Gene Expression and Cleavage Arrest in the Human Embryo

A Dissertation for the Degree of Doctor of Medicine

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Preface

In vitro fertilisation and embryo transfer (IVF–ET) is now an accepted part of clinical practice for the treatment of infertility offering live birth rates in the region of 24% per cycle when three embryos are returned to the uterus However analysis of success in terms of the number of established conceptions per embryo returned reveals that only 10.9% of embryos transferred implant. Achieving improved success for IVF–ET, especially following the replacement of a single embryo in order to reduce the risk of multiple pregnancy is undoubtedly dependent on an improved understanding of the mechanisms that lead to embryonic failure.

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Abbreviations used

hpa	hours post activation
hpi	hours post insemination
mmol L^{-1}	millimoles per litre
DAPI	diamadino–2–phenylindole
IVF–ET	In–vitro fertilisation and embryo transfer
GIFT	Gamete Intra–Fallopian Transfer
LH	luteinising hormone
FSH	follicle stimulating hormone
hCG	human chorionic gonadotrophin
USS	ultrasound scan
GnRH	gonadotrophin releasing hormone
TUDOR	trans–urethral ultrasound directed oocyte retrieval
VLA	Voluntary Licensing Authority
HFEA	Human Fertilisation and Embryology Authority
DNA	deoxy ribonucleic acid
mRNA	(messenger) ribonucleic acid
SDS–PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
EBS	Earle's Balanced Salt Solution
HEBS	Hepes buffered Earle's Balanced Salt Solution
Hepes	N–2–hydroxyethylpiperazine–N–2–ethanosulfonic acid
HIS	heat inactivated (maternal) serum
mgL^{-1}	milligrams per litre
BSA	bovine serum albumin
DDW	double distilled water
NaCl	sodium chloride
KCl	potassium chloride

$CaCl_2.2H_2O$	calcium chloride
$MgCl_2.6H_2O$	magnesium chloride
$MgSO_4.7H_2O$	magnesium sulphate
$NaHCO_3$	sodium carbonate
$NaH2PO_4.2\ H_2O$	sodium hydrogen phosphate
g	grams
HCl	hydrochloric acid
TEMED	tetramethylethylenediamine
µl	microlitres
µg	micrograms
hMG	human menopausal gonadotrophin
i.u	international units
pg	picograms
CO_2	carbon dioxide
mCi	millicuries
mA	milliamps
cpm	counts per minute
mEV	millielectron volts
kD	kilodaltons
FISH	fluorescent in situ hybridisation
rnp	ribonuclear protein

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Chapter One

Introduction

1.1 History of assisted conception procedures

The history of human *in vitro* fertilisation and embryo transfer (IVF–ET) is marked by the birth in 1978 (Edwards, Steptoe et al. 1980) of Louise Brown, the first baby to be born from an embryo created outside the body. This first clinical success, achieved by the embryologist Robert Edwards and the gynaecologist Patrick Steptoe drew upon the knowledge gained by many years of integrated and collaborative work on domestic and laboratory species by scientists from a wide variety of disciplines such as embryology, physiology and endocrinology. Indeed at the time of this first success in the human, IVF–ET had already been possible in domestic and laboratory species for a number of years. The delay in applying these techniques to the human was due in part to the unique ethical and moral problems surrounding the 'creation' of human life *in vitro*, issues which will be discussed in detail later. The unique respect conferred upon the human embryo has also meant that experimental information is scant and hence our knowledge of the mechanisms of pre–implantation development in the human is poor when compared to that in some laboratory and domestic species.

Despite the first successful pregnancy being achieved little more than a decade ago, there have been enormous advances in the clinical practice of IVF–ET, the most profound of these being in the monitoring and control of ovulation, the methods of oocyte recovery and the widening of the clinical applications for the technique of IVF–ET.

1.1.2 Monitoring and control of ovulation.

From the experience of IVF–ET in other mammalian species it was

recognized that the chances of establishing a pregnancy following embryo transfer were improved by the transfer of more than one embryo. Thus the initial attempts at IVF in the human were directed towards obtaining multiple oocytes after ovarian stimulation using gonadotrophins, a process termed "superovulation", which could be fertilised to provide more than one embryo for transfer. Failure to achieve pregnancy despite successful embryo transfer in stimulated cycles led Steptoe and Edwards to abandon ovarian stimulation blaming the effects of the hormones being used in the same cycle as the replacement procedure as the reason for their lack of success (Edwards, Steptoe et al. 1980). They therefore returned to attempts using monitored natural menstrual cycles where only one egg is collected. This technique produced the first successful birth in 1978. Other groups however, most notably Trounson's group in Australia, and Jones' group in the USA, persisted in their attempts at superovulation using either gonadotrophins alone (Jones, Acosta et al. 1984) or in combination with clomiphene citrate (Trounson, Leeton et al. 1981); the method which was to provide the first success during a superovulated cycle in 1980. Oestrogen antagonists such as clomiphene citrate and tamoxifen act by reducing the negative feedback of oestrogen on the pituitary gland, which responds with an increased release of follicle stimulating hormone (FSH). Thus the administration of oestrogen antagonists during the follicular phase of the cycle augments the natural release of FSH, increasing the number of developing follicles and hence oocytes. However the response of the pituitary gland to oestrogen blockade is limited, tending to cause the development of only two or three dominant follicles thus although their use for superovulation in IVF–ET, in combination with gonadotrophins reduces the requirement for the expensive gonadotrophins, more oocytes and hence a better choice of embryos for transfer are usually provided

using gonadotrophins alone (Jones, Acosta et al. 1984). As eggs could be obtained more reliably and in greater number when superovulation was used, Trounson's group was able to achieve better success rates per cycle of treatment commenced than those that could be achieved during natural cycles where it was not always possible to recover the single oocyte. Thus the use of superovulation became more popular and has remained the strategy of choice for most IVF–ET centres.

The experience of IVF–ET in laboratory animals had also revealed that oocyte maturity had a significant effect on the potential for subsequent fertilisation, with immature or post–mature oocytes showing a reduced potential for fertilisation. In the early days of IVF–ET, the timing of oocyte recovery was based on the spontaneous LH surge as assessed by 3 hourly analysis of urine samples from day 8 of the cycle. It was found, however, that in stimulated cycles the LH surge was often delayed (Hodgen 1982). In practice this had considerable advantages since it meant that pre–ovulatory events could be controlled by the administration of human chorionic gonadotrophin (hCG) which mimicked the LH surge and allowed oocyte recovery to be planned. What was and to some extent remains uncertain, however, was, in the absence of the LH surge, when the hCG should be administered in order to allow the recovery of mature fertilisable oocytes which would provide pregnancies. It had been felt that the pattern of oestradiol synthesis would provide a useful guide but this subsequently proved not to be the case (Pepperell, Gronow et al. 1982). Lopata et al (Lopata, Kellow et al. 1982) were able to show, however, that more embryos were obtained from oocytes that came from follicles which were 18–22mm in diameter when the hCG was administered and furthermore that it was embryos derived from these oocytes that gave the highest subsequent pregnancy rate. Thus determining the optimal time for hCG administration is now largely assessed by measuring follicular

diameter with ultrasound scanning.

Despite the suppression of the LH surge in most stimulated cycles, 25% of women still demonstrated evidence of a spontaneous surge (Kerin 1982). This made it essential to monitor all cycles for an LH surge. This involved daily analysis of serum or urine samples in the early phase of the cycle, increasing to 3 hourly analysis later in the cycle when the LH surge was more likely to occur. This frequent monitoring meant that patients had to be available freely, which often required that they be treated as in–patients admitted to the treating centre. Staff also had to be available around the clock to analyse the samples and to perform the oocyte retrieval at the appropriate time, 26 hours (Edwards, Steptoe et al. 1980) following the detection of the spontaneous surge. Although this was expensive and wasteful in terms of resources and the use of hCG to mimic the surge in spontaneous cycles provided a degree of predictability, the possibility of a spontaneous LH surge meant that at that time there was little alternative. The expense of the monitoring, in–patient care and constant availability of staff also limited access to treatment to those who could afford it, and to centres which could devote the time and resources of medical and embryological staff.

In 1985, Fleming et al (Fleming, Haxton et al. 1985) described the use of gonadotrophin releasing hormone (GnRH) agonists to prevent the spontaneous LH surge in patients undergoing ovulation induction with gonadotrophins for anovulatory infertility. GnRH agonists had been developed for use in sex hormone sensitive tumours. When administered continuously they desensitise the pituitary creating a state of temporary, reversible hypophysectomy. The state of pituitary inactivity which they induce provides an ideal environment in which to use exogenous gonadotrophins with little or no risk of a spontaneous LH surge. Thus the

level of biochemical monitoring previously required during superovulation can be dramatically reduced, and in many centres has been completely abandoned in favour of ultrasound surveillance. This has allowed the superovulation phase of treatment to be carried out on an out–patient basis. Abolishing the risk of a spontaneous LH surge also proved to have other advantages. Gonadotrophin therapy could be prolonged until it was felt that a sufficient number of follicles had reached their optimal size. Thus the average number of mature oocytes which were recovered in cycles where GnRH agonists were administered was increased when compared to those where they were not used. This in turn lead to each patient having a greater number of better quality embryos available for transfer, which probably accounted for an improvement in pregnancy rates (Antoine, Salat–Baroux et al. 1990) in GnRH agonist controlled cycles.

Another advantage of the use of GnRH agonists lay in the fact that the state of hypogonadotrophic hypogonadism which they induced, abolished the patient's menstrual cycle thus allowing the gonadotrophin therapy for the superovulation to be commenced at any time convenient to the patient and the treating centre. Furthermore, since final oocyte maturation always had to be induced by the administration of hCG, superovulation and hCG administration could be planned to allow procedures to take place at convenient times. Thus it became possible for oocyte recoveries to be timed to coincide with routine operating lists when the required staff would be available. Planned oocyte retrievals and embryo transfer assisted further in reducing the overhead costs of IVF–ET and also made it possible for centres without dedicated facilities and staff to offer treatment.

The substantial advantages conferred by the use of GnRH agonists have resulted in wide acceptance, and most centres now use them as part of

their ovarian stimulation protocol. However, their introduction has been so recent that there is as yet no clear consensus as to the optimal regimen for their use. In Fleming's original paper the agonist was administered as a sole agent for two to three weeks prior to commencing gonadotrophin therapy, was continued throughout the period of gonadotrophin therapy and was stopped only when the hCG was administered to induce ovulation. The aim of this 'long course' desensitisation was to induce the maximum degree of pituitary desensitisation and ovarian inactivity prior to commencing the gonadotrophins. However it has become clear more recently that the response of the pituitary, and hence the risk of a spontaneous LH surge, can be attenuated by a much shorter courses of treatment. Thus administration of GnRH agonists for as little as three days commencing at the same time as the gonadotrophin therapy, the so called 'ultra–short' protocol, appears to be effective in preventing spontaneous LH surges for the usual 9–15 day period of ovarian stimulation. However in IVF–ET where the patients are usually menstruating spontaneously, as compared to anovulatory infertility where they are not, the ultra short protocol requires that there are staff available seven days a week in order to begin the treatment on the first or second day of the spontaneous menses. Furthermore, as normal pituitary function returns approximately seven days after GnRH agonist therapy ceases (De Ziegler, Steingold et al. 1989), the risk of a spontaneous LH surge is still present in patients where ovarian stimulation has to be prolonged beyond 15 to 16 days in order to obtain an adequate number of follicles of optimal size. Hence the superior cycle control offered by the long term protocol, makes the long protocol the regime of choice for smaller units with limited facilities where procedures must be planned to coincide with available staff and facilities. The ultra short protocol may be better suited to those centres that have dedicated staff and facilities.

The advantages of reduced monitoring, greater cycle reliability, higher pregnancy rates and treatment planning, have meant that although the first clinical success was achieved following intensive monitoring of a natural menstrual cycle, pituitary desensitisation and ovarian stimulation are now routine in IVF–ET treatment.

1.1.3 Oocyte retrieval

Originally needle aspiration of ovarian follicles was performed under direct vision using laparoscopy (Steptoe and Edwards 1970). Since laparoscopy is performed under general anaesthesia, a dedicated theatre and anaesthetist were required, adding further to the expense of the treatment. Furthermore laparoscopy is associated with a moderate degree of post–operative discomfort and a small but significant risk of damage to other intraperitoneal structures such as bowel and blood vessels. A risk which is increased in patients with intra–abdominal scarring, which is, for various reasons, not uncommon in those seeking IVF–ET treatment.
As ultrasound scanning was being used routinely to measure follicular size during the monitoring of patients' response to ovarian stimulation, it was a logical step to use ultrasound imaging to guide the needle to the ovary for egg retrieval, and thus to avoid the potential trauma and post–operative discomfort of laparoscopy. Since transabdominal ultrasound imaging of the pelvis is routinely carried out using the full bladder as an ultrasonically lucent 'window', access to the ovaries could only be gained through the bladder, thus this approach is termed transvesical oocyte recovery. Initially these ultrasound guided oocyte recoveries were also carried out under general anaesthesia (Lenz, Lauritson et al. 1981), however, as experience with the technique increased, it became apparent that the procedure was well tolerated by patients using

local anaesthesia (Lenz and Lauritson 1982) in combination with intravenous administration of a sedative and an analgesic. Immediate postoperative recovery was much quicker than that following general anaesthesia, allowing oocyte retrievals to be safely carried out on an out–patient basis. The transvesical approach was further modified by Parsons et al (Parsons, Booker et al. 1985) who gained access to the ovaries through the urethra of the full bladder rather than percutaneously. This approach was given the acronym TUDOR (**t**rans–urethral **u**ltrasound **d**irected **o**ocyte **r**etrieval), the rationale behind this modification being a reduced risk of infection and reduced patient discomfort.

The ability to carry out oocyte retrieval procedures on an out–patient basis meant that when GnRH agonists began to be used on a routine basis, removing as they did the requirement for in–patient monitoring, it was possible to carry out IVF–ET treatment on a wholly out–patient basis, allowing treatment to be offered by centres which had hitherto been unable to do so due to lack of dedicated in–patient facilities.

During the time that transabdominal ultrasound guided oocyte recovery was being developed, ultrasound imaging of the pelvis was evolving towards transvaginal scanning using transvaginal probes. The transvaginal approach has the advantage that scans can be performed when needed without having to wait for the patient's bladder to fill. This in turn means that the procedure is more comfortable for patients. Furthermore, the ultrasound images of the pelvic structures are greatly improved when the probe is placed in the vagina rather than when the probe is held on the abdomen as the images and resolution provided by ultrasound improve the nearer the probe can be placed to the structure being viewed. Ovaries enlarged by stimulation, or ovaries affected by adhesions following pelvic inflammatory disease, which is common in

many of the patients seeking IVF treatment, tend to lie in the pouch of Douglas. In this position the ovaries lay directly against the vaginal wall, in most cases with only 3 to 4 mm of tissue separating them from an ultrasound probe placed in the vagina. It was thus logical to attempt to pass the oocyte aspirating needle this short distance through the vaginal wall into the ovary rather than the longer distance through the abdominal wall (Gleicher, Friberg et al. 1983). Transvaginal oocyte recovery not only had the advantages of transabdominal ultrasound guided procedures of reduced trauma and anaesthetic requirements, but also appeared to cause even less patient discomfort.

These considerable advantages have meant that ultrasound guided transvaginal oocyte recovery is now the preferred method of oocyte recovery and is carried out routinely on an out–patient basis.

1.1.3 Widened clinical application

Although IVF was initially developed to assist those couples whose infertility was as a result of damaged fallopian tubes, it is now used in infertility due to other causes, such as sperm abnormalities, polycystic ovarian disease, endometriosis and in cases of unexplained infertility (Hull, Glazener et al. 1985). Furthermore, IVF can have a useful diagnostic role. For instance, in a number of cases of unexplained infertility, it can be demonstrated that the inability to conceive arises from an inability of apparently normal sperm to fertilise oocytes, and in the case of male factor infertility poor sperm quality may still be associated with retention of fertilising capacity. The ability to test fertilising capacity meant that a cycle of IVF–ET could be used not only for therapy, but also as a diagnostic test, the results of which might be used to encourage the couple to have further attempts at IVF–ET if fertilisation was successful, or to direct the couple towards alternative treatments, such as donor insemination or assisted

fertilisation techniques, if fertilisation failed.

In addition to the widened application of IVF–ET itself, a number of variations of the technique were developed, the most commonly performed of which is Gamete Intra–Fallopian Transfer (GIFT) (Asch, Ellsworth et al. 1985). In contrast to IVF where fertilisation takes place outside the body, GIFT requires aspiration of oocytes at laparoscopy and immediately replacing them, mixed with prepared sperm, into the cannulated fallopian tube so that fertilisation occurs *in vivo* (see methods below). Thus GIFT requires that the female partner has at least one patent functioning fallopian tube which limits its use in the treatment of infertility when compared with IVF–ET. Indeed many practitioners limit its use to those couples who have unexplained infertility. Although in theory the possibility of pregnancy might be improved as a result of fertilisation being allowed to take place in the appropriate anatomical site *in vivo*, namely the fallopian tube, there have been few prospective randomised trials which have shown any significant increase in pregnancy rates for GIFT over IVF–ET. Furthermore, since IVF no longer requires laparoscopy and general anaesthesia and gives more diagnostic information, GIFT is placed at a disadvantage in terms of the cost required for theatre and personnel time and patient discomfort.

Although it is apparent that great progress in the methods of clinical practice of IVF–ET have been made in a relatively short time, the final report of the Interim Licensing Authority (1991) suggests that improvements in the success rate of the treatment have been less dramatic. The crude live birth rate in 1985 was 8.6% per treatment cycle and by 1989 had only risen to 11.1% for all licensed centres. Although it may be argued that crude success rates are a poor indicator of progress as it is seldom possible to ensure that the groups being compared are

equivalent, particularly since IVF–ET is now offered to couples with an increased range of reasons for their infertility, rather than the well defined discrete problem of tubal blockage, the fact remains that IVF–ET is not a very successful medical treatment. The reason or reasons for the poor success of the procedure per cycle of treatment remain unknown. However the fact that only 11% of embryos transferred result in a pregnancy (The sixth report of the Interim Licensing Authority for Human in Vitro Fertilisation and Embryology, 1991, see Table 1) strongly suggests that embryonic failure is a major contributory factor. The possible reasons for embryonic failure are discussed below, but it is not unreasonable to suggest that an understanding of the mechanisms responsible for this high rate of embryonic failure must be sought in order to improve the technique further.

1.2 Ethical and legal considerations

The study of human embryos in vitro, however, raises considerable moral and ethical dilemmas which have had to be addressed as IVF–ET has emerged as an acceptable clinical treatment. The publicity and excitement that accompanied the announcement of the first successful use of IVF–ET in the human undoubtedly gave hope to many couples who had hitherto had to be resigned to childlessness. However the announcement also made clear to the lay public for the first time that the creation of human life in a test–tube, previously the stuff of science fiction was now science fact. Despite the recognition of the obvious clinical benefits of such treatment, there was public concern as to what actually was happening in the laboratory. Religious and moral groups, faced with observable facts about fertilisation and the development of human life during it's very earliest stages, were forced to consider anew fundamental philosophical

Table 1 overleaf

Table 1

Figures from the sixth report of the Interim Licensing Authority (published 1991) showing the pregnancy rates per replacement and the pregnancy rate per embryo transferred.

Table 1

Number of embryos replaced	Number of Replacements	Pregnancies per replacement	Pregnancies per embryo replaced
1	1030	8.3 %	8.4 %
2	1380	15.6 %	9.6 %
3	3869	24.0 %	10.9 %

Figures from the sixth report of the Interim Licensing Authority for Human Fertilisation and Embryology (1991)

questions about when human life begins. As a multicultural and multireligious society the views expressed were diverse, ranging from the concept that life and individuality commenced at fertilisation (Congregation for the Doctrine of Faith, 1987), to those who took the more scientific view of embryogenesis being part of a continuum of development with no easily defined point at which "life" or individuality could be said to begin (Braude and Johnson 1990). Naturally those who felt that life began at fertilisation had great concerns about the maintenance of human embryos *in vitro* beyond fertilisation and were particularly concerned as to what happened to those supernumery embryos that were created *in vitro* but not transferred.

In response to the questions of what regulations and limitations should be placed on the laboratory practices and research potential of IVF–ET, the government took the first step towards statutory control by establishing a Commission of Inquiry under the chairmanship of Dame Mary Warnock. The remit of this commission was, 'To consider recent and potential developments in medicine and science related to human fertilisation and embryology; to consider what policies and safeguards should be applied, including consideration of the social, ethical and legal implications of these developments; and to make recommendations'. The commission took evidence from a wide range of sources publishing its report in 1984 (Report of the Committee of Enquiry into Human Fertilisation and Embryology, 1984). The report acknowledged that in a pluralist society it was difficult to impose strict guidelines on questions of morality. However amongst a number of important recommendations concerning infertility services, it made two key recommendations which were directly relevant to the practice of IVF–ET, namely that a statutory body should be established to oversee and license the practice of centres offering treatment and carrying out research and that the human embryo should be afforded a

special status, with experimentation only being allowed up until the fourteenth day following fertilisation. The choice of the fourteenth day following fertilisation as a limit to controlled experimentation was based not only on a pragmatic need to impose some limitation on the boundaries of research but also the fact that it is at this stage of development that the primitive streak appears, signifying the first sign of the embryological phase of development and also marking the end of the process of implantation in vivo.

However, although a consultation document was published, the government of the day failed to take any further action to implement the recommendations of the commission. In the absence of any formal direction The Royal College of Obstetricians and Gynaecologists and the Medical Research Council formed a Voluntary Licensing Authority (VLA) to implement some of the recommendations of the report pending government action. Despite lacking statutory power, the VLA proved to be a model of self regulation. It carried out the functions of issuing licenses for treatment and research with full and regular monitoring of all centres involved with IVF–ET. The influence of the VLA was such that, although compliance was entirely voluntary, no centre in this country practiced IVF–ET or carried out research involving human embryos without a licence from the Authority. This 'temporary' arrangement remained in existence until August 1991 when, after a great deal of heated and emotional debate during it's passage through the House of Commons, the Human Fertilisation and Embryology Act received Royal Assent. The Act followed the recommendations of the Warnock Report allowing research up until 14 days following fertilisation and establishing a statutory body known as the Human Fertilisation and Embryology Authority (HFEA) with responsibility for inspecting treatment centres and issuing licenses for

treatment and research. Amongst other statutory requirements, the Act also made it a criminal offence to carry out research or offer treatment involving the creation of an embryo outside the human body without a licence.

This latter point is currently being interpreted by the body responsible for implementing the Act as meaning that a licence is not required to carry out GIFT since the sperm and eggs are transferred back to the fallopian tube before fertilisation has been seen to take place, hence no embryo is created outside the body. However, others would argue that this interpretation is too narrow and that the benefits of patient protection that the Act offers should be extended to include GIFT (Braude, Johnson et al. 1990), since GIFT has at least as great a potential for clinical harm as IVF–ET in terms of the risk of multiple births.

Having established the background to the study of the human embryo in the laboratory I wish to examine the current state of our knowledge of human embryogenesis.

