2AT)_{19}$. Hereafter figures on the right represent transcript length in bases and figures on the bottom represent the ratio of intensity between the full-length transcript (390 NMPs) and that generated by the internal control template pRen-1$^d(C_2AT)_{19}\triangle 150$ (240 NMPs). C, comparison of *Ren-1^d* and *Ren-2^d* transcription ratios (see text for details).


A
1
–
2
ML
3
R1
4
S
5
X
6
B
390
240
(b)
0.2
0.2
0.3
0.3

B



(a)		0.4	0.3	0.3	0.3

C

$(\frac{a}{b})$		2.0	1.5	1.0	1.0

within either the *Ren–1^d* (Figure 4.10A) or *Ren–2^d* (Figure 4.10B) promoters. Indeed a promoter of just 122bp (templates pRen–1$^d\triangle$B$(C_2AT)_{19}$ and pRen–2$^d\triangle$B$(C_2AT)_{19}$) was sufficient for accurate initiation of transcription *in–vitro*. However, when the transcription ratios for the *Ren–1^d* deletions were compared with those for the *Ren–2^d* deletions respectively, an interesting trend emerged. The full–length *Ren–1^d* promoter was found to be 2–fold less active than the corresponding full–length *Ren–2^d* promoter and this difference was decreased to 1.4–fold with deletions to the *Sst*I restriction sites respectively and eliminated with deletions to the *Xba*I and *Bal*I restriction sites respectively (Figure 4.10C). Thus activity of the *Ren–1^d* promoter increased to a level comparable to that of the *Ren–2^d* promoter with a deletion to the *Xba*I restriction site. Furthermore, when the ratios for the *Ren–1^d* deletions were compared, a slight increase in activity was observed when the promoter was deleted to the *Xba*I restriction site. These observations are entirely consistant with those made by Nakamura *et al.* (1989), suggesting a negative control element(s) (which was shown to down–regulate CAT–expression from the TK promoter of pUTKAT1 by >60%) to be present upstream to the *Xba*I restriction site in the *Ren–1^d* promoter; that is within fragment 340 (see Chapter 7).

However, it must be stressed that the small differences between ratios obtained with the deletion–mapping are at the limits of reproducability. Attempts to achieve consistancy in the measurement of ratios which had less than a 2–fold difference have so far been unsuccessful. Thus the small differences observed in the *in–vitro* transcriptions may be the result of experimental error, but were of sufficient interest with respect to recent observations by Nakamura *et al.*, (1989) to be discussed.

4.4 Fine Deletion–Mapping of the 122bp *Ren–1^d* Promoter using Polymerase–Catalysed Chain Reaction Technology

To determine the minimum size of promoter required to drive the G–free cassette *in–vitro*, fine deletion–mapping of the 122bp *Ren–1^d* promoter was carried out. Instead of using the exonuclease activity of *Bal*31 or Mung–Bean nuclease to generate a nested set of deletions, the recently developed polymerase catalysed chain reaction (PCR) (Mullis and Faloona, 1987) was employed for its simplicity and speed (precise

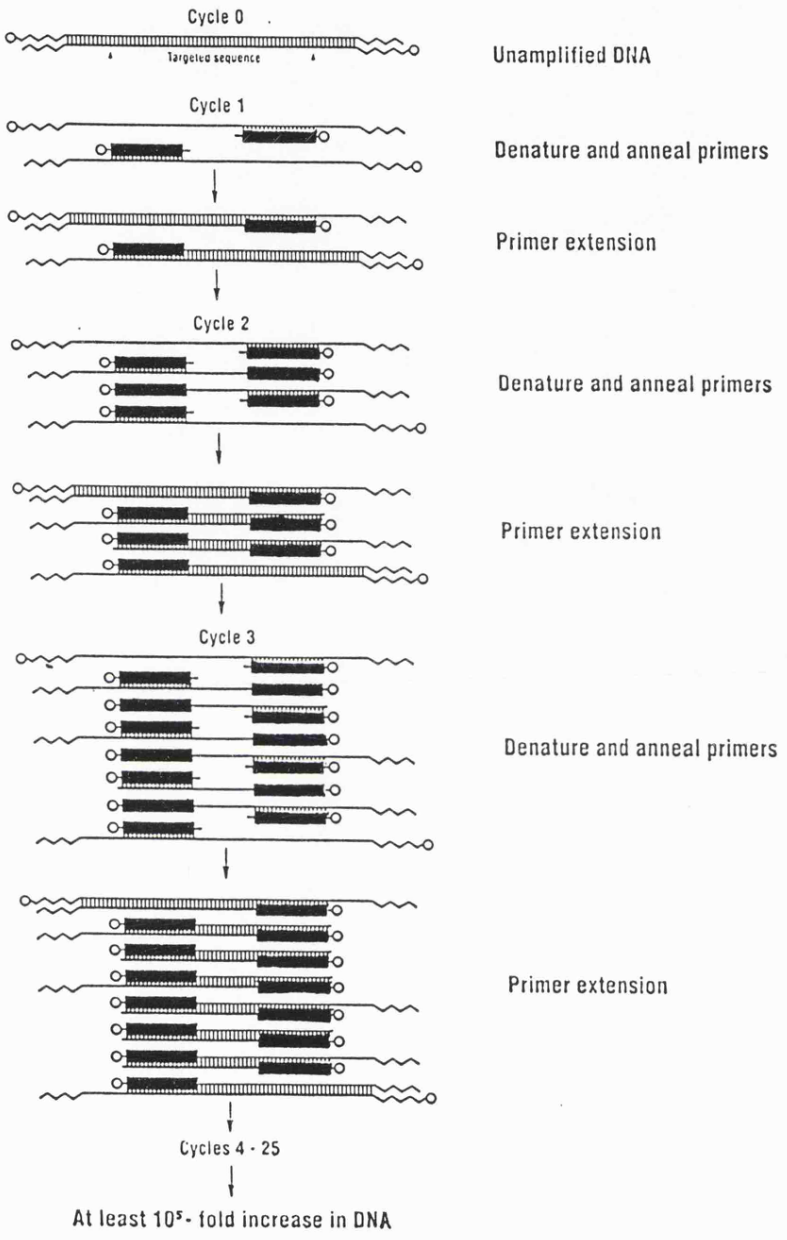


Figure 4.11

Scheme showing the amplification of a specific DNA fragment using PCR technology.

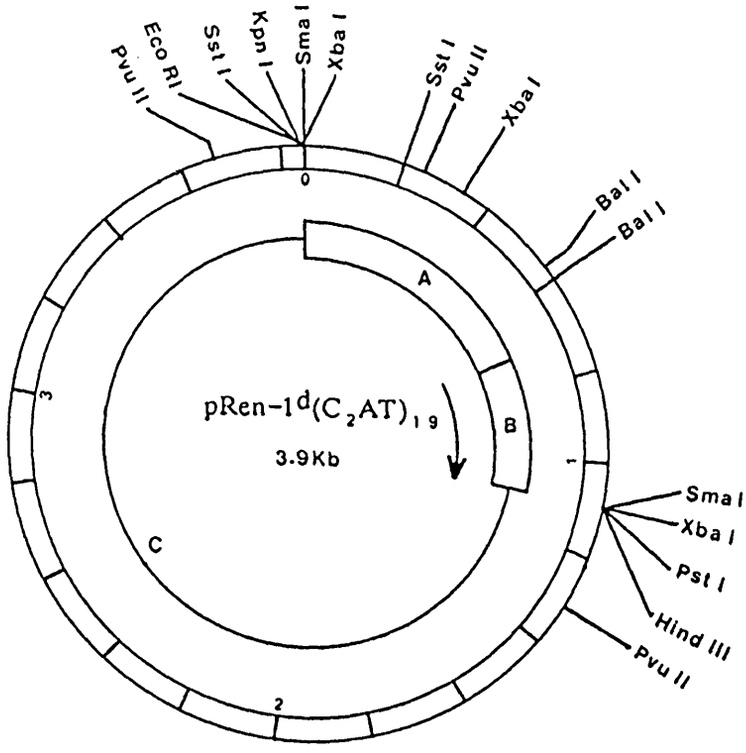


Figure 4.12

Restriction site map of pRen-$1^d(C_2AT)_{19}$. A, *Ren-1^d* (717bp) promoter; B, G-free cassette; C, pUC13.

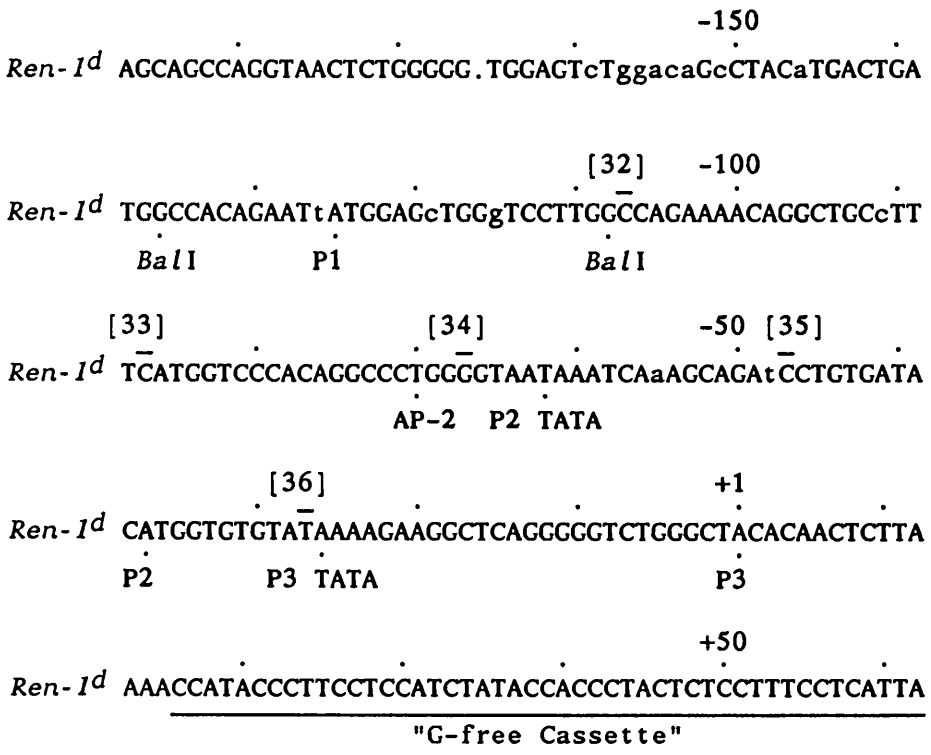


Figure 4.13

Nucleotide sequence of the *Ren-1*^{d} promoter showing annealing positions of the oligonucleotide primers ADS32 ([32]) to ADS36 ([36]) respectively.

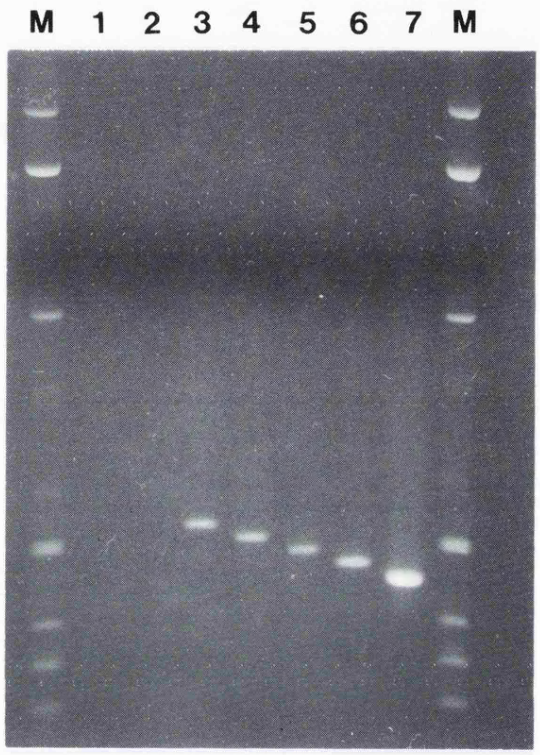


Figure 4.14

PCR generated deletions in the *Ren-1^d* promoter. 1, minus primers; 2, minus enzyme; 3-7, L32- L36-Ren-1^d$(C_2AT)_{19}$ respectively.

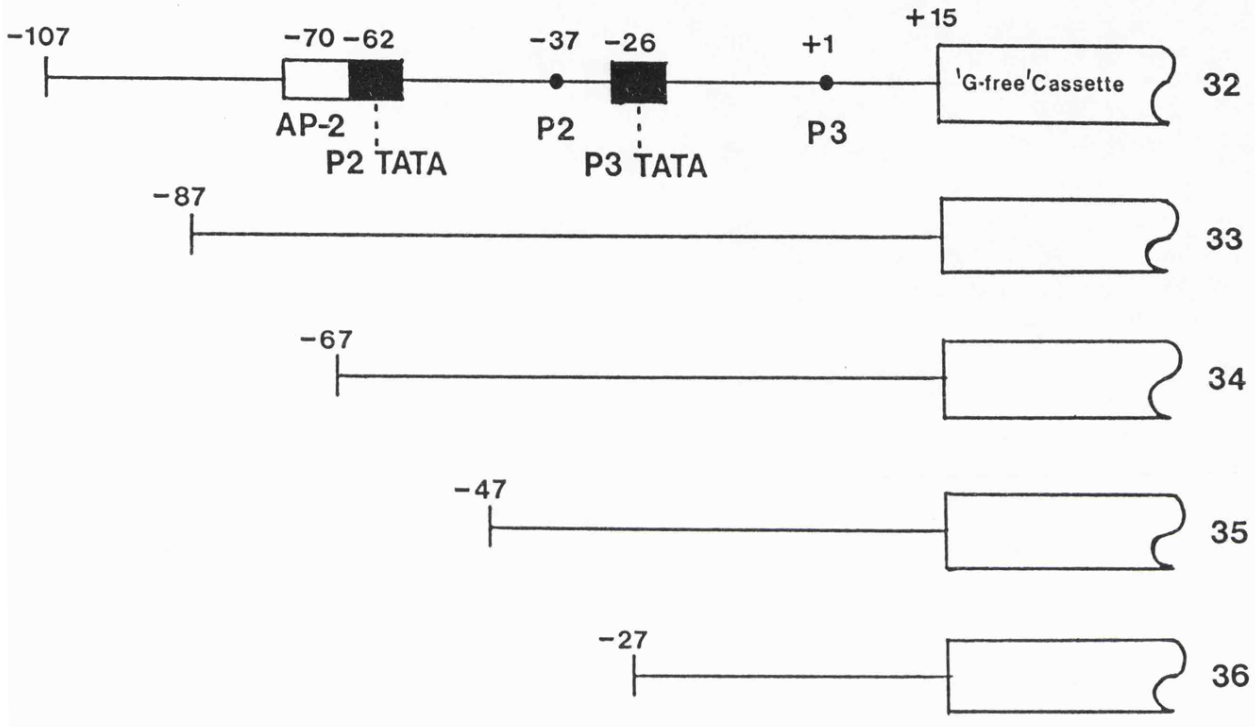


Figure 4.15

PCR generated deletion-mutant linear templates. 32-36, L32- to L36-Ren-1$^{d}(C_2AT)_{19}$ respectively.

deletions may be generated overnight). In each reaction two oligonucleotide primers that flank the region to be amplified were used. One primer, X, is made complementary to the (+)-strand and the other, Y, is made complementary to the (-)-strand. The annealing of X to the (+)-strand of denatured DNA followed by extension with *Taq*I DNA polymerase (Saiki *et al.*, 1988) and dNTPs, results in the synthesis of a (-)-strand fragment containing the target sequence. At the same time, a similar reaction occurs with Y, creating a new (+)-strand. Since these newly synthesised DNA strands are themselves template for the PCR primers, repeated cycles of denaturation, primer-annealing and extension results in the exponential accumulation of the target sequence defined by the primers (Figure 4.11).

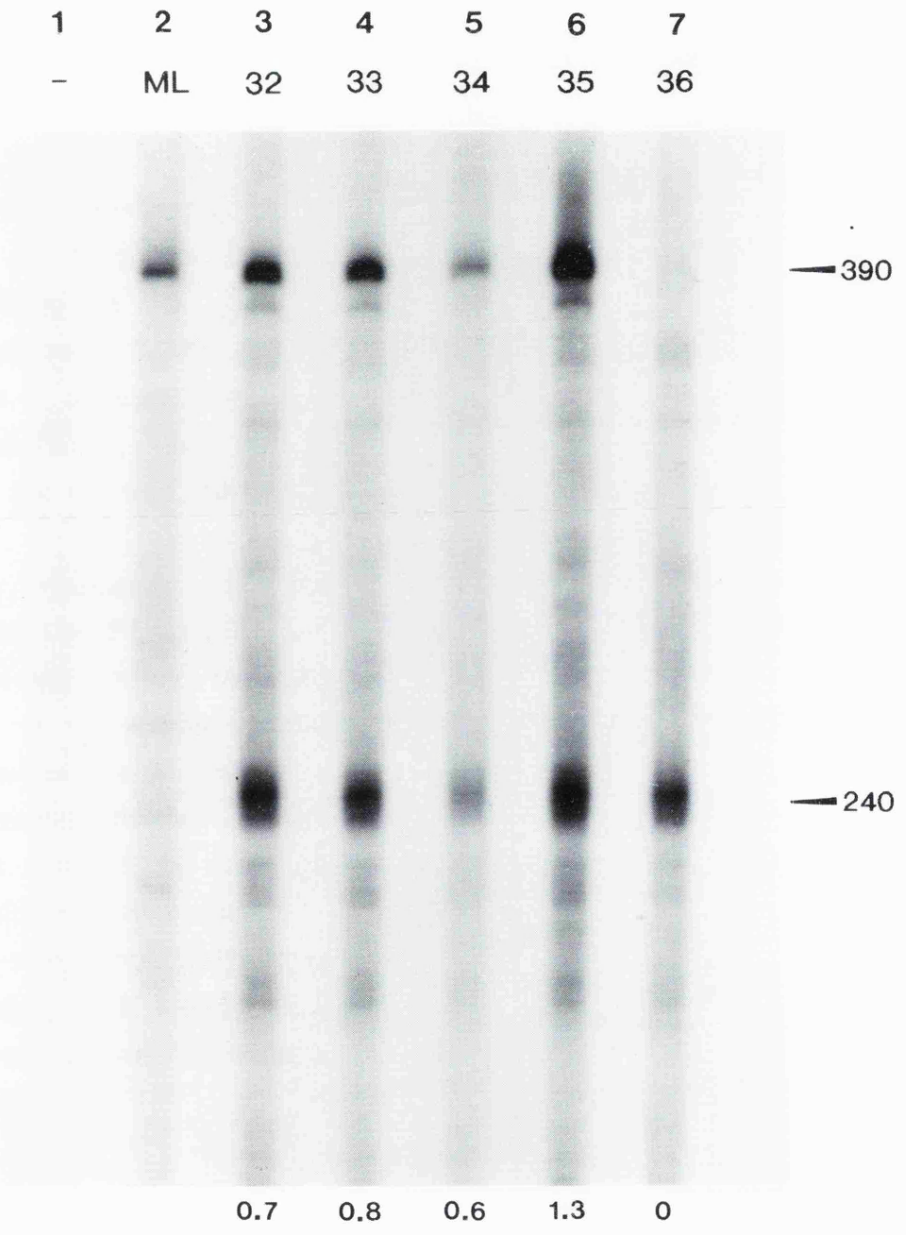
The *Eco*RI-*Hin*dIII restriction fragment, containing the full-length *Ren-1*^{d} promoter/G-free cassette fusion, of pRen-1^d(C_2AT)$_{19}$ (Figure 4.12), was purified via LMP agarose gel-electrophoresis and used as template DNA. Deletions within the *Ren-1*^{d} promoter (from the *Bal*I restriction site) were generated by the use of a successive set of 26 base oligonucleotide primers (ADS32, ADS33, ADS34, ADS35 and ADS36) each of which annealed to the (+)-strand 20 bases downstream of the previous primer (Figure 4.13). Each of these primers when used in conjunction with the 26 base oligonucleotide primer ADS37, which annealed to the (-)-strand downstream of the G-free cassette, produced consecutive deletions in the *Ren-1*^{d} promoter of 20bp (Figure 4.14). PCRs were carried out by L. Sheahan (Leicester University) to give the templates L32- to L36-Ren-1^d(C_2AT)$_{19}$ (Figure 4.15)

4.5 The Minimum *Ren-1*^{d} Promoter Fragment Capable of Driving Transcription *In-Vitro*

In-vitro transcriptions were carried out as outlined in Section 2.26 with the exception that 1.1pmol of linear test template DNA was used in each reaction. Nuclear-protein extract was prepared from adult DBA/2 male mouse kidney. Comparison of the transcription ratios showed that deletions of up to 40bp downstream of the *Bal*I restriction site had little effect on the activity of the *Ren-1*^{d} promoter, whereas with deletions of 60bp and 80bp downstream the activity increased 2-fold and was abolished altogether respectively (Figures 4.15 and 4.16).

Figure 4.16

Effect of PCR generated deletion-mutants on the activity of the *Ren-1^d* promoter in mouse kidney nuclear-protein extract. 1, p$(C_2AT)_{19}$; 2, pML$(C_2AT)_{19}$; 3-7, L32- to L36-Ren-1$^d(C_2AT)_{19}$ respectively. (See Figure 4.10 for details).


1
2
3
4
5
6
7
–
ML
32
33
34
35
36
390
240
0.7
0.8
0.6
1.3
0

The 2-fold increase in activity observed with L35-Ren-1^{d}(C_2AT)$_{19}$ may be explained by considering the availability of RNA polymerase II for driving transcription from the *Ren-1^{d}* P3 promoter. One should recall that only transcripts arising from initiation at the P3 promoter will be detected by the *in-vitro* transcription assay, because of the presence of the G-free cassette. With L32- to L34-Ren-1^{d}(C_2AT)$_{19}$ both the P2 and P3 promoters were available for binding of RNA polymerase II *in-vitro*. When the P2 TAATAAA sequence was deleted, as in L35-Ren-1^{d}(C_2AT)$_{19}$, the possible competitive binding of RNA polymerase II to a non-functional promoter was eliminated. As a result, activity of the P3 promoter might be expected to increase. A further deletion of 20bp, as in L36-Ren-1^{d}(C_2AT)$_{19}$, disrupted the P3 TATAAAA sequence itself and as expected activity was abolished altogether. Thus it appears that, *in-vitro*, a fragment of just 62bp (from -47 to +15) containing only the P3 promoter is sufficient for driving transcription of the G-free cassette.

In the above hypothesis one assumed that the binding of RNA polymerase II to the P2 and P3 promoters occurred with roughly equal efficiency. However, using nuclease S1 mapping and primer-extension analysis, Field *et al.* (1984) demonstrated that *in-vivo* only the P3 promoter is used in the kidney. Whether RNA polymerase II binds only to the P3 promoter in the kidney or whether binding occurs to both promoters, but control is such that only transcription from the P3 promoter is allowed, is unknown. The data presented here tentatively suggest that *in-vitro* RNA polymerase II was able to bind to both the P2 and P3 promoters, with kidney nuclear-protein extract, but whether the expression pattern *in-vitro* correlates with that observed *in-vivo* has not been determined.