1.3 Overview and comparative study of embryogenesis

1.3.1 From oocyte to embryo

Current evidence suggests that oocyte development or oogenesis in the human follows a similar pattern to that seen in domestic and laboratory mammals. Oocytes are derived from primordial germ cells which migrate to the genital ridges in the embryo where they initially undergo a period of proliferation and differentiation into primary oocytes. This takes place mid way through fetal development and initially the ovaries contain approximately 7 million of these primary oocytes which enter but do not complete their first meiotic division. The oocytes remain in the dictyate stage of the first meiotic division until puberty when the number of

oocytes remaining has fallen to approximately 400 000 (Johnson and Everitt 1989). Following puberty and throughout the individual's reproductive years a constant trickle of these oocytes surrounded by follicular cells leave this state of suspended animation and undergo the first stages of follicular development. Complete follicular development leading to ovulation depends on the hormonal environment in which these pre–antral follicles find themselves. Many will undergo atresia but for some the circulating levels of follicle stimulating hormone (FSH) and luteinising hormone (LH), as determined by the woman's menstrual cycle, will be sufficient to promote further development. Meiosis resumes if one of these more developed antral follicles is exposed to a surge of LH when it is just entering its terminal phase of development. The resumption of meiosis is characterised by the breakdown of the nuclear membrane surrounding the chromosomes, still each consisting of two chromatids. These then separate to form two nuclei, each nucleus having half the original chromosome complement. Cell division is then completed by the plasma membrane forming a deep furrow (see cleavage below) to separate the two nuclei. However, as it forms this furrow the cytoplasm is divided unequally to give one large cell containing almost all the cytoplasmic components, and hence developmental potential, and one extremely small cell, the first polar body. At this stage, oocyte development is again suspended and the oocyte is released at ovulation in this form with the chromosomes in the oocyte arranged in preparation for the next meiotic division, this structure being called the meiotic spindle (Pickering, Johnson et al. 1988). Meiosis only resumes normally if and when the sperm penetrates the plasma membrane of the oocyte. During this second meiotic division there is also an unequal division of the cytoplasm resulting in the oocyte proper, which has most of the cytoplasm, and the much smaller second polar body. In practice in the human it is rare to see

both first and second polar bodies together as the first usually degenerates rapidly after fertilisation. There is evidence from frogs where sperm penetration has been simulated by 'pricking ' oocytes with fine glass needles which results in the resumption of meiosis (Alberts, Bray et al. 1983b), that it is the physical act of the sperm penetrating or fusing with the oocyte membrane that is the stimulus to the resumption of meiosis. Furthermore the fact that these parthenogenetically activated oocytes, oocytes stimulated to develop in the absence of male gametes, go on to cleave suggests that, apart from initiating the resumption of meiosis and subsequent cell division, the act of fertilisation is, at least in the early stages of development, of little significance. This ability to induce the resumption of meiosis by mechanical means would suggest that the underlying mechanism must be physico–chemical in nature and indeed analysis of the potential difference and movement of ions across the plasma membrane of the oocyte (Epel 1978) at the time of fertilisation has demonstrated that sperm penetration or similar physical stimuli induce rapid changes in the calcium permeability of the plasma membrane. Sperm penetration has also been shown to induce the release of the contents of granules which lie just beneath the cortex of the egg (Schuel 1978). The contents of these granules act to alter the physical structure of the the zona pellucida, the thick coat that surrounds the oocyte. This so called zona reaction renders the oocyte impermeable to further sperm penetration preventing fertilisation by multiple sperm (polyspermy) (Johnson and Everitt 1989) which if allowed to happen would result in a non–viable embryo with multiple sets of chromosomes.

Approximately 19–22 hours after fertilisation, the male and female DNA within the zygote condenses and a nuclear membrane forms around each set of chromosomes. These nuclear structures, the pronuclei, can be seen

using light microscopy and in clinical practice their presence is taken as the first sign of successful fertilisation (Johnson and Everitt 1989). Between 22 and 26 hours after fertilisation the nuclear membranes surrounding the pronuclei breakdown and the chromosomes fuse, during syngamy. The process of DNA replication and cell division then commences.

The early rounds of cell division are associated with an increase in the cell number but little change in mass of the embryo (Johnson and Everitt 1989). Hence the use of the term cleavage to describe pre–implantation cell division until the blastocyst stage. The number of cleavage divisions the embryo has undergone is often used to describe the developmental stage of the embryo and implies that a certain cell number should have been reached. For instance, an embryo said to have undergone its second cleavage division should have four cells. In the human and other mammals (Kelly, Mulnard et al. 1978), however, co–ordination of cell division is usually lost between between the 8 and 16–cell stages and, on occasions, at stages even earlier than this. Thus, this terminology is of limited value in the human and other experimental mammals.

1.3.2 Cleavage and gene activity in domestic and laboratory species

1.3.2.1 Cleavage

Because of their ready availability and ease of handling, many of the investigations into the mechanisms of cleavage have involved the use of embryos from lower order animals such as sea urchins and frogs (Alberts, Bray et al. 1983a). It is uncertain, therefore, whether the mechanisms discovered as a result of these studies can be assumed to apply to higher order animals such as mammals.

Cleavage requires the co–ordination of two separate processes, namely nuclear division or mitosis, also referred to as karyokinesis, and

cytoplasmic division, or cytokinesis.

A cleavage division commences with replication of the centriole, followed by DNA replication to form chromatids. The chromatids are then separated by the action of microtubules which appear to originate in the dense material surrounding the centriole rather than in the centriole itself. The array of microtubules radiating from the pole of the cell is called the polar spindle. These 'centriolar' tubules interact with other microtubules which take their origin from the centromere of the chromosome, the function of these being to orientate the chromosomes and attach them to the polar spindle. The whole assembly of the two polar spindles with the attached chromosomes is called the mitotic spindle. The precise mechanism which leads to separation of the chromosomes is unclear, although proteins that have been shown in other circumstances to be capable of generating force and movement (Warner and Mitchell 1980) have been found to be associated with the microtubules. During the separation of the chromosomes the surface membrane of the cell is furrowed by the action of further microtubules. The furrow forms along an axis perpendicular to the mitotic spindle and deepens gradually until it meets the remains of the mitotic spindle. At this point, the process often halts for a time leaving a cytoplasmic 'bridge' between the daughter cells, the midbody. Although this midbody usually breaks down it is of practical importance in treatment as remnants may be encountered during attempts at embryo biopsy making the removal of single cells for analysis difficult.

1.3.2.2 Gene activity

At the time of the first studies into the genetic mechanisms of cell division in cleavage stage embryos, the perceived dogma was that cellular function relied on the transcription of genetic information from the nucleus of the cell, the DNA, to form messenger RNA (mRNA) and the subsequent, often immediate translation of this mRNA to form proteins which acted as the effector in the system. However, when Xenopus (Briggs, Green et al. 1951) and sea urchin eggs were enucleated physically (Denny and Tyler 1964), it was found that they remained capable of cleavage and were also capable of synthesising the same proteins as intact controls for their developmental stages. Furthermore similar results (Gross and Cousineau 1964) were found when physical enucleation was simulated by treating cleavage stage embryos with chemical inhibitors of transcription such as actinomycin D.

This ability of embryos to continue to develop and synthesise proteins without nuclei, and thus presumably unable to synthesise new mRNA, led to the hypothesis that the early stages of development were independent of the embryonic genome and that, until such time as the embryonic genome became active and was capable of supporting the metabolic needs of the organism, embryonic development was directed by proteins synthesised on a store of mRNA. Since there were minor differences between the proteins synthesised by the oocyte immediately before and immediately after fertilisation it was also proposed that at least some of this mRNA was stored in an inactive form in a complex with ribonuclear proteins (RNP) for later activation, this complex being termed the 'informosome'. Further experiments confirmed the presence of these stored mRNAs by extracting them from sea urchin (Maggio, Vittorelli et al. 1964), frog (Davidson, Crippa et al. 1966) and mouse (Braude and

Pelham 1979) oocytes, and after translation in a cell free system, demonstrating synthesis of similar proteins to those synthesised by the intact organism.

Qualitative analysis of protein synthesis using radioactive amino–acid labelling and one dimensional polyacrylamide gel electrophoresis (see below) at various points throughout the cleavage stage in mouse (Flach, Johnson et al. 1982), pig (Davis 1985), sheep (Crosby, Gandolfi et al. 1988), cow (Frei, Schultz et al. 1989) and goat (Sakkas, Batt et al. 1989) embryos revealed that, apart from the minor differences following fertilisation, the pattern remained constant in oocytes and during early cleavage stages. At a consistent stage for each species, namely the 2 cell stage in the mouse, the 4 cell stage in the pig, 8–16 cell stage in sheep, cows, and goats, there was a change in the pattern, with the appearance of new proteins and a loss of others. Since the new proteins were not identified following the extraction and translation of mRNAs from oocytes, it seemed likely that their synthesis depended on new transcriptional activity rather than stored mRNA (Flach, Johnson et al. 1982). Furthermore, the appearance of these new proteins and hence the change in protein synthetic pattern was suppressed when the embryo was exposed to α–amanitin, a specific inhibitor of RNA polymerase II, the enzyme responsible for mRNA synthesis (Lindell, Weinburg et al. 1970). Thus, it was concluded that these new proteins were derived from transcription of new mRNA, and therefore that their appearance in the protein synthetic pattern could be used as a marker for embryonic genome activity. This enabled the division of protein synthetic patterns of cleavage stage embryos from all species into an 'early' pre–gene activation pattern and a 'late' post–gene activation pattern (Flach, Johnson et al. 1982; Davis 1985; Crosby, Gandolfi et al. 1988; Frei, Schultz et al. 1989; Sakkas, Batt et al. 1989) The subject of transition

from maternal to embryonic control in mammalian species is extensively reviewed by Telford et al; (Telford, Watson et al. 1990).

1.3.3 Cleavage and gene activity in the human embryo

1.3.3.1 Introduction

The refinements in the clinical practice of IVF–ET over the last decade (see 1.1.1) have resulted not only in improved patient access to treatment at a reduced cost, but also that more human embryos are being created *in vitro*. Despite this apparent increase in accessibility to human embryos *in vitro*, most studies been confined to observation and description and there have been few experimental investigations of the mechanisms of cleavage and pre–implantation development. This bias towards observational research is not surprising as planned experiments rely largely on human embryos being donated as surplus to the therapeutic requirements of patients undergoing assisted conception procedures. However this in itself confers certain restraints on the amount and type of work that can be undertaken. This reliance for material on patients undergoing treatment has a number of important implications for the potential for experimental embryo research. Firstly, since for ethical reasons, all zygotes obtained as the result of treatment must be allowed to progress until embryo transfer in order to allow the patient the benefit of the maximum choice of embryos for transfer few embryos are available for any experiments other than simple observation prior to this stage, usually 48 hours post–fertilisation. Secondly, the validity of any results obtained from the research research might be influenced by the selection of the 'best' 2 or 3 embryos for transfer to the patient, as selection could imply that those remaining in vitro after transfer are possibly of inferior quality. Thus, the 'spare' embryos may be more likely to have inherent abnormalities. Third, improvements in cryopreservation of embryos for

transfer in subsequent cycles has reduced the number of embryos being donated for research.

The problem of access to embryos at early stages of development can be overcome to some extent by the use of oocytes surplus to GIFT procedures, which could then be fertilised specifically for research purposes. However, in common with therapeutic IVF–ET, there is also pressure to cryopreserve these embryos for subsequent transfer. Furthermore the potential bias of selection is still present since selection is applied to oocytes for GIFT replacement. Hence, embryos resulting from this process could still be a morphologically inferior group.

Thus the study of cleavage and cellular mechanisms in human embryos is often limited to observation alone, and it may often be necessary to draw conclusions from experiments that have involved the study of fewer embryos than would be considered ideal when compared with those that can be conducted using laboratory species.

1.3.3.2 Cleavage

When compared to amphibians and sea urchins which reach the blastocyst stage within 24 hours, in common with other mammals, the rate of cleavage in human embryos is slow, with a cell doubling time ranging from 8 to 51 hours (Fishel 1986). The wide range of timing for the human probably reflects the variation in quality of the group being studied and may reflect underlying embryonic abnormalities. Indeed a considerable morphological heterogeneity between embryos has been demonstrated (Winston, Braude et al. 1991), the number of cells contained within each embryo varying substantially (10–60 cells at 120–124 hours post insemination). Gross abnormalities of the nuclear morphology and nuclear to cytoplasmic ratio have also been demonstrated with many cells

having no nucleus, or others may have multiple nuclear structures. This variation in growth rate and morphology makes it difficult to establish the boundaries of what may be considered a 'normal' pattern of development.

1.3.3.3 Gene activity

Braude et al (Braude, Bolton et al. 1988) studied the protein synthetic patterns from human oocytes and embryos at various stages throughout cleavage. In keeping with the findings from similar studies in other mammalian systems, they were able to demonstrate that a consistent qualitative change occurred in the protein synthetic pattern between the 4 and 8–cell stages. Since this change could be suppressed by exposure to the transcriptional inhibitor α–amanitin it was concluded that activation of the genome in the human embryo occurs between the 4 and 8–cell stages of development. This timing of genome activation has been supported by studies which have examined the intra–cellular sites of radioactive uridine incorporation into cleavage stage human embryos (Tesarik, Kopecny et al. 1986). Uridine is one of the purines which is used only in the synthesis of RNA but not DNA thus active incorporation of uridine is indicative of RNA synthesis. These studies revealed that there was extranucleolar incorporation of radioactive uridine, indicating synthesis of all types of RNA and hence genome activity, at the 4–cell stage. It was also found that there was evidence of nucleolar incorporation of uridine, which is indicative of the synthesis of ribosomal RNA, at the 8–cell stage. Taken together these data suggest strongly that, in a similar pattern to other mammals studied, human embryonic development is maternally directed until the 4 to 8–cell stage whereafter there is a transition to embryonic control.

That the 4 to 8–cell stage should be the stage of genome activation is of

particular relevance to studies of human development *in vitro*.Various reports suggest that only between 17 and 40% of a cohort of fertilised human oocytes will go on to form blastocysts in vitro (Bolton, Hawes et al. 1988; Hardy, Handyside et al. 1989) and that the stage at which embryos are most likely to undergo cleavage arrest is between the 4 and 8–cell stages. This could suggest a possible link between genome activity and cleavage arrest *in vitro*.

1.3.4 Cleavage arrest in laboratory and domestic mammals

Cleavage arrest is unusual in sea urchins and amphibians, and seems to be a peculiar feature of the in vitro development of mammals. Although cleavage arrest is observed at various stages of development, there is a tendency for cleavage to arrest more commonly at specific stages. In certain strains of mice arrest is almost universal at the 2–cell stage (the "2 cell block"(Flach, Johnson et al. 1982)). Pig embryos tends to arrest at the 4–cell stage (Davis 1985), and in sheep (Crosby, Gandolfi et al. 1988), cow (Frei, Schultz et al. 1989) and goat (Sakkas, Batt et al. 1989) embryos demonstrate a tendency to arrest between the 8 and 16–cell stages. It can be seen therefore, that in all of the mammalian species mentioned above, including the human, the observed stage of maximal cleavage arrest is coincident with the demonstrated stage of activation of the genome (see 1.3.2), giving rise to the hypothesis that spontaneous cleavage arrest might result from spontaneous failure of activation of the genome (Braude, Bolton et al. 1988)

This phenomenon of cleavage arrest has probably been most extensively studied in the mouse (Goddard and Pratt 1983). In this extensive study of the "2–cell block", they found that blocked embryos had an appropriate nuclear to cytoplasmic ratio, suggesting that the arrest of cleavage was complete in that both karyokinesis and cytokinesis are halted. Electron

microscopy showed that blocked embryos had underdeveloped mitochondria and endoplasmic reticulum when compared to non–arrested embryos. As a result of these findings they suggested that cleavage arrest might result from functional immaturity of the cytoplasmic components of the cells, which they postulated might have been induced by inadequate in vitro culture conditions. The possibility that cleavage arrest might be a result of cytoplasmic inadequacy is supported by the observation that blocked embryos resume cell division following the intra–cytoplasmic injection of cytoplasm taken from embryos derived from strains of mice which do not demonstrate the 2–cell block (Muggleton-Harris, Whittingham et al. 1982). Furthermore, the theory that cytoplasmic inadequacy might be induced by sub–optimal culture conditions is supported by more recent experiments where culture conditions have been manipulated successfully to overcome the tendency to cleavage arrest. In the mouse, Nasr Esfahani et al (Nasr-Esfahani, Johnson et al. 1990; Nasr-Esfahani, Aitken et al. 1990) have demonstrated that the addition of transferrin to the culture medium overcame the high rate of cleavage arrest observed in certain strains of mouse. The total block to development in vitro beyond the 16–cell stage in the sheep (Gandolfi and Moor 1987) has been overcome by co–culture of the embryos with cells derived from the sheep oviduct.

It is believed that transferrin acts to prevent cellular damage by chelating iron in the culture medium, free iron being an essential co–factor in the destructive action of oxygen free radicals which are released by decaying cells. Inhibiting their action by the addition of transferrin could be expected to be beneficial. The mode of action of cell co–culture is less clear. It has been postulated that co–culture might exert its effect by simulating in vivo conditions. It was for this reason that the initial attempts at co–culture

used cells derived from the genital tract. However, as well as demonstrating that sheep embryos could be encouraged to divide if cultured with cells from the oviduct, it was also demonstrated that they developed just as well when cultured with ovine fibroblasts (Gandolfi and Moor 1987). A similar lack of specificity for cells from the genital tract has also been reported in pigs (Allen and Wright 1984) and cows (Kim, Roussel et al. 1989). This lack of specificity would suggest that co–culture works in a non–specific way. This raises the possibility that rather than adding specific factors to the medium, as was initially expected, the co–cultured cells may be acting in a similar way to transferrin by 'mopping up' toxins.

Goddard et al (Goddard and Pratt 1983) went on to investigate the role of gene activation in the aetiology of the 2–cell block in the mouse. They were able to demonstrate that 'blocked' 2–cell embryos were capable of synthesising some of the transcriptionally dependent proteins usually only synthesised by embryos which had progressed beyond the 2–cell stage, but the synthesis of these proteins took place later than in controls. However the arrested embryos did not appear to be capable of synthesising a group of proteins that were typically associated with morulae and blastocysts. They concluded from these findings that abnormality of cleavage was more likely to be caused by cytoplasmic anomalies induced by culture in vitro, rather than by failure of genome activation.

To date the study by Goddard and Pratt of the 2–cell block in the mouse represents the most in depth study of the phenomenon of cleavage arrest in any species. What evidence is available then, from the human, to support a role for either of the two proposed aetiologies of sub–optimal culture conditions and failure of gene activation.

1.3.5 Cleavage arrest in human embryos

As has already been stated (see 1.3.3), the in vitro development of the human embryo is characterised by a marked tendency towards the spontaneous arrest of development during early cleavage stages. Only a minority of fertilised oocytes reach advanced stages of pre–implantation development *in vitro* (Bolton, Hawes et al. 1988; Hardy, Handyside et al. 1989). It is also a consistent feature in the human that arrest tends to be most frequent at the 4 to 8–cell cleavage division. This tendency towards developmental arrest has important clinical and experimental implications.

As embryo transfer as part of a therapeutic procedure is usually carried out at the 4–cell stage, it is inevitable that some embryos that are destined to undergo cleavage arrest and therefore unable to establish a pregnancy, are being transferred. This transfer of embryos with restricted developmental potential undoubtedly contributes to the low conception rate of 11% per embryo transferred (Table 1). It may also explain why increased pregnancy rates are observed when multiple embryos are transferred, as presumably the chances of transferring at least one embryo that is destined to reach the blastocyst stage are increased.

Experimentally the tendency for embryos to undergo cleavage arrest makes it difficult to determine what constitutes a 'normal' embryo. It is quite possible for an embryo judged to be normal at one stage of development, having a cell number appropriate to the time elapsed since fertilisation with minimal evidence of cell breakdown, still to undergo cleavage arrest. Since the mechanisms responsible for subsequent cleavage arrest might be active in the embryo prior to the arrest becoming evident, it is always possible to question the 'normality' of an embryo. To overcome this weakness, observations and experiments need to be performed on

relatively large numbers of embryos and rather than referring to 'normal' embryos, it may be preferable instead to refer to 'non–cleavage arrested' or simply 'non–arrested' embryos.

To date there has been no single in depth investigation of the mechanisms responsible for cleavage arrest in the human embryo. Tesarik et al (Tesarik, Kopecny et al. 1988) studied the ultrastructure of a number of embryos using electron microscopy, comparing those which had shown evidence of uridine incorporation, and hence were deemed to have activated their genome and *presumably* were non–arrested, with those *presumed* to be cleavage arrested by having failed to incorporate uridine. It was found that those embryos which had incorporated uridine showed increased numbers of organelles such as lysosomes and endoplasmic reticulum when compared to those embryos that failed to incorporate uridine. Although these findings are consistent with those from the mouse, where arrested embryos were also shown to have poorly developed mitochondria and endoplasmic reticulum, Tesarik et al could not relate their findings to whether or not the embryos had shown definite evidence of abnormal or arrested development.

As with other species, attempts have been made to manipulate culture conditions in an attempt to reduce the rate of cleavage arrest in human embryos (Menezo, Testart et al. 1984; Quinn, Kerin et al. 1985). However, preliminary studies in our own laboratory have failed to show any significant reduction in the rate of arrest in human embryos following the addition of transferrin to the standard currently used culture medium. Furthermore, co–culture of human embryos with cells derived from the fallopian tube has only been shown to result in a somewhat subjective improvement in the quality of blastocysts obtained with no increase in the proportion of embryos reaching this stage (Sathananthan, Bongso et al.

1990). Thus, at this stage it can only be concluded that although inadequate culture conditions might still have a role in the aetiology of cleavage arrest in the human embryo, the relationship, if one does exist, is not as simple as that which has been observed in other mammals.

A number of studies have been carried out to examine the gross genetic structure of oocytes and embryos. It has been demonstrated that a high proportion of oocytes retrieved after superovulation are chromosomally abnormal (Wramsby, Fredga et al. 1987; Macas, Floersheim et al. 1990), and that over half of human embryos cultured *in vitro* show karyotypic (Plachot, de Grouchy et al. 1988) or nuclear abnormalities (Winston, Braude et al. 1991). The drugs used as part of the superovulation strategy have been implicated as a possible cause for the abnormalities seen in oocytes and embryos, but the absence of a suitably sized control group of oocytes and embryos resulting from unstimulated cycles makes it difficult to address this point appropriately. Furthermore, although these abnormalities might cause or contribute to embryonic failure it may equally well be argued that they are secondary events occurring as a result of other phenomena such as poor culture conditions or deranged cell cycle control in embryos about to undergo cleavage arrest from another cause.

As stated earlier (1.2.3.3) the high rate of cleavage arrest which occurs at a stage coincident with the stage of activation of the genome has led to the suggestion that failure of activation of the genome in the embryo as a whole (Braude, Bolton et al. 1988) or in a significant proportion of blastomeres within the embryo (Tesarik, Kopecny et al. 1988) may be the cause of the cleavage arrest. To date only Tesarik's group have investigated this possibility further using the incorporation of radioactive uridine to indicate RNA synthesis and hence genome activity. When each blastomere was examined individually it was found that only some of the blastomeres within an embryo showed evidence of incorporation of

uridine and that the proportion of blastomeres which showed evidence of incorporation varied from embryo to embryo. From this variation it was suggested that cleavage arrest might result from failure of activation of the genome in a critical proportion of the blastomeres However, they were unable to relate their findings as to whether the embryos examined were cleavage arrested or destined to undergo cleavage arrest. Thus despite the experimental evidence from domestic and laboratory mammals of the link between cleavage arrest and suppression of transcriptional activity (1.2.4) The role of gene activity in cleavage arrest in human embryos remains unclear .

1.3.6 Parthenogenesis

A recurring problem for those attempting any studies of human embryonic development is the paucity of material available. Recently Winston et al (Winston, Johnson et al. 1991) have described a method for parthenogenetically activating human oocytes which could provide a source of 'embryos' which may be suitable for subsequent experimental study.

Parthenogenesis, or the activation of embryological development in the absence of male gametes, is a method of reproduction routinely employed by some lower order animals such as drosophila, certain lizards, crustaceans and teleost fish (White 1954). It can be readily induced in the laboratory in lower order species such as sea urchins where oocytes are commonly activated using an electric shock to provide large highly synchronous populations of 'embryos' for experiments (Evans, Rosenthal et al. 1983). Parthenogenesis has been observed to occur spontaneously in vitro in the oocytes of most laboratory and domestic species where it has been assumed that the physical handling of the oocytes has been the

activating agent (Kaufman 1978). It can also be induced in many of these species using various physical stimuli such as electric shocks and pricking the oocyte with a fine needle (Alberts, Bray et al. 1983a) or even by mimicking the calcium fluxes induced by the penetrating sperm by the use of a calcium ionophore (Kaufman 1978).

The use of parthenogenetically activated human oocytes as an experimental model for the study of embryogenesis would have particular advantages. First, with no process of fertilisation occurring, the ethical and moral dilemmas raised by the creation of embryos for experimentation would be avoided. Second, it would utilise a currently unused source of material as further attempts to fertilise oocytes that have failed to fertilise after initial insemination are rarely successful and when successful the embryos rarely result in pregnancy. Thus failed fertilised oocytes are often discarded.

However, although parthenogenesis has occasionally been observed to occur spontaneously in human oocytes cultured in vitro (Braude and Johnson 1988), until recently there has been no reliable system for inducing activation. Winston et al (Winston, Johnson et al. 1991) used the calcium ionophore A23187 to induce activation in an average of 60% of fresh and aged human oocytes, a rate that compares favorably with the fertilisation rate achieved in vitro. However before parthenogenetically activated oocytes can be accepted as a suitable model for the investigation of embryogenesis in the human it is necessary to determine whether they follow a normal pattern of development including timing and pattern of gene activation.

1.4 Summary and aims of the study

It is evident that failure of embryos to reach full developmental potential is one of the main causes of the poor success rate of IVF–ET. Furthermore the strategy routinely employed to overcome the high rate of embryonic failure, namely the transfer of multiple embryos, exposes the patient to an unacceptably high rate of multiple pregnancy (The sixth report of the Interim Licensing Authority for Human in Vitro Fertilisation and Embryology, 1990). In order to improve the success rate of IVF–ET it is essential that a greater understanding is gained of the basic cellular processes in the cleavage stage embryo, in the hope that methods can be devised either to improve the potential for development of embryos in vitro, or to allow embryos to be chosen for transfer that are likely to have full developmental potential.

From studies in a number of species, including the human, the transcriptionally sensitive change in protein synthetic pattern that takes place as development progresses is widely accepted as a sensitive and reliable indicator of genome activity. I have therefore attempted to define further the role of gene activity in cleavage arrest in human embryos by comparing the protein synthetic patterns obtained from spontaneously cleavage arrested embryos with the patterns obtained from a number of oocytes and non–arrested embryos analysed at various stages of development. I have attempted to determine whether cleavage arrested embryos have patterns of protein synthesis similar to the 'early' pre–genome activation pattern associated with oocytes and early cleavage stage embryos, or whether they have patterns similar to the 'late' post–genome activation pattern associated later cleavage stage embryos. To examine further the possibility that embryonic failure may be due to failure of activation of the embryonic genome in some but not all

blastomeres within the embryo, I have also analysed the protein synthetic patterns of individual blastomeres from both cleavage arrested and non–arrested embryos.

Since one of the features of a normal embryological developmental pattern in the human is activation of the embryonic genome, I have also analysed the protein synthetic pattern obtained from parthenogenetically activated oocytes that had reached various stages of development for evidence of genome activity in order to determine whether parthenogenetically activated oocytes might be a suitable experimental model for the study of early human development.

Chapter Two

Materials & Methods

2.1 Recipes

2.1.1 Culture Media

2.1.1.1 Modified Earle's Balanced Salt Solution (EBS) (provided as 10x stock solution Flow Laboratories, Irvine, UK)

Compound	Amount mgL^{-1}
$CaCl_2.2\,H_2O$	264.9
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6800
$NaHCO_3$	2200
$NaH_2PO_4.2\,H_2O$	158.3
glucose	1000
sodium phenol red	17.0

For use in in vitro fertilisation this was further modified by the addition of

penicillin 60 units

(Glaxo Laboratories, Greenford UK)

sodium pyruvate $2.2\ mgL^{-1}$

(Sigma Chemical Co, Poole, UK.)

gentamicin $4.0\ mgL^{-1}$

(Flow Labs, Irvine, UK)

osmolarity adjusted to 280-284 mosm/kg with sterile double distilled water (Boots Chemical Co., Nottingham,UK)

2.1.1.2 Earles Balanced Salt Solution + Heat Inactivated Patient's Serum (EBS + HIS)

As for EBS with 10% by volume heat inactivated, sterile filtered maternal serum. The maternal serum was prepared from blood collected from the patient by venepuncture. The plasma was separated by centrifugation at 3000 x g. for 10 minutes. This was then poured off and allowed to stand to allow coagulation. The coagulum was then removed with a glass rod and the resulting serum sterile filtered before being put into a heated block at 60°C for at least one hour.

This was the standard culture medium used for long term incubation of the embryo and for fertilisation where the ambient carbon dioxide levels are controlled at 5%.

2.1.1.3 Earles Balanced Salt Solution + Bovine Serum Albumin (EBS + BSA)

As for EBS with the addition of 400 mgL^{-1} fraction V dried bovine serum albumin (BSA) (Sigma, Poole).

This was used for embryo culture where the presence of maternal serum was not desirable.

2.1.1.4 Earles Balanced Salt Solution + Albuminar (EBS + Albuminar)

As for EBS with the addition of 10% by volume of 10% solution of human albumin (Albuminar 20; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex)

2.1.1.5 Calcium free Earle's Medium

As for EBS+BSA but without the addition of calcium chloride.

This medium was used to disaggregate embryos for the analysis of single

cells.

2.1.1.6 Hepes Buffered Earle's Balanced Salt Solution (HEBS)

to make 1 Litre

10x strength EBS*	100ml
sodium pyruvate	8.8mg
water	725.0 ml
sodium bicarbonate*	33.7mg

*10x strength EBS (see 2.1.1.1) is supplied without bicarbonate which must be added when making up the solutions.

To this was added 84 ml of a solution of a 100ml stock solution of 5.957g HEPES (Ultrol buffers, Calbiochem, Nottingham, UK) which had been adjusted to a pH of 7.4 with 5M sodium hydroxide

The osmolarity was adjusted to 280-285 mosm/kg with double distilled water and the solution then sterile filtered.

2.1.1.7 Hepes Buffered Earle's Balanced Salt Solution + Heparin (HEBS +HEP)

As above with the addition of 1000 units per litre of mucus heparin (Leo Laboratories, Princes Risborough, UK) and the solution then sterile filtered This was used as the medium for flushing follicles at egg recovery and for immediate storage thereafter.

2.1.1.8 Hepes Buffered Earle's Balanced Salt Solution + Heat Inactivated Serum (HEBS + HIS)

As for HEBS with the addition of 10% by volume heat inactivated maternal serum (see 2.1.1.2).

This was used as the medium for the replacement of embryos into the uterus.

2.1.1.9 Acid Tyrode's Solution

To make 100ml

NaCl	0.8g
KCl	0.02g
$CaCl_2.2H_2O$	0.0265g
$MgCl_2.6H_2O$	0.01g
Glucose	0.1g
polyvinylpyrrolidone (PVP) (Sigma Lab)	0.4g

Made up to 100ml with DDW and pH adjusted to 2.5 with 1M HCl

This medium was used to remove the zona pellucida from embryos prior to disaggregation.

2.1.1.10 Isotonic Percoll

To make 1 litre

10X strength EBS	100.0ml
bovine serum albumin	2.84g
sodium pyruvate	0.104mg
gentamicin	18.9mg
penicillin	56.8mg
sodium lactate (Sigma lab)	3.4 ml of a 60% syrup
Percoll	900ml

This was prepared as discontinuous gradients for centrifugation of sperm. These gradients were composed of 4mls of 90% Percoll onto which was layered 4 mls of 50% percoll. The relevant concentrations were achieved by the addition of appropriate volumes of EBS + BSA. These gradient columns were used for preparation of motile sperm prior to fertilisation.

2.1.2 Electrophoresis

All the following chemicals were of specially pure electrophoretic grade where appropriate and supplied by either BDH Laboratory supplies or Northumbria Biologicals. The various solutions were originally prepared as stable stocks which were then combined in appropriate quantities just prior to use.

Stock Solutions .

2.1.2.1 Acrylamide Stock

acrylamide	292.0g.
bis acrylamide	8.0g.

made up to 1 litre with double distilled water (DDW).

2.1.2.2 Separating Gel Buffer

trizma Base	181.6g.
sodium dodecyl sulphate (SDS)	4.0g.

pH adjusted to 8.8 with conc. HCl and then made up to 1 litre with DDW.

2.1.2.3 Stacking Gel Buffer

trizma base	60.4g.
SDS	4.0g.

pH adjusted to 6.8 and then made up to 1 litre with DDW.

2.1.2.5 Sample/Lysis buffer

To make 10 ml

stacking gel buffer	0.625ml
SDS	0.2g
glycerol	1.0ml

β- mercaptoethanol 0.5ml

0.4 ml of a 0.05% solution of Bromophenol blue

all then made up to 10 ml with DDW.

2.1.2.6 10% Separating gel

separating gel buffer	8.0ml
acrylamide Stock	8.0ml
DDW	8.0ml
tetramethylethylenediamine (TEMED)	45µl.

This was mixed by swirling and then 15µl of a 10% solution of ammonium persulphate was added. These last two reagents act as the polymerising catalysts and so are added immediately prior to casting the gel.

2.1.2.7 Stacking gel

stacking gel buffer	1.25ml
acrylamide stock	0.75ml
DDW	3.0ml
TEMED	5µl

This was mixed by swirling and then 50µl of a 10% solution of ammonium persulphate was added. This was then poured onto the pre-cast separating gel.

Note the above quantities were sufficient to cast one gel in the system used. Larger volumes were used for a larger capacity system.

2.2 Obtaining oocytes and embryos

2.2.1 Subjects

All oocytes and embryos were donated as surplus to therapeutic requirements of patients undergoing treatment as part of an MRC funded, Interim Licensing Authority approved clinical assisted conception programme. The median age of the patients during the period of study was 36 years (mean 32 years, range 21–40 years). The duration of infertility ranged from a minimum of 12 months up to several years with a wide range of diagnoses being represented including blocked or damaged fallopian tubes, male factor and unexplained infertility. All patients had been assessed by at least two medical practitioners as being suitable for treatment.

2.2.2 Superovulation

Multiple follicular development was induced with a fixed programme of pituitary desensitisation using a gonadotrophin releasing hormone analogue (buserelin acetate; Suprefact, Hoechst, Hounslow, UK) (Fleming and Coutts 1989), 500μg administered by intra-nasal spray daily in divided doses. This was followed with human menopausal gonadotrophin (hMG; Pergonal, Serono, Welwyn Garden City, UK) administered daily for 10 days commencing at 150 i.u. per day rising to an absolute maximum of 600 i.u. daily (Sher, Knutzen et al. 1984), the dose being adjusted according to patient response monitored by vaginal ultrasound scanning and serum oestradiol assays. 10,000 units of human chorionic gonadotrophin (hCG; Profasi, Serono, Welwyn Garden City, UK) was administered, 34 -36 hours before oocyte retrieval, when there was evidence of at least three ovarian follicles of 17-19mm diameter and the serum oestradiol concentration had reached a minimum of 1000pg/ml, but not greater than 3000pg/ml.

2.2.3 Oocyte recovery

Oocytes were recovered 34-36 hours after the injection of hCG. In the case of IVF-ET this was performed transvaginally using ultrasound guidance. The patients were sedated with an intravenously administered mixture of pethidine, up to 100mg, and midazolam (Hypnoval: Roche, Hounslow, UK), up to 10mg. In the case of GIFT, eggs were recovered laparoscopically under general anaesthesia (Braude 1987). All follicles were methodically aspirated and then flushed with HEBS + Hep. The fluid was examined immediately to determine if an oocyte was present. Oocytes were then kept in HEBS at 37°C in a heated block pending the return of two or three oocytes to the patient in the case of GIFT, or transfer to an incubator in the laboratory in the case of IVF and for those oocytes surplus to therapeutic requirements in GIFT procedures.

2.2.4 Preparation of spermatozoa and fertilization *in vitro*

A semen specimen was produced by the patient's partner 1-2 hours prior to the egg recovery by masturbation into a sterile container. After initial assessment, an aliquot of the semen (1 or 2ml) was layered on a discontinuous Percoll gradient (see 2.1.1.9) and spun at 400 X G for 10 minutes. After centrifugation the supernatant was discarded and the sperm pellet remaining washed twice with HEBS + HIS. A further assessment of the quality and concentration of the separated sperm was made at this stage. For IVF–ET, 100,000 to 150,000 spermatozoa were added to 1ml drops of EBS+HIS containing the oocytes. In cases of GIFT 20–40µl of sperm suspension was loaded into the GIFT catheter along with 2 or 3 of the recovered oocytes.

2.2.5 Incubation and observation

All incubations were carried out in drops of medium under light paraffin oil (FSA Laboratories, Loughborough, UK) in Falcon tissue culture dishes (Becton Dickinson Labware, New Jersey, USA) at 37.5°C in an atmosphere of 5%CO_2 /95% air. In the case of IVF the presence of pronuclei 19-22 hpi was taken as evidence of fertilisation, after which all oocytes, both fertilised and unfertilised, were transferred to individual 100µl drops of EBS+HIS for further culture. A maximum of three embryos was returned to the patient transcervically 44-46 hpi using a Wallace–Edwards embryo replacement catheter (Wallace Ltd., Colchester,UK). The quality of the embryo as noted by overall appearance, appropriate cell number, regularity of cells and degree of cell fragmentation was used to select embryos for replacement. Those not used for replacement were maintained in culture and their morphology assessed daily. Those oocytes that failed to show evidence of pronuclear structures at 19-22 hpi were deemed to have failed to fertilise.

2.3 Radiolabelling with ^{35}S methionine

Studies in the mouse have reported a relatively low rate of protein synthesis in cleavage arrested embryos and embryos prior to the onset of gene activity (Goddard and Pratt 1983) with a consequent poor uptake of precursors. Assuming this to be similar in the human, the uptake of the radioactively labelled amino acid precursor was enhanced by performing the labelling in a medium devoid of other sources of precursor. Thus, embryos and unfertilised oocytes were washed through an excess of EBS + BSA prior to labelling to remove all traces of the maternal serum contained in the original culture medium. A final resting period of at least 15 minutes in a 100µl drop of EBS + BSA prior to addition of the

radioactive label was also found to improve the reliability of labelling, particularly in the case of failed fertilised oocytes, possibly because this allowed the small residue of maternal serum beneath the zona pellucida to become even further diluted. The specimens were then transferred to a 50µl drop of EBS+BSA to which was added 5µl of high specific activity ^{35}S-methionine (15mCi ml^{-1}, Amersham International, UK) and cultured therein for 30 minutes. Although methionine is only present in relatively small concentrations in most proteins, it was chosen as the amino acid substrate as the ^{35}S isotope can be obtained with a very high specific activity (>1000 Ci/mmol). However with a half-life of only 80 days, samples had to be analysed shortly after labelling. 0.0125mg of the vital stain DAPI (4,6 Diamadino-2-phenylindole; Sigma) was added to the same drops and a further 30 minutes of culture allowed. DAPI is a vital stain which preferentially stains the nuclear histone proteins and fluoresces when exposed to ultraviolet light. This allowed the protein synthetic patterns to be related to the apparent nuclear number as well as the morphology of the embryo. At the end of the end of the total culture period (1 hour) the embryos were washed through an excess of EBS + BSA to remove excess DAPI and radioactive label. The nuclear number assessed by visualising the specimen under ultraviolet light using filter set A (allowing the transmission of light between 450 and 490 nm). The specimens were then washed through an excess of EBS to remove the BSA and collected into individual tubes and instantly frozen using dry ice. The tubes were then stored at -80°C until required for analysis.

2.4 Disaggregation of embryos

After radiolabelling and DAPI staining, but prior to examination under ultraviolet light, the zonae pellucidae were removed by placing the

embryos for 1–3 minutes into a 1ml drop of acid Tyrode's solution in a cavity block at 37°C. Each embryo was then gently drawn up and down a pipette until the zona was seen to disappear. The embryo was then washed through three drops of HEBS+BSA to remove any remaining traces of the acid solution. The embryos were transferred to 100µl drops of calcium free culture medium under oil and incubated for five minutes. Although the blastomeres of the human embryo are relatively easier to disaggregate than those of the mouse, removal of calcium from the medium encourages the breakdown of cell to cell bridges making the process of disaggregation easier. Separation of individual blastomeres was encouraged by gently drawing the embryo up and down a micro–pipette with a bore slightly larger than that of the blastomeres. They were then immediately washed through three drops of EBS containing calcium as prolonged exposure to calcium free medium can lead to cell lysis. Each blastomere was placed into an individual 5µl drop of EBS+BSA and examined under ultraviolet light for the presence and structure of nuclei before a further wash through 3 drops of EBS to remove the BSA. Each was then collected into individual tubes and treated in the same way as whole embryos (see above). The origin of all blastomeres was recorded for subsequent analysis. Due to the complexity and number of steps involved, it was not uncommon for some blastomeres to be lost by lysis and damage during pipetting and transfer.

2.5 Parthenogenetic activation

Oocytes surplus to GIFT procedures and some of the failed fertilised oocytes from IVF procedures, were entered into a protocol aimed at inducing parthenogenetic activation. The protocol followed was that described by Winston et al (Winston, Johnson et al. 1991). The cumulus mass was removed by exposure for 0.5 - 2 minutes to 0.05% hyaluronidase (type II from ovine testes, Sigma, UK) in HEBS before rinsing in an excess

of EBS + BSA. After grading and scoring for the presence of the first polar body, the oocytes were held in a 1ml drop of HEBS+BSA at 37°C in air for at least 30 minutes before exposure to 5μM calcium ionophore in a 1ml drop of HEBS+BSA without oil for exactly 5 minutes. The oocytes were then rinsed in an excess of EBS+Albuminar before transfer to a fresh 1ml drop of EBS+Albuminar. They were inspected for evidence of pronuclear formation at 19-22 hours after activation. If pronuclei were present the oocyte was deemed to have been successfully activated. However because of the altered timing of events in parthenotes, (some may undergo immediate cleavage rather than pronuclear formation, and others may have development retarded) all oocytes were allowed to remain in culture for at least 48 hours following attempted activation and inspected for evidence of cleavage in which case they were also deemed to be successfully activated. They were then transferred to individual drops for further culture.

2.6 α–Amanitin Treatment

In order to determine the transcriptional dependence of the proteins synthesised, some oocytes and non–arrested embryos were exposed to a transcriptional inhibitor, α–amanitin (Lindell, Weinburg et al. 1970) derived from the mushroom *amanita phylloides.* This has been shown in appropriate but not excessive concentrations (1– 100 μg/ml) to be an inhibitor of the enzyme RNA polymerase II, and thus inhibits specifically the synthesis of mRNA.

The embryos and oocytes were placed in 100μl drops of EBS + BSA to which was added 10μl of a stock solution of α–amanitin (1mg of α–amanitin (Boehringer Mannheim, Lewes, UK) in 990μl of EBS). The addition of 10μl of this to a 100μl drop gave a final concentration of

approximately 100μg/ml which has been shown to result in 100% inhibition of RNA polymerase II activity (Lindell, Weinburg et al. 1970) whilst not affecting RNA polymerase I and III *in vitro*. Appropriate untreated controls were labelled at the same time for comparison.

2.7 Gel electrophoresis

2.7.1 Introduction

Gel electrophoresis is an established technique for the separation of protein mixtures obtained from various biological systems (Van Blerkom and Brockway 1975). When placed in an electric field (electrophoresis) proteins will migrate as a function of their molecular weight and electrochemical properties. Electrophoresis can exploit one or other or both of these properties. Since this study was concerned with detecting qualitative differences in proteins synthesised at various stages of development, separation in one dimension under reducing conditions was used. The SDS and mercaptoethanol denature the proteins, resulting in an unfolding of the secondary and tertiary structure, and the detergent nature of SDS overcomes the charge differences between proteins causing most to migrate as anions. Thus in this system the proteins are separated by molecular weight alone with an inverse linear relationship between the relative migration distance and the log of the molecular weight. This method has been shown to be the most effective in producing high resolution of the proteins and provides consistency of results.

The degree of resolution can be further enhanced by varying the concentration of the polyacrylamide in the gel (Van Blerkom 1977). As there is such a wide variation in the molecular weight of cellular proteins no single concentration of gel is entirely satisfactory. A 10% acrylamide gel was chosen as it consistently provided reproducible results, was simple to

produce and gave a satisfactory resolution of proteins in the weight range of 10 to 200 KD, which has been shown in murine studies (Howlett 1986; Pratt, Bolton et al. 1983) to be a region of great activity in cleavage. An exponential gradient gel with an acrylamide concentration of 8-18% could retain and resolve more cellular proteins but these gels are technically more difficult to produce consistently, and thus reproducibility of results may suffer. Limited studies were performed using a modern horizontal system (Ultraphor II, LKB) which used ultrathin pre-cast SDS-PAGE gels with an 8-18% gradient with a running distance of only 6-8 cm. The majority of the embryos were analysed using a traditional discontinuous vertical gel system of the type used in previous studies of human protein synthetic patterns (Braude, Bolton et al. 1988)

2.7.2 Gel preparation

In the case of the vertical gel system the separating gel was prepared as documented in recipes and then formed between two glass plates separated by 0.8 mm thick Teflon spacers and sealed with narrow silastic rubber tubing. The glass plates (approximately 15cm square) were thoroughly cleaned before use as grease and dirt can hamper the polymerisation. Once poured the gel was gently overlaid with distilled water which ensured a level surface for the subsequent stacking gel and also prevented the surface of the gel from drying out. Once polymerisation was complete (approximately 1 hour), the distilled water was poured off and the excess removed with blotting paper taking care not to touch the surface of the gel. The stacking gel was prepared according to recipes, and poured on top of the separating gel. A 0.8 mm thick teflon well former (comb), with 20 slots (4mm wide and 15 mm deep) was then placed into the still liquid gel. After a further hour the comb was gently removed and the remaining unpolymerised gel washed out with DDW. The excess water was then

removed by shaking and the gel loaded with the samples.
The gels for the horizontal system came ready prepared and fixed to a flexible plastic backing in a sealed foil packet. These gels were 0.3mm thick with a running distance of 8cm (the distance the samples have to migrate during the electrophoresis) and a width of 25 cm The reduced thickness and short running distance meant that these gels took less time to run with the added advantage that the increased width allowed more samples to be analysed simultaneously.

2.7.3 Sample preparation

Just prior to electrophoresis the samples were thawed and 15µl of lysis buffer added to the tube. The tube was agitated to ensure full mixing and then briefly spun in a benchtop microfuge at 6000rpm to ensure that all the sample was at the bottom of the tube. The tube was placed in boiling water for 3 minutes to ensure complete denaturation of the proteins. The samples were then allowed to cool before loading onto the gel.