4.5.1 Construction of p35-Ren-1^{d}(C_2AT)$_{19}$ and p36-Ren-1^{d}(C_2AT)$_{19}$

Because linear templates were used for deletion-mapping of the *Ren-1^{d}* promoter one might argue that the absence of activity with L36-Ren-1^{d}(C_2AT)$_{19}$ was not due to disruption of the P3 promoter but was a consequence of the ever-decreasing size of the template DNA. In order to counter this argument, L35-Ren-1^{d}(C_2AT)$_{19}$ and

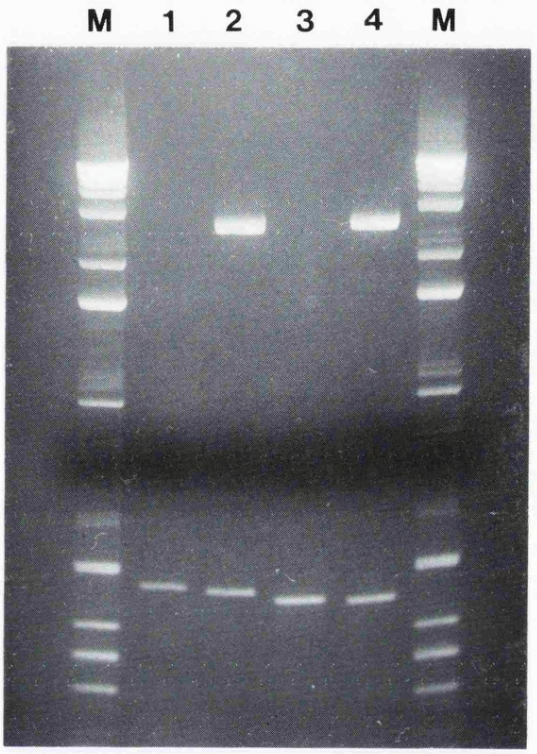
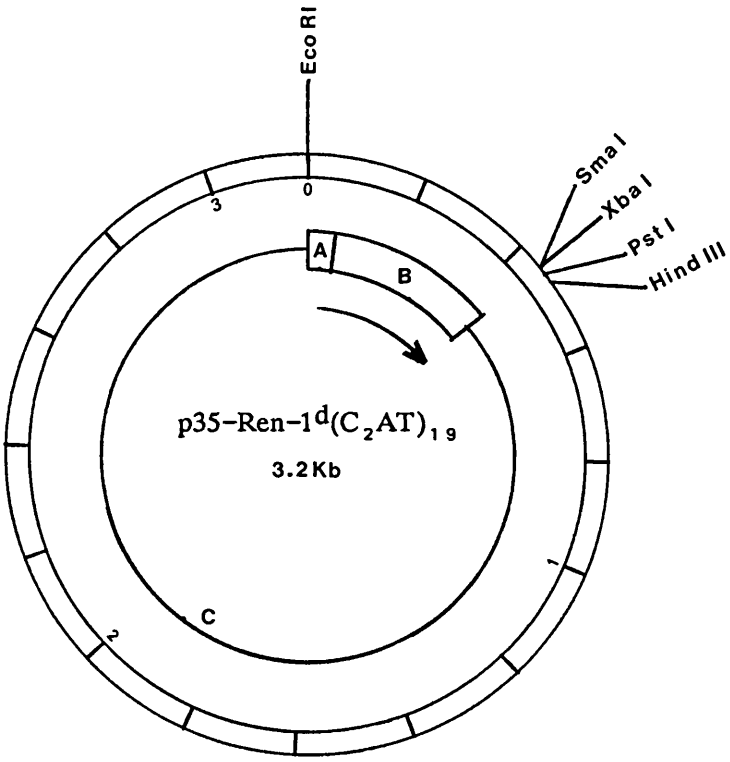


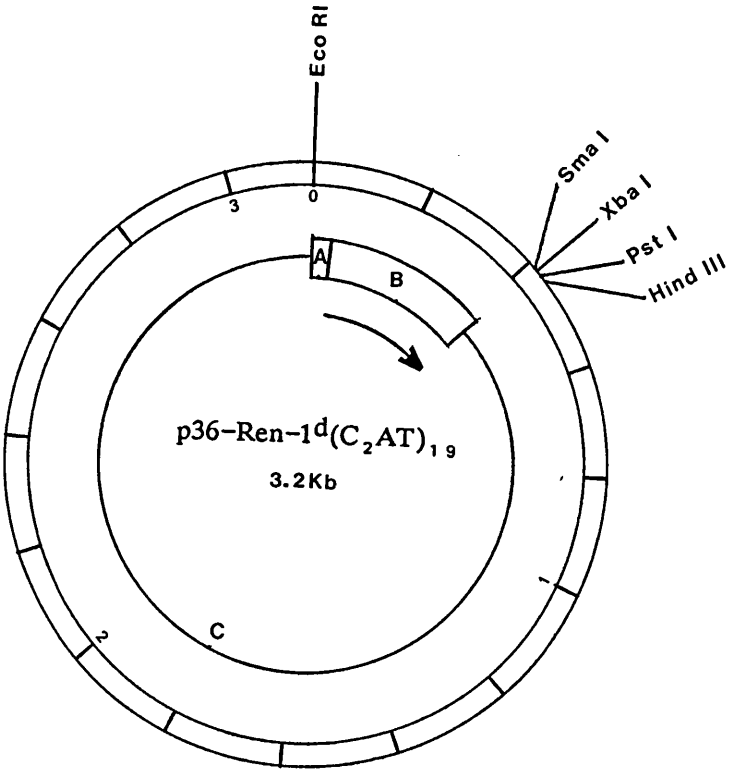
Figure 4.17

Restriction endonuclease digestion of p35-Ren-1^{d}(C_2AT)$_{19}$ and p36-Ren-1^{d}(C_2AT)$_{19}$. 1, L35-Ren-1^{d}(C_2AT)$_{19}$; 2, *Eco*RI-*Hin*dIII digestion of p35-Ren-1^{d}(C_2AT)$_{19}$; 3, L36-Ren-1^{d}(C_2AT)$_{19}$; 4, *Eco*RI-*Hin*dIII digestion of p36-Ren-1^{d}(C_2AT)$_{19}$.

Figure 4.18

Restriction site maps of p35-Ren-1^{d}(C_2AT)$_{19}$ and p36-Ren-1^{d}(C_2AT)$_{19}$. A, *Ren-1^{d}* (62bp) and *Ren-1^{d}* (42bp) promoters respectively; B, G-free cassette; C, pUC13.


Eco RI
Sma I
Xba I
Pst I
Hind III
0
1
2
3
A
B
C
p35-Ren-1^{d}(C$_2$AT)$_{19}$
3.2Kb


Eco RI
Sma I
Xba I
Pst I
Hind III
0
1
2
3
A
B
C
p36-Ren-1^{d}(C$_2$AT)$_{19}$
3.2Kb

L36-Ren-$1^d(C_2AT)_{19}$ were subcloned into a pUC13 background and assayed as covalently closed circular templates.

The large restriction fragment of *Eco*RI-*Hin*dIII digested pRen-$1^d(C_2AT)_{19}$ was purified, via LMP agarose gel-electrophoresis, and ligated with *Eco*RI-*Hin*dIII digested L35-Ren-$1^d(C_2AT)_{19}$ and L36-Ren-$1^d(C_2AT)_{19}$ respectively. The mixtures were used to transform *E. coli* JM101 cells and the transformants were screened for the presence of insert by restriction endonuclease digestion (Figure 4.17) to give the two templates p35-Ren-$1^d(C_2AT)_{19}$ and p36-Ren-$1^d(C_2AT)_{19}$, respectively (Figure 4.18).

4.5.2 Activity of p35-Ren-$1^d(C_2AT)_{19}$ and p36-Ren-$1^d(C_2AT)_{19}$ as *In-Vitro* Transcription Templates

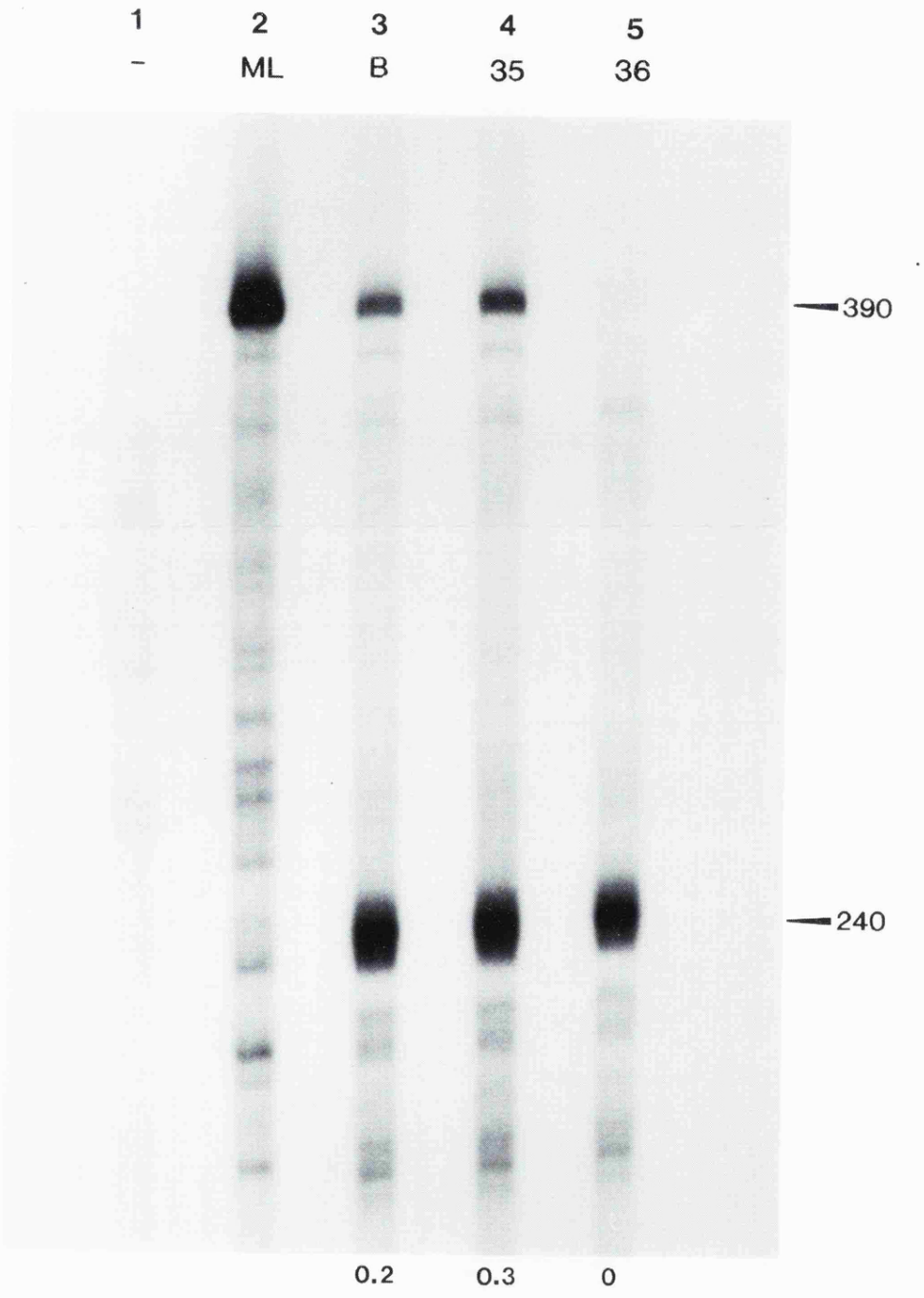
In-vitro transcriptions were carried out with nuclear-protein extract prepared from adult DBA/2 male mouse kidney. Even when L36-Ren-$1^d(C_2AT)_{19}$ DNA was in the form of a covalently closed circular template, that is p36-Ren-$1^d(C_2AT)_{19}$, no full-length transcripts were observed (Figure 4.19). Previously active templates remained active in the closed circular form. Therefore the loss of activity was indeed due to disruption of the P3 promoter rather than a consequence of template size. Furthermore, the data confirmed that only the P3 promoter was able to drive the G-free cassette *in vitro*. The 2-fold increase in promoter activity with L35-Ren-$1^d(C_2AT)_{19}$ was not observed with p35-Ren-$1^d(C_2AT)_{19}$, possibly because of the increased number of promoters present in the latter template. As a result the titration of RNA polymerase II by the P2 promoter was decreased, and thus its deletion caused a negligable effect on P3 promoter activity.

4.6 Association of a Transcription Factor Activator Protein 2 (AP-2) Consensus Sequence with the Renin Promoters

Sequence analysis has revealed the presence of an inverted AP-2 consensus sequence (CCCCAGGGC on the (-)-strand) within the promoter region of the *Ren-1^d* gene (Burt *et al.*, Gene; in press) and *Ren-2^d* gene at position -74 to -66 respectively (Figure 4.13). Purified

Figure 4.19

In-vitro transcription of the circular forms of L35-Ren-1^{d}(C_2AT)$_{19}$ and L36-Ren-1^{d}(C_2AT)$_{19}$ in mouse kidney nuclear-protein extract. 1, p(C_2AT)$_{19}$; 2, pML(C_2AT)$_{19}$; 3, pRen-1$^{d}\triangle$B(C_2AT)$_{19}$; 4, p35-Ren-1^{d}(C_2AT)$_{19}$; 5, p36-Ren-1^{d}(C_2AT)$_{19}$. (See Figure 4.10 for details).


1
-
2
ML
3
B
4
35
5
36
390
240
0.2
0.3
0

AP-2 has been shown to activate transcription *in-vitro* from a hybrid promoter containing human metallothionein II_A ($hMT-II_A$) upstream sequences, and AP-2 also recognises control elements of the human growth hormone, c-*myc* and $H-2K^b$ genes, and the SV40 and bovine papilloma virus enhancers (Imagawa *et al.*, 1987). AP-2 is thought to play an important role in activation of the SV40 enhancer because mutations of its binding-site lead to a severe decrease in enhancer activity (Zenke *et al.*, 1986). The binding-site for AP-2 forms a cell-type-specific enhancer element whose activity is increased in response to treatment of cells with phorbol esters and agents that elevate cAMP levels (Imagawa *et al.*, 1987; and Mitchell *et al.*, 1987). These results suggest that AP-2 is a transcription factor that mediates the effects of two distinct signal-transduction systems known to be involved in growth control, one activated by phorbol esters and involving protein kinase C, and the second activated by cAMP and involving protein kinase A (Nishizuka *et al.*, 1986; and Cohen, 1985).

4.7 Construction of Templates with AP-2 Consensus Sequence and Control Sequence Deletions Respectively

To establish whether the AP-2 consensus sequence associated with the *Ren-1^d* gene was functional *in-vitro*, a template, pRen-$1^{d*}\triangle X(C_2AT)_{19}\triangle$AP-2 was constructed which contained a deletion of the AP-2 consensus sequence. As a control a deletion of a sequence (at position -103 to -96) which showed no homology with other regulatory sequences was generated (pRen-$1^{d*}\triangle X(C_2AT)_{19}\triangle$CD).

The EcoRI-HindIII restriction fragment of pRen-$1^{d*}\triangle X(C_2AT)_{19}$ (containing the *Ren-1^d* promoter/G-free cassette fusion) (Figure 4.9) was purified, via LMP agarose gel-electrophoresis, and ligated into the polylinker of *Eco*RI-*Hin*dIII digested M13mp19. The mixture was used to transform *E. coli* JM101 cells and after screening DNA from white plaques for insert, by preparing RF DNA followed by restriction endonuclease digestion, single-stranded template DNA for mutagenesis was prepared. The mutagenesis protocol used was one developed by Kunkel (1985) (see Section 3.3.1). After annealing of mutant oligonucleotides ADS19 (AP-2 deletion) and ADS21 (control deletion) to the single-stranded template respectively followed by synthesis and

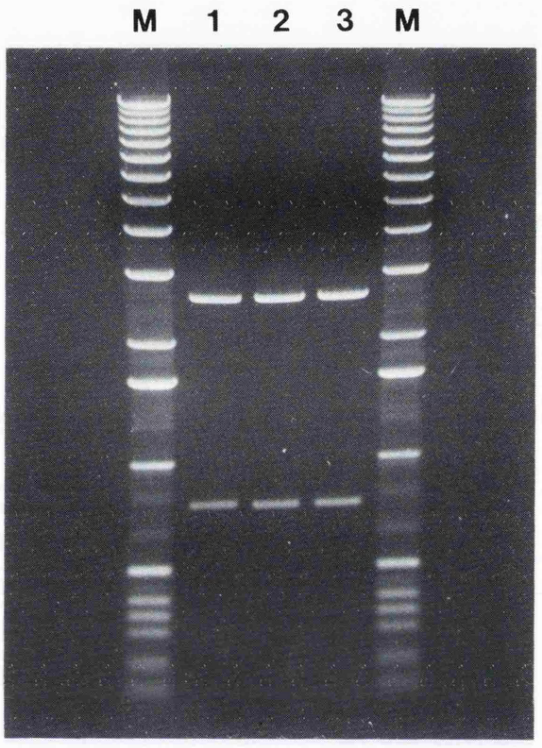


Figure 4.20

*Eco*RI–*Hin*dIII digestion of 1, pRen–1^{d*}△X(C_2AT)$_{19}$; 2, pRen–1^{d*}△X(C_2AT)$_{19}$△CD; 3, pRen–1^{d*}△X(C_2AT)$_{19}$△AP–2.

Figure 4.21

Plasmid sequencing of A, pRen-1^{d*}△X(C_2AT)$_{19}$; B, pRen-1^{d*}△X(C_2AT)$_{19}$△AP-2; and C, pRen-1^{d*}△X(C_2AT)$_{19}$△CD: showing wild-type promoter, AP-2 consensus sequence deletion and control sequence deletion respectively.

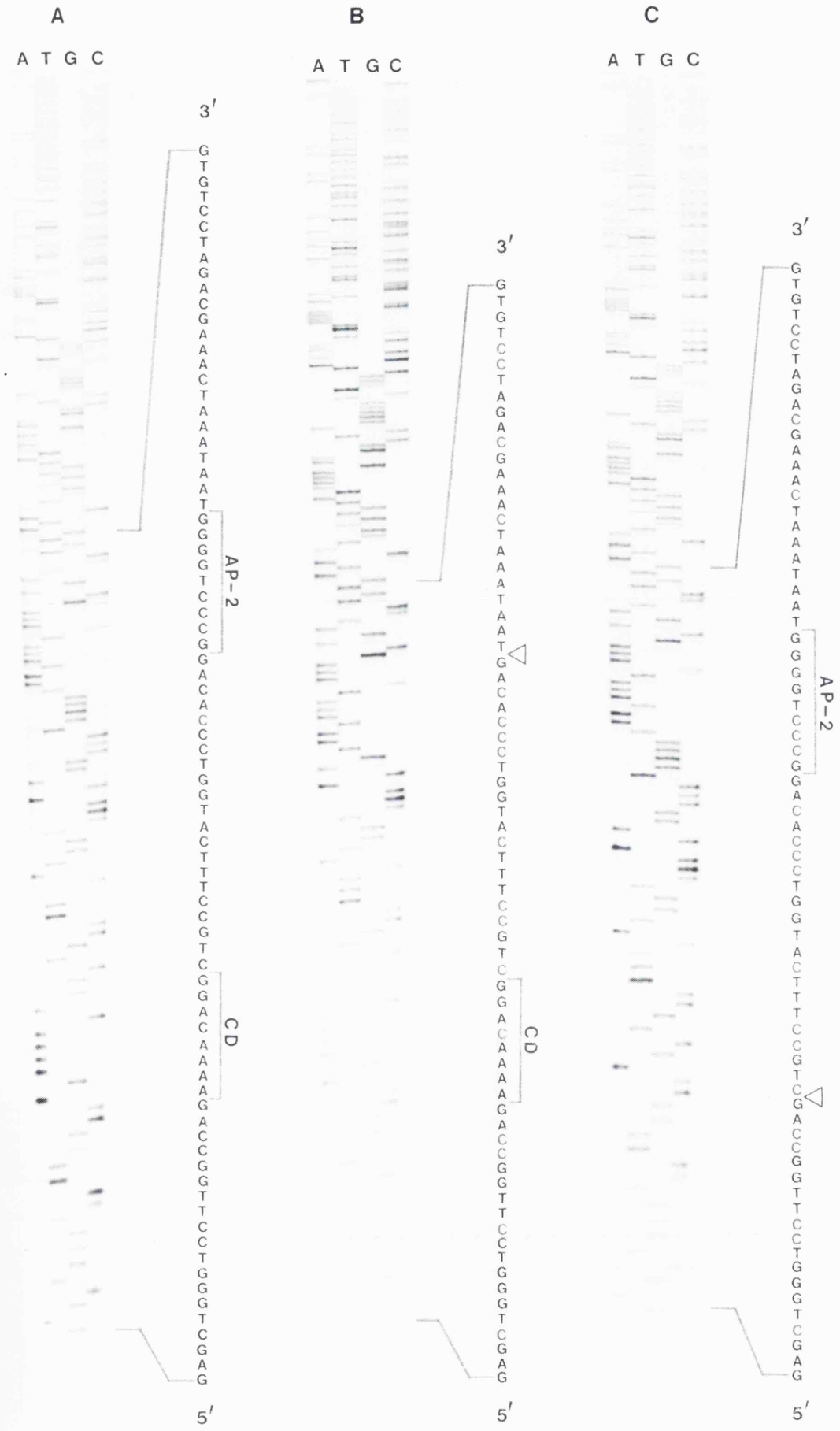
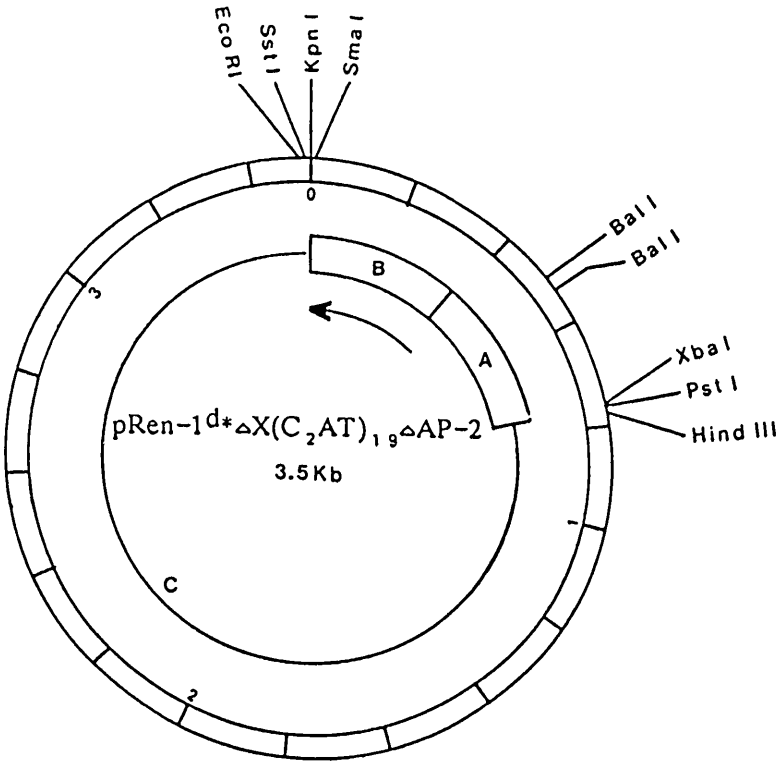
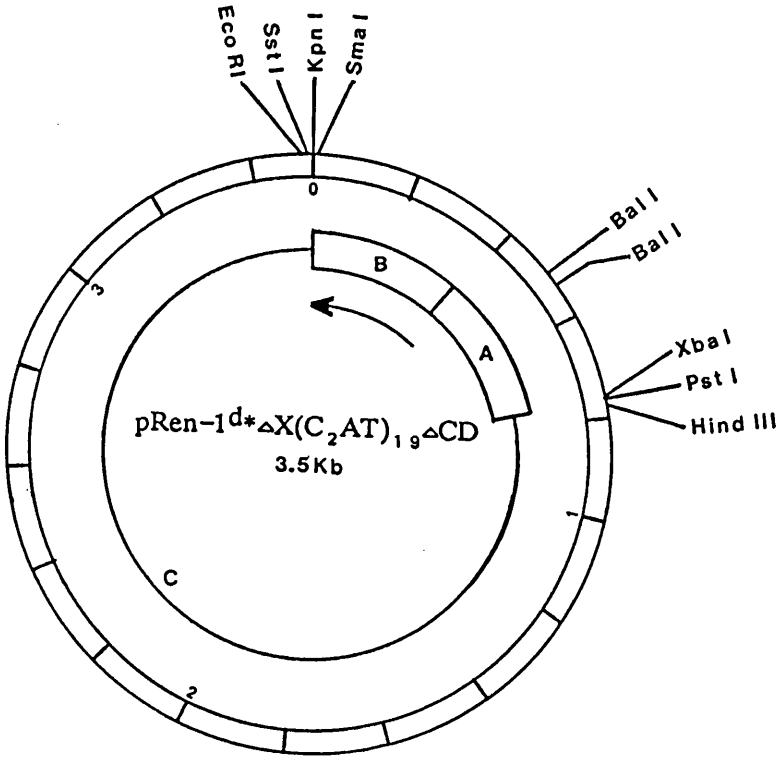

A
A T G C
3′
GTGTCCTAGACGAAACTAAATAATTGGGGTCCCGGACACCCTGGTACTTTCCGTCGGACAAAAGACCGGTTCCTGGGTCGAG
AP-2
CD
5′
B
A T G C
3′
GTGTCCTAGACGAAACTAAATAATTGACACCCTGGTACTTTCCGTCGGACAAAAGACCGGTTCCTGGGTCGAG
CD
5′
C
A T G C
3′
GTGTCCTAGACGAAACTAAATAATTGGGGTCCCGGACACCCTGGTACTTTCCGTCGACCGGTTCCTGGGTCGAG
AP-2
5′

Figure 4.22

Restriction site maps of pRen-1^{d*}△X(C$_2$AT)$_{19}$△AP-2 and pRen-1^{d*}△X(C$_2$AT)$_{19}$△CD. A, *Ren-1^d* promoter; B, G-free cassette; C, pUC13.