2.7.4 Running the gel

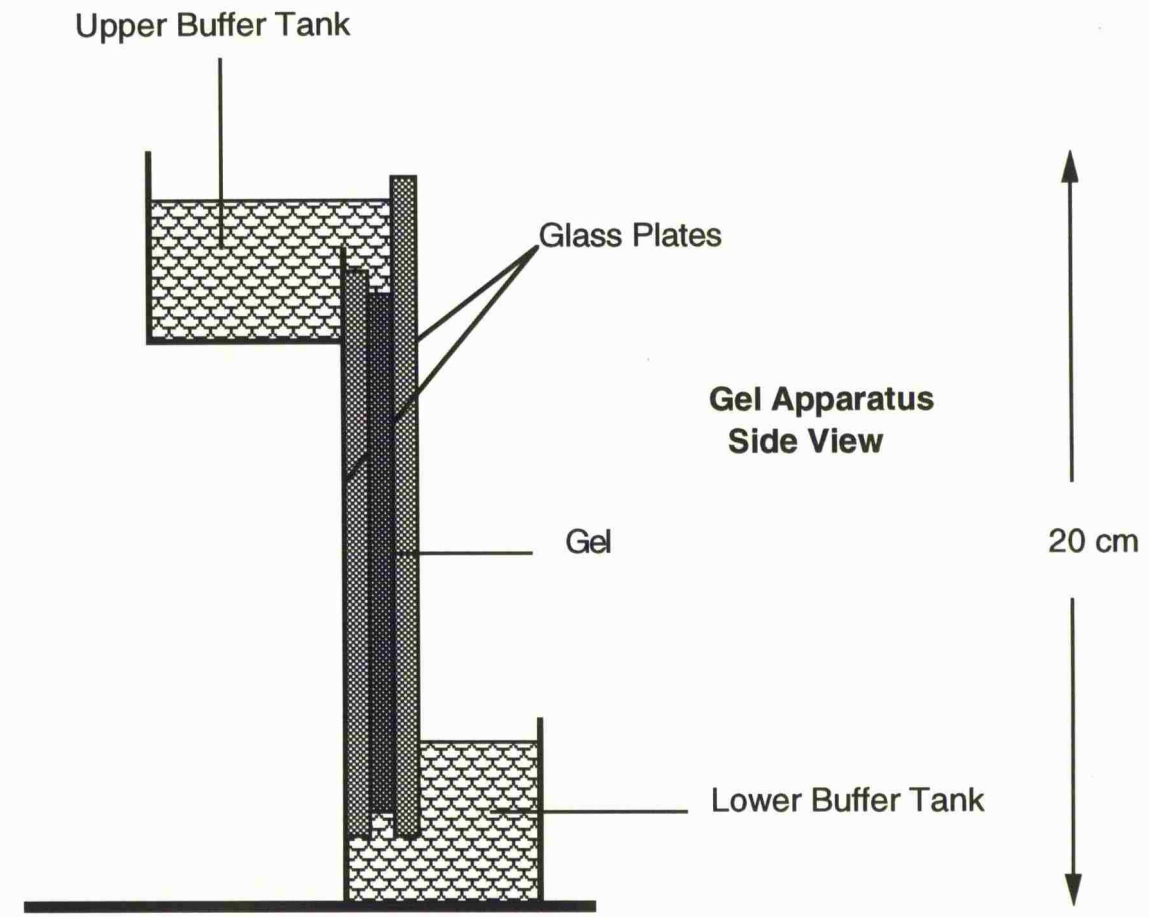
In the vertical gel system the silastic tubing seal was removed from between the glass plates and the assembly fixed into the gel tank. The gel tank was constructed as shown in Fig 1. There are essentially two reservoirs each filled with running buffer. The upper reservoir forms the cathode and the lower the anode. The gel forms the contact between the two. After fixing the gel assembly into the tank the reservoirs were flooded with running buffer. Any bubbles trapped between the glass plates were carefully removed using a syringe and a long curved needle. The individual samples were loaded *in toto* into the wells under the running buffer with a careful note being kept of the sequence of loading. The

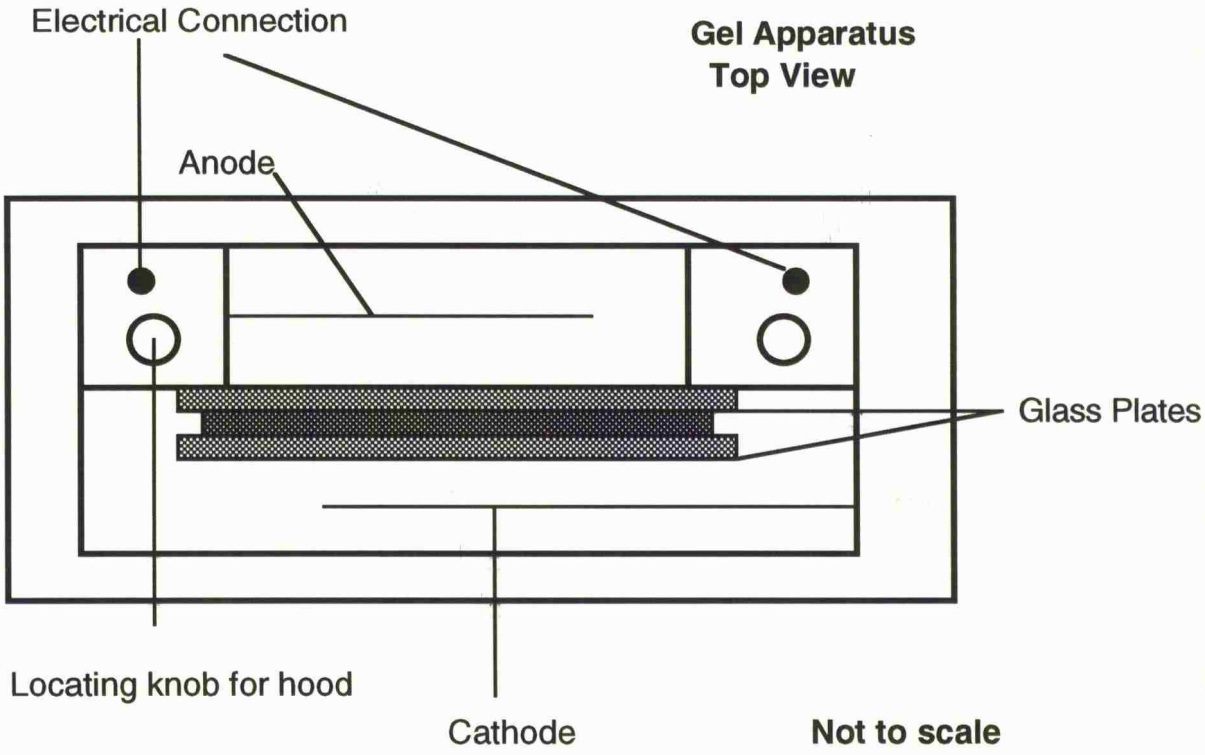
Figure 1 overleaf

Figure 1.

Diagram of the vertical gel apparatus as constructed and used in our laboratory. The tank is made of perspex and fixed with silicon glue. For clarity the hood which is placed over the apparatus whilst it is running has been omitted. The hood is vented to allow heat and any released vapours to escape but it is not essential for the reliable running of the gel.

Figure 1.


Upper Buffer Tank
Glass Plates
Gel Apparatus
Side View
Gel
20 cm
Lower Buffer Tank


Electrical Connection
Gel Apparatus
Top View
Anode
Glass Plates
Locating knob for hood
Cathode
Not to scale

glycerol present in the lysis buffer increases the density of the sample and keeps it within the well. However, care must be taken not to allow samples to spill over wells. ^{14}Carbon labelled protein markers of known molecular weight were added to one well on each gel to allow comparative size analysis. Once loading was completed, the electrodes were connected and a fixed voltage of 240V applied across the gel. The current was allowed to vary but generally remained about 30mA. The progression of the samples through the gel could be seen by the progression of the blue marker dye (bromophenol blue) contained in the lysis buffer. The current was switched off once this marker dye had just run off the gel, which generally took about four hours. The gel assembly was then removed from the tank and the gel gently removed from between the glass plates. The softer stacking gel was cut away and discarded, and, to allow subsequent orientation, the corner of the gel corresponding to the side at which the loading had started was removed.

In the case of the horizontal gel system, after removing the gel, from its wrapping, it was placed, still attached to the plastic backing strip, onto a water cooled ceramic plate in the gel tank. In this system the liquid running buffer of the vertical system was replaced by gel buffer strips. These came ready prepared and were laid onto each end of the gel. Small, 0.5 x 1 cm pieces of filter paper were laid 1cm apart directly onto the gel surface towards the cathode and the samples loaded directly onto the filter paper. The use of filter paper was simply to confine the spread of the sample and is not necessary when the samples being loaded have a volume of less than 5µl. The thinner gel and the use of a cooling plate allowed this system to run at a higher current of 50mA with a variable voltage 300 and 600V, resulting in a running time of less than one hour at the end of which the buffer strips were removed and the gel, still attached

to its backing strip, could be processed.

2.7.5 Processing the gel for autoradiography

As the proteins within the gel could diffuse from their position on the gel, the proteins had to be fixed using a solution of 30% acetic acid, 40% methanol and 30% distilled water for a minimum of one hour. As there was so little radioactivity in the samples, autoradiography was enhanced by using light emission stimulated by the release of radiation. To achieve this the gel was placed in a proprietary scintillant solution (Amplify, Amersham International, UK). Although development time is reduced, fluorography results in some loss of quality and definition of the signal. Initially this step was carried out in accordance with the manufacturer's instructions with the gel being in the solution for between 15 and 30 minutes but the definition of the bands on the autoradiogram was improved by reducing the time to a maximum of 5 minutes (see Fig 2). The above steps were identical for both gel systems but because of the differing thickness of the gels the drying step was different for each system. The vertical gel was 'blotted' onto blotting paper (Whatman 3mm) and dried under heat and vacuum. The horizontal gel, being much thinner, was dried using a hairdryer for between 15 and 20 minutes. After drying, the gels were placed against pre-flashed(Laskey and Mills 1975) Fuji Rx X-ray film, the gel and film were placed in a light proof film cassette and stored at -80°C to improve the efficiency of the light detection(Laskey and Mills 1975).

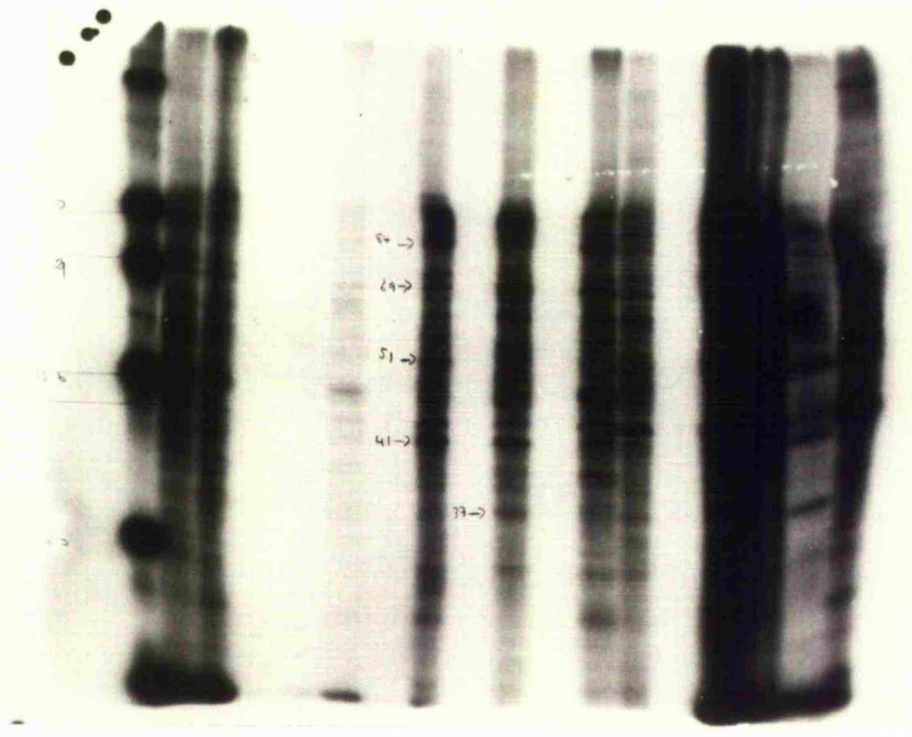
After 2 to 4 weeks the X–Ray film was developed in a semi–automatic processor (PLH Medical equipment, Watford, U.K.) at 22°C using Photosol developer and fixer (Photosol, Basildon, U.K.). The developing regime consisted of 4 minutes in the developer, rinsing in water followed by 2 minutes in the fixing solution followed by a further wash in water.

Figure 2 overleaf

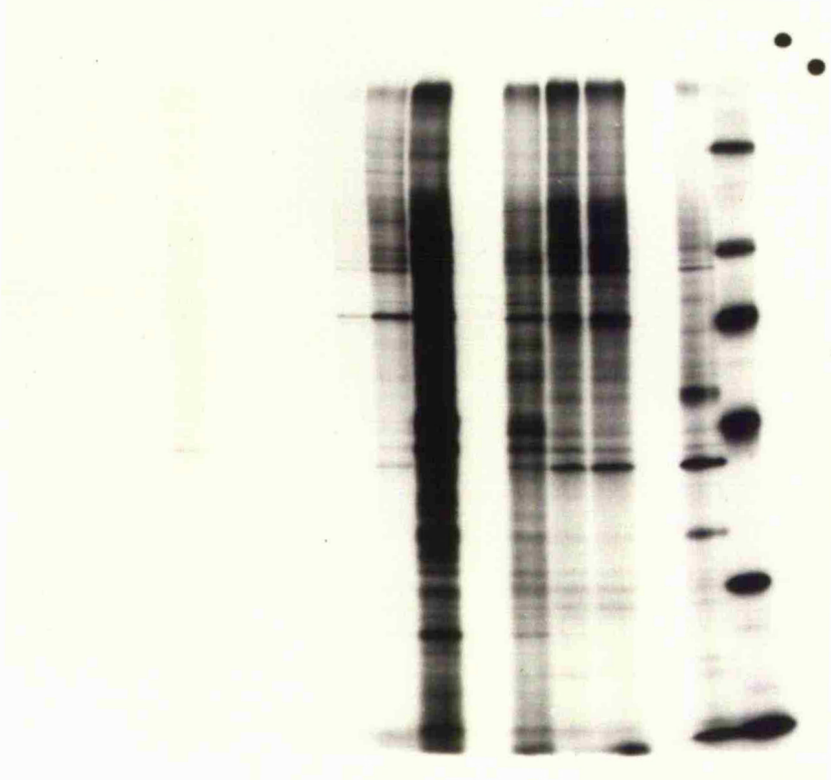
Figure 2.

Autoradiograms from gels which were exposed to the scintillant solution Amplify for the recommended 15 minutes before drying (A) and an autoradiogram from a gel which was exposed to the scintillant solution for only 5 minutes before drying (B). It can be seen that the bands in autoradiogram A appear to be poorly focused and are generally indistinct when compared to those in the autoradiogram B where the bands are much more tightly focused, allowing better visual resolution of protein bands.

Figure 2



A
After 15 minutes
in Amplify



B
After 5 minutes
in Amplify

2.8 Analysis

Following electrophoresis, the proteins in each sample had become separated along a linear track along the length of the gel with each protein or group of proteins becoming focussed into a band. The distance migrated by the proteins is inversely proportional to its molecular weight. As each embryo synthesizes a large number of proteins of varying molecular weights, each track was made up of many bands. The relative molecular weight of the proteins in the bands was estimated by comparison with the standard markers run at the same time. Visual inspection of the autoradiograms should reveal whether a particular band is present or absent when compared to adjacent tracks. However when proteins are analysed in one dimension it is often the case that a change in protein synthesis may reveal itself as a relative weakening or strengthening of the intensity of a band rather than simply the appearance or complete disappearance of the band. These relative changes in intensity might represent the addition or loss of a protein to a group of proteins migrating at the same molecular weight of the band which could be demonstrated if the sample to be analysed was separated in two dimensions (see above). Thus incomplete disappearance produced a problem of interpretation since some estimate of the relative strength of the bands on the gel had to be made.

2.8.1 Visual analysis

By pre–flashing the film (see above), the non-linear response of the film to photons or radioactive exposure is converted to a more linear response. Thus, providing that maximal density has not been reached, the density of a band will be proportional to the amount of radioactivity emitted by the

radioisotopes in the gel which is proportional to the amount of amino acid incorporated during protein synthesis. Thus a dark band on the autoradiogram will reflect a relatively large amount of newly synthesised protein present within the gel, and a light band a lesser amount of protein synthesis. In this study a four point scoring scale was used : absent, weakly present, strongly present and uninterpretable. In order to determine which were the most consistent changes in protein pattern, in the vertical gel system 27 bands were identified initially to be strongly present at some time. However as more gels were analysed it became clear that many of the bands were present in all or a majority of the oocytes and embryos and were unlikely to be of assistance in discriminating between different synthetic patterns. Eventually 7 bands that appeared to have discriminatory potential were selected. Each track was analysed subsequently only in respect to the changes of these 7 bands. The bands identified subsequently were compared with those previously identified by Braude et al (Braude, Bolton et al. 1988). Fig 3 shows how this assessment of the bands on a gel was converted to the diagrammatic format. The autoradiogram in this figure shows that the intensity of each track can vary within the gel, which in part reflects the variation in the amount of radioactivity incorporated between individual embryos, thus further increasing the difficulty in assessment. This variation in track density can be taken into account when making the visual inspection by only comparing the strengths of the bands within any one track and not from one track to another unless of similar overall density. There are two other strategies that can be used to minimize this problem and to assist with visual analysis. First relative differences between individual embryos can be overcome by grouping large numbers of synchronous embryos together in each sample. Thus it is likely that each sample will contain embryos

Figure 3 overleaf

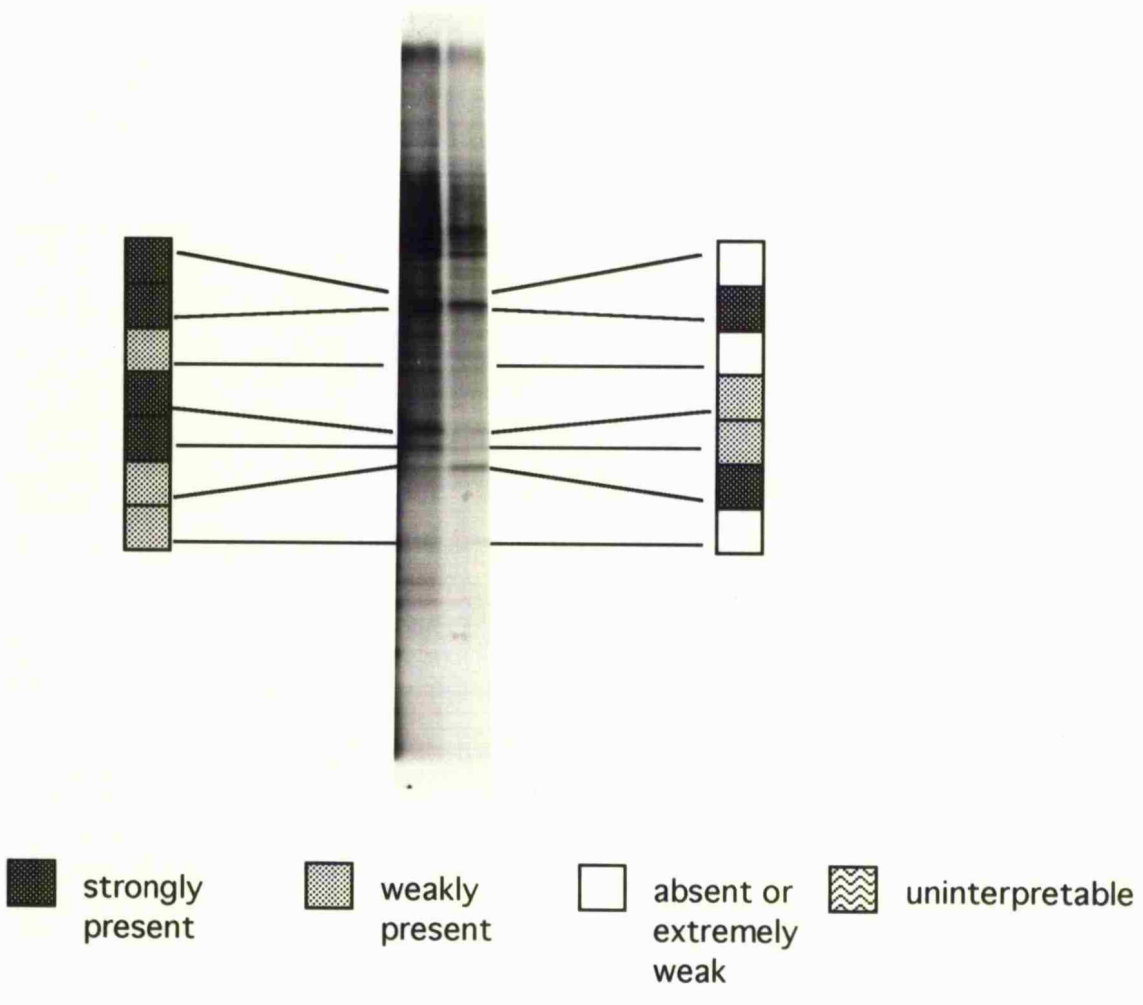
Figure 3

This figure shows how the information from the gel was converted to a diagrammatic format. The density of the band was assessed visually with reference to the overall density of the track and a four point scoring scale used: strongly present, weakly present, absent or uninterpretable.

These were in turn represented diagrammatically as a dark shaded box, a lightly shaded box, a blank box and a box filled with wavy lines.

Converting the information to this format facilitated comparison between gels and avoided having to constantly refer back to the autoradiograms.

Figure 3


strongly present
weakly present
absent or extremely weak
uninterpretable

which have incorporated high and low levels of radioactivity. This results in samples which have high levels of radioactivity but with the levels varying little from sample to sample. This is useful where one has access to large amounts of synchronous material but is not applicable to the study of human embryos which are available only in small numbers and can be highly heterogeneous. Alternatively an attempt can be made to load equal amounts of radioactivity onto each track by measuring the amount of radioactivity incorporated into individual samples. Although there is no comparative literature of using this technique in human embryo analysis, it has been extensively used in studies of laboratory species, often when more than one embryo or oocyte is included in each sample.

2.8.2 Assessment of radioactive label incorporated

The method followed was that described by Howlett 1987(Howlett 1987). A 5µl aliquot of the sample was added to 1ml of a 25% solution of trichloroacetic acid (TCA) to which had been added 0.25 ml of a 10% solution of bovine serum albumin (BSA) in DDW to act as bulking protein to aid precipitation. The samples were then stored at 4°C for a minimum of 1 hour before being filtered individually through glass fibre filters (GF/C; Whatman) and washed with an excess of a 10% solution of TCA. The TCA precipitates the proteins which are then retained on the filter disk. The filter was allowed to dry overnight before being placed in a scintillation vial with Cocktail–N scintillation fluid (Fisons Plc, UK) and then counted for 5 mins in an LKB scintillation counter (windows set to cover standard energy range for ^{35}S methionine of 0–0.17 mEV). These results expressed as counts per minute (c.p.m.) were used to calculate the volume of each of the original samples which needed to be loaded onto each track in order to give tracks with equal amounts of radioactivity.

2.8.3 Scanning Densitometry

The short running distance of the horizontal gel system and use of the exponential gradient gel, although resulting in very sharp focussing of the bands, also resulted in very tight packing of the bands making visual analysis very difficult. However the short running distance and tight focussing also made these autoradiograms ideal for analysis using a scanning densitometer.

The autoradiogram was loaded onto the carriage of a Joyce–Loebel scanning densitometer (Vickers Scientific Instruments, UK) and the absorption level zeroed against the general background darkness of the film. The film was then adjusted on the carriage so that the beam of light lay at the beginning of a track, and the machine set to automatically move the carriage in a straight line for the total distance of the track. Peaks and troughs of absorption produced by the bands in the track were produced as a graph by the machine (see Fig 4). It also calculated and produced a table of the degree of absorption by each band (expressed as a percentage of the total absorption with reference to the overall strength of the track), which overcome the effect of loading unequal amounts of radioactivity. By comparing the absorption profile with a visual interpretation of the autoradiograph it appeared that a band could be assigned to be weakly present when the absorption was less than 1% of the total, and appeared to be strongly present if the absorption was greater than this. A four point scale similar to that used in the visual analysis was constructed: not detectable (no absorption), weakly detectable(<1% absorption), strongly detectable (>1% absorption) and uninterpretable. The graph produced from each track was analysed and each peak marked according to its relative strength. A graph from an oocyte analysed 24 hours following failed fertilisation and from an expanded blastocyst analysed at 120 hours post

Figure 4 overleaf

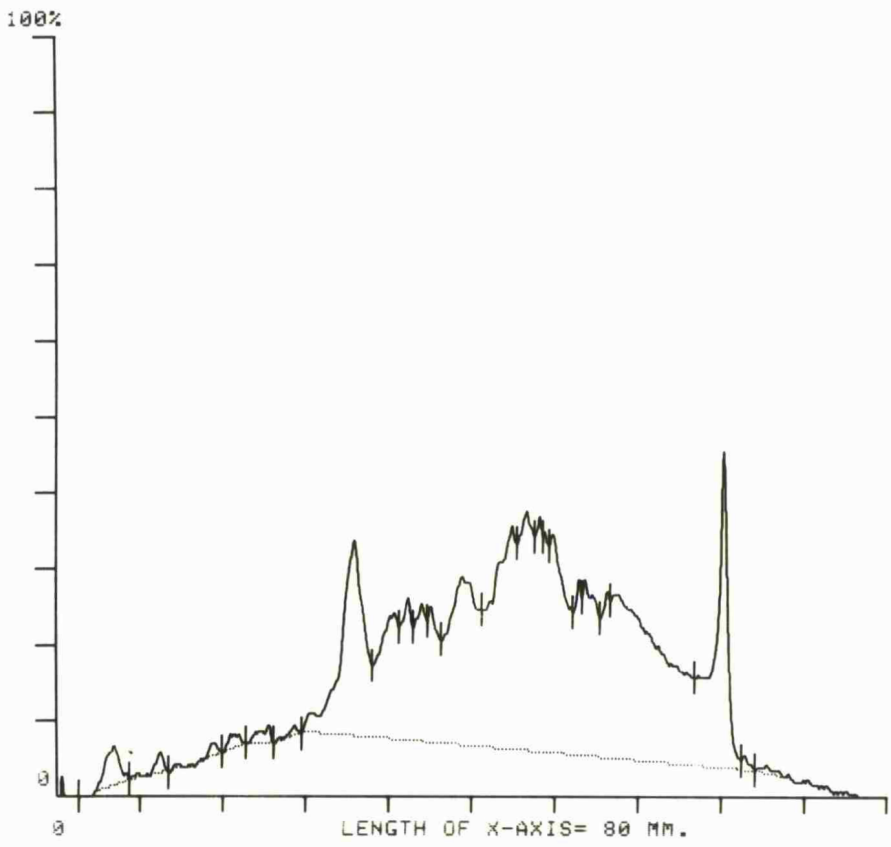
Figure 4.

Photograph of the absorption graph produced by the scanning densitometer.

Figure 4

LINEAR SCAN RUNNING...

SCAN NUMBER= 18
SCAN LENGTH= 80 MM. APERTURE WIDTH= 0.1 MM.



NUMBER OF PEAKS= 25 TOTAL INTEGRAL= 38448

PEAK	POSITION	HEIGHT	REL%	INTEGRAL	PEAK	POSITION	HEIGHT	REL%	INTEGRAL
1	0.5	7	0.06	24	2	5.5	13	1.07	414
3	10.0	7	0.32	124	4	15.2	4	0.21	82
5	16.8	5	0.29	112	6	20.5	6	0.39	152
7	23.0	3	0.14	54	8	28.8	66	8.92	3432
9	32.7	43	4.7	1808	10	34.1	48	3.07	1182
11	35.4	46	3.07	1184	12	36.2	46	2.72	1048
13	39.2	57	9.64	3710	14	44.1	75	10.53	4050
15	45.5	80	6.61	2542	16	46.7	79	3.12	1202
17	47.2	74	2.22	856	18	47.9	74	6.81	2622
19	50.4	60	2.49	960	20	51.0	59	4.7	1808
21	53.1	57	2.69	1038	22	54.0	55	17.42	6698
23	64.4	106	8.29	3190	24	66.3	5	0.18	72
25	68.4	3	0.21	84					

insemination were photocopied to transparent sheets which were laid on top of each other for direct comparison (see fig 5). There was little variation in the overall length of the tracks in the horizontal system due to the uniformity of construction of the factory produced horizontal gels and the short running distance. Peaks of absorption could be clearly seen to occur at regular points.

The use of the scanner for the vertical system was considerably more difficult. The greater width of the bands, 2-4 mm in the vertical system compared to less than 1mm in the horizontal system, and variation in width of the bands when compared to the uniformly narrow bands seen when using the horizontal gels, resulted in persistently bizarre and often uninterpretable absorption graphs even in well developed gels that were easily interpretable visually. The longer running distance of the tracks in the vertical system also resulted in some of the tracks being slightly curved due to the dragging effect of the edge of the gel on the advancing proteins, and also to the heat which built up from the high current. These unwanted effects were reduced by avoiding the placing of samples in the wells nearest to the edge of the gel and carrying out the electrophoresis at as low a current, compatible with overseeing the running of the gel etc. Despite these precautions, the small curvature of the track meant that since the densitometer scans in straight lines, a track often had to be scanned twice at different angles in order to obtain a complete absorption profile. These technical difficulties often resulted in uninterpretable scans. In the light of this, the use of the scanner for the vertical gel system was abandoned although it remained a satisfactory method for analysing the autoradiograms from the horizontal system.

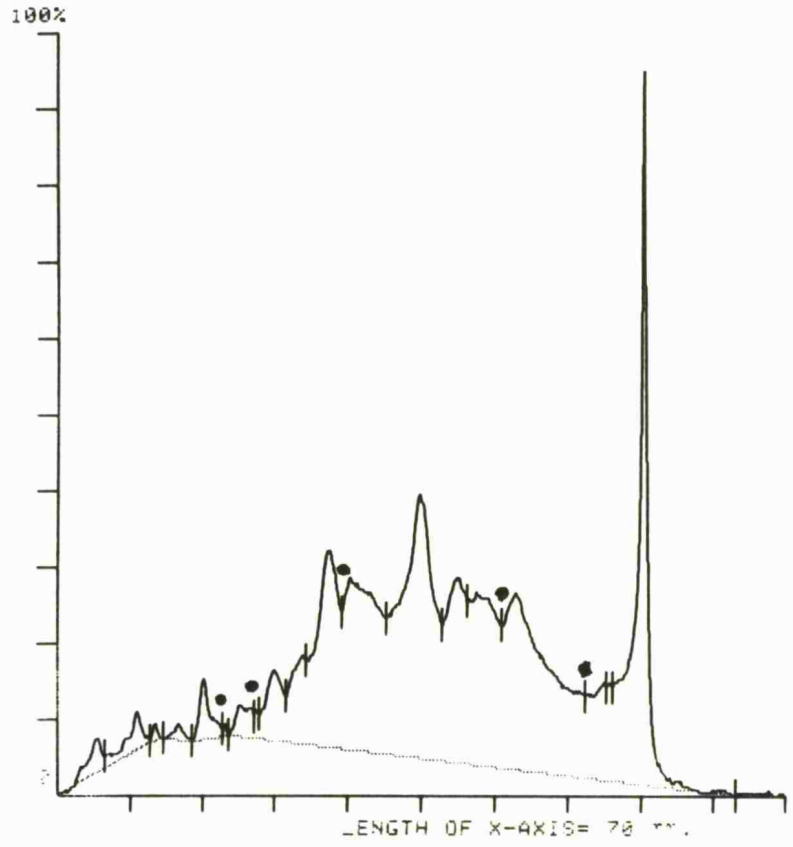
Comparison of the graphs of the oocyte and blastocyst obtained using this

Figure 5 overleaf

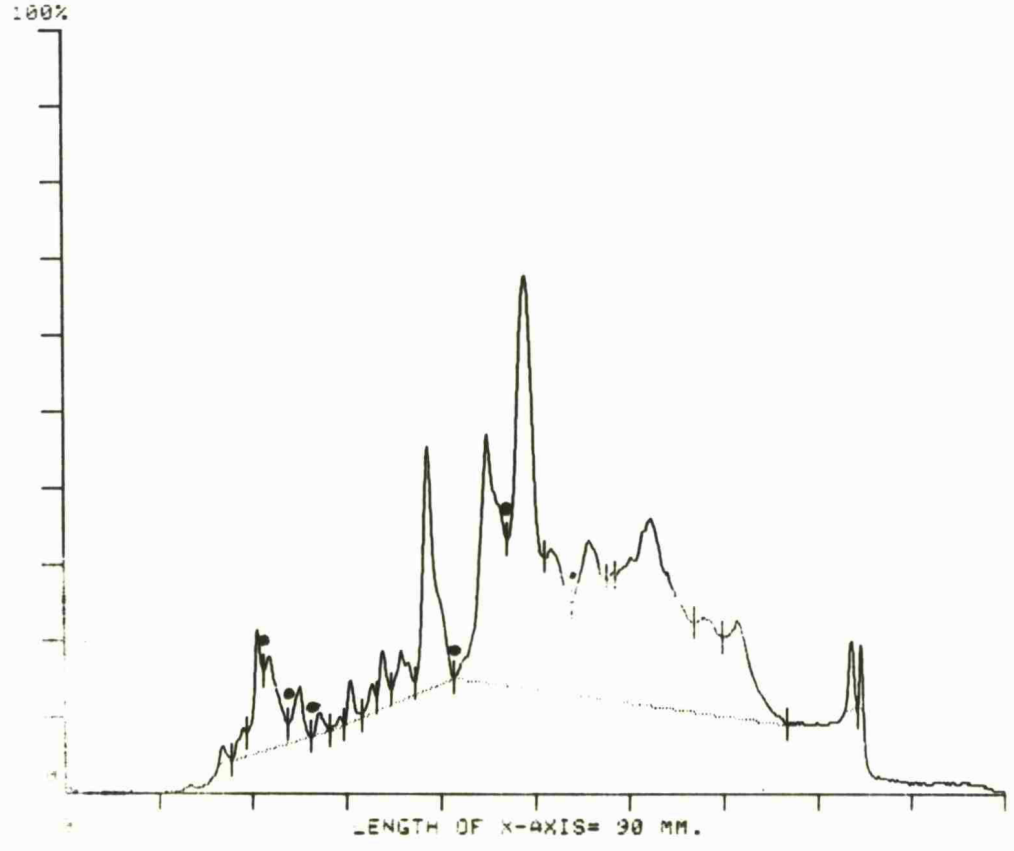
Figure 5

A photograph of graphs produced by the scanning densitometer. These represent the protein synthetic pattern of an oocyte and a blastocyst when analysed using the horizontal gel system. Obvious differences can be seen which represent different bands within each pattern. Those points that were discriminatory for the early (pre–activation) pattern and the late (post–activation) pattern are marked.

Figure 5



Graph from Blastocyst



Graph from Failed fertilised oocyte

technology with the horizontal system revealed obvious differences in the populations of proteins being synthesised at the two developmental stages. From this initial examination 12 points of discrepancy were noted (see fig 5); 6 peaks that appeared in the graph from the oocyte but not the blastocyst, and 6 that appeared in the graph from the blastocyst but not the oocyte. All the graphs were then studied for the presence of these 12 peaks and, using the value given for the degree of absorption, a diagrammatic protein synthetic profile constructed for each oocyte and embryo

Chapter Three

Changes in protein synthetic pattern associated with non–arrested embryos

3.1 Introduction

Studies of laboratory and domestic species have suggested a common pattern of control of protein synthesis in the mammalian pre–implantation embryo, namely a dependence on maternal mRNA inherited in the oocyte until a species specific stage when control shifts to transcripts from the embryonic genome. This change in control of protein synthesis can be shown to be associated with a qualitative change in the electrophoretic pattern of proteins synthesised during early cleavage, which is sensitive to transcriptional inhibition. Such a change has been demonstrated in the human to occur between the 4 and 8–cell stages of embryonic development (Braude, Bolton et al. 1988). Incorporation of radioactive uridine into nucleoli, indicating mRNA synthesis, has also been detected in human embryos at the 4-cell stage (Tesarik, Kopecny et al. 1986). Both of these studies therefore strongly indicate that in the human embryo the genome activates between the 4 and 8–cell stages.

These studies have demonstrated also that gene activation can be investigated either by autoradiographic assessment of the incorporation of radioactive uridine into nucleoli, or by qualitative analysis of the pattern of protein synthesis as obtained using one dimensional polyacrylamide gel electrophoresis.

As the analysis of protein patterns has shown itself to be a robust, reliable and reproducible technique, and the technology and expertise was readily available in our laboratory, this was the method chosen to examine the relationship between gene activity and cleavage arrest.

Before an examination of cleavage arrested embryos could be undertaken it was necessary to confirm previous findings in order to define, using current equipment, which proteins or groups of proteins could be used as an indicator of gene activity.

To do this the protein synthetic patterns from oocytes and non–arrested embryos were examined at various stages of development. As discussed in the previous chapter (2.8.2), attempts were made to produce gels with tracks of even intensity by assessing the amount of radioactivity incorporated into the samples and the results of these experiments are also presented here.

3.2 Results

Since the two gel systems were analysed by different methods, the results from each are presented separately. Fresh uninseminated oocytes were not available for study, but as previous work had not shown significant differences in the protein synthetic pattern between fresh oocytes and early failed fertilised oocytes (Braude, Bolton et al. 1988), oocytes which had failed to show evidence of fertilization by 24 hpi were analysed in their stead.

3.2.1 Vertical gel system

42 failed fertilised oocytes, 24 of which were less than 48 hours post insemination (88 hours post hCG) and 29 embryos which had cleaved appropriately for their time in culture were analysed using the vertical gel system. Assessment of their morphology by light microscopy showed that 2 of the embryos were at the early 2 cell stage (31 hpi), 13 were between 3 and 5 cells (43-48 hpi), 4 between 6 and 8–cells (72-89 hpi), 5 were morulae (88–90 hpi), and 5 had reached the blastocyst stage (89-120 hpi). DAPI

staining was also used to assess the number of nuclear structures in 13 of the embryos. This showed that 8 had appropriate nuclear to cytoplasmic ratios, 4 appeared to have fewer nuclei than cells and 1 appeared to have more.

Seven protein bands (A-G) (Fig 6) were identified by visual analysis as showing major changes in intensity during the development from oocyte to blastocyst. The relative density of each of these bands is shown diagrammatically in fig 7 for each individual oocyte or embryo analysed. Band A (37kD) was present in the majority of failed fertilised eggs and in embryos up until the 2–cell stage. A band in this position was also present in some later stage embryos. Band B (41kD) was usually strongly present in the oocyte and early cleavage stages, but appeared to weaken in intensity after the 2–cell stage. Bands C and F, (44kD and 69kD) were seen predominantly from fertilisation onwards and were usually absent or present only inconsistently in unfertilised oocytes. Bands D, E and G (46, 51 and 74 kD respectively) were strongly present in most embryos from the 4–cell stage onwards although they too were present in some failed fertilised oocytes and embryos prior to this stage.

The protein synthetic pattern of the 17 oocytes analysed at times greater than 48 hours after attempted fertilisation is shown in Fig 8. In general they showed a consistent strong presence of bands A and B, although 9, mainly those at later times, showed the presence of either or both of bands E and F.

3.2.2 Horizontal gel system

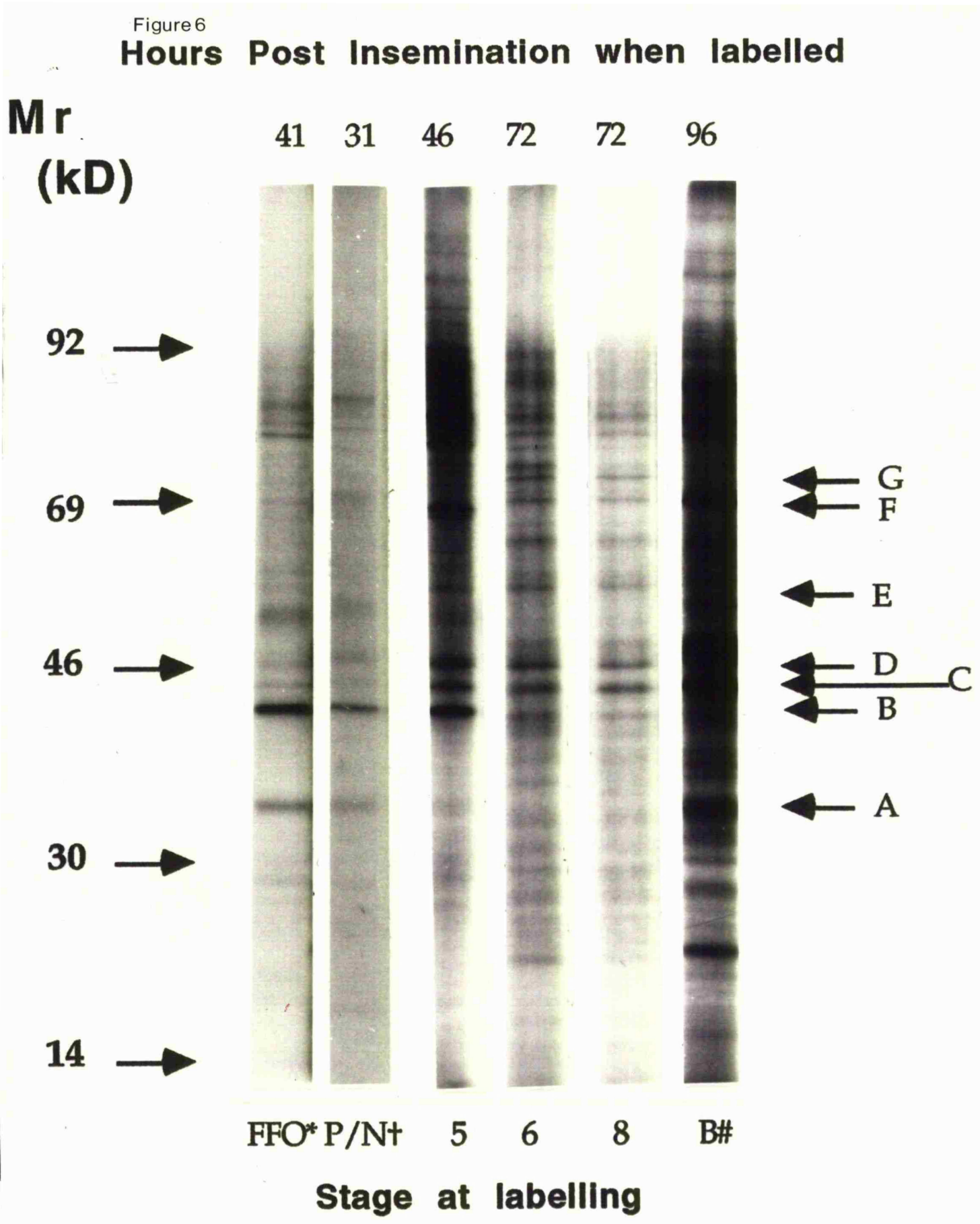
13 failed fertilized eggs were analysed of which 11 were less than 48 hpi (88 hours post HCG). Twenty embryos which had cleaved appropriately for their time in culture were also analysed using this system; 8 of these had either 3 or 4 cells (up to 48 hpi) and 6 had either 7 or 8–cells (69–93 hpi).

Figure 6 overleaf

Figure 6

Composite autoradiogram of S^{35} methionine labelled polypeptides from embryos at the pronucleate stage (P/N, 31 hpi), 5 cell (46 hpi), 6 cell (72 hpi), 8 cell (72 hpi) and blastocyst (B, 96 hpi), separated by one dimensional PAGE. The pattern of a failed fertilised oocyte (FFO) labelled at 41 hpi is shown in lane 1. Approximate molecular weights derived from C^{14} labelled marker proteins run with each gel are shown on the left hand side. The bands felt to show major changes during development are marked A to G.

Figure 6



* Failed fertilised oocyte

† Pronucleate stage pre-embryo

\# Blastocyst

Figure 7 overleaf

Figure 7

Pictorial representation of protein bands A-G (Fig 7) from individual normal embryos, and failed fertilised oocytes prior to 48 hpi. Each column represents the data obtained from the autoradiogram from a single oocyte or embryo between 2–cells (2c) and blastocyst (B) The number of cells, and the number of nuclei seen by fluorescence microscopy under U.V. illumination after DAPI staining is noted for each embryo or oocyte.

Figure 7

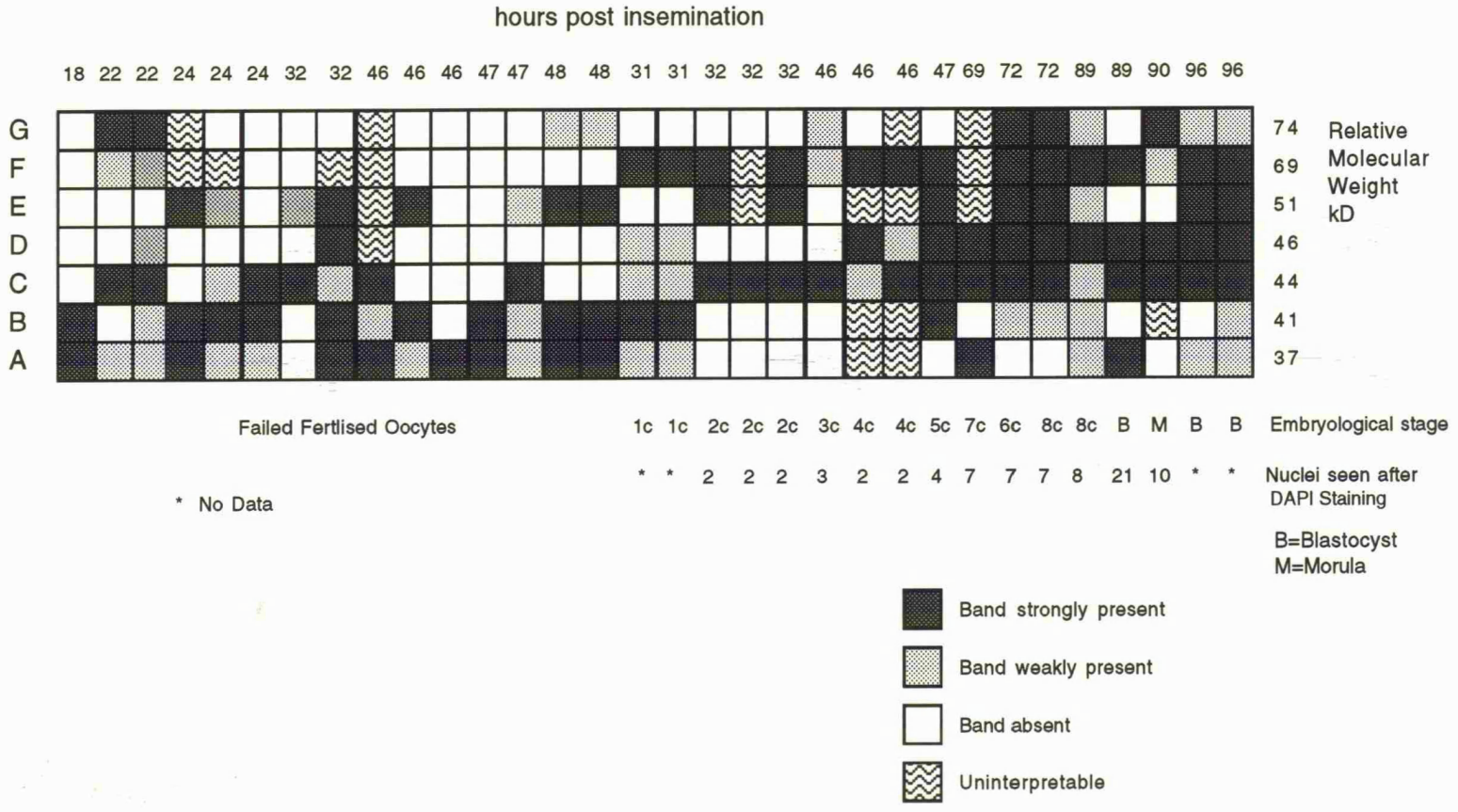
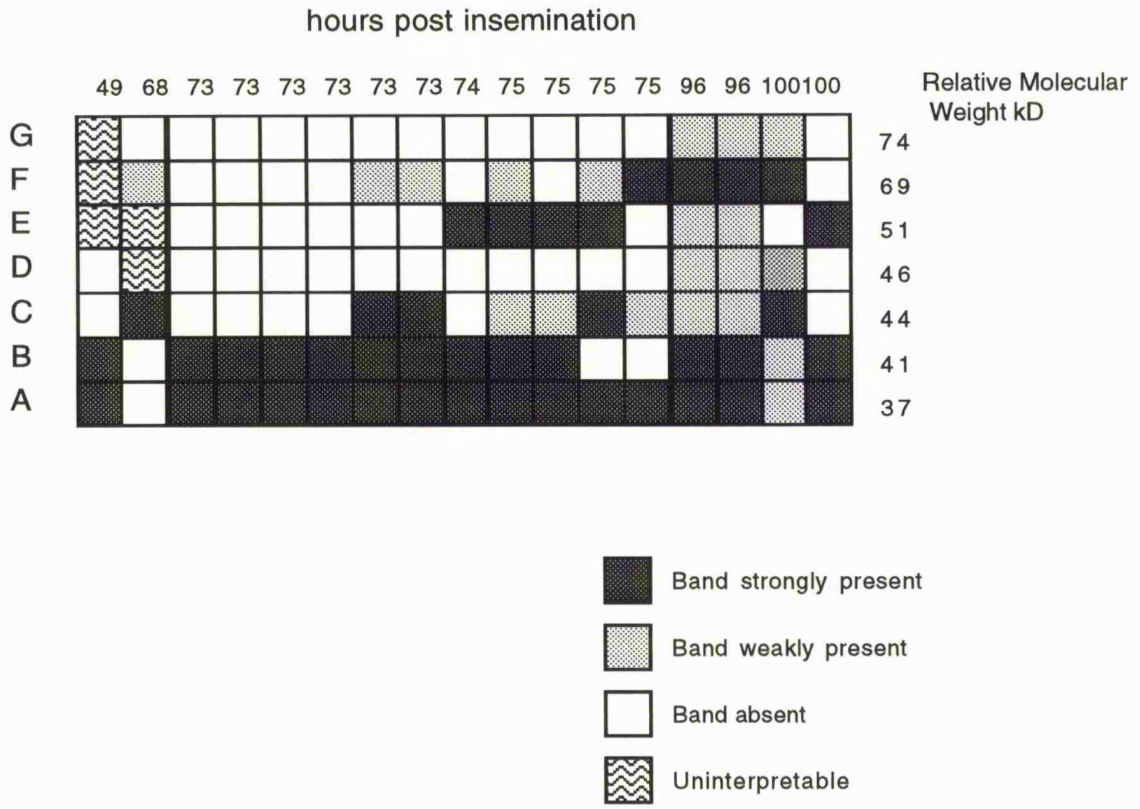

hours post insemination
18 22 22 24 24 24 32 32 46 46 46 47 47 48 48 31 31 32 32 32 46 46 46 47 69 72 72 89 89 90 96 96
G
F
E
D
C
B
A
74
69
51
46
44
41
37
Relative Molecular Weight kD
Failed Fertlised Oocytes
1c 1c 2c 2c 2c 3c 4c 4c 5c 7c 6c 8c 8c B M B B
Embryological stage
* * 2 2 2 3 2 2 4 7 7 7 8 21 10 * *
Nuclei seen after DAPI Staining
* No Data
B=Blastocyst
M=Morula
Band strongly present
Band weakly present
Band absent
Uninterpretable