Eco RI
Sst I
Kpn I
Sma I
Bal I
Bal I
Xba I
Pst I
Hind III
0
1
2
3
A
B
C
pRen-1d*ΔX(C2AT)19ΔAP-2
3.5Kb


Eco RI
Sst I
Kpn I
Sma I
Bal I
Bal I
Xba I
Pst I
Hind III
0
1
2
3
A
B
C
pRen-1d*ΔX(C2AT)19ΔCD
3.5Kb

ligation of the second strand, the mixtures were used to transform *E. coli* JM101 cells. Single-stranded template was prepared from several of the transformants and screened by M13 sequencing for correct deletion of the AP-2 consensus sequence and control sequence respectively.

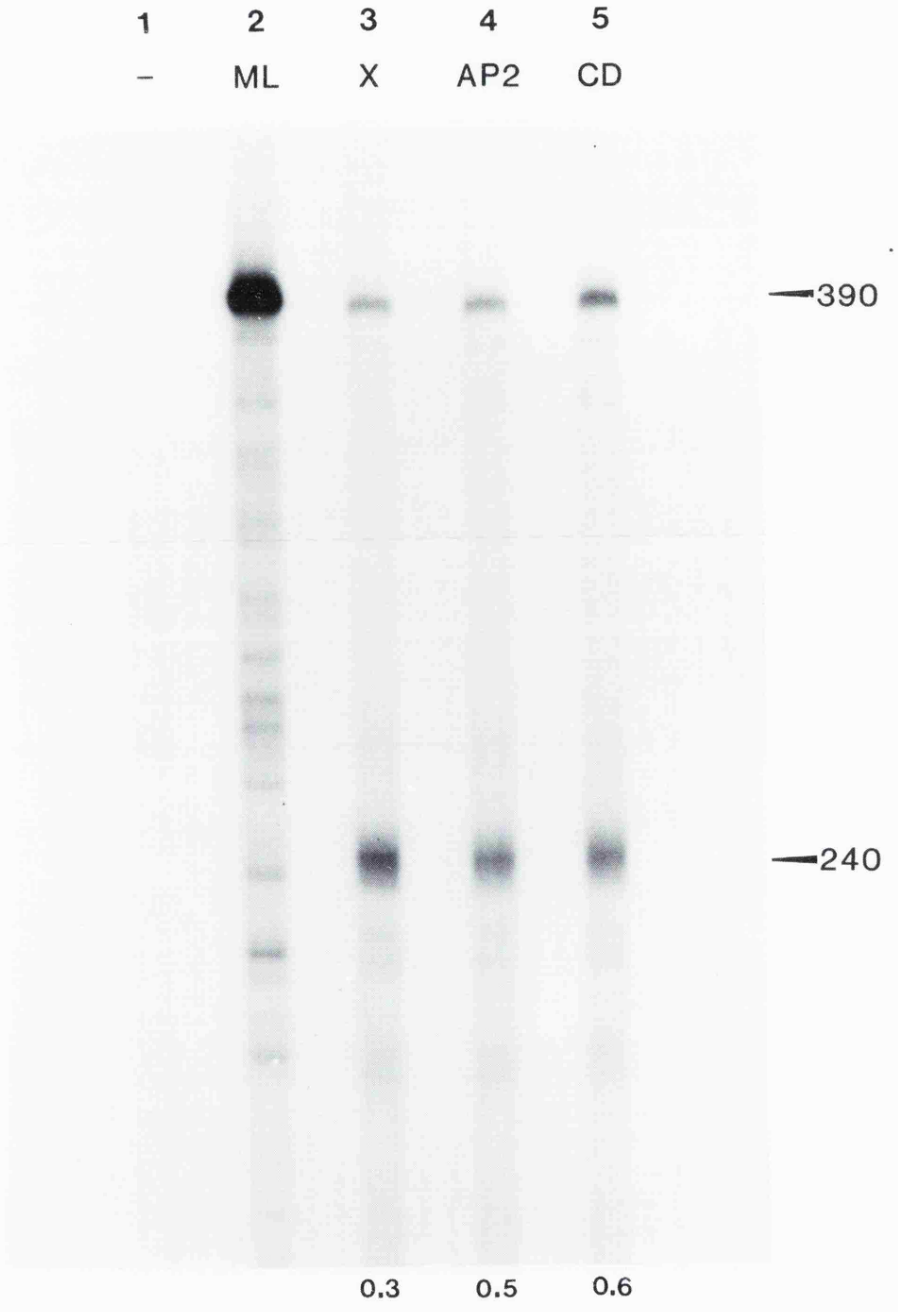
After large-scale purification of RF DNA by caesium chloride/ethidium bromide density-gradient centrifugation, the mutated fragments were cut out of M13mp19 by an *Eco*RI-*Hin*dIII digestion. After purification, via LMP agarose gel-electrophoresis, they were ligated into the large fragment of *Eco*RI-*Hin*dIII digested pRen-1^{d}*$\triangle$X$(C_2AT)_{19}$ respectively. Following transformation of *E. coli* JM101 cells, transformants were screened for the presence of insert by restriction endonuclease digestion (Figure 4.20) and final confirmation of the absence of the AP-2 consensus and control sequences was by plasmid sequencing (Figure 4.21). Correct deletions gave the two templates pRen-1^{d}*$\triangle$X$(C_2AT)_{19}\triangle$AP-2 and pRen-1^{d}*$\triangle$X$(C_2AT)_{19}\triangle$CD respectively (Figure 4.22).

4.8 Effect of Deleting the AP-2 Consensus Sequence on *Ren-1^{d}* Promoter Activity

In-vitro transcriptions were carried out with nuclear-protein extract prepared from adult DBA/2 male mouse kidney. Comparison of the ratios showed that both the control deletion and AP-2 consensus sequence deletion resulted in approximately a 2-fold increase in transcription rate above that seen for the intact *Ren-1^{d}* promoter (Figure 4.23). This result was somewhat surprising, since deletion of the control sequence in L33-Ren-$1^{d}(C_2AT)_{19}$ and disruption of the AP-2 consensus sequence in L34-Ren-$1^{d}(C_2AT)_{19}$ (Figures 4.13 and 4.15), had no effect on *in-vitro* transcription (see Section 4.5). Furthermore AP-2 normally acts as an enhancer rather than a silencer, and one would expect the removal of its binding-site to be accompanied by a loss rather than a gain in promoter efficiency. Nevertheless since both the control and AP-2 consensus sequence deletions produced the same effect, and since no effect on transcription was observed with the deletion-mapping experiments, AP-2 appeared to have a minimal influence on the *Ren-1^{d}* promoter *in-vitro*. One may hypothesise that AP-2 influences activity of the renin genes only in the presence of phorbol esters or cAMP or both, since these agents have been shown to increase AP-2 activity (Imagawa *et al.*, 1987;

Figure 4.23

Effect of deletion of the AP-2 consensus sequence on *Ren-1^d* promoter activity in mouse kidney nuclear-protein extract. 1, p$(C_2AT)_{19}$; 2, pML$(C_2AT)_{19}$; 3, pRen-1$^{d*}\triangle X(C_2AT)_{19}$; 4, pRen-1$^{d*}\triangle X(C_2AT)_{19}\triangle$AP-2;
5, pRen-1$^{d*}\triangle X(C_2AT)_{19}\triangle$CD. (See Figure 4.10 for details).


1
2
3
4
5
–
ML
X
AP2
CD
390
240
0.3
0.5
0.6

and Mitchell *et al.*, 1987). However this seems unlikely since purified AP-2 has been shown to stimulate transcription *in-vitro* of a plasmid (pMK$\triangle$3'-129) in the absence of these agents (Imagawa *et al.*, 1987). (pMK$\triangle$3'-129 contains three AP-2 sites located 5' to position -129 of the hMT-II_A promoter fused to a truncated herpes simplex virus thymidine kinase promoter.)

CHAPTER 5

RESULTS

Effect of Downstream Sequences on the Activity of the *Ren-1^d* Promoter *In-Vitro*

5.1 Downstream Regulatory Sequences

There have been several well-defined reports of regulation of gene-expression by DNA sequences located 3' to the cap-site. These include the internal promoter of the *Xenopus laevis* 5S RNA gene (Bogenhagen *et al.*, 1980); the 3' enhancer of the human β-globin gene (Trudel and Constantini, 1987; and Trudel *et al.*, 1987) and chicken adult β-globin gene (Choi and Engel, 1986; and Hesse *et al.*, 1986); the 3' enhancer of the T-cell receptor β-chain gene (Krimpenfort *et al.*, 1988); the tissue-specific enhancer of the immunoglobulin gene (Queen and Baltimore, 1983); and finally the transcriptional enhancer associated with the first intron of the mouse α_2 (type I) collagen gene (Rossi and De Crombrugghe, 1987).

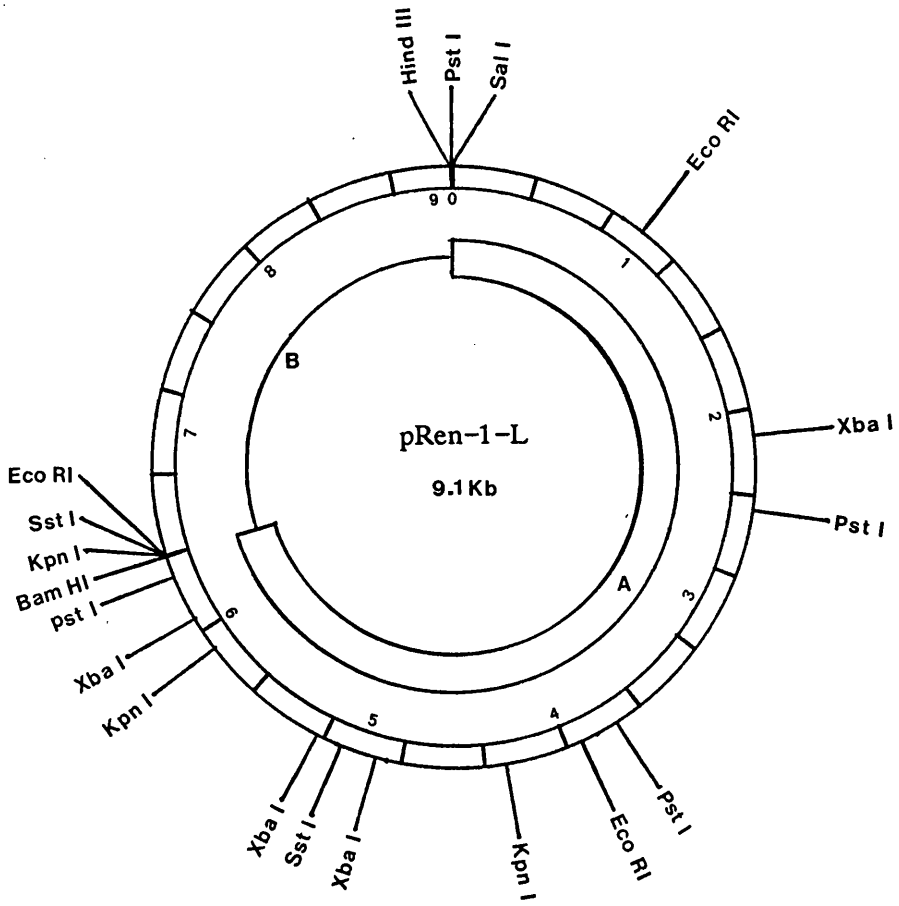
Miller *et al.* (1989) proposed that elements downstream of the renin promoter are required for optimal renin gene-expression (see Section 7.1). To investigate this possibilty several templates were prepared and tested in the *in-vitro* transcription assay developed in Chapter 3.

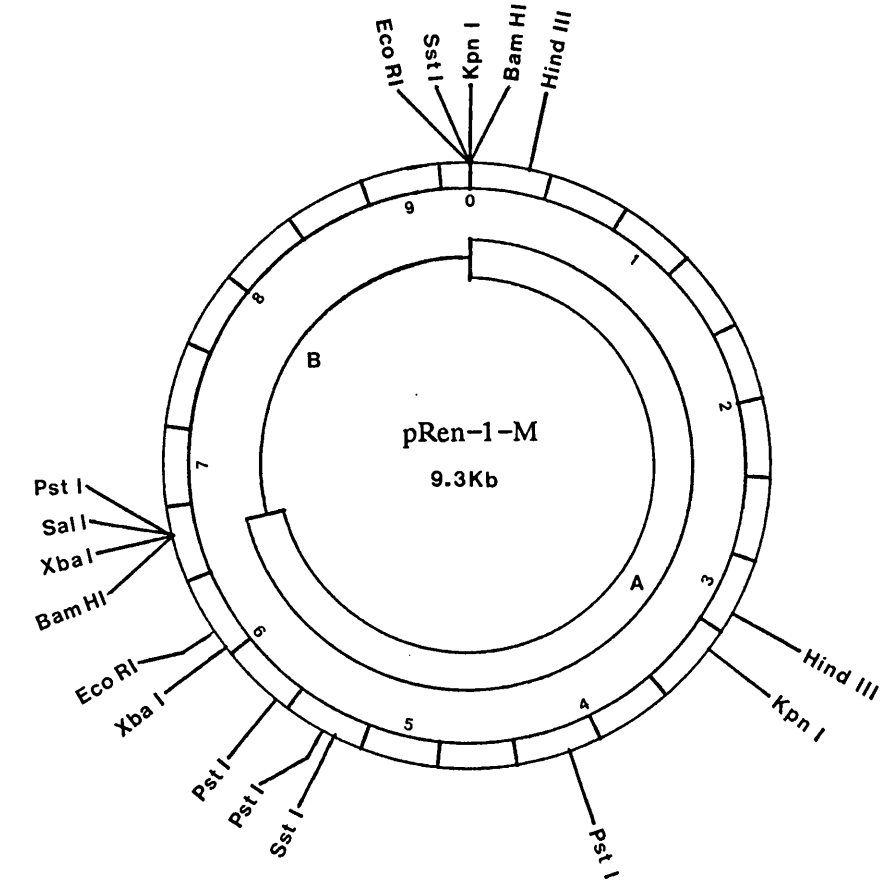
5.2 Preparation of Templates Containing Sequences Located 3' to the *Ren-1^d* Promoter

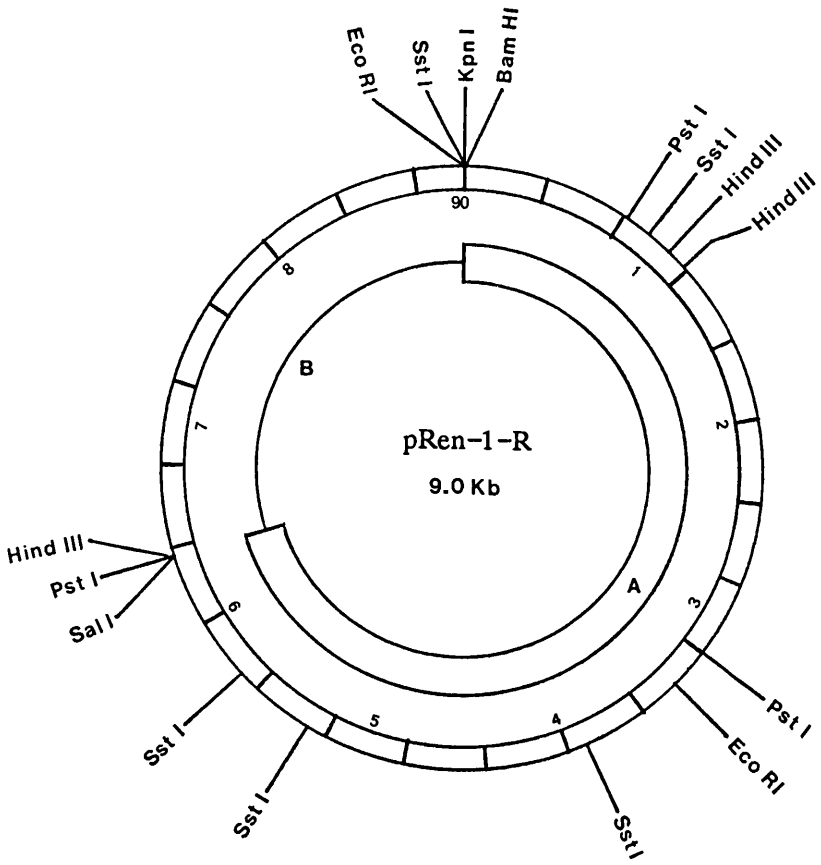
The *Ren-1^d* gene comprising of 5Kb of upstream sequence, 10Kb of coding and non-coding sequence and 4Kb of downstream sequence, was obtained collectively on the three plasmids pRen-1-L, pRen-1-M and pRen-1-R (which were a generous gift from C.C.J Miller and A.T. Carter, ICI Joint Laboratory, Leicester University) (Figure 5.1). Each of these plasmids contains approximately 6.4Kb of *Ren-1^d* sequence ligated into the polylinker of pUC18. pRen-1-L contains 5Kb of upstream

Figure 5.1

Restriction site maps of pRen-1-L, pRen-1-M and pRen-1-R. A, L,M and R fragments respectively; B, pUC18.


Hind III
Pst I
Sal I
Eco RI
Xba I
Pst I
Pst I
Eco RI
Kpn I
Xba I
Sst I
Xba I
Kpn I
Sst I
Bam HI
Pst I
Xba I
Eco RI
pRen-1-L
9.1Kb
A
B
0
1
2
3
4
5
6
7
8
9


Eco RI
Sst I
Kpn I
Bam HI
Hind III
Hind III
Kpn I
Pst I
Pst I
Sst I
Pst I
Eco RI
Xba I
Pst I
Sal I
Xba I
Bam HI
pRen-1-M
9.3Kb
A
B
0
1
2
3
4
5
6
7
8
9


Eco RI
Sst I
Kpn I
Bam HI
Pst I
Sst I
Hind III
Hind III
90
1
2
3
4
5
6
7
8
B
pRen-1-R
9.0 Kb
A
Hind III
Pst I
Sal I
Sst I
Sst I
Sst I
Pst I
Eco RI

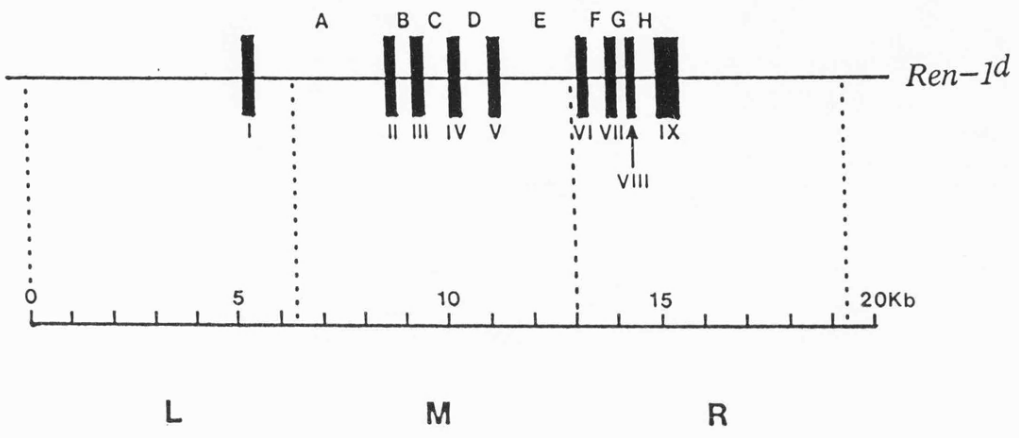


Figure 5.2

Mouse *Ren-1*^{d} gene structure, showing origin of L,M and R fragments. Exons are labelled I–IX and introns are labelled A–H.

sequence, exon I and part of intron A; pRen-1-M contains the remainder of intron A, introns B, C, and D, part of intron E and exons II, III, IV and V; pRen-1-R contains the remainder of intron E, introns F, G and H, exons VI, VII, VIII and IX together with 4Kb of downstream sequence (Figure 5.2). These plasmids, containing the left (L); middle (M); and right (R) fragments of the *Ren-1^d* gene respectively, were used in the construction of the following templates.

5.2.1 Construction of Templates Containing the L, M and R Fragments Downstream of the *Ren-1^d* Promoter/G-free Cassette Fusion

The L, M, and R fragments were cut out of pRen-1-L, pRen-1-M and pRen-1-R by *Sal*I-*Bam*HI, *Bam*HI-*Bam*HI and *Bam*HI-*Sal*I digestions respectively (Figure 5.1). After purification, via LMP agarose gel-electrophoresis, each fragment was blunt-ended using the 5' to 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I.

pRen-1^d(C_2AT)$_{19}$ was digested with the restriction endonuclease *Hin*dIII, and after purification by direct extraction, was blunt-ended using the 5' to 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. Following a second round of purification by direct extraction the 5' phosphate groups were removed, to prevent re-ligation of the vector, by treatment of the plasmid with calf-intestinal phosphatase.

The purified blunt-ended L, M, and R fragments were now ligated into the blunt-ended and phosphatased *Hin*dIII site of pRen-1^d(C_2AT)$_{19}$ respectively, and the mixtures used to transform *E. coli* HB101 cells (for the L fragment) and *E. coli* JM101 cells (for the M and R fragments). (HB101 is a *RecA*$^-$ strain and was used in transformations with the L fragment to prevent homologous recombination between the *Ren-1^d* promoter of pRen-1^d(C_2AT)$_{19}$ and homologous sequences present in the L fragment). Transformants were initially screened for the presence of insert by colony hybridisation using oligonucleotide-labelled L, M and R fragments as probes respectively. Plasmid DNA was then prepared from positive

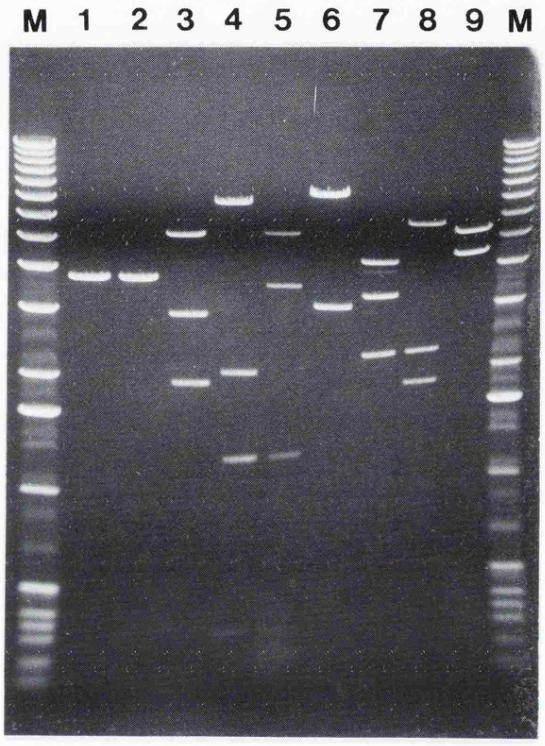
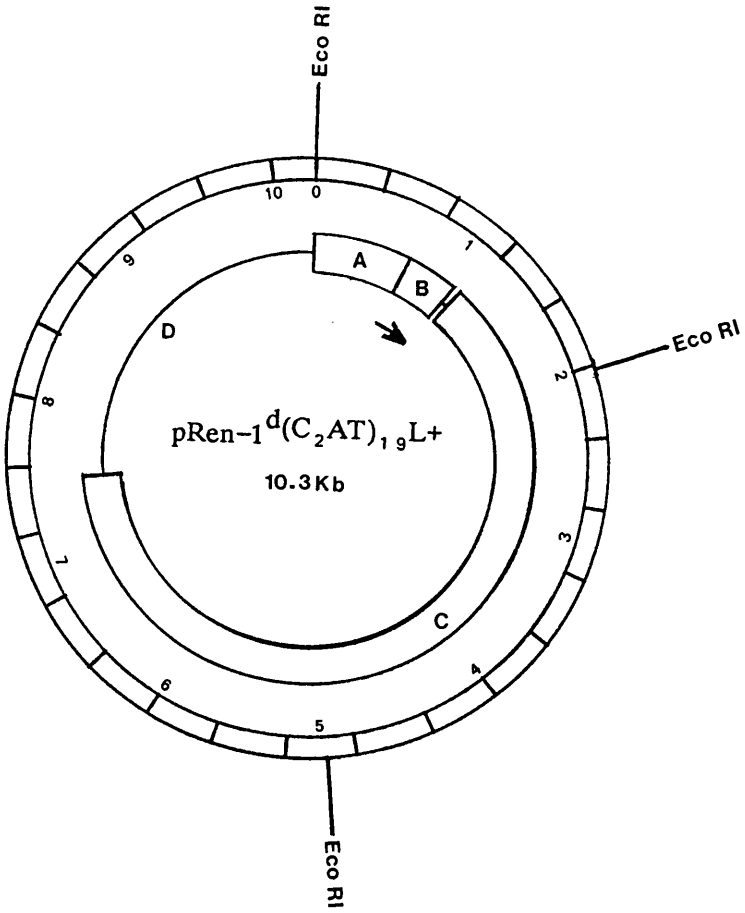


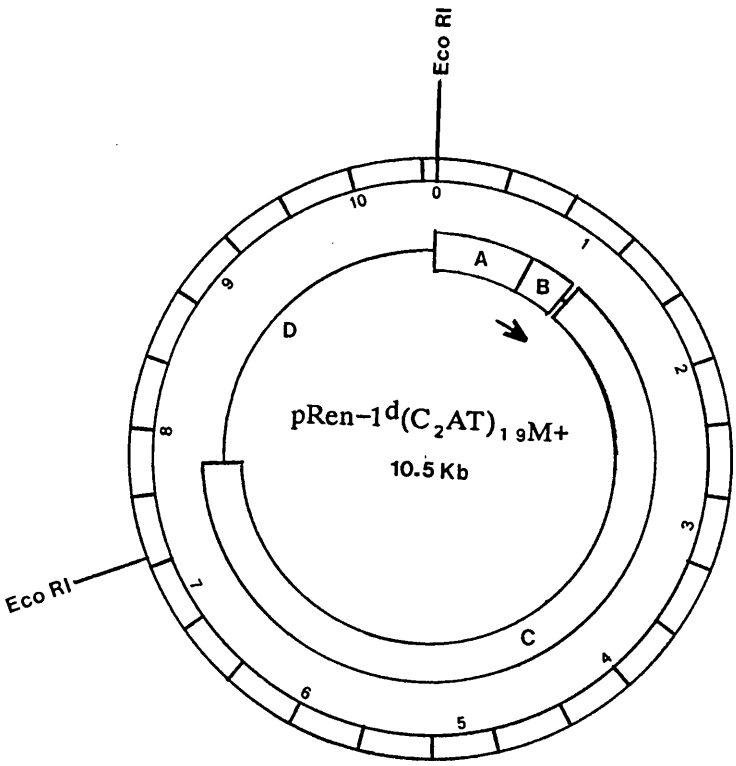
Figure 5.3

Restriction endonuclease digestion of templates with the L, M and R fragments in the sense (+) orientation downstream or both orientations upstream of the *Ren-1*^{d} promoter/G-free cassette fusion. 1 and 2, pRen-1^{d}(C_2AT)$_{19}$; 3, pRen-1^{d}(C_2AT)$_{19}$L+; 4, pM+Ren-1^{d}(C_2AT)$_{19}$; 5, pM-Ren-1^{d}(C_2AT)$_{19}$; 6, pRen-1^{d}(C_2AT)$_{19}$M+; 7, pR+Ren-1^{d}(C_2AT)$_{19}$; 8, pR-Ren-1^{d}(C_2AT)$_{19}$; 9, pRen-1^{d}(C_2AT)$_{19}$R+. 1,3,6 and 9, *Eco*RI digestion; 2,4,5,7 and 8, *Pst*I digestion.

Figure 5.4

Restriction site maps of templates with the L, M and R fragments in the sense (+) orientation downstream of the *Ren-1^d* promoter/G-free cassette fusion. A, *Ren-1^d* (717bp) promoter; B, G-free cassette; C, L,M and R fragments respectively; D, pUC13.


Eco RI
Eco RI
Eco RI
A
B
C
D
pRen-1$^{d}(C_2AT)_{19}L+$
10.3Kb
0
1
2
3
4
5
6
7
8
9
10


Eco RI
Eco RI
A
B
C
D
pRen-1$^{d}(C_2AT)_{19}M+$
10.5Kb
0
1
2
3
4
5
6
7
8
9
10

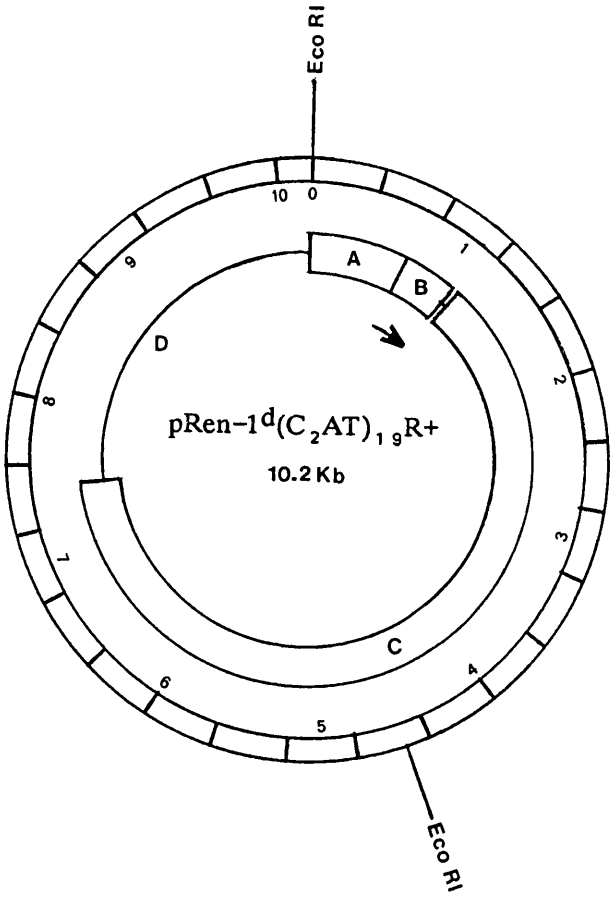
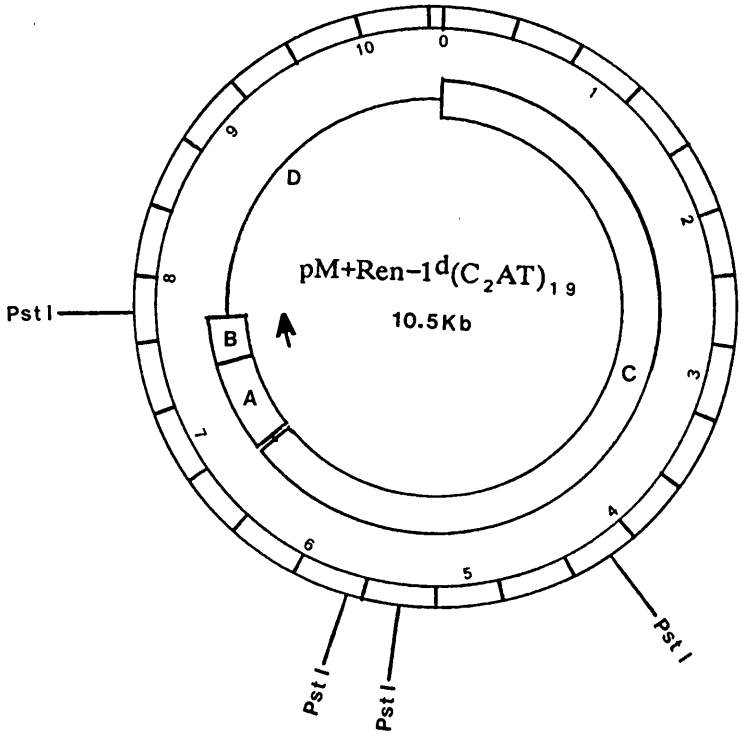
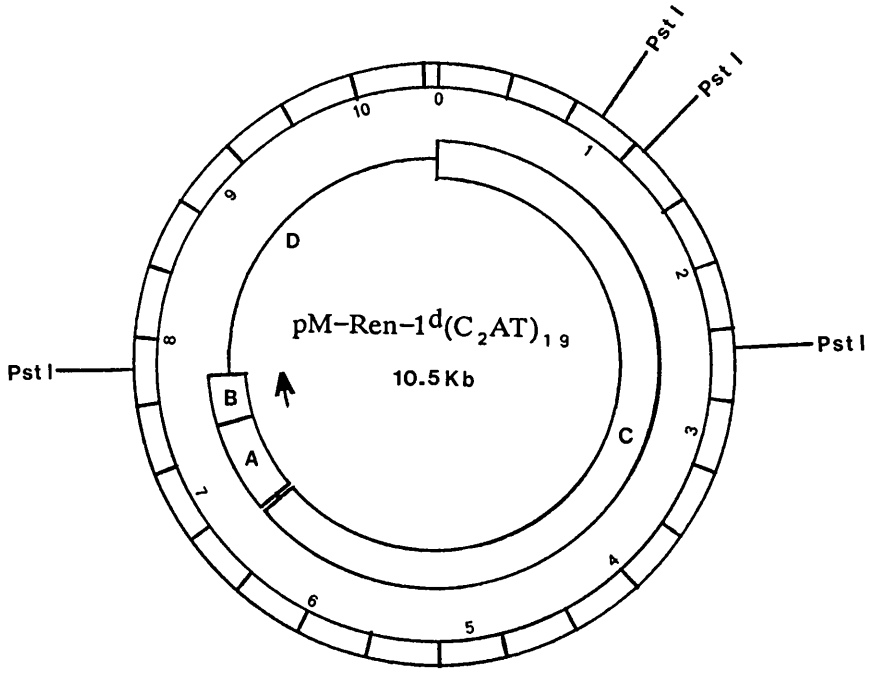
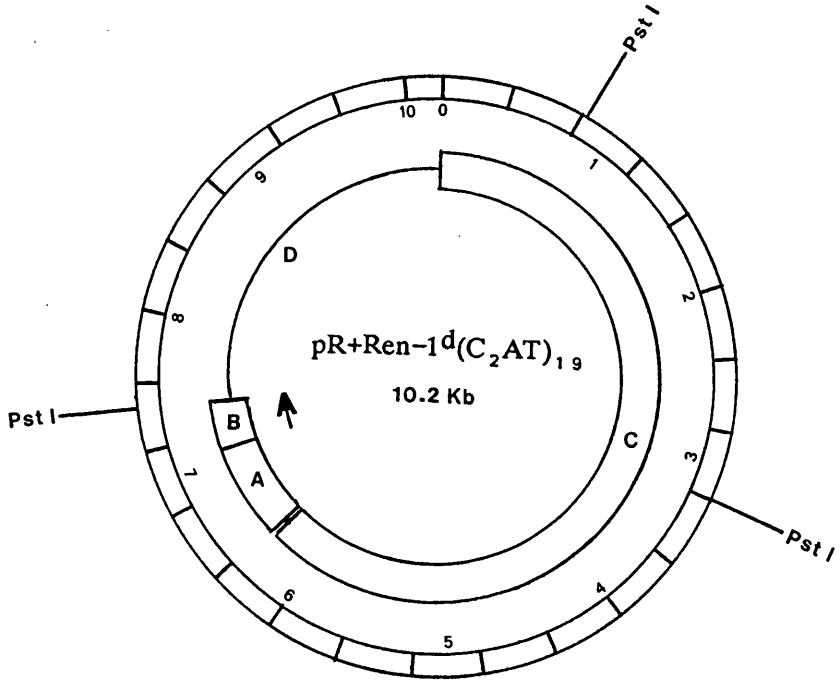

Eco RI
10
0
1
2
3
4
5
6
7
8
9
A
B
C
D
pRen-1d(C2AT)19R+
10.2 Kb
Eco RI

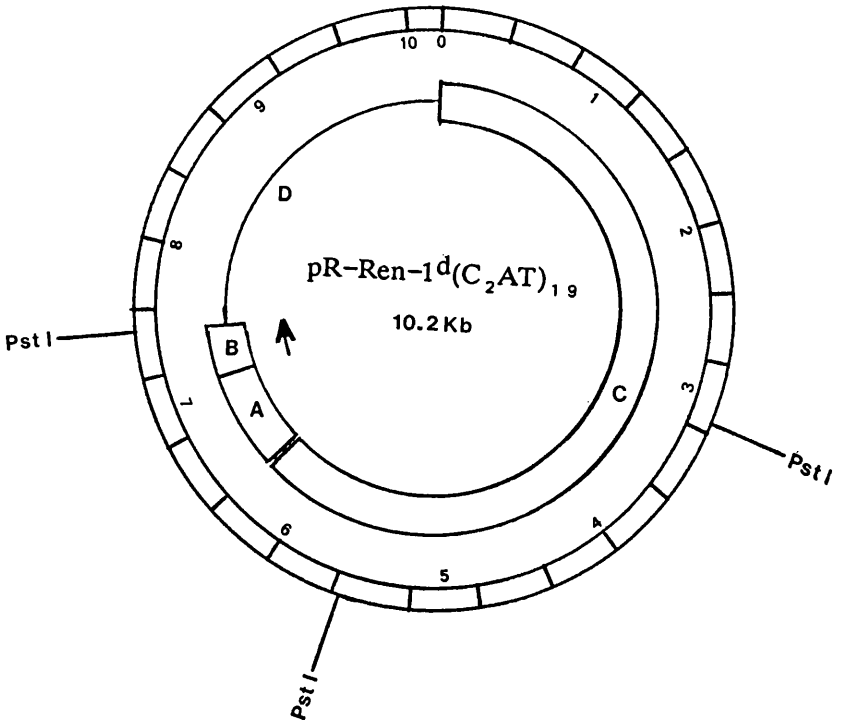
Figure 5.5

Restriction site maps of templates with the M and R fragments in the sense (+) and anti-sense (-) orientations upstream of the *Ren-1^d* promoter/G-free cassette fusion. A, *Ren-1^d* (717bp) promoter; B, G-free cassette; C, M and R fragments respectively; D, pUC13.


pM+Ren-1d(C2AT)19
10.5Kb
Pst I
Pst I
Pst I
Pst I
A
B
C
D
0
1
2
3
4
5
6
7
8
9
10


pM-Ren-1d(C2AT)19
10.5Kb
Pst I
Pst I
Pst I
Pst I
A
B
C
D
0
1
2
3
4
5
6
7
8
9
10


Pst I
10 0
1
2
3
4
5
6
7
8
9
D
pR+Ren-1^{d}(C$_2$AT)$_{19}$
10.2 Kb
B
A
C
Pst I
Pst I


10 0
1
2
3
4
5
6
7
8
9
D
pR-Ren-1^{d}(C$_2$AT)$_{19}$
10.2 Kb
B
A
C
Pst I
Pst I
Pst I

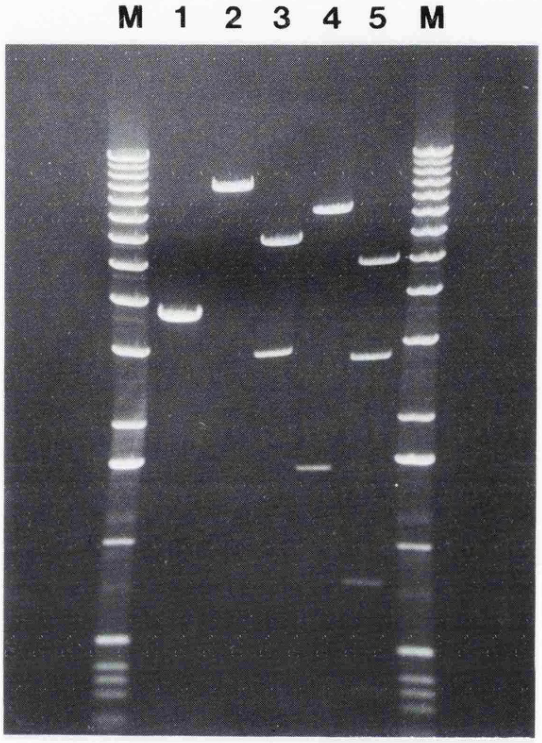


Figure 5.6

Restriction endonuclease digestion of "pL+Ren-1^{d}($C_2AT)_{19}$". 1, pRen-1^{d}($C_2AT)_{19}$; 2-5, "pL+Ren-1^{d}($C_2AT)_{19}$". 1 and 3, *Eco*RI digestion; 2, *Sst*I digestion; 4, *Kpn*I digestion; 5, *Xba*I digestion. Note that the plasmid length was approximately 9Kb (track 2) compared to the expected size of 10.3Kb (Figure 5.7).

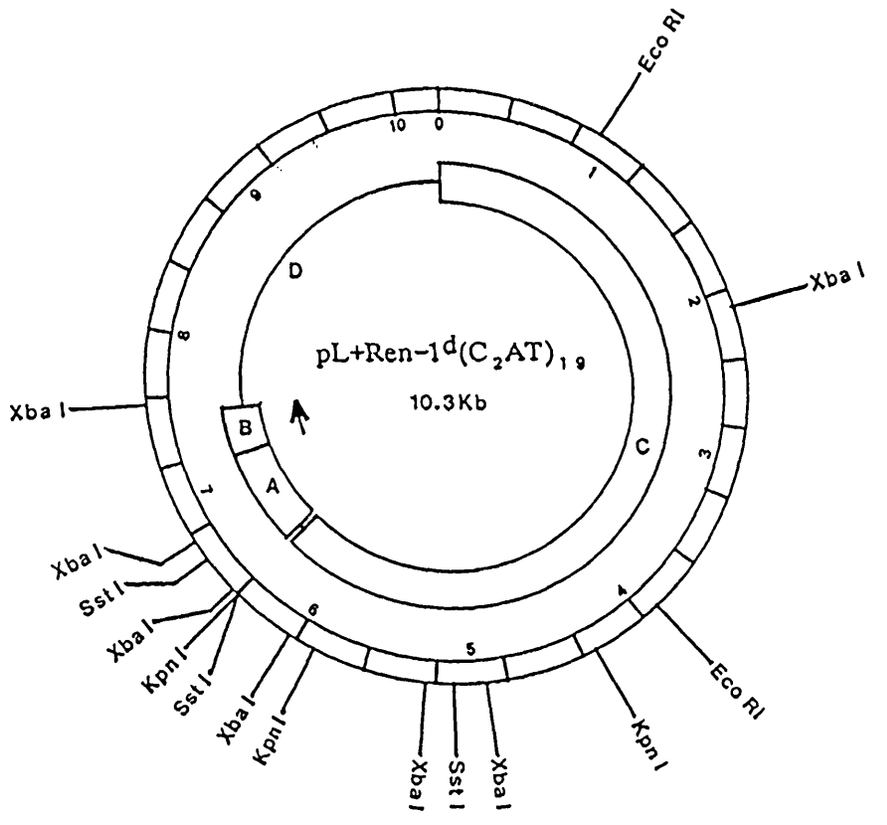


Figure 5.7

Restriction site map of "pL+Ren-$1^{d}(C_2AT)_{19}$", a template with the L fragment in the sense (+) orientation upstream of the *Ren-1^{d}* promoter/G-free cassette fusion. A, *Ren-1^{d}* (717bp) promoter; B, G-free cassette; C, L fragment; D, pUC13.

colonies and screened further by restriction endonuclease digestion (Figure 5.3). L, M and R fragments in the sense (+) orientation downstream of the *Ren–1^d* promoter/G–free cassette fusion gave the templates pRen–1^d(C_2AT)$_{19}$L+, pRen–1^d(C_2AT)$_{19}$M+ and pRen–1^d(C_2AT)$_{19}$R+ respectively (Figure 5.4).