Figure 8 overleaf

Figure 8

Pictorial representation of protein bands A-G (Fig 7) from individual failed fertilised oocytes in culture for greater than 48 hpi. Each column represents the data obtained from a single oocyte, as for Fig 7

Figure 8


hours post insemination
49 68 73 73 73 73 73 73 74 75 75 75 75 96 96 100100
Relative Molecular Weight kD
G
F
E
D
C
B
A
74
69
51
46
44
41
37
Band strongly present
Band weakly present
Band absent
Uninterpretable

There were 3 morulae (88 hpi), and 3 blastocysts (89-120 hpi). Fig 9 shows a typical autoradiogram from the horizontal gel system. As can be seen the running distance is short which results in dense packing of the bands, although the bands tend to be very tightly focussed. These autoradiograms were analysed using a scanning densitometer, which enabled identification of 10 protein bands which showed major changes in intensity during the development from oocyte to blastocyst. The relative density of each of these bands is shown diagrammatically for each individual oocyte or embryo analysed (see Fig 10). Five bands at relative molecular weights of 14, 14.5, 19.5, 35.5 and 56.5kD (group I proteins) are present in the majority of failed fertilised eggs and in embryos up until the four cell stage, although bands in these positions are also present occasionally in later stage embryos. From the four cell stage onwards 5 bands at 20, 27.5, 37, 40 and 69 kD (group II proteins) are seen more commonly, although some of these are also seen occasionally in failed fertilised oocytes and embryos prior to this stage.

3.2.3 Measurement of incorporated radioactivity

Interpretation of the changes in intensity of protein bands on autoradiograms is complicated by the variation in density of the tracks. To assist with interpretation an attempt was made to load samples of equivalent radioactivity by first counting the amount of radioactivity present in a small aliquot and adjusting the amount of the sample loaded proportionally.

The amount of radioactivity incorporated into the protein fraction of 66 samples was assessed. These samples were analysed on 3 gels using the vertical system. The values obtained for the amount of radioactivity incorporated showed a large variation, ranging from 4 to 4502 cpm. This

Figure 9 overleaf

Figure 9

Photograph of an autoradiogram obtained from the horizontal gel system. The 10 discriminatory bands are indicated with their respective molecular weights. Standard molecular weight markers are included in the left hand column for reference. The number of cells as assessed by light microscopy is noted for each embryo as well as the number of hours that had elapsed since insemination until the time of radioactive labelling.

Figure 9

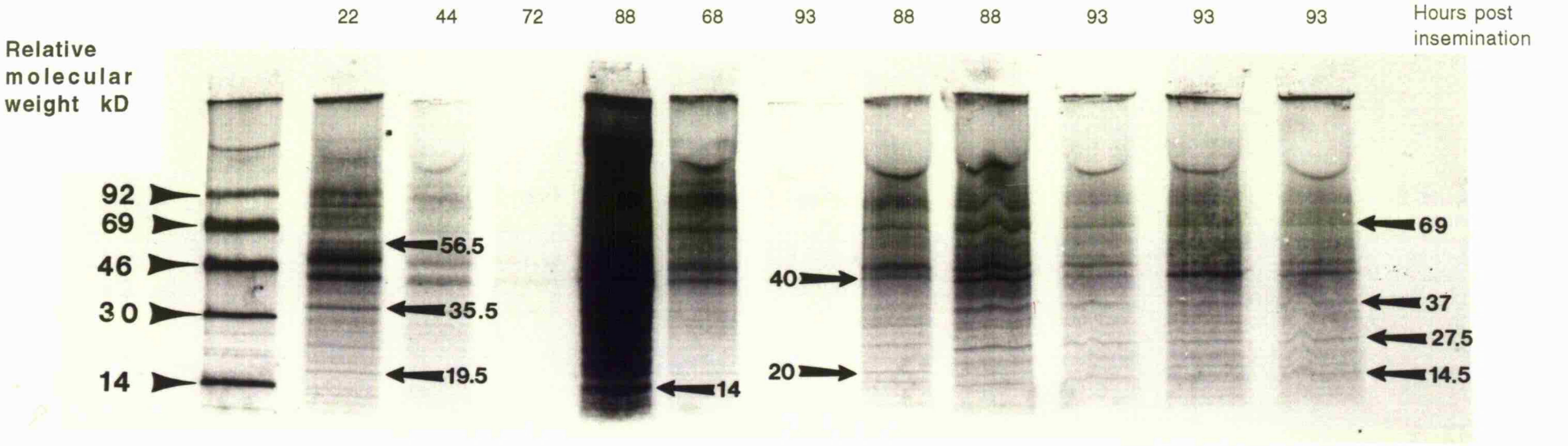

22
44
72
88
68
93
88
88
93
93
93
Hours post insemination
Relative molecular weight kD
92
69
46
30
14
56.5
35.5
19.5
14
40
20
69
37
27.5
14.5
FFO
FFO
FFO
3c
3c
4c
5c
6c
8c
8c
7c
Morphology at time of labelling
FFO = Failed fertilised oocyte

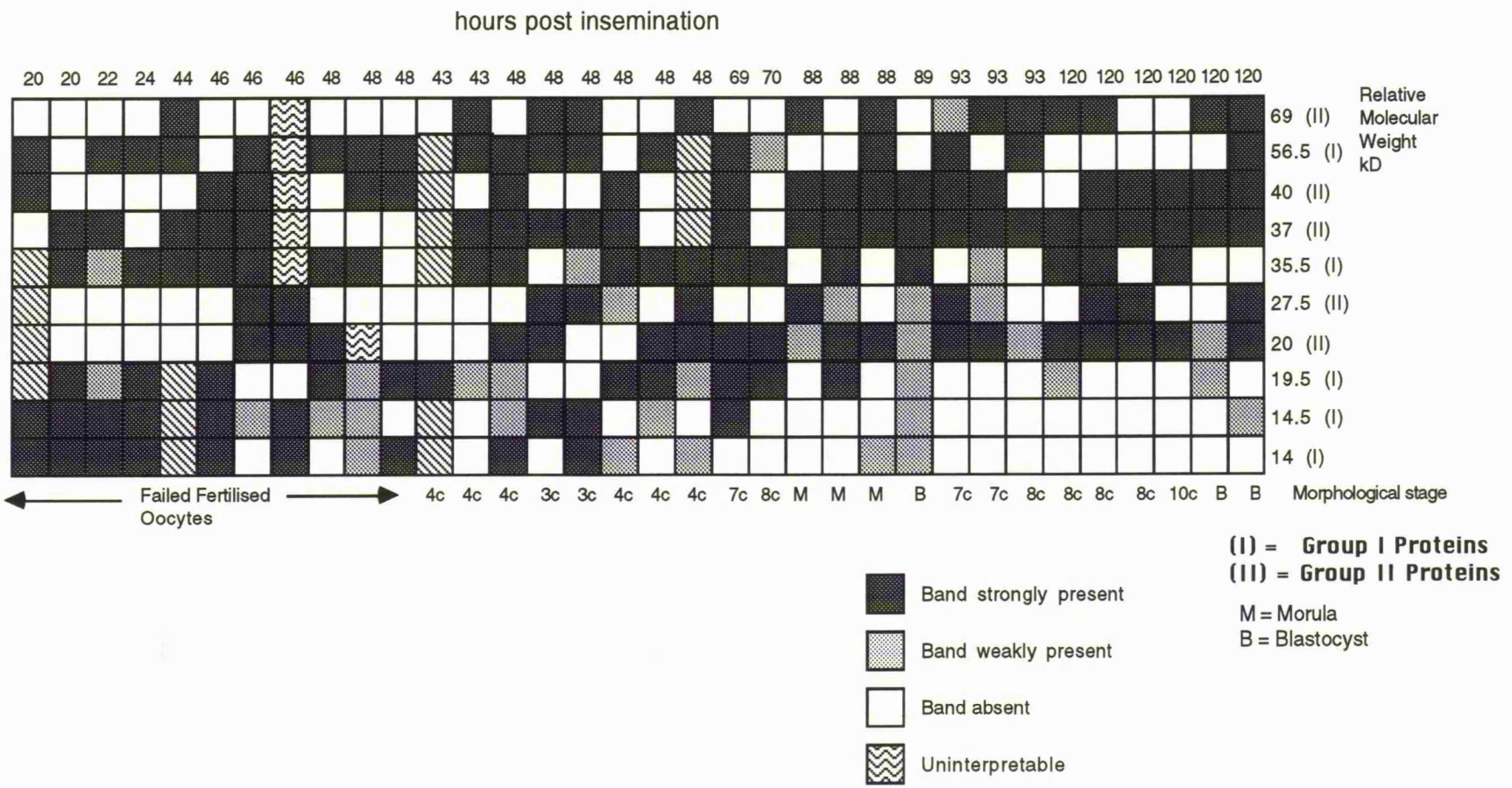
Figure 10 overleaf

Figure 10

Pictorial representation of the density of 10 protein bands analysed using the horizontal gel system and scanning densitometry, 5 bands tended to be present at early stages of development (group I : 14, 14.5, 19.5, 35.5 and 56.5 kD), and 5 tended to be associated with later stages (group II : 20, 27.5, 37, 40 and 69 kD) from individual normal embryos, and failed fertilised oocytes prior to 48 hours after insemination.

As in Fig 7, each column represents the data obtained from the autoradiogram from a single oocyte or embryo. The number of cells as assessed by light microscopy is noted for each embryo as well as the number of hours that had elapsed since insemination until the time of radioactive labelling.

Figure 10


hours post insemination
20 20 22 24 44 46 46 46 48 48 48 43 43 48 48 48 48 48 48 69 70 88 88 88 89 93 93 93 120 120 120 120 120 120
69 (II)
56.5 (I)
40 (II)
37 (II)
35.5 (I)
27.5 (II)
20 (II)
19.5 (I)
14.5 (I)
14 (I)
Relative Molecular Weight kD
Failed Fertilised Oocytes
4c 4c 4c 3c 3c 4c 4c 4c 7c 8c M M M B 7c 7c 8c 8c 8c 8c 10c B B
Morphological stage
Band strongly present
Band weakly present
Band absent
Uninterpretable
(I) = Group I Proteins
(II) = Group II Proteins
M = Morula
B = Blastocyst

degree of variation did not allow adjustment of the volume of sample loaded to give equal activity for each track. Therefore a level of 100 cpm was chosen arbitrarily as a threshold level for equivalent loading as this made subsequent calculations easier. Below this value the whole sample was loaded onto the gel.

Fig 11 shows the results of this technique for one of the three gels in which it was used. It can be seen that there was still a large discrepancy between the density of the tracks despite adjusting of the amount of sample loaded. Furthermore the results were not consistent in so far as the tracks that appeared to be weak on the autoradiogram were not always from those samples that had given the lowest values for incorporation of radioactivity. When these autoradiograms were compared to other gels where no attempt was made to assess the incorporated radioactivity (see Figs 12 or 18) it was found that adjusting the amounts loaded resulted in autoradiograms of poorer quality and so this method was abandoned.

3.3 Interpretation of Results

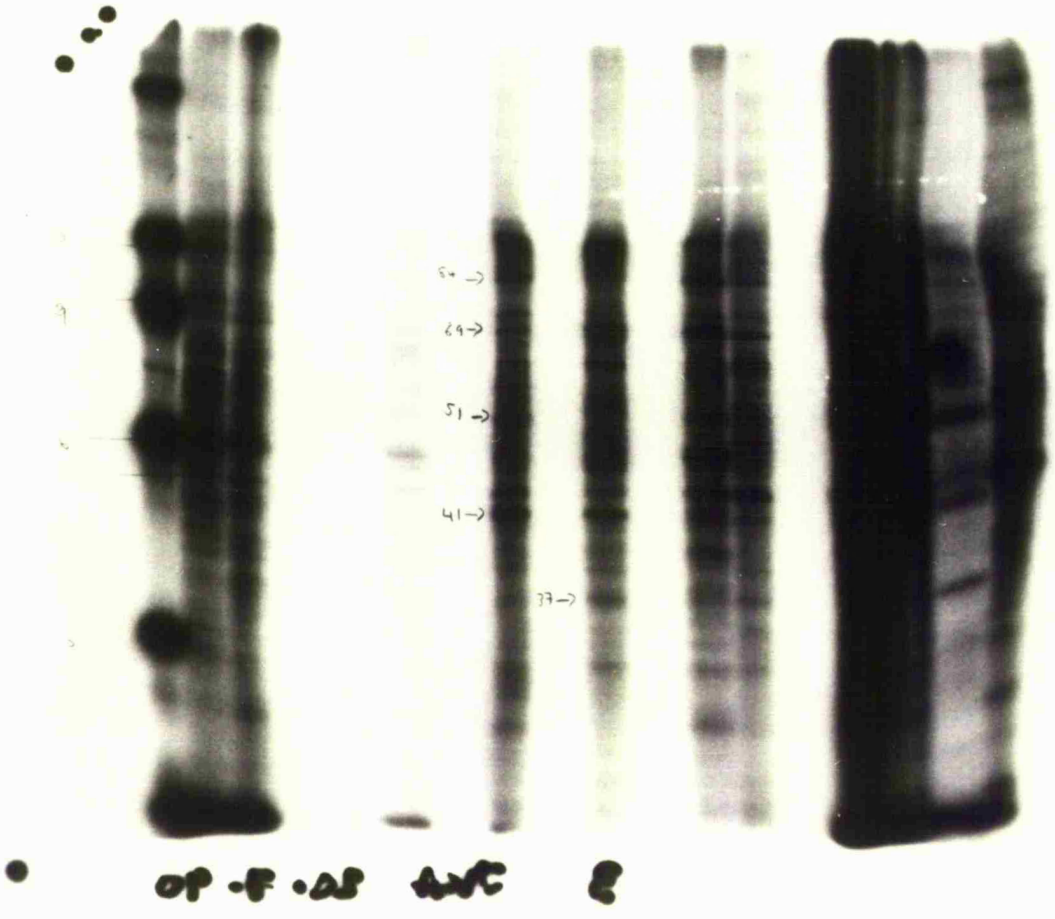
To our knowledge this was the first attempt to use the newer short running distance, horizontal gel system as opposed to the larger, more traditional, vertical gel system, for this particular type of protein analysis. The aim of using the horizontal system was to use the short running distance to expedite analyses and also to use the mass produced pre–packed gels to provide uniform conditions, which might have allowed better comparison of results between gels. However, although it seemed possible to obtain reproducible results from the horizontal system when the autoradiographs were analysed using the scanning densitometer the extra workload this analysis involved obviated the advantage of the faster running time

Figure 11 overleaf

Figure 11

Photograph of an autoradiogram obtained from a gel on the vertical gel system where the amount of sample loaded onto the gel had been adjusted using the value obtained for radioactive incorporation in an attempt to produce tracks of equal intensity.

Figure 11



The use of the two systems was further complicated by the fact that, for a number of reasons, comparison of the results from the two systems was difficult. Firstly, the estimated molecular weights of the proteins from gel electrophoresis can only be approximate. This was particularly so in the horizontal system where the shorter running distance meant that a migrated distance of 1mm on the gel was equivalent to a change of 5 kD in the estimated molecular weight of the protein band. Thus, a band estimated at 50kD on one system could not be said to be equivalent to a band at 50kD on the other system. Secondly, 7 of the 10 discriminatory bands in the horizontal system had relative molecular weights of less than 37kD, in contrast to the vertical system where this was the lowest molecular weight protein used as part of the analysis. This was probably the result of the superior resolution over a broader range of molecular weights allowed by the use of the pre–cast exponential gels in the horizontal system. The amount of activity found in these low molecular weight proteins using the horizontal gel system might, however, question what has previously been a basic assumption about protein synthesis in the human embryo, namely that the changes in protein synthesis in the human embryo take place over the same range of protein weights as in the mouse.

The inability to compare results from the two systems meant that in order to gain the maximum amount of consistent data a choice had to be made to use only one system. Thus, although a number of normal and cleavage arrested embryos were analysed using both systems, the fact that in practice the use of the horizontal gel system did not result in any savings in time and the inability to compare the results from this system with data from previous studies eventually lead to the decision to abandon the horizontal system and persist with the more traditional vertical system.

The results of the attempts to use quantitative assessment of incorporated

radioactivity as an aid to visual interpretation of the vertical gels were disappointing. However similar difficulties have been described before (Braude 1979) where it was suggested that the discrepancy between the activity of the loaded sample and the track density might have arisen due to radioactivity being incorporated into proteins that were outside the range of the gel such as histones. Furthermore the wide range in activity between samples has also been found in studies in the mouse (Goddard and Pratt 1983) and sheep (Crosby, Gandolfi et al. 1988) where radioactive uptake was found to be very poor as the stage of transition from maternal to embryonic control was reached.

Allowing for small differences in interpretation of relative molecular weight, the changes in pattern observed here using the vertical gel system are directly comparable to the original paper by Braude et al (1988). Bands A and B corresponding directly to the same bands in the original paper, bands D and G here probably corresponding to bands C and E in the original paper. A further band at 44kD (band C) is also described here. This is present at most stages but shows a decline in intensity from the four cell stage onwards, in parallel with the increase in intensity of band D. Thus it might be concluded that bands B, C and D could be related and their variation in density the result of post-translational modification. The possibility of post–translational changes in intensity of proteins is supported by data from the mouse where similar changes have also been described (Van Blerkom 1981; Pratt, Bolton et al. 1983).

The difficulties in establishing what constitutes a 'normal' embryo and hence a 'normal' protein synthetic pattern have already been discussed The increased tendency for embryos to arrest or develop aberrant cleavage in vitro between the 4 and 8-cell stages (Bolton, Hawes et al. 1988; Hardy, Handyside et al. 1989) and the fact that all the embryos analysed were

surplus to therapeutic requirements once the "best" 2 or 3 of the cohort had been returned to the patient might suggest that those analysed here were of a poorer quality. Similarly the use of failed–fertilised oocytes to examine unfertilised oocyte patterns may be less than ideal since their failure to fertilise may have been due to inherent oocyte abnormalities and not only to sperm related events. However the use of embryos with appropriate cell numbers for their time in culture, with the majority also having appropriate nuclear to cytoplasmic ratios, and the analysis of a relatively large number of embryos and oocytes from different patients might be expected to overcome these difficulties and enable trends to be discerned in the protein synthetic pattern during early cleavage.

The marked changes demonstrated in the protein synthetic patterns following the passage through the third cleavage division when analysed using the horizontal gel system or the vertical gel system and the appearance of several new protein bands associated with the loss of others at the same stage when analysed using the vertical gel system would suggest that some major change in protein synthesis is taking place at or around this stage which accords with previous data (Braude, Bolton et al. 1988; Tesarik, Kopecny et al. 1988).

The nature and significance of these changes required further elucidation.

Chapter Four

Determination of the significance of the changes in protein synthetic pattern associated with non–arrested embryos

4.1 Introduction

Qualitative analysis of the protein synthetic patterns of non–arrested human embryos had shown a number of defined changes associated with development from the unfertilised oocyte to the blastocyst stage.

There are a number of possible causes for these changes. Studies of the mouse have shown that mRNA may be inherited from the oocyte in an inactive, masked form (Braude, Pelham et al. 1979) to be activated at later stages in development with a consequent change in the protein synthetic pattern. It has also been demonstrated in the mouse that changes in protein synthetic pattern can arise as a result of changes at the post–translational level with activation of proteins by processes such as phosphorylation (Goddard and Pratt 1983). Alternatively these changes could be associated with the onset of embryonic gene activity and the switch from utilization of maternal mRNAs to embryonically coded mRNAs.

To determine which if any of the changes observed in the protein synthetic pattern of the non–arrested embryos were transcriptionally dependent, a series of experiments were carried out to analyse the protein synthetic patterns of morphologically normal embryos after exposure to the transcriptional inhibitor α–amanitin.

4.2 Results

Ten apparently normally developing embryos at stages ranging from the late pronucleate stage (31 hpi) to early four cell stage (48 hpi) were exposed to α-amanitin and then analysed on the vertical gel system. These were analysed over 9 gels with all results being consistent. Fig 12 is an autoradiogram using the vertical gel system in which ^{35}S–methionine labelled proteins synthesised by 5 embryos after exposure to α-amanitin can be compared with those synthesised by untreated embryos at similar stages. It can be seen that apart from the disappearance of band A, the pattern of the pronucleate stage embryo exposed to α-amanitin (b) is the same as that seen in untreated embryos labelled at the early 2–cell stage stage (a & c). The expected change in intensity of bands B and D and the appearance of bands E and G, seen in the untreated embryos (d & e) between the 4 and 8-cell stages, is suppressed in the embryos which were exposed to α-amanitin at the early 4–cell stage (f to i), despite their continued cleavage to the 8-cell stage. The pattern in the embryos exposed to α-amanitin at the 4–cell stage is similar to that seen in an early 2-cell embryo (a, c).

13 apparently normally developing embryos at stages ranging from the late 2–cell stage (45 hpi) to early four cell stage (48 hpi) were exposed to α-amanitin and then analysed on the horizontal gel system. These were analysed on 4 gels with the autioradiograms being analysed using the scanning densitometer. The results are shown in diagrammatic format in Fig 13. All of these embryos showed strong synthesis of at least one of the group II (putatively transcriptionally dependent) proteins. However no particular protein seemed to be synthesised more commonly and no consistent pattern could be discerned.