5.2.2 Construction of Templates Containing the L, M and R Fragments Upstream of the *Ren–1^d* Promoter/G–free Cassette Fusion

The strategy for creating templates with the L, M and R fragments in the sense (+) and anti–sense (–) orientation upstream of the *Ren–1^d* promoter/G–free cassette fusion was similar to that outlined in Section 5.2.1 with the exception that the vector pRen–1^d(C_2AT)$_{19}$ was digested with the restriction endonuclease *Eco*RI instead of *Hin*dIII. Final screening by restriction endonuclease digestion (Figure 5.3) revealed the correct construction of the following templates: pM+Ren–1^d(C_2AT)$_{19}$; pM–Ren–1^d(C_2AT)$_{19}$; pR+Ren–1^d(C_2AT)$_{19}$; and pR–Ren–1^d(C_2AT)$_{19}$ (Figure 5.5). Attempts to prepare pL+Ren–1^d(C_2AT)$_{19}$ and pL–Ren–1^d(C_2AT)$_{19}$ templates have so far failed. Screening of colony hybridisation positives by restriction endonuclease digestion (compare Figure 5.6 with Figure 5.7), revealed that a deletion/recombination event was occuring in pL+Ren–1^d(C_2AT)$_{19}$ templates, which resulted in the absence of approximately 1.2Kb of DNA downstream of the second *Kpn*I restriction site present in the L fragment. The consequence was that the whole of the *Ren–1^d* P3 promoter driving the G–free cassette was deleted and therefore such templates were transcriptionally incompetent (Figure 5.8A, track 4). Since templates with the L fragment were grown in a *RecA*$^-$ strain the deletion/recombination event described here appeared to be *Rec* independent (Matfield *et al.*, 1985).

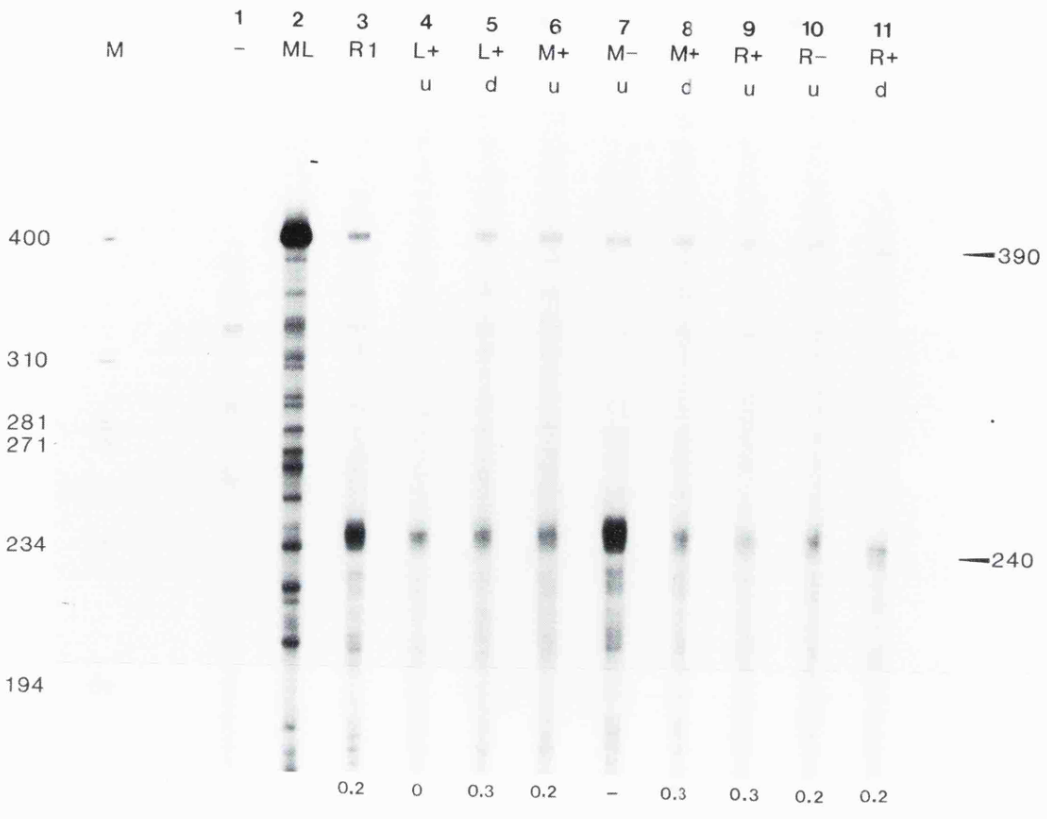
5.3 Effect of L, M and R Fragments on the Activity of the *Ren–1^d* Promoter

In–vitro transcriptions were carried out with nuclear–protein extracts prepared from adult DBA/2 male mouse kidney and liver respectively. In

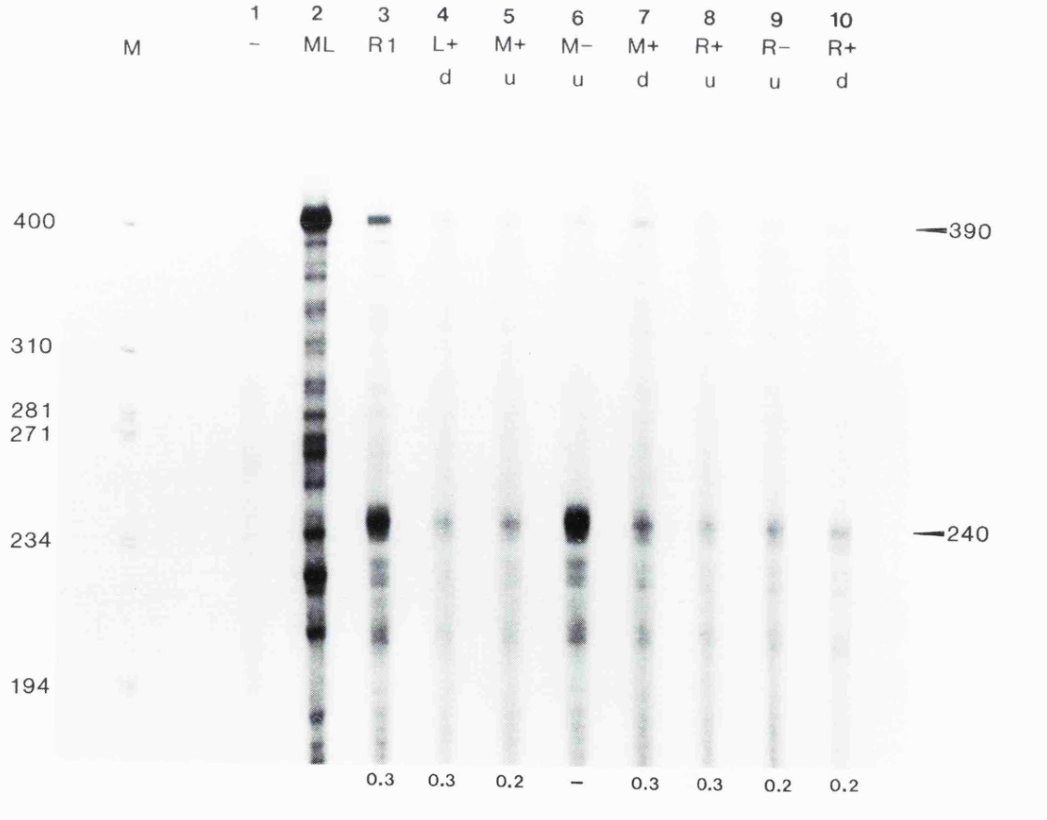
Figure 5.8

Effect of L,M and R fragments on the activity of the *Ren-1^d* promoter in A, mouse kidney and B, mouse liver nuclear-protein extracts. A1 and B1, p($C_2AT)_{19}$; A2 and B2, pML($C_2AT)_{19}$; A3 and B3, pRen-1^d($C_2AT)_{19}$; A4, "pL+Ren-1^d($C_2AT)_{19}$"; A5 and B4, pRen-1^d($C_2AT)_{19}$L+; A6 and B5, pM+Ren-1^d($C_2AT)_{19}$; A7 and B6, pM-Ren-1^d($C_2AT)_{19}$; A8 and B7, pRen-1^d($C_2AT)_{19}$M+; A9 and B8, pR+Ren-1^d($C_2AT)_{19}$; A10 and B9, pR-Ren-1^d($C_2AT)_{19}$; A11 and B10, pRen-1^d($C_2AT)_{19}$R+. M, *Hae*III digest of $\varphi X174$ DNA and figures on the left indicate the size of each marker band in base pairs. (See Figure 4.10 for details).

A


M
1 –
2 ML
3 R1
4 L+ u
5 L+ d
6 M+ u
7 M– u
8 M+ d
9 R+ u
10 R– u
11 R+ d
400
310
281
271
234
194
390
240
0.2 0 0.3 0.2 – 0.3 0.3 0.2 0.2

B


M
1 –
2 ML
3 R1
4 L+ d
5 M+ u
6 M– u
7 M+ d
8 R+ u
9 R– u
10 R+ d
400
310
281
271
234
194
390
240
0.3 0.3 0.2 – 0.3 0.3 0.2 0.2

both extracts, with the exception of pM-Ren-1^{d}(C_2AT)$_{19}$ (see below), the ratio of intensity between the control and test transcripts (as measured by laser densitometry) showed negligible difference in promoter activity, regardless of whether the L, M and R fragments were downstream in the sense (+) orientation, upstream in both orientations, or absent altogether, with respect to the *Ren-1*^{d} promoter (Figure 5.8A, tracks 3 to 11 and Figure 5.8B, tracks 3 to 10). These data suggest, at least *in-vitro*, that sequences 3' to the cap-site have little effect on transcription from the *Ren-1*^{d} promoter. Indirect evidence for downstream elements being involved in renin gene-expression was reported by Miller *et al.* (1989). These workers showed that a complete *Ren-1*^{d} transgene was expressed normally in mice containing only the *Ren-1*^{c} gene whereas a truncated *Ren-1*^{d} promoter/CAT fusion transgene failed to be expressed. However, as yet there have been no direct reports in the literature, be it in mouse, rat, or human, of downstream regulatory elements being involved in the control of renin gene-expression.

However, an interesting phenomenon was observed with the template pM-Ren-1^{d}(C_2AT)$_{19}$ with both kidney (Figure 5.8A, track 7) and liver (Figure 5.8B, track 6) nuclear-protein extracts. This template has the M fragment in the anti-sense (-) orientation upstream to the *Ren-1*^{d} promoter/G-free cassette fusion. When assayed, the activity of pM-Ren-1^{d}(C_2AT)$_{19}$ appeared no different to that of other related test templates whereas the activity of the control template pRen-1^{d}(C_2AT)$_{19}\triangle$150 increased 3- to 4-fold above that observed for other incubations (Figure 5.8A, compare track 7 with tracks 5 to 11 and Figure 5.8B, compare track 6 with tracks 4 to 10). When the M fragment was in the sense (+) orientation, either upstream or downstream to the *Ren-1*^{d} promoter/G-free cassette fusion (as in pM+Ren-1^{d}(C_2AT)$_{19}$ and pRen-1^{d}(C_2AT)$_{19}$M+ respectively), elevated activity of the control template was <u>not</u> observed (Figure 5.8A, tracks 6 and 8 and Figure 5.8B, tracks 5 and 7). Thus the apparent *trans* stimulation of pRen-1^{d}(C_2AT)$_{19}\triangle$150 in the presence of pM-Ren-1^{d}(C_2AT)$_{19}$ appeared to be orientation-dependent with respect to the M fragment (see Chapter 7). Further investigation will be required to understand the full implications of the above data.

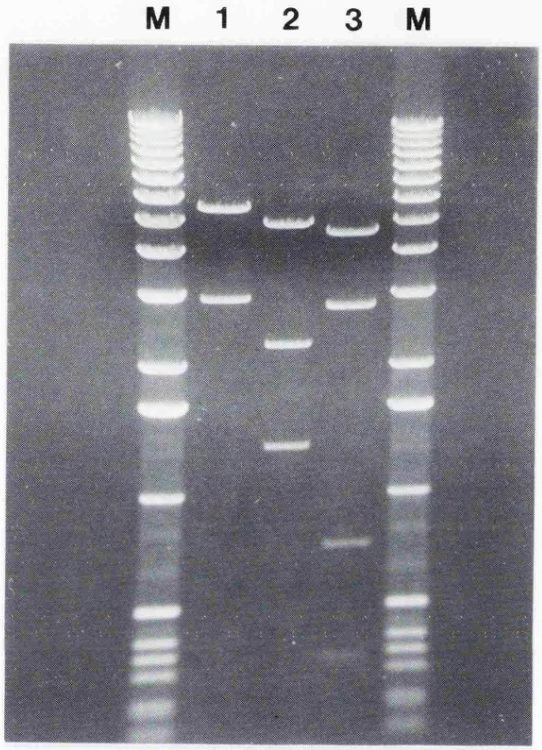


Figure 5.9

Restriction endonuclease digestion of pL+'Ren-1$^{d}(C_2AT)_{19}$. 1, *Eco*RI digestion; 2, *Pst*I digestion; 3, *Xba*I digestion.

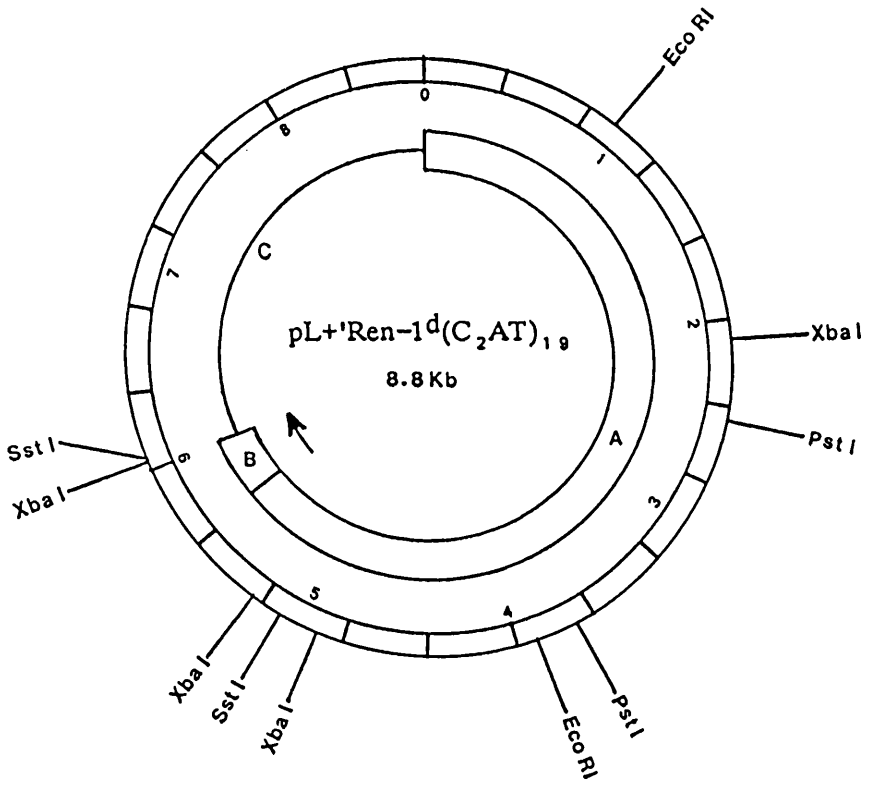


Figure 5.10

Restriction site map of pL+'Ren-$1^d(C_2AT)_{19}$. A, L+' fragment fused to the *Ren-1^d* promoter; B, G-free cassette; C, pUC13.

5.4 Construction of pL+'Ren-$1^d(C_2AT)_{19}$

In order to establish whether sequences 5' to nucleotide -704 of the *Ren-1^d* promoter fragment influences *in-vitro* transcription, the template pL+'Ren-$1^d(C_2AT)_{19}$ was constructed. This template has essentially a continuous promoter of approximately 5.6 kilobases fused directly to the G-free cassette and therefore correct spacings between potential regulatory elements are maintained.

pRen-$1^d(C_2AT)_{19}$ was digested with the restriction endonuclease *Eco*RI, and after purification by direct extraction, was blunt-ended using the 5' to 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. Following a second round of purification by direct extraction, pRen-$1^d(C_2AT)_{19}$ was cut with the restriction endonuclease *Sst*I and the large fragment purified via LMP agarose gel-electrophoresis.

The L fragment of pRen-1-L was cut out by a *Sal*I-*Bam*HI digestion, and after purification via LMP agarose gel-electrophoresis, was blunt-ended using the 5' to 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. Following purification by direct extraction, the blunt-ended L fragment was digested with the restriction endonuclease *Sst*I and the larger L' fragment purified via LMP agarose gel-electrophoresis.

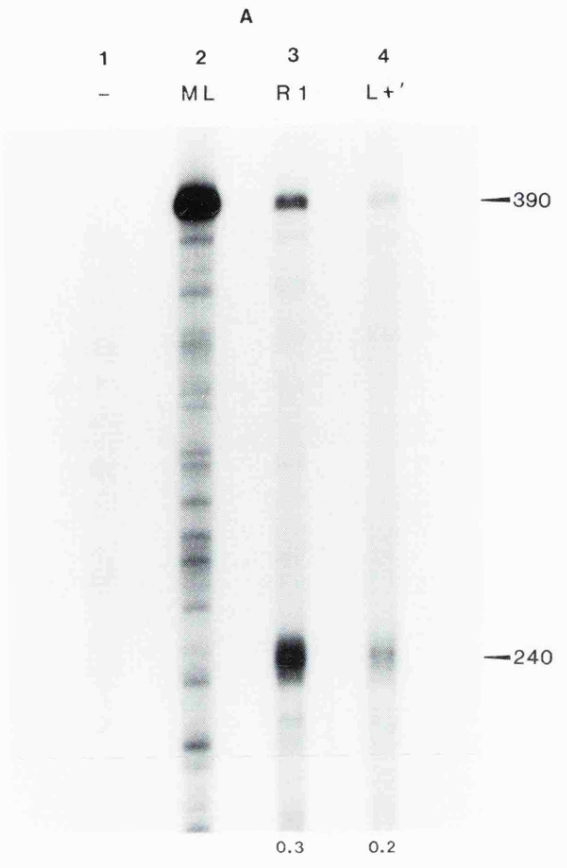
The L' fragment was now ligated into the blunt-ended and *Sst*I sites of pRen-$1^d(C_2AT)_{19}$ and the mixture used to transform *E. coli* JM101 cells. Transformants were screened for the correct fusion between the L' fragment and the *Ren-1^d* promoter by restriction endonuclease digestion (Figure 5.9) to give pL+'Ren-$1^d(C_2AT)_{19}$ (Figure 5.10).

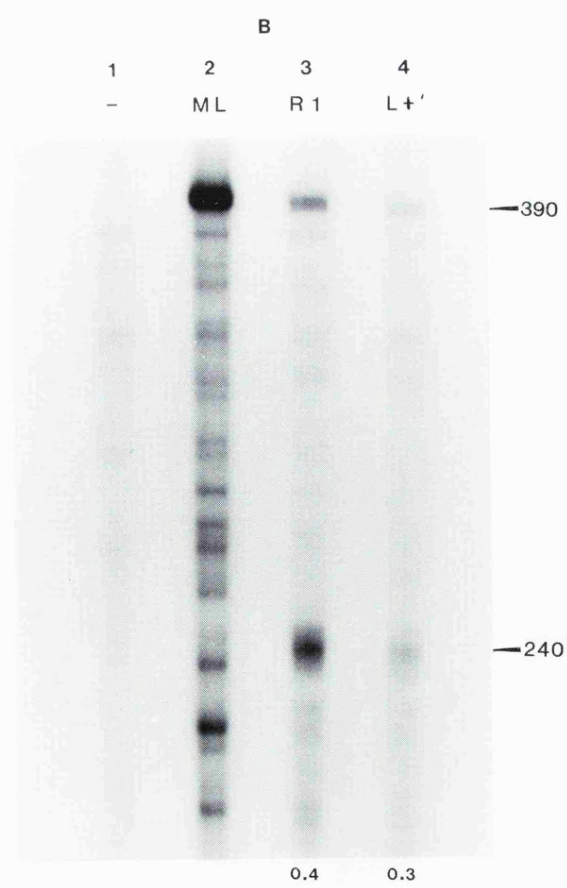
5.5 Activity of pL+'Ren-$1^d(C_2AT)_{19}$ in Driving the G-free Cassette *In-Vitro*

In-vitro transcriptions were carried out with nuclear-protein extracts prepared from adult DBA/2 male mouse kidney and liver. In both extracts the ratio of intensity between the control and test transcripts, for pRen-$1^d(C_2AT)_{19}$ and pL+'Ren-$1^d(C_2AT)_{19}$ showed negligible difference in promoter activity suggesting that *in-vitro*, sequences 5' to

Figure 5.11

In-vitro transcription of pL+'Ren-1^{d}(C_2AT)$_{19}$ in A, mouse kidney and B, mouse liver nuclear-protein extracts. 1, p(C_2AT)$_{19}$; 2, pML(C_2AT)$_{19}$; 3, pRen-1^{d}(C_2AT)$_{19}$; 4, pL+'Ren-1^{d}(C_2AT)$_{19}$. (See Figure 4.10 for details).


A
1
2
3
4
–
M L
R 1
L + ′
390
240
0.3
0.2


B
1
2
3
4
–
M L
R 1
L + ′
390
240
0.4
0.3

nucleotide −704 do not significantly influence transcription from the *Ren-1*^{d} promoter (Figure 5.11). It is possible that the presence of an "enhancer element" being down-regulated by a "silencer element" or vice versa would not be detected, unless fine deletion-mapping was embarked upon. However in gross terms the contributory effect of upstream sequences on *Ren-1*^{d} promoter activity appeared to be minimal.

CHAPTER 6

RESULTS

Protein-DNA Complexes Associated with the *Ren-1^d* and *Ren-2^d* Promoter Fragments

6.1 Mobility-Shift Assay

Protein-DNA interactions have been investigated by two principal methods: the filter-binding assay (Riggs *et al.*, 1970) and the mobility-shift assay (Garner and Revzin, 1981; and Fried and Crothers, 1981). The filter-binding assay relies on the ability of proteins to induce attachment of DNA to a nitrocellulose filter. A weakness of this method is that it does not generally allow analysis of the detailed composition of the products of the binding reaction. Interaction of a single protein with a DNA molecule is usually sufficient to cause filter retention, so it is frequently impossible to determine directly the extent to which a single DNA species binds several proteins. In contrast, the mobility-shift assay is based on the fact that protein-DNA complexes are resolved from free DNA species, as discrete bands, by electrophoresis through low ionic strength polyacrylamide gels. The low ionic strength conditions and the gel-matrix itself, stabilise the complex against dissociation (Fried and Crothers, 1981). Furthermore, microdensitometry allows for quantitation of the protein and DNA present in each complex. The mobility-shift assay has been elegantly used for the kinetic and equilibrium analysis of a number of prokaryotic DNA binding proteins (Hendrickson and Schleif, 1984, 1985; Fried and Crothers, 1984a, 1984b; and Bushman *et al.*, 1985) and has also been used to detect and purify eukaryotic proteins that were believed to recognise specific DNA sequences (Strauss and Varshavsky, 1984; Piette *et al.*, 1985; Carthew *et al.*, 1985; Singh *et al.*, 1986; and Lichtsteiner *et al.*, 1987).

In this work the mobility-shift assay was used to search for specific *trans*-acting factors which not only may influence transcription of the renin genes but may also be responsible for the differential expression of

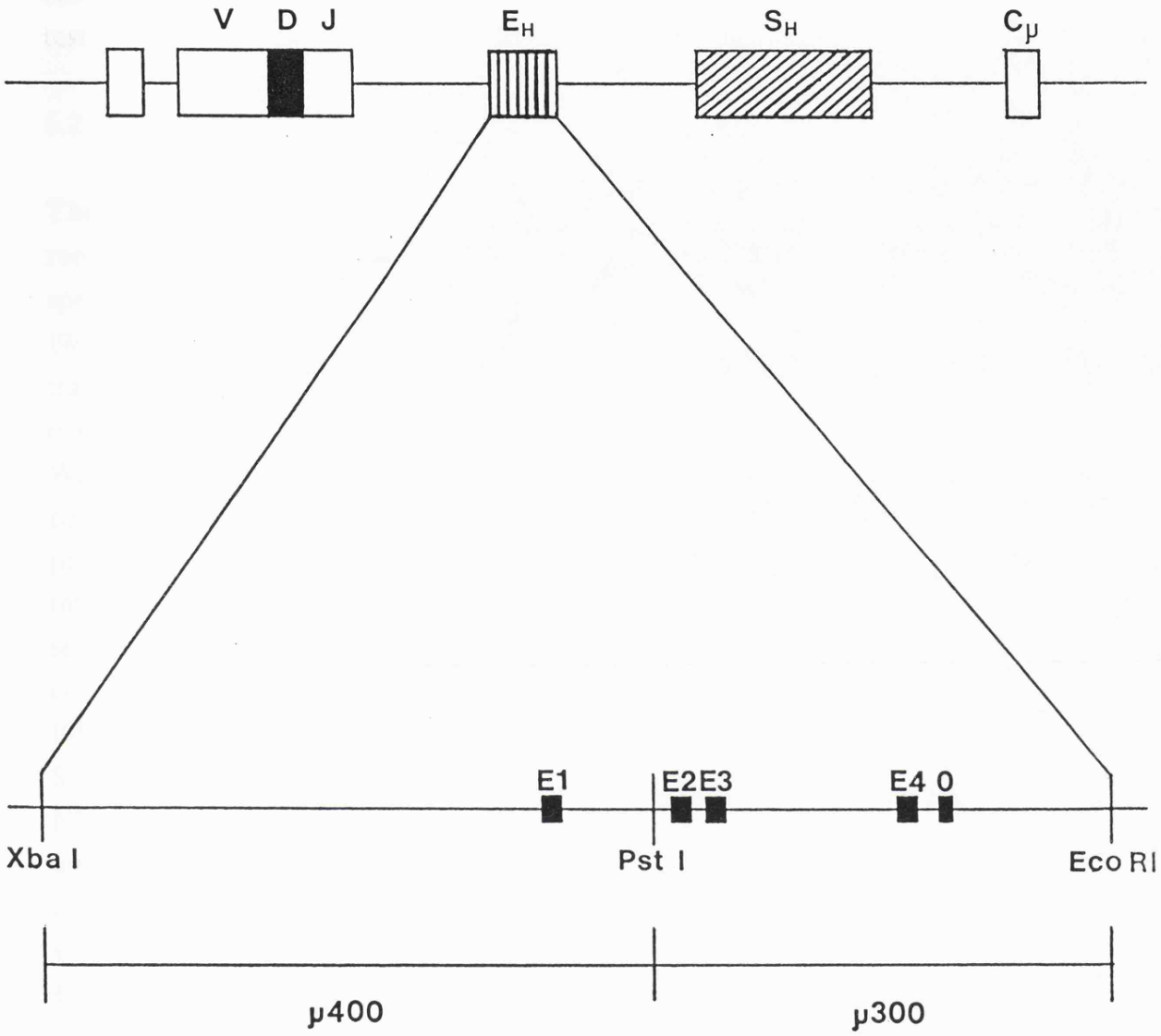


Figure 6.1

Organisation of the immunoglobulin heavy-chain enhancer. The immunoglobulin heavy-chain locus is shown with the intron containing the enhancer (E_H) and the μ switch region (S_H) separating the rearranged V-D-J (variable-diversity-joining) exons and the constant region μ chain gene (C_μ). The enhancer is located in a 678bp *Xba*I-*Eco*RI fragment and contains the specific binding sites E1-E4 and O (see text for details).

Ren-1^d and *Ren-2*^{d} in mouse tissues such as SMG, liver, kidney and testis (Dzau *et al.*, 1987).

6.2 The Immunoglobulin Heavy-Chain Enhancer as a Model System

The intron separating the variable- and constant-region exons of the rearranged immunoglobulin heavy-chain locus contains a lymphocyte-specific transcriptional enhancer (Banerji *et al.*, 1983; and Gillies *et al.*, 1983). The enhancer is a member of a class of *cis*-acting tissue-specific transcriptional control elements, which are characterised by orientation-independent and relatively position-independent function. Within this enhancer is an octomer motif, ATTTGCAT, which is also conserved between the mouse immunoglobulin heavy- and light-chain promoters and situated approximately 70bp upstream from the site of initiation (Parslow *et al.*, 1984; and Falkner and Zachau, 1984). This motif seems to be essential for efficient use of immunoglobulin promoters in B cells (Bergman *et al.*, 1984; Mason *et al.*, 1985; and Falkner and Zachau, 1984). Using a modified mobility-shift assay Singh *et al.* (1986) and Staudt *et al.* (1986) have identified protein nuclear-factors, NF-A1 and NF-A2, which bind to the said octomer motif. Furthermore *in-vivo* analysis of the position of DNA binding factors, by assessing the availability of specific bases to chemical modification, has identified four sequence clusters within the heavy-chain enhancer, denoted E1 to E4 (Ephrussi *et al.*, 1985; and Church *et al.*, 1985). Again using the mobility-shift assay Weinberger *et al.* (1986) have identified a mouse B-cell nuclear factor (NF-μE1) which binds specifically to E1 and binds poorly, if at all, to the other related motifs. Conversely, a different factor (NF-μE3) binds with high affinity to E3 but does not bind E1 to a significant degree (Sen and Baltimore, 1986). Thus the immunoglobulin heavy-chain enhancer must be recognised by at least three and probably more different factors.

The heavy-chain enhancer is located in a 678bp *Xba*I-*Eco*RI fragment and can be split internally at a *Pst*I site to generate approximately 400bp (μ400) and approximately 300bp (μ300) fragments respectively (Figure 6.1). Within μ400 is the binding site E1 and similarly within μ300 there are four binding sites, E2; E3; E4 and O (octomer motif). Thus it is possible to distinguish between specific binding to μ400 or μ300 and

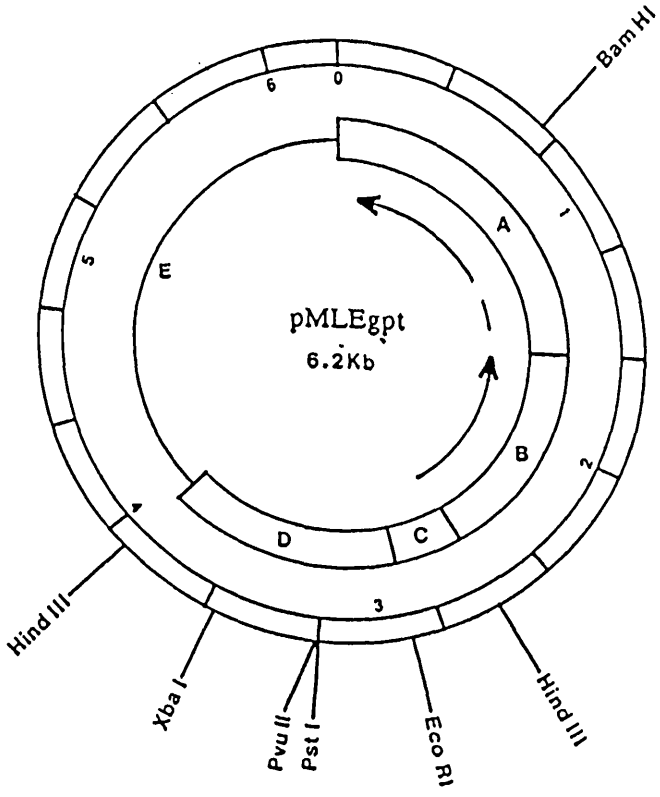


Figure 6.2

Restriction site map of pMLEgpt. A, SV40 T-antigen; B, guanine phosphoribosyltransferase (gpt); C, adenovirus-2 major-late promoter; D, immunoglobulin heavy-chain enhancer; E, pBR322.

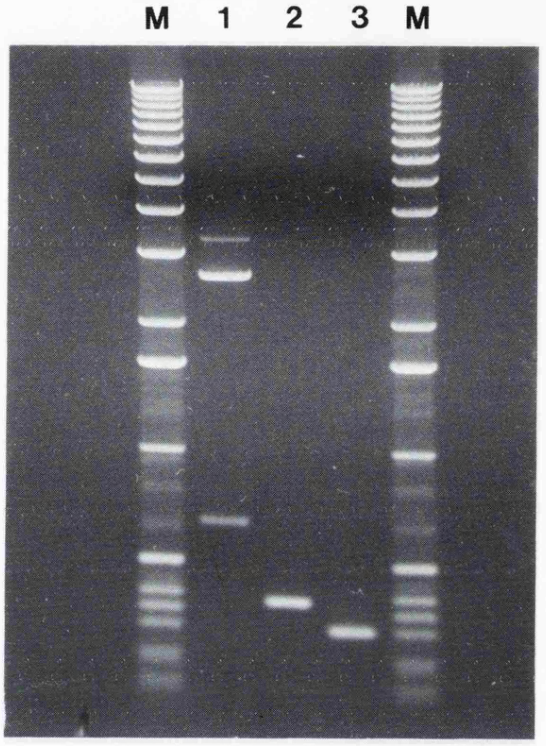


Figure 6.3

Preparation of μ400 and μ300 fragments from the immunoglobulin heavy-chain enhancer. 1, *Xba*I–*Eco*RI digestion of pUC19(μ700); 2, μ400; 3, μ300.

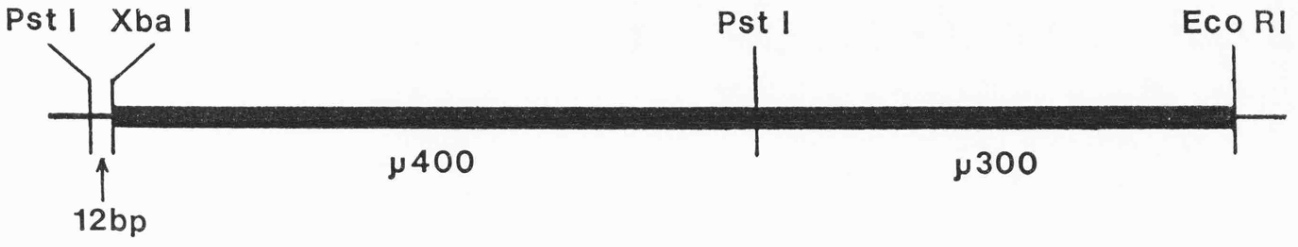


Figure 6.4

Scheme showing the rationale behind the exision of μ400 and μ300 by a *Pst*I–*Eco*RI digestion. The bold line represents the immunoglobulin heavy–chain enhancer and the thin line the pUC19 polylinker.

non-specific binding by the use of competing DNA fragments, since the binding of NF-μE1, NF-μE3 and NF-A are specific for E1, E3 and O respectively.

6.2.1 Preparation of μ400 and μ300 Fragments

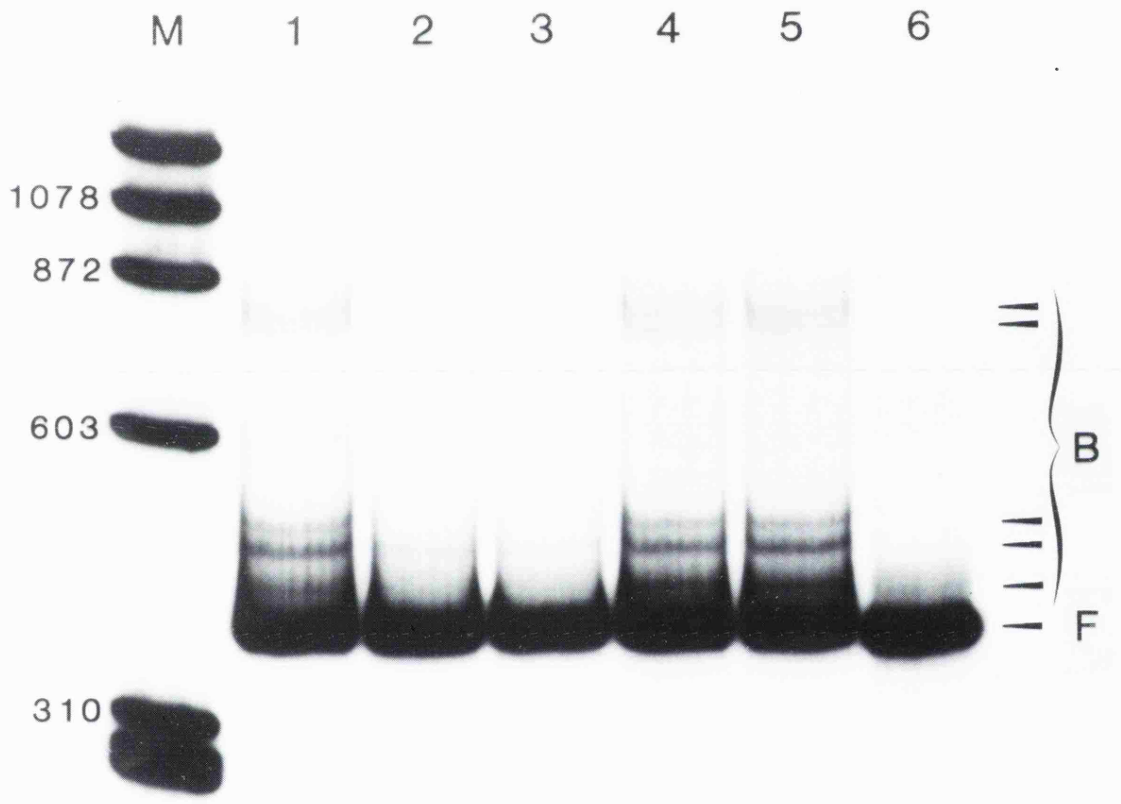
The immunoglobulin heavy-chain enhancer was obtained on the plasmid pMLEgpt (which was a generous gift from T.M. Harrison, Leicester University) (Figure 6.2). The *Xba*I-*Eco*RI restriction fragment, containing the enhancer element, was ligated into the polylinker of *Xba*I-*Eco*RI digested pUC19 and the mixture used to transform *E. coli* JM83 cells. Transformants were screened for the presence of the enhancer element by restriction endonuclease digestion (Figure 6.3) to give the plasmid pUC19(μ700). After large-scale plasmid purification by caesium chloride/ethidium bromide density-gradient centrifugation, the μ400 and μ300 fragments were cut out of pUC19 by a *Pst*I-*Eco*RI digestion (Figure 6.4) and purified via LMP agarose gel-electrophoresis (Figure 6.3). A μ400 probe was prepared by digesting the *Pst*I-*Pst*I μ400 fragment with the restriction endonuclease *Xba*I and the recessed 3' end was subsequently labelled with [α^{32}P]dCTP using the Klenow fragment of *E. coli* DNA polymerase I.

6.3 Establishing the Mobility-Shift Assay

The mobility-shift assay was established with DNA containing the heavy-chain enhancer of the immunoglobulin locus and nuclear-protein extract prepared from tumours grown by injecting Balb/c mice subcutaneously with J558L cells. The tumours were kindly grown by T.M. Harrison. In order to minimise non-specific protein-DNA interactions all incubations contained 2μg of the simple alternating copolymer duplex poly(dI-dC).poly(dI-dC) (Singh *et al.*, 1986; Weinberger *et al.*, 1986; and Sen and Baltimore, 1986). This simple alternating copolymer has the advantage over heterologous competitor DNA (*E. coli*) (Strauss and Varshavsky, 1984) in that the former probably competes less efficiently than the latter for binding of a sequence-specific factor thereby significantly increasing the sensitivity of the assay (Singh *et al.*, 1986).

Figure 6.5

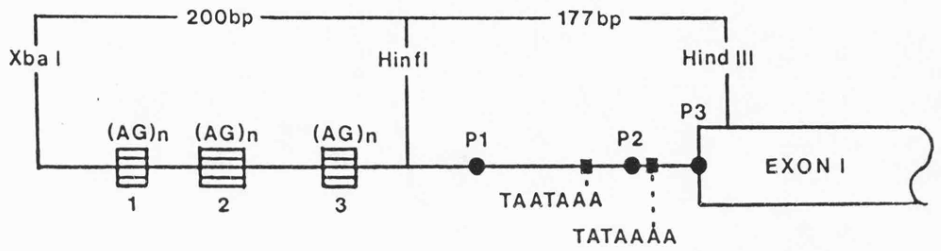
Nuclear factor binding to μ400 and competition analysis. Tracks 1–5 each contained 4fmol μ400 probe DNA and 8μg J558L nuclear–protein extract. In addition tracks 2 and 3 contained 200fmol and 400fmol cold μ400 respectively and tracks 4 and 5 contained 200fmol and 400fmol cold μ300 respectively. Track 6 contained only 4fmol μ400 probe DNA as a control. M, *Hae*III digest of φX174 DNA and the figures on the left indicate the size of each marker band in base pairs; B, bound probe; F, free probe.


M
1
2
3
4
5
6
1078
872
603
310
B
F

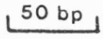
The mobility-shift assay can distinguish between specific and non-specific protein-DNA interactions (Figure 6.5). Clearly cold μ400 was able to compete for the binding of nuclear factor NF-μE1 (tracks 2 and 3), whereas cold μ300 which lacks the specific binding site E1, for NF-μE1 binding, showed no competition even when the concentration of μ300 was 100x greater than the μ400 probe (track 5). All the complexed bands observed are competed by μ400, but not by cold μ300, and therefore do not represent non-specific protein-DNA interactions. The data were different to those reported by Weinberger *et al.* (1986) who observed a single complexed species with NF-μE1 binding. However, similar discrepancies, with the octomer motif, have been reported. Singh *et al.* (1986) observed two complexed species with nuclear-protein extracts of a human B lymphoma cell-line (EW) and a human carcinoma cell-line (HeLa). Landolfi *et al.* (1986) also observed two complexes with HeLa cell extracts but with lymphoid cell extracts upto four distinct protein-DNA complexes were reported. Landolfi *et al.* (1986) suggested that the discrepancy may reflect technical differences in extract preparation and/or assay conditions. Each species may represent multimers of a single factor bound to the DNA. A single protein, differentially postranslationally modified (e.g. phosphorylation), could be responsible for generating some of the species. It was also possible that one or more of these complexes represented the interaction of one protein with another protein which was bound to the DNA. The putative second protein need not possess sequence-specific DNA-binding capability and may have affinity for the other nuclear factor only when the latter was bound to the DNA. It may well be that the formation of protein-DNA complexes observed in this work was the result of a combination of the above mechanisms and not the result of any single mechanism.

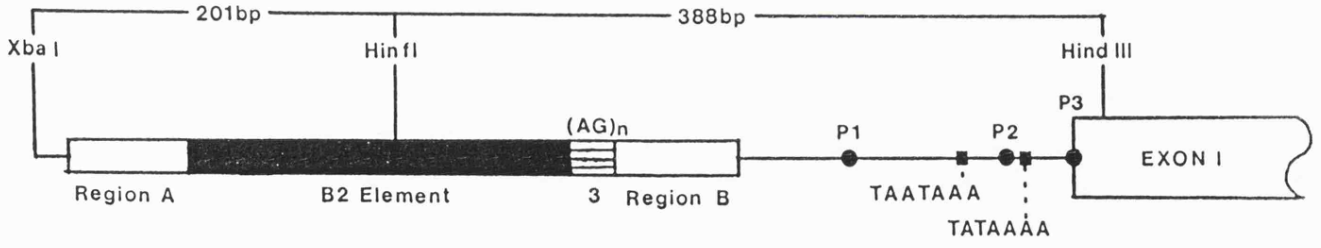
6.4 Preparation of the *Ren-1^d* and *Ren-2^d* Promoter Fragments as Probes for the Mobility-Shift Assay

The promoter organisation of *Ren-1^d* and *Ren-2^d* of high producer mice is illustrated in Figure 6.6. A detailed description of the two promoters is given in Section 1.5.3. The 5' flanking sequences of *Ren-1^d* (1872bp) and *Ren-2^d* (4881bp) were obtained on the plasmids pRen-1-Cat-2 and pRen-2-Tag-2 respectively (Figure 6.7). Probe fragments (Figure 6.6) for the mobility-shift assays were obtained from both plasmids by the



Ren–1^d





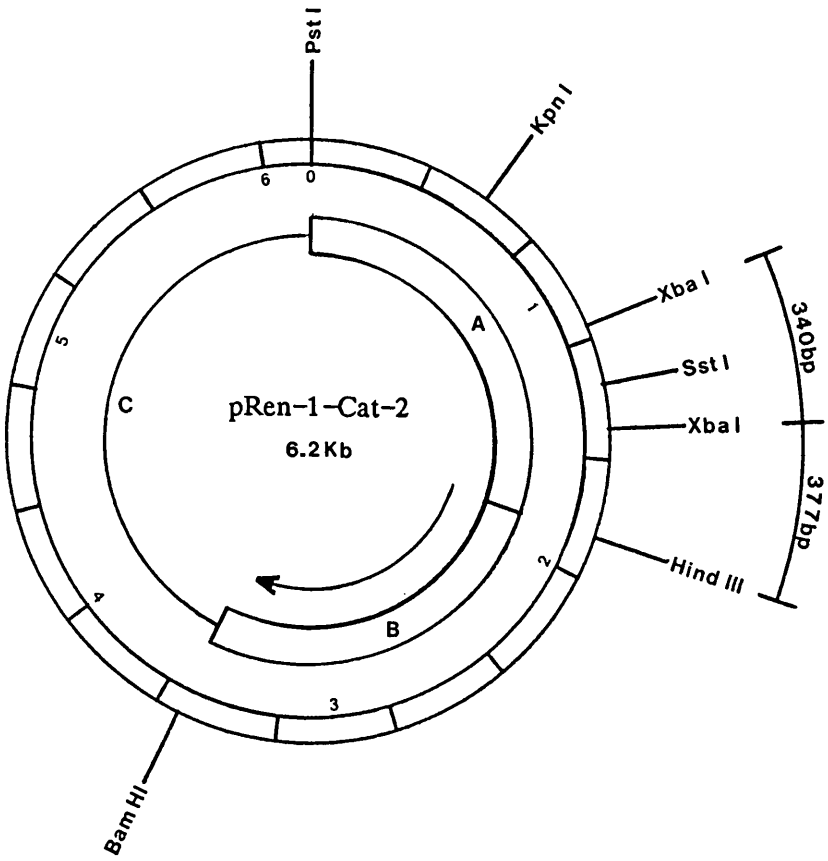
Ren–2^d

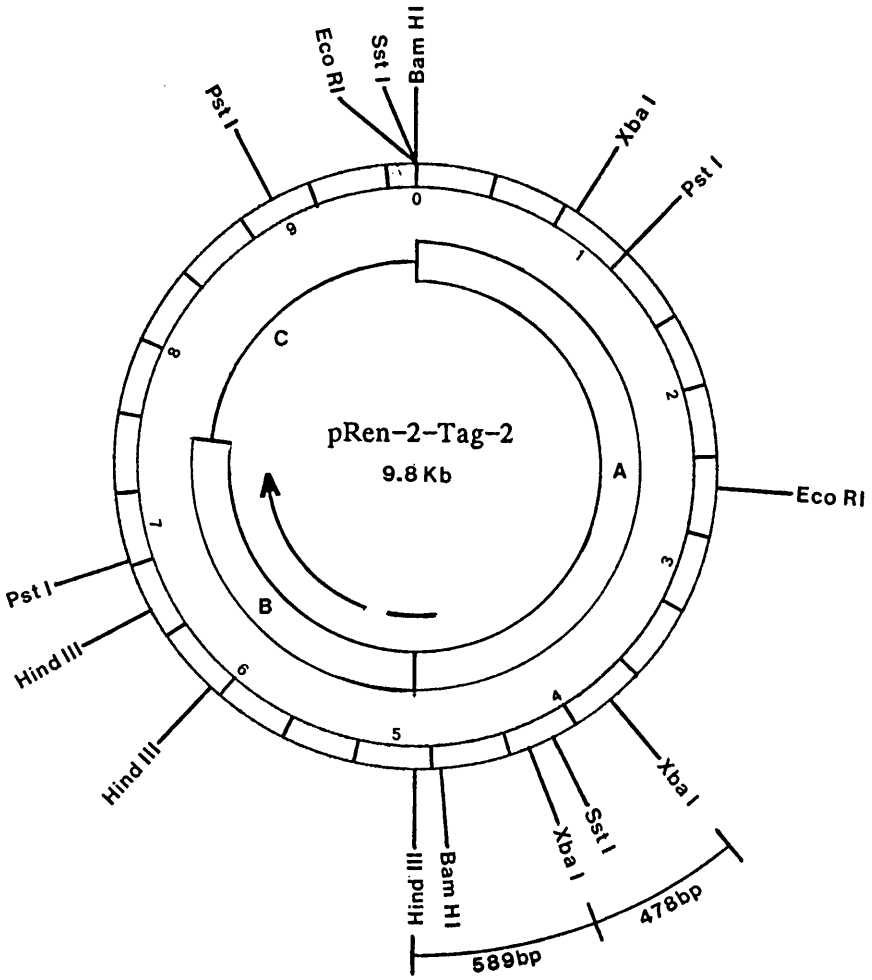
Figure 6.6

Detailed comparison of *Ren–1^d* and *Ren–2^d* promoter structure. P1, P2, P3, start–sites for mRNA transcription; TATAAAA, P3 TATA box; TAATAAA, P2 TATA box; (AG)n, polypurine tracts; Region A and Region B, rearranged sequence flanking the B2 *Alu* equivalent mobile element.