Figure 12 overleaf

Figure 12

Autoradiogram of one dimensionally separated ^{35}S-methionine labelled polypeptides from embryos at the 4-cell stage (47 hpi lane b), and 8-cell stages (72 hpi, lanes f-i), after culture in the presence of α-amanitin (100 μg/ml) from the late pronucleate stage (31 hpi, b) and early 4 cell stage (48hpi f-i). Untreated embryos labelled at the early 2-cell stage (31 hpi lanes a and c), 7 cell stage (72 hpi, lane d) and 8 cell stage (72 hpi, lane e), are shown for comparison. Approximate molecular weights derived from ^{14}C labelled marker proteins run with each gel are shown on the left hand side.

Figure 12

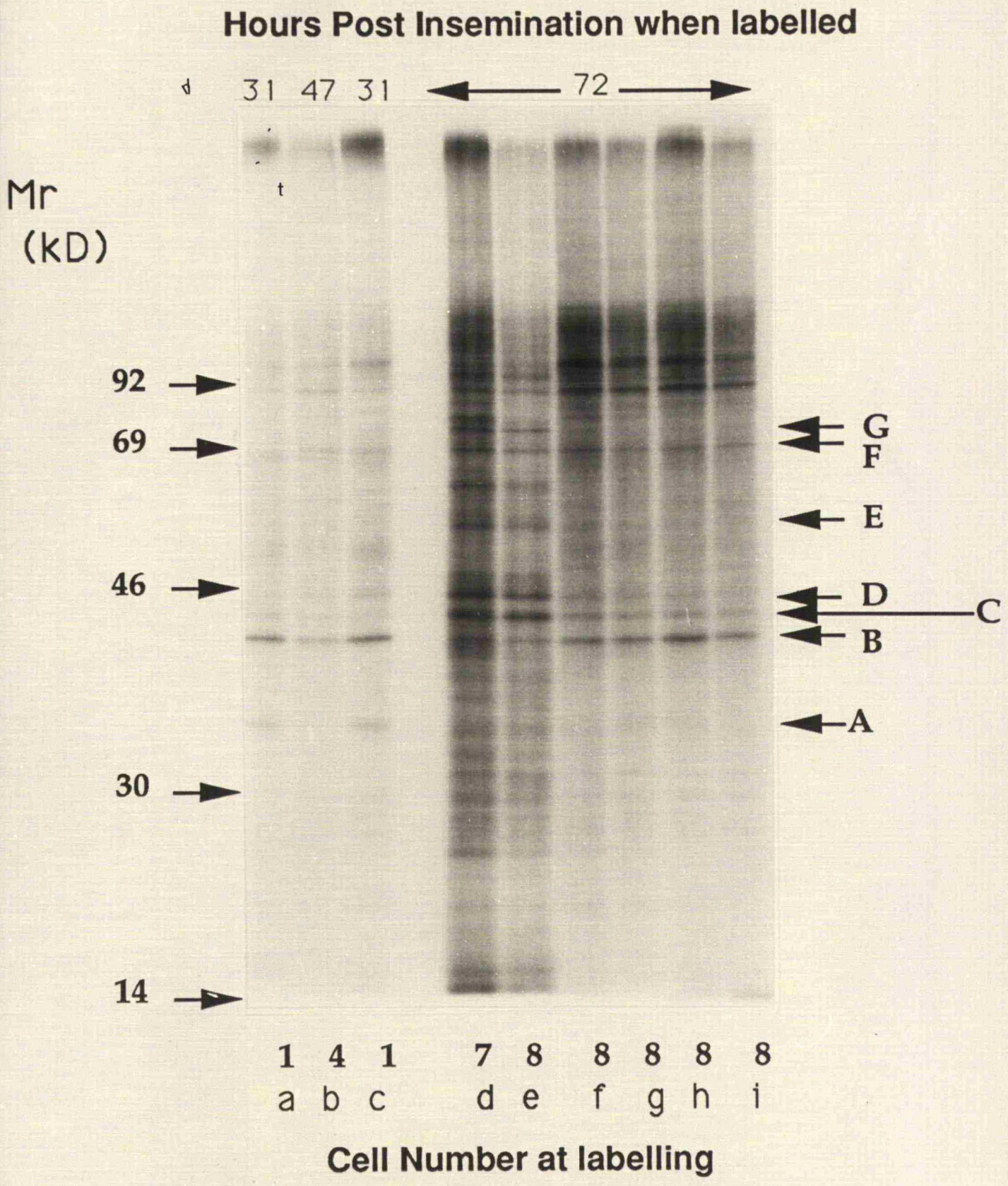
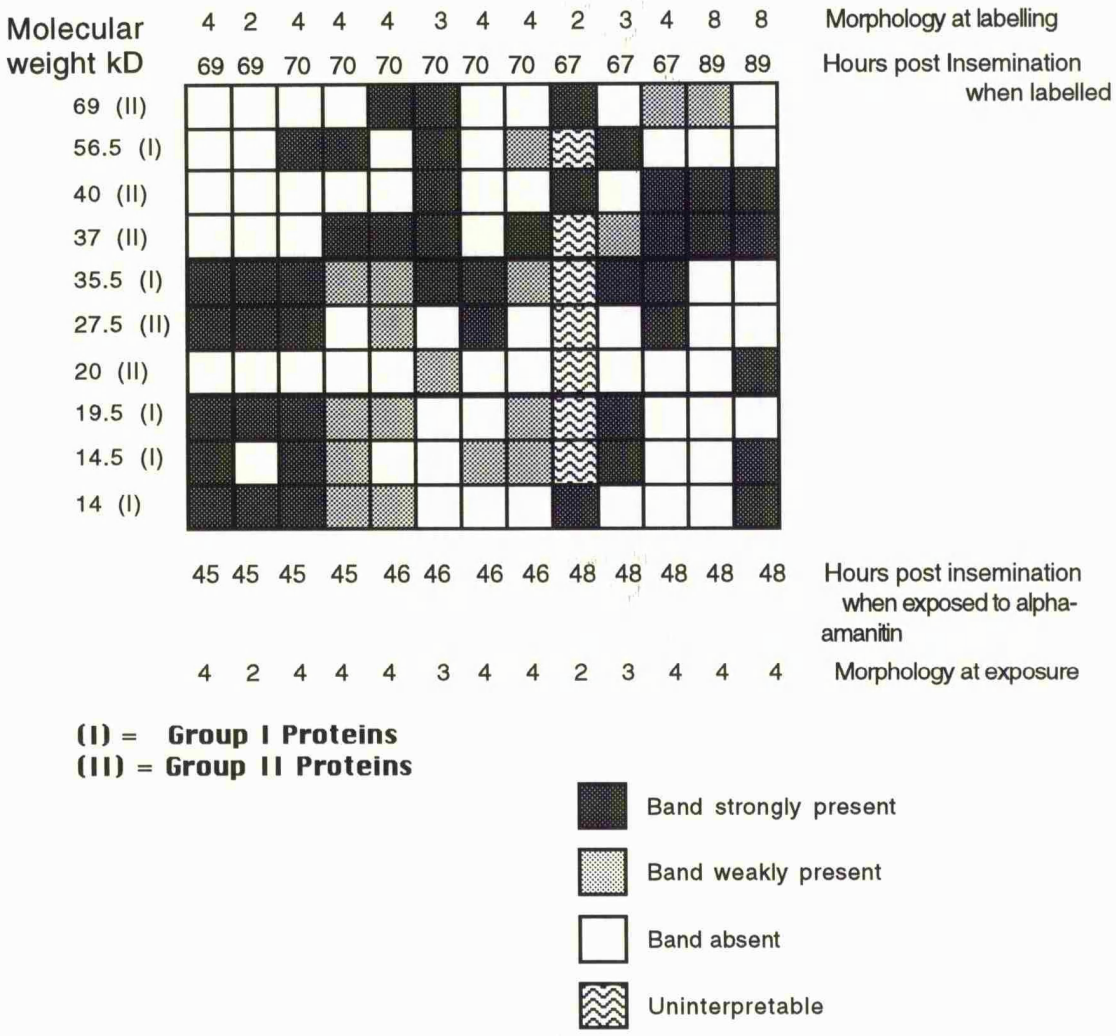

Hours Post Insemination when labelled
31 47 31
72
Mr (kD)
92
69
46
30
14
G
F
E
D
C
B
A
1 4 1 7 8 8 8 8 8
a b c d e f g h i
Cell Number at labelling

Figure 13 overleaf

Figure 13

Pictorial representation of the density of the ten protein bands associated with the early and late stages of embryo development as assessed by scanning densitometry from 13 individual embryos analysed using the horizontal gel system after addition of α-amanitin (100 μg/ml) to the culture medium between the late 2 cell stage (45 hpi) and early 4 cell stage (48 hpi).

Figure 13


Molecular weight kD
4 2 4 4 4 3 4 4 2 3 4 8 8
Morphology at labelling
69 69 70 70 70 70 70 70 67 67 67 89 89
Hours post Insemination when labelled
69 (II)
56.5 (I)
40 (II)
37 (II)
35.5 (I)
27.5 (II)
20 (II)
19.5 (I)
14.5 (I)
14 (I)
45 45 45 45 46 46 46 46 48 48 48 48 48
Hours post insemination when exposed to alpha-amanitin
4 2 4 4 4 3 4 4 2 3 4 4 4
Morphology at exposure
(I) = Group I Proteins
(II) = Group II Proteins
Band strongly present
Band weakly present
Band absent
Uninterpretable

4.3 Interpretation of results

The degree to which proteins synthesised during early cleavage are dependent on transcription can only be demonstrated conclusively by an examination of the patterns of synthesis in embryos enucleated shortly after fertilisation, or created from enucleated oocytes fertilised by sperm which have previously been irradiated to destroy or inactivate the paternal genetic complement (Briggs, Green et al. 1951). Because of the technical difficulties of these manoeuvres in small mammalian embryos, alternative strategies using transcriptional inhibitors to achieve chemical enucleation have been employed.

Although Lindell's (Lindell, Weinburg et al. 1970) original work on the mechanism of action of α–amanitin was based on it's action on isolated nuclear preparations and not on intact cell systems such as embryos at low concentrations, the consistency of the effects on cleavage and protein synthesis in other species (Flach, Johnson et al. 1982; Sakkas, Batt et al. 1989; Crosby, Gandolfi et al. 1988; Frei, Schultz et al. 1989; Davis 1985) would suggest that α–amanitin treatment could be used to test the transcriptional dependence of protein synthesis and cleavage in the human.

General comparison of the protein synthetic patterns obtained from the α–amanitin treated embryos with those obtained from untreated controls reveals two fundamental features. Firstly, many of the same protein bands are seen both in the treated and untreated embryos. Secondly, a number of bands which were found to be associated with embryos at later stages of development failed to appear in later stage embryos treated with α–amanitin.

Examining these in turn. The persistence of bands despite α–amanitin treatment might be a result of α–amanitin not entering the cell in

sufficient quantity to exert its full effect. Unfortunately there are no established techniques to allow the intra–cellular concentration of α–amanitin to be assessed. However failure of α–amanitin to enter the cells would seem unlikely since the concentration used was well in excess of that shown to be effective in other mammalian species (Flach, Johnson et al. 1982; Crosby, Gandolfi et al. 1988). Furthermore the ability of α–amanitin treatment to suppress the appearance of some protein bands in later stage embryos is consistent with α–amanitin being present in the cells to exert an effect.

Thus it must be concluded that the persistence of protein bands following α–amanitin treatment reflects continuing protein synthesis which is independent of transcription. The most likely source of this would be translation from a maternal store of mRNA. Although it has not been proven in the human that such a store exists, there is evidence from the mouse and other lower order species that embryos inherit such a store which is able to direct and control cleavage and protein synthesis until the embryonic genome becomes active. Alternatively, some of the continued protein synthesis could derive from embryonic mRNA synthesised before the embryos were exposed to α–amanitin. For reasons discussed earlier, 'normal' early cleavage stage human embryos are difficult to obtain since embryos could only be regarded as surplus to therapeutic requirements after embryo transfer took place at 48 hpi. The few analysed here at stages earlier than this came from oocytes surplus to GIFT procedures which were fertilised in vitro as a diagnostic test of fertilising capability. However Braude et al (Braude, Bolton et al. 1988) found no difference between the protein synthetic patterns of oocytes which had been fertilised after exposure to α–amanitin when compared to those of early (up to 2–cell) embryos. This is also consistent with the findings here, with no significant difference between the protein synthetic patterns of treated and untreated

embryos at early stages (tracks a, b and c Fig 12). Thus it could be concluded that those bands that persist after α–amanitin treatment derive from maternal transcripts. Furthermore, although it might be that certain essential proteins, originally synthesised using maternal mRNA, continue to be synthesised on zygotic transcripts following gene activation, the maternal transcripts must be relatively long lived, since many of the bands persist into the late 8–cell stage (72 hpi) even following α–amanitin treatment.

The absence of some bands following treatment might have resulted from a non–specific effect on protein synthesis, particularly given the high concentration of α–amanitin used. However, the absence of any effect on the protein synthetic pattern in early cleavage stage embryos and the fact that α–amanitin has been used at this concentration in other species such as the rabbit (Van Blerkom and Manes 1974), with no suggestion of a non–specific action, would mitigate against this. It could be concluded therefore that the synthesis of those bands which failed to appear after α–amanitin exposure namely bands D, E & G was transcriptionally related. This therefore allowed the patterns of synthesis to be divided into a pre-transcriptional "early" pattern (a-c and f-i Fig 12) and a post–transcriptional "late" pattern (d and e, Fig 12).

In summary, the response to α-amanitin exposure of the proteins which constituted bands D, E and G, on the vertical system had been sufficiently consistent to conclude that their synthesis was dependent upon the process of transcription, and therefore that their presence in the protein synthetic pattern could be used as a marker of genome activity. The appearance of these bands between the 4–8 cell stage also confirms previous findings that this is the likely stage when the human genome becomes active.

Having identified the protein markers of genome activity, it was possible

to analyse the protein synthetic patterns from cleavage arrested embryos for evidence of genome activation.

Chapter Five

Protein synthetic patterns in cleavage arrested embryos

5.1 Introduction

The *in vitro* development of the human embryo is characterised by a marked tendency towards the spontaneous arrest of development during the cleavage stage, with only a minority of fertilised oocytes reaching advanced stages of development (Bolton, Hawes et al. 1988; Hardy, Handyside et al. 1989). Most tend to arrest between the 4 and 8–cell cleavage division. Evidence from domestic and laboratory species has implicated inadequate culture conditions as a potential cause of cleavage arrest (Crosby, Gandolfi et al. 1988; Goddard and Pratt 1983) and indeed manipulation of culture conditions has met with some success in reducing the rate of cleavage arrest in some species (Crosby, Gandolfi et al. 1988; Nasr-Esfahani, Johnson et al. 1990). However manipulation of culture conditions for human embryos has been less successful (Sathananthan, Bongso et al. 1990), suggesting that inadequate culture conditions might not be the sole cause of cleavage arrest. Since all mammalian species studied show an increased tendency to arrest at the developmental stage coincident with genome activation, it has been proposed that failure of genome activation might be a significant cause of cleavage arrest. A role for failure of genome activation in cleavage arrest is also supported by the finding that exposure to inhibitors of gene expression such as α–amanitin, induces cleavage arrest at the same stage.

To determine the role of embryonic gene expression in the aetiology of cleavage arrest in human embryos cultured in vitro, the protein synthetic patterns of a number of embryos that had undergone spontaneous cleavage arrest in vitro were examined for evidence of embryonic genome

activity using the previously identified transcriptionally related proteins.

5.2 Results

5.2.1 Vertical gel system

54 cleavage arrested embryos yielded interpretable data. 42 of these were analysed on the third day post insemination (64-75 hpi) and 11 on the fourth day (90-96 hpi). Semi–quantitative visual analysis (see Analysis Chapter 2) revealed that 27 showed little evidence of the transcriptionally related changes described in Chapter 3 (Fig 14) with neither of bands D or E being strongly present and band G only seen strongly in 8. The remaining 27 (Fig 15) showed clear evidence of transcriptionally related changes ; all but 4 of these embryos having reached the 4–cell stage or beyond, 2 having arrested during the second cleavage division (at 3 cells) and 2 failing to cleave. Only 4 of those with late patterns had an appropriate cell to nucleus ratio with 3 embryos having more nuclear structures visible than cells counted but most (15) having less. Two pronucleate stage embryos which had failed to cleave also showed evidence of transcriptionally related changes. In the one of these for which data are available there appeared to be six nuclear structures on DAPI staining.

Of the group which did not show strong synthesis of the transcriptionally related bands D and G, none had progressed beyond the 5 cell stage. They also exhibited abnormal cell to nucleus ratios with only 4 having an appropriate ratio, 3 had more nuclear structures than cells, and 20 had more cells evident than nuclear structures. 10 of the embryos failed to incorporate the DAPI stain.

Figure 14 overleaf

Figure 14

Pictorial representation of protein bands A-G (Fig 7), as analysed using the vertical gel system, from individual cleavage arrested embryos showing weak or absent transcriptionally related protein synthesis. Each column represents the data obtained from a single embryo. The number of cells and the number of nuclei seen by fluorescence microscopy under U.V. illumination after DAPI staining is noted for each embryo.

Figure 14

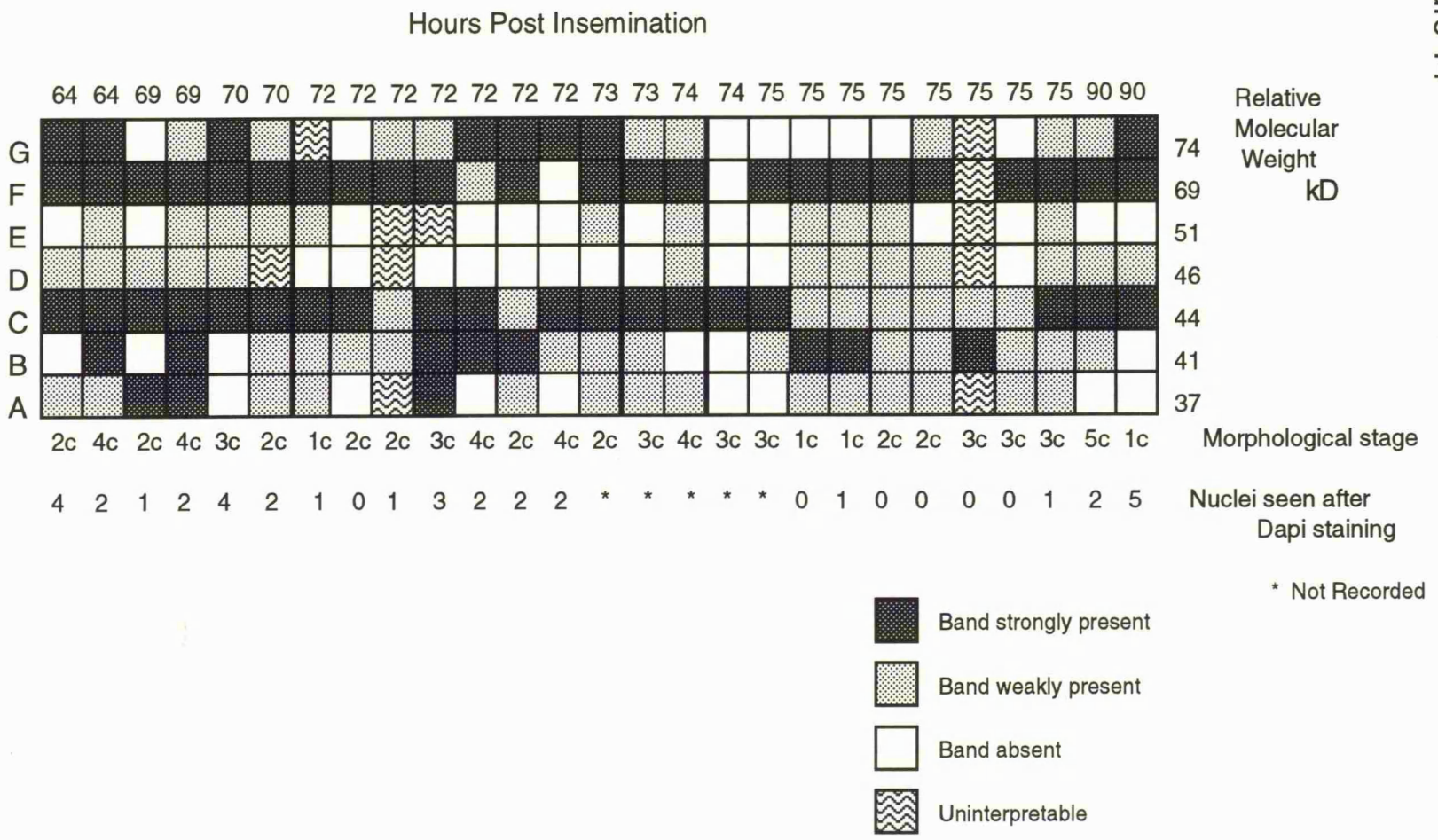
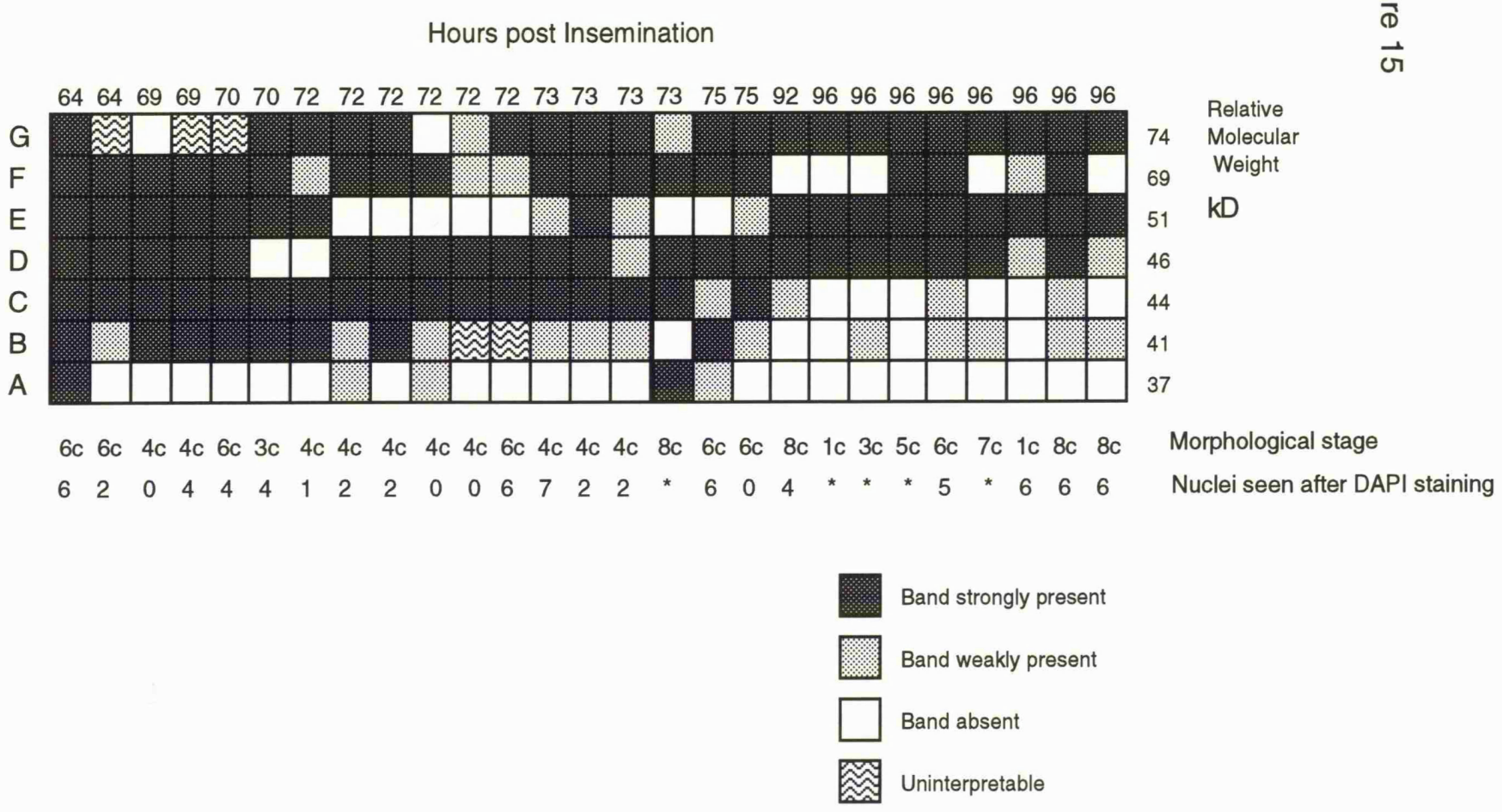

Hours Post Insemination
64 64 69 69 70 70 72 72 72 72 72 72 72 73 73 74 74 75 75 75 75 75 75 75 75 90 90
G
F
E
D
C
B
A
Relative Molecular Weight kD
74
69
51
46
44
41
37
2c 4c 2c 4c 3c 2c 1c 2c 2c 3c 4c 2c 4c 2c 3c 4c 3c 3c 1c 1c 2c 2c 3c 3c 3c 5c 1c
Morphological stage
4 2 1 2 4 2 1 0 1 3 2 2 2 * * * * * 0 1 0 0 0 0 1 2 5
Nuclei seen after Dapi staining
* Not Recorded
Band strongly present
Band weakly present
Band absent
Uninterpretable

Figure 15 overleaf

Figure 15

Pictorial representation of protein bands A-G (Fig 7), as analysed using the vertical gel system, from individual cleavage arrested embryos showing strong evidence of synthesis of transcriptionally related protein synthesis Each column represents the data obtained from a single embryo. The number of cells and the number of nuclei seen by fluorescence microscopy under U.V. illumination after DAPI staining is noted for each embryo.