Figure 6.7

Restriction site maps of I, pRen-1-Cat-2 and II, pRen-2-Tag-2. I: A, *Ren-1*^{d} 5' sequence; B, chloramphenicol acetyltransferase (CAT); C, pUC8. II: A, *Ren-2*^{d} 5' sequence; B, SV40 T-antigen; C, pAT153.


Pst I
Kpn I
Xba I
Sst I
Xba I
Hind III
Bam HI
340bp
377bp
pRen-1-Cat-2
6.2Kb
A
B
C
0
1
2
3
4
5
6


Eco RI
Sst I
Bam HI
Pst I
Xba I
Pst I
Eco RI
Pst I
Hind III
Hind III
Hind III
Bam HI
Xba I
Sst I
Xba I
478bp
589bp
pRen-2-Tag-2
9.8Kb
A
B
C
0
1
2
3
4
5
6
7
8
9

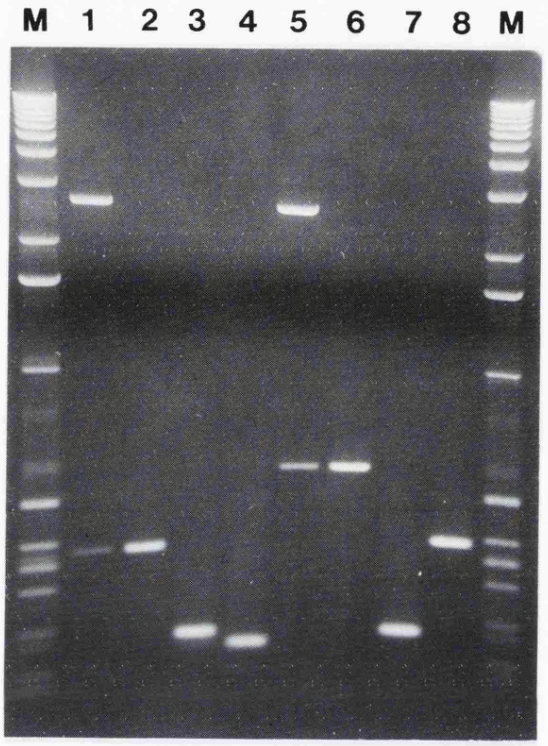


Figure 6.8

Preparation of fragments 200 and 177 (*Ren-1*^{d}) and 201 and 388 (*Ren-2*^{d}) for the mobility-shift assay. 1, *Xba*I-*Hin*dIII digestion of pRen-1^{d}(377); 2, fragment 377; 3, fragment 200; 4, fragment 177; 5, *Xba*I-*Hin*dIII digestion of pRen-2^{d}(589); 6, fragment 589; 7, fragment 201; 8, fragment 388.

following strategy. The *Xba*I–*Hin*dIII restriction fragments of both *Ren–1*^{d} (377bp) and *Ren–2*^{d} (589bp) (hereafter called 377 and 589 respectively) were ligated into the polylinker of *Xba*I–*Hin*dIII digested pUC19, and the mixtures used to transform *E. coli* JM83 cells respectively. Transformants were screened for the presence of insert by restriction endonuclease digestion (Figure 6.8) to give the plasmids pRen-1^d(377) and pRen-2^d(589) respectively. After large scale plasmid purification by caesium chloride/ethidium bromide density-gradient centrifugation, fragments 377 and 589 were cut out of pUC19 by an *Xba*I–*Hin*dIII digestion and purified via LMP agarose gel-electrophoresis (Figure 6.8). The purified 377 and 589 fragments were then digested with the restriction endonuclease *Hin*fI, and the resulting fragments (200bp and 177bp for *Ren–1*^{d} and 201bp and 388bp for *Ren–2*^{d}) (hereafter called 200, 177, 201 and 388 respectively) were purified via LMP agarose gel-electrophoresis (Figure 6.8).

200, 177, 201 and 388 probe fragments were prepared by labelling the recessed 3' ends of each restriction site with [α^{32}P]dATP plus cold dNTPs using the Klenow fragment of *E. coli* DNA polymerase I.

6.5 Protein–DNA Complexes Associated with the Renin Promoter Fragments

The mobility-shift assay was carried out with nuclear-protein extracts prepared from adult DBA/2 male mouse SMG and liver.

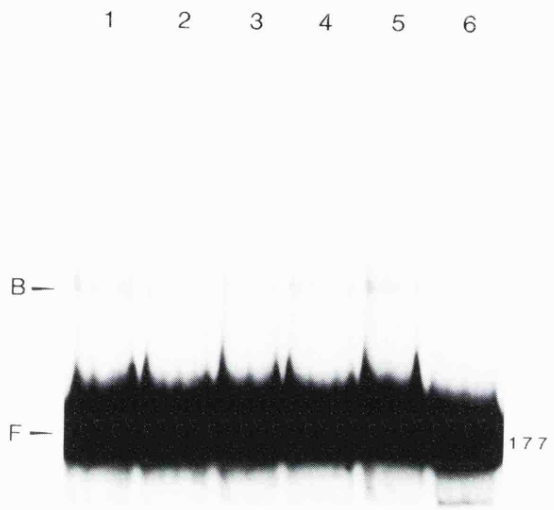
6.5.1 Complexes with SMG Nuclear-Protein Extract

With probe fragments 177 (*Ren–1*^{d}) and 388 (*Ren–2*^{d}) (Figure 6.9A and B respectively) a shift band (B) was observed which did not appear to be competed out by cold fragment (tracks 2 and 3) even when the cold fragment was in excess by 100x (track 3). The result was somewhat anomalous since a sequence-specific interaction would be expected to be competed out by cold fragment and a non-sequence specific interaction would be expected to produce a smear rather than a discrete band. A sequence-specific protein-DNA interaction was however observed with probe fragment 200 (*Ren–1*^{d}) but not with probe fragment 201 (*Ren–2*^{d}) (Figure 6.9C and D

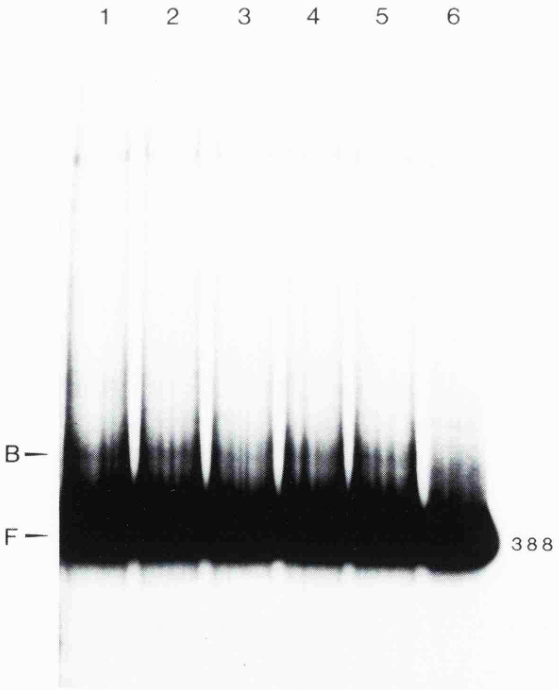
Figure 6.9

Binding of mouse SMG nuclear-protein factors to the *Ren-1*^{d} (A and C) and *Ren-2*^{d} (B and D) probe fragments (177, 200, 388, and 201 respectively). Tracks 1-5 each contained 4fmol probe fragment and 8μg mouse SMG nuclear-protein extract. In addition tracks 2 and 3 contained 200fmol and 400fmol cold fragment (competitive DNA) respectively and tracks 4 and 5 contained 200fmol and 400fmol cold μ400 (non-competitive DNA) respectively. Track 6 contained only 4fmol probe fragment as a control. Figures on the right represent the respective probe fragments. B, bound probe; F, free probe.

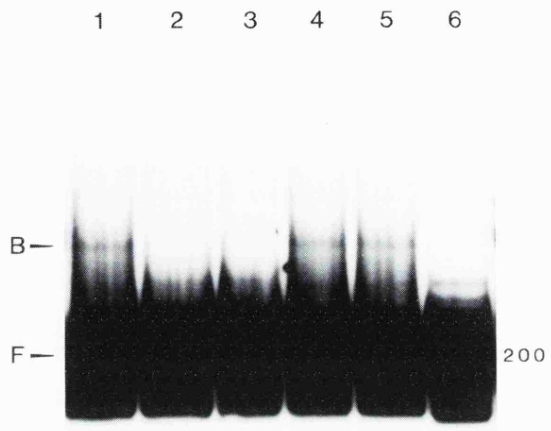
A



B



C



D

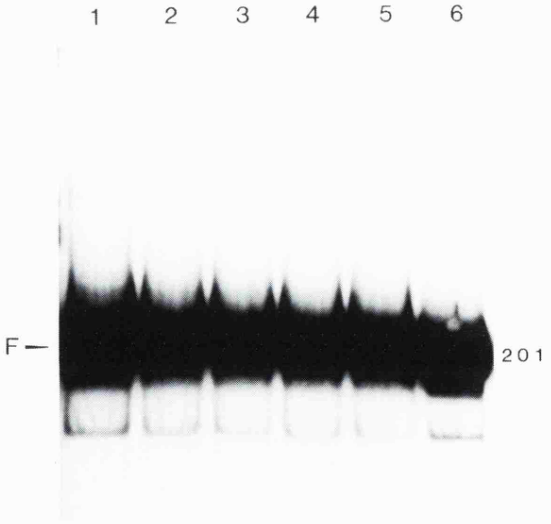
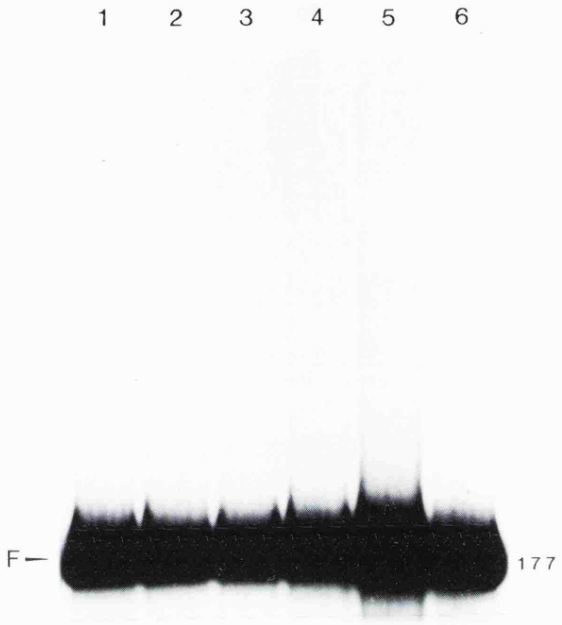


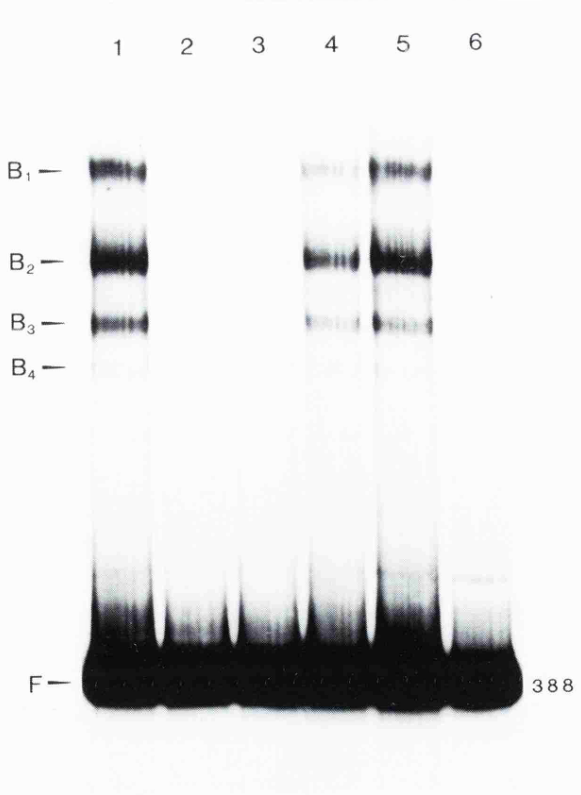
Figure 6.10

Binding of mouse liver nuclear-protein factors to the *Ren-1*^{d} (A and C) and *Ren-2*^{d} (B and D) probe fragments (177, 200, 388, and 201 respectively). Tracks 1-5 each contained 4fmol probe fragment and 8μg mouse liver nuclear-protein extract. In addition tracks 2 and 3 contained 200fmol and 400fmol cold fragment (competitive DNA) respectively and tracks 4 and 5 contained 200fmol and 400fmol cold μ400 (non-competitive DNA) respectively. Track 6 contained only 4fmol probe fragment as a control. Figures on the right represent the respective probe fragments. B, bound probe; F, free probe.

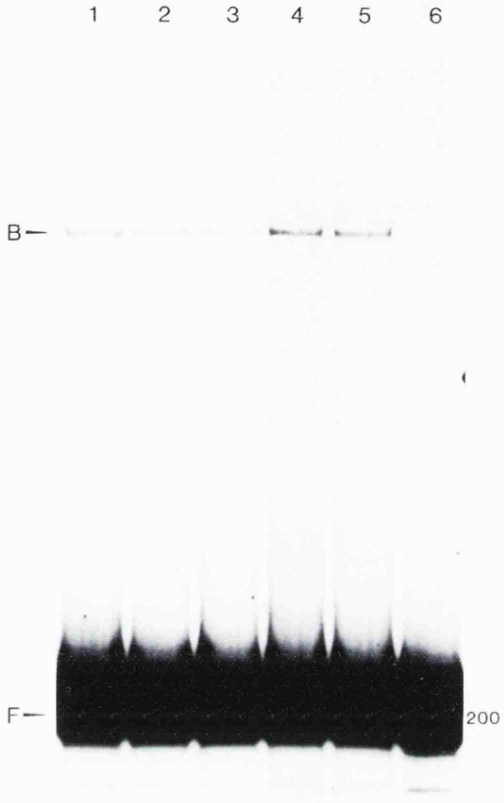
A



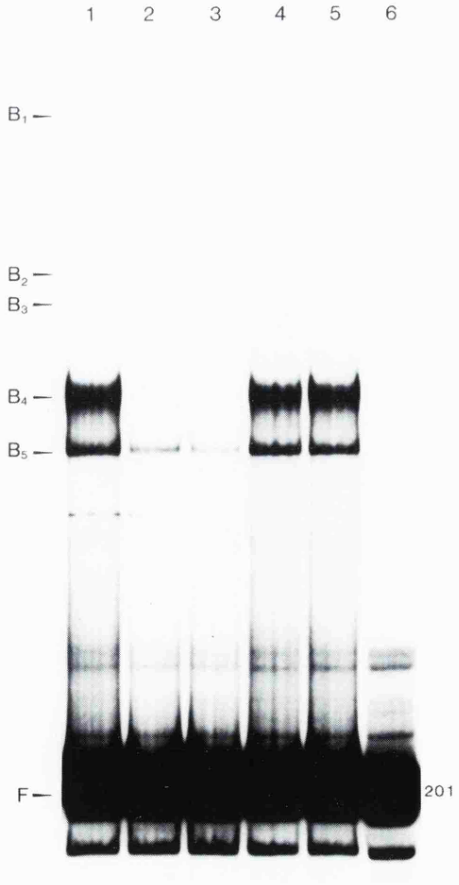
B



C



D



respectively).

6.5.2 Complexes with Liver Nuclear-Protein Extract

With the *Ren-2*^{d} probe fragments 388 and 201 (Figure 6.10B and D respectively), multiple sequence-specific interactions were observed (B1-B3 and B1-B5) but this was not the case for the *Ren-1*^{d} probe fragments 177 and 200 (Figure 6.10A and C respectively) which failed to show any sequence-specific protein-DNA binding. As with SMG nuclear-protein extract non-competed shift bands were also present with probe fragments 388 (B4) and 200.

In the SMG of high producer mice the accumulation of *Ren-2*^{d} transcripts is approximately 100x greater than those for the *Ren-1*^{d} gene, whereas in the liver only *Ren-1*^{d} transcripts are detected (see Section 1.4.3). Field and Gross (1985) argued that the reduced level of *Ren-1*^{d} gene-expression in the SMG relative to *Ren-2*^{d} was due to non-responsiveness of the *Ren-2*^{d} gene to a negative control. Indeed, Nakamura *et al.* (1989) demonstrated that a negative control element(s) was functional in *Ren-1*^{d} but was non-functional in *Ren-2*^{d}, and they went on to suggest that the 160bp insertion in the *Ren-2*^{d} gene interfered with such control (see Section 7.1). In this work the proteins associated with the complexes produced with the SMG and liver extracts not only appeared to be tissue-specific but also appeared to have distinctly different binding patterns suggesting the involvement of different proteins. Furthermore, the pattern of protein-DNA binding supported by these data was consistent with a negative control hypothesis, where the binding of tissue-specific *trans*-acting factors switched off gene-expression. For example with respect to the *Ren-2*^{d} gene, protein-DNA binding was only observed with liver nuclear-protein extract where *in-vivo* this gene is silent. With respect to the *Ren-1*^{d} gene, Miller *et al.* (1989) has shown that *Ren-1*^{c} is expressed approximately 10-fold higher than *Ren-1*^{d} in the SMG and that *Ren-1*^{d} is expressed to a slightly higher level in the liver compared to SMG. In this work protein-DNA binding to the *Ren-1*^{d} gene was only observed with SMG nuclear-protein extract where *in-vivo* this gene is very poorly expressed. Furthermore the presence of *cis*-acting negative control element(s) in *Ren-1*^{d} has been reported by Nakamura *et al.* (1989) (see

Chapter 7) but further work is required to determine the different controlling mechanisms of the renin genes. Footprinting analysis may give some information about which DNA sequences are involved in protein-binding and the proteins associated with those binding-sites.

CHAPTER 7

DISCUSSION

High renin-producing mouse strains harbour two genes *Ren-1*^{d} and *Ren-2*^{d}, whereas low renin producing strains have just one gene, *Ren-1*^{c}. However the differential production of renin seen between high and low producer mice cannot be explained simply in terms of gene-dosage and much effort has been made to try and understand how these genes are regulated. Furthermore the observation that renin gene-expression is under genetic, developmental, hormonal and tissue-specific controls suggests that the renin genes may provide a system in which to study aspects of these controlling mechanisms and their interaction during different stages of development.

Much of the initial work on renin regulation has involved the measurement of its mRNA and/or the protein product itself under various conditions and in various tissues. From such studies evidence has accumulated which suggests that the renin genes are controlled principally at the level of transcription (Catanzaro *et al.*, 1985; Karen and Morris, 1986; Nakamura *et al.*, 1989; and Burt *et al.*, in press). Extensive structural analyses, by restriction endonuclease mapping, heteroduplex analysis and DNA sequencing, have revealed differences between *Ren-1*^{d} and *Ren-2*^{d} which may account for their differential expression (Figure 1.6). These differences include the presence of a mouse B2 (*Alu* equivilent) mobile element 3' to exon IX in *Ren-1*^{d} and 5' to exon I in *Ren-2*^{d} (I3) (Field *et al.*, 1984; Burt *et al.*, in press; and Krayev *et al.*, 1982); two insertions I1 and I2, which lack homology with any known insertion sequence, 5' to exon I in *Ren-2*^{d} (Burt *et al.*, in press); and a 3Kb element, which has homology to IAP genomes, 3' to exon IX in *Ren-2*^{d} (Burt *et al.*, in press). The mouse renin genes have also been shown to have three transcription start-sites P1, P2 and P3 whose utilisation is tissue-specific. However P3 is the major transcription start-site, and renin tissue-specificity is thought to be due to relative utilisation of P3 rather than to utilisation of different promoters (Field *et al.*, 1984). In this work an attempt has been made to determine those

regions involved in the control of renin gene-expression in the mouse using an *in-vitro* transcription assay (Sawadogo and Roeder, 1985a; and Gorski *et al.*, 1986) (see Section 3.1).

7.1 Deletion-Mapping

In pRen-1$^{d}(C_2AT)_{19}$ and pRen-2$^{d}(C_2AT)_{19}$ the respective renin promoters have been fused to the G-free cassette in such a way as to allow only those transcripts initiating from the P3 promoter to be detected (see Section 3.3). Transcription in various nuclear-protein extracts (Figure 3.11) showed that these templates were able to initiate accurately and produce full-length transcripts. Therefore those *cis*-acting elements required for accurate *in-vitro* initiation at P3 were present within these promoter fragments. In mouse kidney, liver and testis nuclear-protein extracts both the *Ren-1^{d}* and *Ren-2^{d}* promoters were active, whereas *in-vivo* only *Ren-1^{d}* transcripts can be detected in the liver (Miller *et al.*, 1989; and Lillycrop, unpublished data). These results suggested that either the *in-vitro* assay did not fully mimic the *in-vivo* situation or that the regions involved in tissue-specific control were located outside of the promoter fragments used. However limited tissue-specificity was observed, since the *Ren-2^{d}* promoter was found to be approximately 2-fold more active than the *Ren-1^{d}* promoter in all extracts except mouse liver extract where activity was equal for both promoters. The 2-fold difference may reflect either a positive effect on the *Ren-2^{d}* promoter or a negative effect on the *Ren-1^{d}* promoter. Results from deletion-mapping of both promoters (Figure 4.10), via restriction endonuclease digestion, appeared to favour the presence of a negative control element(s) within the *Xba*I fragment (fragment 340) of the *Ren-1^{d}* gene.

The presence in *Ren-1^{d}* of such a negative control element(s) was reported by Nakamura *et al.* (1989). These workers have cloned in either orientation the 340bp *Ren-1^{d} Xba*I fragment (in this work called fragment 340) from pRn34 into the *Xba*I site of pUTKAT1, a chloramphenicol acetyltransferase (CAT) expression vector that contains the truncated thymidine kinase (TK) promoter. Similarly they have also cloned the 480bp *Ren-2^{d} Xba*I fragment (in this work called fragment 478) from pBa6 in either orientation into the *Xba*I site of pUTKAT1.

Transfection of these chimeric templates into mouse pituitory tumor AtT-20 cells and human choriocarcinoma JEG-3 cells, revealed that the *Ren-1^d* 5' flanking sequence in the sense orientation inhibited basal CAT expression from the TK promoter of pUTKAT1 by greater than 60%, whereas the same sequence in the anti-sense orientation did not. The 5' flanking sequence of *Ren-2^d* had no inhibitory effect on basal CAT expression. The data presented by Nakamura *et al.* (1989) demonstrated that a negative control element(s) was functional in *Ren-1^d* but was non-functional in *Ren-2^d*, and they went on to suggest that the 160bp insertion in the *Ren-2^d* gene (Figure 7.1) interferes with the function of the negative control element(s). Indeed the renin DNA fragments used by these workers were shown to contain two sequences homologous to the negative element of the chicken lysozyme gene (Steiner *et al.*, 1987) (Figure 7.1). However since negative control only functioned in one orientation, the putative *Ren-1^d* control sequences were not by definition silencer elements as observed in other genes (Brand *et al.*, 1985; and Steiner *et al.*, 1987).

However, in this work the differences in promoter activity obtained with the deletion-mapping analysis were too small to be accurately and consistantly determined. Part of the problem may be due to the fact that in the kidney, for example, only the juxtaglomerula cells, which constitute <1% of the total cell population, synthesise renin (Field and Gross, 1985). Therefore *trans*-acting factors specifically produced by only these cells would be in relatively low concentration, and since only the P3 TATAAAA need be present for transcription initiation *in-vitro* to occur (see below), their effects on renin promoter activity would be diluted by the transcriptional competence of the remaining cell population. Therefore it is important to establish whether the *in-vitro* data can be supported *in-vivo* by using a more sensitive method such as a transient expression assay in cultured cells with for example CAT as a reporter gene or more favourably using transgenic mouse technology.

Deletion-mapping by restriction endonuclease digestion also showed that for both *Ren-1^d* and *Ren-2^d* a promoter fragment of just 122bp was sufficient for transcription *in-vitro*. Finer deletion-mapping of the *Ren-1^d* promoter fragment using PCR technology (Figure 4.16) revealed that the presence of only the P3 promoter was sufficient for accurate

Figure 7.1

Nucleotide sequence of *Ren-1*^{d} and *Ren-2*^{d} showing the 160bp insertion in the *Ren-2*^{d} gene and sequence homologies to the negative element of the chicken lysozyme gene (underlined).

```
          -700        -690        -680        -670        -660
Ren-1d TCTAGAGTCATTGGGCTCAGcCACCCTTCCCACACCCCCATGCCTGCCAC
Ren-2d TCTAGAGTCATTGGGCTCAG.CACCCTTCCCACACCCCCATGCCTGCCAC
          -1050       -1040       -1030       -1020       -1010

          -650        -640        -630        -620
Ren-1d CACTCTGCTCTGCGACCAG.GTtcTGCTTATCCTATACCT.....
Ren-2d CACTCTGCTCTGCGACCAGtGTctTGCTTATCCTATACCTcacctaagct
          -1000       -990        -980        -970        -960


Ren-1d .....
Ren-2d acatgccataaatcaatgcttgttattcaaggcatcatctcctttgggggg
          -950        -940        -930        -920        -910


Ren-1d .....
Ren-2d gcacttctcaaaaagtcctctcctgtccctagatatctgagactcctcag
          -900        -890        -880        -870        -860

                                                      -610
Ren-1d .....ACcTaAcTTgg
Ren-2d tggccttccatttagaagggtgttgtctctgaagtaaaaACtTcAgTTcc
          -850        -840        -830        -820        -810

          -600        -590        -580        -570        -560
Ren-1d TCTCACAGGCTAgaATTTATCAGgaCTGcCCTGCCATGGGCCTTGTTGGg
Ren-2d TCTCACAGGCTAagATTTATCAGagCTG.CCTGCCATGGGCCTTGTTGG.
          -800        -790        -780        -770        -760

          -550        -540        -530        -520        -510
Ren-1d CAcCTGGCaTGGGgaAATAAaGGaGGTGGgCGAGAcCTGTGTCtCacCAG
Ren-2d CA.CTGGCgTGGG..AATAA.GGgGGTGG.CGAGA.CTGTGTC.CcgCAG
             -750        -740        -730        -720

          -500        -490        -480        -470        -460
Ren-1d AGCTCAGAgtGACTGGatgGGCAGACAGCAGGgGAAGgCACTGGGTGGTC
Ren-2d AGCTCAGA..GACTGGgcaGGCAGACAGCAGGaGAAGaCACTGGGTGGTC
          -710        -700        -690        -680        -670

          -450        -440        -430        -420        -410
Ren-1d TGGCAGCTGGAAACGCTGGGAGGCCTTCTTGgGGGAaATTAGATGAaGCT
Ren-2d TGGCAGCTGGAAACGCTGGGAGGCCTTCTTGcGGGAgATTAGATGAgGCT
          -660        -650        -640        -630        -620
```

transcription initiation and that disruption of the P3 TATAAAA resulted in total loss of promoter activity. Thus it appeared that *in–vitro* a fragment of just 62bp from -47 to +15 (Figure 4.13) was sufficient for driving the G-free cassette. Furthermore the data confirmed that the transcripts produced in the *in–vitro* transcription assay were only initiating from the P3 promoter as expected. Interestingly, Miller *et al.* (1989) reported that transgenic founder lines containing fusion genes with approximately 5 Kb of upstream renin promoter DNA driving sequences encoding the reporter gene CAT failed to express CAT protein. Since the expression pattern of a complete *Ren–2^d* transgene, containing only 2.5 Kb of upstream sequence, has been shown to be identical to that observed in wild-type animals in which *Ren–1^d* and *Ren–2^d* are closely linked on chromosome 1 (Tronik *et al.*, 1987), these workers suggested that elements downstream of the renin promoter are essential for renin gene-expression. This work shows however that *in–vitro*, downstream sequences are not required for accurate transcription initiation from the major transcription start-site P3; only the P3 TATAAAA need be present.

Although PCR technology has been used for amplifying single-copy genomic sequences and other DNA sequences in low concentration, there have been no reports of the direct use of PCR products in *in–vitro* transcription systems. In this work linear templates produced by such technology have been used successfully to drive transcription *in–vitro* and the main advantage has been the speed in which alterations could be generated, within a given template, and assayed compared to conventional methods.

Finally sequence analysis has revealed the presence of an AP-2 consensus sequence within the promoter fragment of both renin genes (Burt *et al.*, in press). In an attempt to determine whether AP-2 influences promoter activity, its consensus sequence was deleted from the *Ren–1^d* gene (see Sections 4.6 to 4.8). The rate of transcription *in–vitro* in the absence of the AP-2 consensus sequence was found to be no different to that of the wild-type promoter, suggesting that the AP-2 site was non-functional. Although AP-2 has been shown to function *in–vitro* in the absence of exogenous cAMP or phorbol esters, agents known to increase AP-2 activity (Imagawa *et al.*, 1987), it would still be interesting

to determine whether such agents are required for AP-2 regulated renin gene-expression. It should be pointed out that although an AP-2 consensus sequence is present within the *Ren-1^d* promoter the question still remains as to whether such a sequence is able to function as an AP-2 binding-site. Footprinting analysis in the presence and absence of purified AP-2 may provide some answers. Furthermore the ability of the renin AP-2 consensus sequence to function as a regulatory element could be investigated by inserting the renin consensus sequence upstream of a well characterised AP-2 regulated promoter, such as the hMT-II$_A$ promoter, and assaying the template in the presence and/or absence of AP-2, cAMP and phorbol esters. Such data could only add to our understanding of renin gene-regulation.

7.2 Effect of Downstream Sequences on Promoter Activity

The ability of DNA sequences located 3' to the cap-site to regulate gene-expression has been well documented (see Section 5.1) but in the case of the *Ren-1^d* gene such sequences appeared to have a minimal influence on P3 promoter activity *in-vitro*. However an interesting phenomenon was observed where activity of the internal control template pRen-1^d(C$_2$AT)$_{19}$$\triangle$150 increased 3- to 4-fold when incubated with pM-Ren-1^d(C$_2$AT)$_{19}$, but remained at a basal level when incubated with pM+Ren-1^d(C$_2$AT)$_{19}$ (Figure 5.8). The apparent *trans* stimulation of pRen-1^d(C$_2$AT)$_{19}$$\triangle$150 in the presence of pM-Ren-1^d(C$_2$AT)$_{19}$ was therefore orientation dependent with respect to the M fragment.

One may suggest a tentative hypothesis where, in the anti-sense (-) orientation only, a binding-site was created at the junction between the M fragment and the vector DNA, which was able to preferentially titrate-out specific down-regulating *trans*-acting factors responsible for suppression of transcriptional activity at the *Ren-1^d* P3 promoter. However because of the close proximity of the "new" binding-site with respect to the native site present within the *Ren-1^d* promoter fragment (Nakamura *et al.*, 1989), down-regulation of pM-Ren-1^d(C$_2$AT)$_{19}$ was maintained whereas activity of pRen-1^d(C$_2$AT)$_{19}$$\triangle$150 increased in the absence of factor-binding (Figure 7.2). The above hypothesis is purely speculative and although association of *cis*-acting negative control element(s) with the *Ren-1^d* gene has been reported (Nakamura *et al.*,

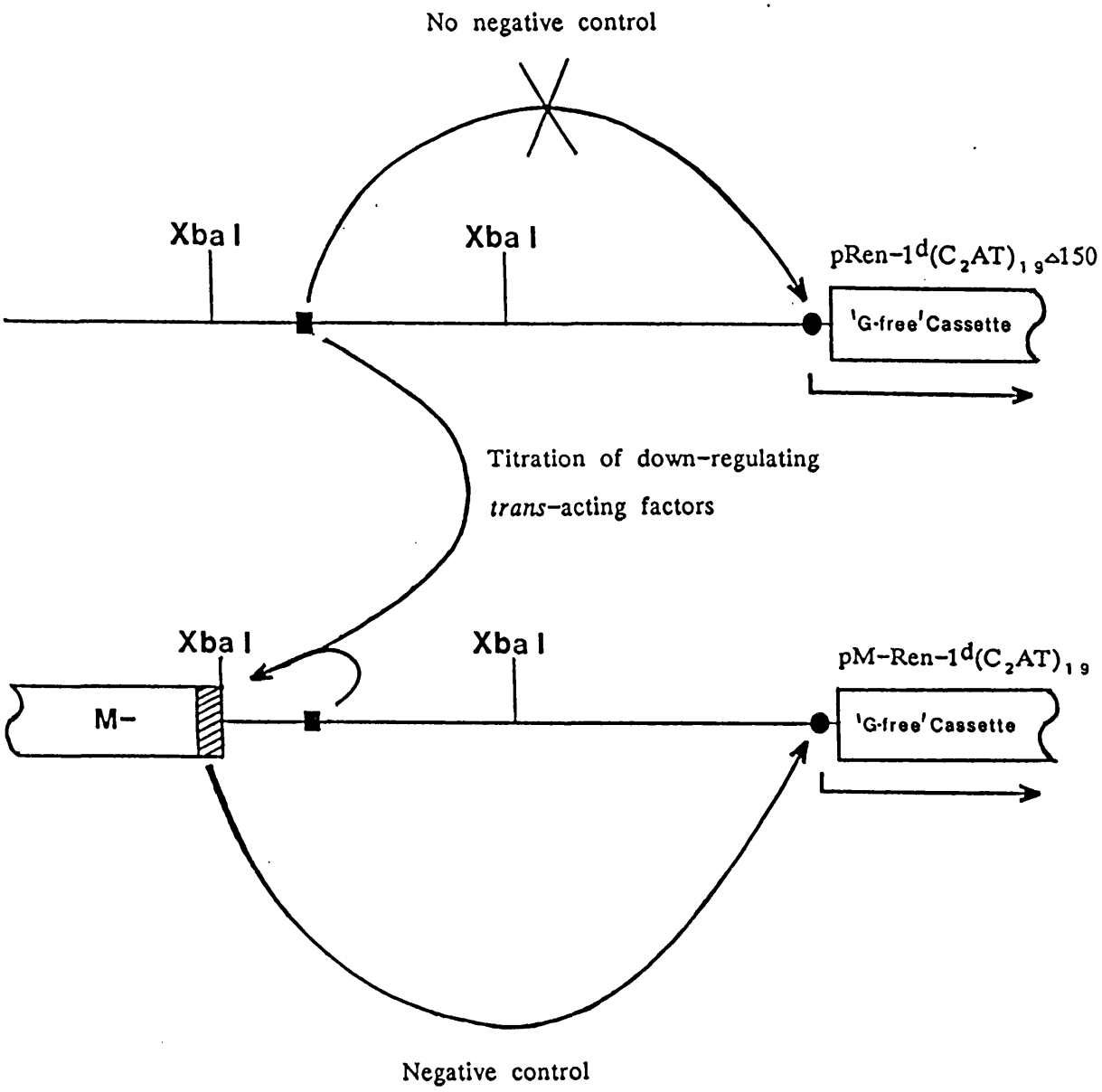


Figure 7.2

Scheme showing the possible mechanism by which the activity of pRen-$1^d(C_2AT)_{19}\Delta150$ is enhanced in the presence of pM-Ren-$1^d(C_2AT)_{19}$. When the M fragment is in the anti-sense (-) orientation only, a binding site is created at the junction between the M fragment and the vector DNA (striped box), which is able to preferentially titrate out down-regulating *trans*-acting factors which normally bind to the native negative control element (black box) of the *Ren-1^d* gene. Due to the close proximity between the "new" and native binding sites in pM-Ren-$1^d(C_2AT)_{19}$, negative control at the P3 promoter (black circle) is maintained whereas the effect of such control is diminished in pRen-$1^d(C_2AT)_{19}\Delta150$.

1989) both site-directed mutagenesis and deletion-mapping will be required to investigate further the apparent *trans* effect of pM-Ren-$1^d(C_2AT)_{19}$ on the activity of pRen-$1^d(C_2AT)_{19}\triangle 150$.

7.3 Mobility-Shift Assays

The mobility-shift assay has also been used to investigate the relationship between the pattern of protein-DNA binding and renin gene-expression. Interestingly the complexes associated with the *Ren-1^d* promoter fragments were distinct from those associated with the *Ren-2^d* promoter fragments, such that protein-DNA binding not only appeared to be tissue-specific but also protein-specific (Figures 6.9 and 6.10). Furthermore the pattern of complex formation was consistent with a negative control hypothesis where the binding of tissue-specific *trans*-acting factors switched-off gene-expression.

The *Ren-1^d* (340bp) and *Ren-2^d* (480bp) *Xba*I fragments used by Nakamura *et al.* (1989) are positioned directly upstream of the *Ren-1^d* (377bp) and *Ren-2^d* (589bp) 5' flanking sequences respectively, used in the mobility-shift assay (Figure 6.7). As with the *Ren-2^d* (480bp) *Xba*I fragment, the *Ren-2^d* 589 fragment has undergone an insertion event with respect to the *Ren-1^d* gene. The insert has been identified as a mouse B2 (*Alu* equivalent) mobile element (Field *et al.*, 1984), but whether such an event also confers *cis*-acting regulatory properties on the *Ren-2^d* promoter is uncertain, since removal of such sequences in the deletion-mapping experiments appeared to have no effect on promoter activity *in-vitro* (Figure 4.10). If such sequences are able to elicit subtle changes in promoter activity these changes may not have been detected due to the limited sensitivity of the *in-vitro* transcription assay. It is unlikely, however, that disruption of a *cis*-acting negative control element(s) alone is responsible for the observed tissue-specific regulation of the mouse renin genes, since renin gene-expression appears to be more complex than can be easily explained by such a system. Indeed in the human renin gene both negative and positive regulatory elements have been identified in the 5' flanking sequence -892 to +13 (Burt *et al.*, in press), using an assay system similar to that employed by Nakamura *et al.* (1989). By deletion analysis these workers have identified a silencer, since it was active in both orientations, at -149 to +13 and

have identified at least two positive control sequences at -892 to -453 and -453 to -149 which acted synergistically to stimulate CAT expression 11-fold. The second element acted like a classical enhancer in that its action was orientation independent. Further investigation will be needed to fully understand the relationship between the different controlling elements and the roles they play in renin gene-expression.

7.4 Summary

These *in-vitro* transcription studies have shown limited tissue-specificity between the *Ren-1^d* and *Ren-2^d* promoters. Furthermore deletion-mapping analysis both by restriction endonuclease digestion and PCR technology, have shown that a promoter fragment (from -47 to +15) containing only the P3 TATAAAA is sufficient for initiating accurate *in-vitro* transcription from the *Ren-1^d* promoter. Also sequences 3' to the *Ren-1^d* P3 start-site when positioned either upstream or downstream to the *Ren-1^d* promoter/G-free cassette fusion appear to have a minimal effect on promoter activity suggesting that these regions are not involved in *in-vitro* regulation of the *Ren-1^d* gene. The presence of an AP-2 consensus sequence in both the *Ren-1^d* and *Ren-2^d* genes hinted towards possible AP-2 regulation but deletion of such sequences from the *Ren-1^d* promoter had no effect on transcription.

Interestingly both the deletion-mapping and the mobility-shift data together with the hypothesis explaining the possible mechanism for the differential expression between pRen-1^d(C_2AT)$_{19}$$\Delta$150 and pM-Ren-1^d($C_2AT$)$_{19}$ (see Section 7.2), are entirely consistant with the presence of a negative control element being present within the *Ren-1^d* promoter. However, further investigation will be required to fully understand the implications with respect to renin regulation of such controlling mechanisms.

In this work investigation of tissue-specific renin gene-expression appears to have been hindered by several factors. First, because only a small proportion of the cells within a given tissue express renin, those tissue-specific *trans*-acting factors produced by only renin expressing cells were in relatively low concentration. As a result their effects were diluted in the *in-vitro* transcription assay. Second, the sensitivity of the

assay was further reduced by the fact that transcription was terminated at a time point (20 minutes, see Figure 3.3) where the reaction was near completion and a maximum signal was obtained. The sensitivity and value of the assay could have been increased by choosing a time point where the rate of product formation was linear. A further problem has been the inability to prepare transcriptionally competent nuclear-protein extracts from SMG where renin is synthesised by 20-30% of the cells (Gresik *et al.*, 1981). Furthermore, extracts produced from testis not only required a large number of animals to be harvested but transcriptional competence of the extract, as measured by Ad-2 ML promoter activity, was found to be relatively low.

Although there have been reports of genes in *in-vitro* transcription systems behaving at least qualitatively as they do *in-vivo* (Gorski *et al.*, 1986; and Monaci *et al.*, 1988), these have been few and far between. Transcription initiation is often achieved, but full tissue-specific regulation of the gene of interest is not always obtained. These observations may reflect the important role which chromatin may play in gene-regulation *in-vivo*, as opposed to *in-vitro* where soluble transcription factors interact with "naked" DNA. Because of the many shortcomings with *in-vitro* transcription assays a more suitable approach may be to do the initial characterisation of the renin promoters in cultured cells. General promoter elements may then be defined by deletion analysis and site-directed mutagenesis. Further investigation could then be extended *in-vitro* by using mobility-shift experiments to define protein-DNA interactions. Furthermore, potential tissue-specific elements could be investigated by transgenic mouse technology. What is apparent is that *in-vitro* transcription systems are not always the most ideal method of carrying out initial characterisations of promoter elements, especially when also investigating tissue-specificity.

Therefore it appears that not only will the *in-vitro* data obtained in this work have to be supported *in-vivo* but that future investigations into renin gene-expression will probably be more fruitful with an *in-vivo* assay system. Transient expression assays, where the renin promoter is fused to a reporter gene, may prove useful in defining general transcription elements and factors but in the absence of a suitable renin secreting cell-line, tissue-specific regulation will have to be investigated

using transgenic mouse technology.

APPENDIX A

The following is a list of the size markers of the BRL 1Kb ladder in base pairs.

12 216
11 198
10 180
9 162
8 144
7 126
6 108
5 090
4 072
3 054
2 036
1 635
1 018
516
506
394
344
298
220
200
154
142
75

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ABSTRACT

In–Vitro Studies of the Control of Renin Gene–Expression in the Mouse

by
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Certain inbred strains of mice can be divided into two groups on the basis of their submandibular gland (SMG) renin activity. High renin producing strains have two genes *Ren–1*^{d} and *Ren–2*^{d}, whereas low produces have a single gene *Ren–1*^{c}. To search for the presence of regulatory elements involved in renin control, approximately 1Kb of both *Ren–1*^{d} and *Ren–2*^{d} 5' flanking sequence containing the major transcription start–site P3 were fused to the "G–free" cassette, and the resulting templates transcribed in various nuclear–protein extracts. In rat liver, mouse kidney and mouse testis extracts the *Ren–2*^{d} promoter was 2–fold more active than the *Ren–1*^{d} promoter whereas in mouse liver, both promoters were transcribed equally well. Deletion–mapping data are consistant with the presence of a functional negative control element(s) within *Ren–1*^{d} but not *Ren–2*^{d}. Polymerase catalysed chain reaction (PCR) generated deletions have shown that a fragment of 62bp, containing only the P3 promoter, was sufficient for accurate *in–vitro* transcription. Disruption of the P3 TATAAAA resulted in total loss of activity. Deletion of an AP–2 consensus sequence from the *Ren–1*^{d} promoter had little effect on *in–vitro* transcription. Furthermore, sequences 3' to the *Ren–1*^{d} P3 start–site when positioned either upstream or downstream to the *Ren–1*^{d} promoter/G–free fusion, appeared to have a minimal effect on promoter activity suggesting that these regions are not involved in *in–vitro* regulation of the *Ren–1*^{d} gene. Finally, patterns of specific protein–DNA interactions observed with the mobility–shift assays, are also consistant with an overall negative control hypothesis.