Figure 15


Hours post Insemination
64 64 69 69 70 70 72 72 72 72 72 72 73 73 73 73 75 75 92 96 96 96 96 96 96 96 96
G
F
E
D
C
B
A
74
69
51
46
44
41
37
Relative Molecular Weight
kD
6c 6c 4c 4c 6c 3c 4c 4c 4c 4c 4c 6c 4c 4c 4c 8c 6c 6c 8c 1c 3c 5c 6c 7c 1c 8c 8c
Morphological stage
6 2 0 4 4 4 1 2 2 0 0 6 7 2 2 * 6 0 4 * * * 5 * 6 6 6
Nuclei seen after DAPI staining
Band strongly present
Band weakly present
Band absent
Uninterpretable

5.2.2 Horizontal gel system

31 embryos produced interpretable data. 14 were analysed on the third day post insemination (67-70 hpi); 12 on the fourth day (74–94 hpi) and 5 on the fifth day (120 hpi). All the embryos analysed using this system showed evidence of synthesis of at least one of the group II proteins. However 13 showed a predominant tendency to synthesise the group I proteins (Fig 16) whilst the remainder, 18, (Fig 17) showed a reduced level of synthesis of these proteins associated with an increased tendency to synthesise the group II proteins.

Morphological assessment of the embryos showed that none had successfully completed the third cleavage division, only 4 having reached the 6–cell stage and the remainder having arrested at the 4-cell stage or earlier. There were no differences in respect of morphology between those embryos predominantly synthesising the group I proteins and those predominantly synthesising the group II proteins, each group having embryos that had reached the 6–cell stage and each with embryos that had failed to cleave.

5.3 Interpretation of results

The results from the vertical gel system suggest that about half of the cleavage arrested embryos studied were capable of synthesising some, but not always all, of the transcriptionally dependent proteins and thus in these cases arrest was unlikely to be the result of a failure of the onset of transcriptional activity. This ability to synthesise transcriptionally dependent proteins did not appear to be linked simply to time in culture, as some arrested embryos in culture for as long as 90 hpi still did not synthesise these proteins.

Analysis of the morphology of the embryos which failed to show evidence

Figure 16 overleaf

Figure 16

Pictorial representation of the ten protein bands (see fig 10) as analysed on the horizontal gel system, from individual cleavage arrested embryos showing a similar pattern of protein synthesis to early cleavage stage embryos. Each column represents the data obtained from a single embryo. The number of cells and the number of nuclei seen by fluorescence microscopy under U.V. illumination after DAPI staining is noted for each embryo.

Figure 16

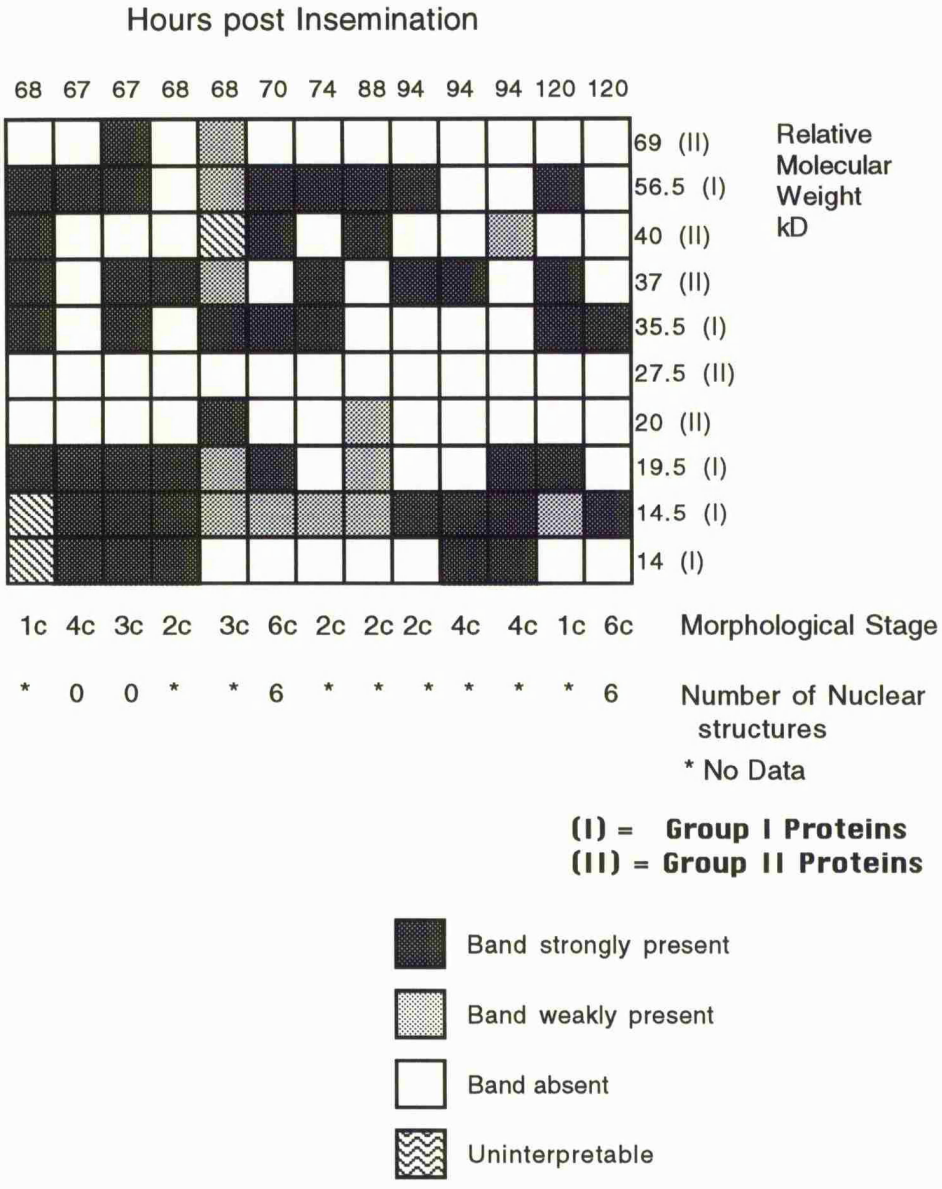
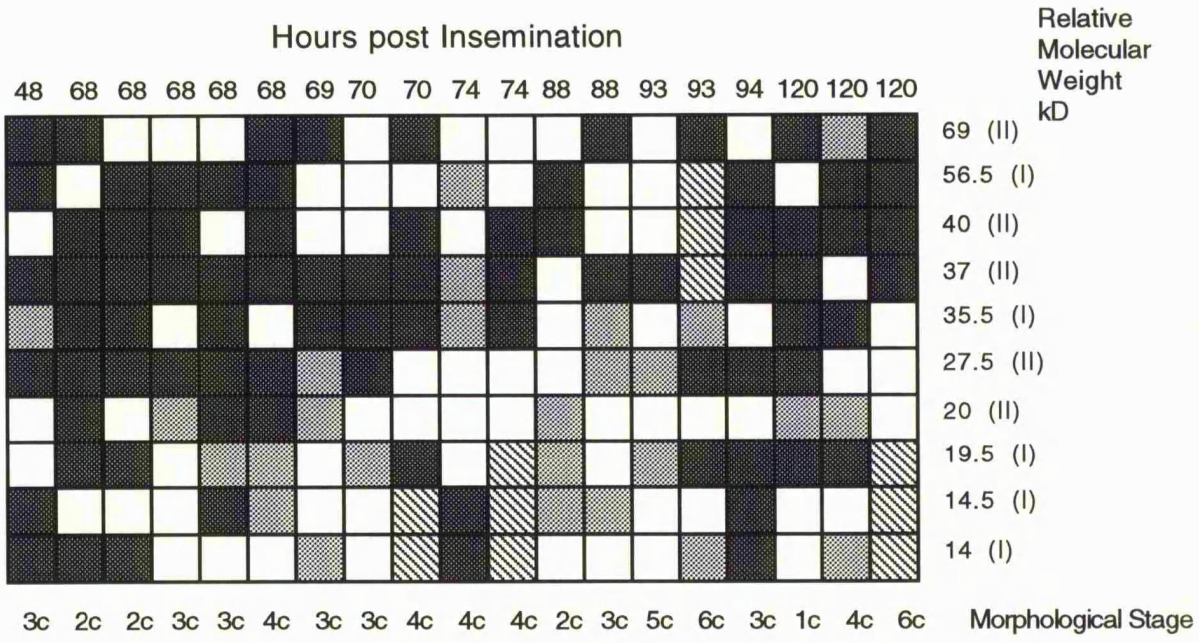

Hours post Insemination
68 67 67 68 68 70 74 88 94 94 94 120 120
69 (II)
56.5 (I)
40 (II)
37 (II)
35.5 (I)
27.5 (II)
20 (II)
19.5 (I)
14.5 (I)
14 (I)
Relative Molecular Weight kD
1c 4c 3c 2c 3c 6c 2c 2c 2c 4c 4c 1c 6c
Morphological Stage
* 0 0 * * 6 * * * * * * 6
Number of Nuclear structures
* No Data
(I) = Group I Proteins
(II) = Group II Proteins
Band strongly present
Band weakly present
Band absent
Uninterpretable

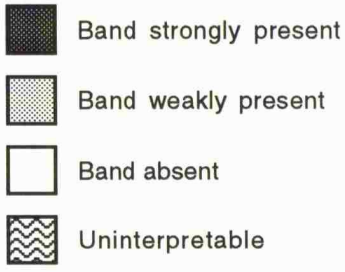
Figure 17 overleaf

Figure 17

Pictorial representation of the ten protein bands (see fig 10) as analysed on the horizontal gel system, from individual cleavage arrested embryos showing strong evidence of transcriptionally related protein synthesis (the group II proteins). Each column represents the data obtained from a single embryo. The number of cells seen by phase contrast microscopy is noted for each embryo.

Figure 17


Hours post Insemination
48 68 68 68 68 68 69 70 70 74 74 88 88 93 93 94 120 120 120
Relative Molecular Weight kD
69 (II)
56.5 (I)
40 (II)
37 (II)
35.5 (I)
27.5 (II)
20 (II)
19.5 (I)
14.5 (I)
14 (I)
3c 2c 2c 3c 3c 4c 3c 3c 4c 4c 4c 2c 3c 5c 6c 3c 1c 4c 6c
Morphological Stage


Band strongly present
Band weakly present
Band absent
Uninterpretable

of synthesis of transcriptionally related proteins shows that none had progressed beyond the second cleavage division. This might suggest that gene expression has a permissive effect on further cleavage, or alternatively that gene expression occurs only once a critical cell number is reached. However the finding that some of the embryos which showed evidence of transcriptionally dependent protein synthesis had not progressed beyond the second cleavage division, some even having failed to cleave at all, would argue against the need for a minimum cell number to be attained before gene expression can occur. Thus it appears unlikely that gene expression is linked simply to cytokinesis.

The presence of multiple nuclear structures, as detected by DAPI staining, in some of the embryos with apparently low cell numbers might suggest that these embryos could have undergone rounds of nuclear replication without cleavage. Thus a minimum nuclear number or DNA content might be required to allow the onset of gene activity. However, there were other embryos in the transcriptionally active group which had only one or two nuclear structures. This would suggest that the attainment of a minimum nuclear number is not necessary before transcriptional activity occurs. However it is possible that the embryos had replicated their DNA but without nuclear division. A formal analysis of the amount of DNA present would be needed in order to determine whether gene expression was linked to DNA content.

The finding of abnormalities of nuclear to cytoplasmic ratio in many of the embryos is consistent with previously described data (Winston, Braude et al. 1991) where it was shown that even apparently morphologically normal embryos can be grossly abnormal, with respect to nucleus to cell ratios. As staining with DAPI and radioactive protein labelling had not been attempted concurrently before, this was not applied to the human

embryos until it had been tested in a model system using mouse embryos (data not shown). Although there was a slight reduction in the amount of radioactivity incorporated, concurrent staining and labelling did not affect the quality of the subsequent autoradiograph. As this system was only used once it had been tested there is no staining data in some of the embryos.

In some embryos, the presence of a nucleus or nuclear material could not be discerned after DAPI staining. Since DAPI efficiently stains histone proteins in the nuclei of all cells, even when the cells are fixed in formalin, the most likely explanation for the failure is that the nuclei or chromatin had broken down secondary to declining cell function, with the nuclear proteins having become too diffuse to produce a discrete area of DAPI staining. As continued protein synthesis in these embryos would have to be independent of nuclear function it might be expected that they would all exhibit the early, maternally directed pattern. However 4 of the 10 embryos in which no nuclei were seen, did show evidence of transcriptionally dependent protein synthesis. Since all of these 4 embryos had reached at least the 4–cell stage it is possible that there had been some genomic transcription prior to nuclear breakdown.

Although half of the embryos analysed appeared capable of transcriptionally dependent protein synthesis the remainder did not. Thus it could be argued that failure of gene expression might still be an important cause of cleavage arrest in some embryos. However as all the embryos showing the early pre–gene activation pattern had arrested before the second cleavage division, the stage at which gene activation would normally occur, it would be reasonable to suggest that the failure of these embryos to synthesise transcriptionally dependent proteins might be due to cleavage arrest occurring before the genome activated.

The results from the horizontal gel system suggest that roughly half of the embryos showed evidence of synthesis of some of the group II proteins

associated with late cleavage stage embryos. Although this proportion is consistent with the 50% of embryos which showed evidence of transcriptionally dependent protein synthesis in the vertical system, the failure of the α–amanitin studies to confirm the transcriptional dependence of the group II proteins in the horizontal system makes it impossible to make definite conclusions about the data derived from the horizontal system.

In summary, at least half of the arrested embryos studied were found to be capable of synthesising at least some of the transcriptionally dependent proteins suggesting that cleavage arrest may still occur following gene activation. Synthesis of these proteins did not appear to be linked simply to time in culture, cytokinesis or karyokinesis although it could be related to DNA content. The absence of transcriptionally dependent protein synthesis in half the embryos studied might still argue in favour of failure of genome activation as a cause cleavage arrest, although the early stage of their arrest might suggest that this had occurred before the genome could activate. Analysis of the patterns from whole embryos, however, does not allow us to draw conclusions as to whether gene activation was complete or whether all the blastomeres within an embryo activate their genome at the same time. Indeed the results of the morphological studies discussed above would tend to suggest that there may be a great deal of heterogeneity between blastomeres within an embryo. In order to answer these questions a series of experiments were undertaken to separate or disaggregate blastomeres and then analyse their protein synthetic patterns individually.

Chapter Six

Protein synthetic patterns from individual blastomeres

6.1 Introduction

Asynchrony of genome activation between blastomeres within an embryo has been suggested by studies of uridine uptake into nucleoli as an indicator of mRNA synthesis (Tesarik, Kopecny et al. 1986). It was found that within an embryo, some blastomeres showed evidence of uridine uptake whereas others did not. It thus was suggested that genome activation between blastomeres was asynchronous, and that failure of genome activation by the majority or a critical proportion of blastomeres, might account for some cases of cleavage arrest.

Asynchrony of genome activation might also explain the persistent synthesis of early, non–transcriptionally dependent proteins in later cleavage stage, non–arrested embryos since some proteins could continue to be synthesised in some of the blastomeres on old mRNA which had escaped degradation. Genome activation in only some blastomeres within an embryo whilst synthesis on maternal mRNA continues but runs down in others would also explain the variable intensity of bands observed in the protein synthetic patterns, since within each embryo a variable number of blastomeres might be expected to be synthesising either transcriptionally dependent or independent proteins.

Since it has been possible to identify a group of proteins whose synthesis appears to indicate genome activation, it should be possible to determine the degree to which the state of genome activation varies between the constituent blastomeres of an embryo by analysing their protein synthetic patterns individually after they have been separated.

Demonstrating such variation would have important implications for

pre–implantation diagnosis and tests for the health of embryos prior to transfer, for if these were based on analysis of metabolites from one or two blastomeres it would not necessarily reflect the condition of the embryo as a whole.

6.2 Results

6.2.1. Morphology and method

Disaggregation of the embryos into individual blastomeres was attempted in a total of 21 embryos; 16 cleavage arrested and 5 non–arrested. The arrested embryos included 5 that had arrested at the 2–cell stage, 5 at the 3 or 4 cell stage, with the remainder arresting between the 5 and 8–cell stages. The non–arrested embryos included one at the 4–cell stage, one 5–cell and three at the 8–cell stage. 91% (30/33) of blastomeres were recovered intact from the non–arrested embryos and 66% (42/64) from the arrested embryos (see table 2).

6.2.2. Protein Synthetic Patterns

34 blastomeres gave interpretable patterns after labelling and electrophoresis. 12 originated from 4 arrested embryos and 22 from 3 non–arrested embryos.

Fig 18 shows the protein synthetic patterns of 16 blastomeres obtained from 2 non–arrested 8 cell embryos. Not only were clear differences noted in the density of tracks, but within these tracks (3,4 and 8) there appeared to be a selective incorporation of radioactivity into certain proteins. In these tracks most bands were absent or faint except for band E (51kD) which was absent or only faintly present in the patterns from the other blastomeres from this embryo (tracks 1,2,5,6,7) and band G (74kD) which was strongly represented in all blastomeres. An identical pattern was observed in tracks

Table 2 overleaf

Table 2

Table showing the recovery rate of intact blastomeres from disaggregated arrested and non–arrested embryos.

Table 2

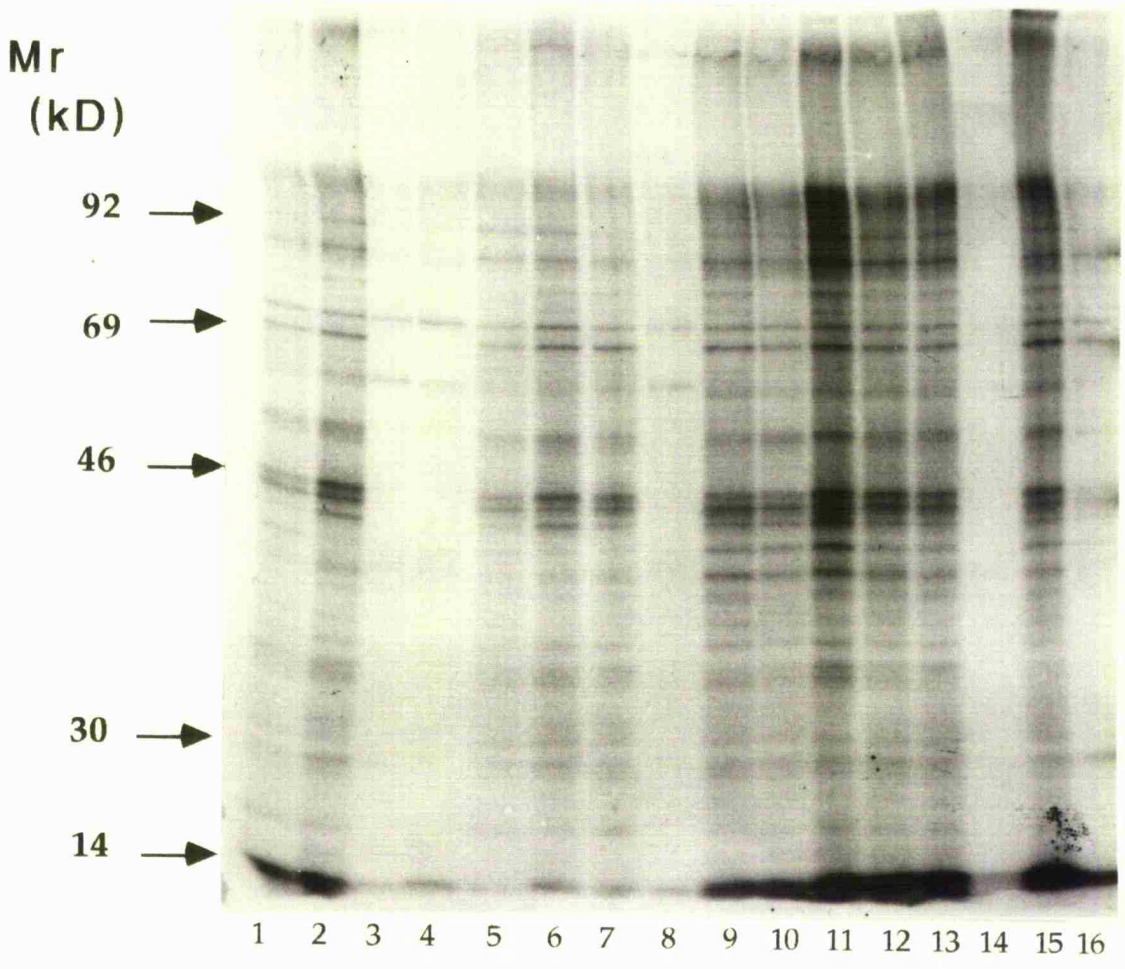
Arrested			Non–Arrested		
Cells	Blastomeres recovered	HPI	Cells	Blastomeres recovered	HPI
2	2	90	4	4	47
2	1	72	5	4	47
2	0	90	8	8	72
2	0	90	8	6	72
2	0	90	8	8	72
3	3	96			
3	2	96			
4	4	72			
4	3	72			
4	0	68			
5	5	90			
5	3	68			
6	4	68			
6	6	96			
7	5	90			
7	4	90			
Total 64	42 (66%)		33	30 (91%)	

Figure 18 overleaf

Figure 18

Photograph of an autoradiogram showing the protein patterns from sixteen blastomeres obtained by disaggregation of two non–arrested eight cell embryos (72 hpi) tracks 1–8 and tracks 9–16.

Figure 18



2 & 4 in Fig 19 which shows the protein synthetic patterns of blastomeres recovered from one of the arrested embryos. However track 1 on this gel was derived from 2 blastomeres which it had not been possible to separate and track 2 was derived from a blastomere which lysed just prior to collection. The remainder of this gel was loaded with 11 blastomeres from 2 arrested embryos but the resulting patterns were so weak as to make interpretation impossible.

It can be seen from Fig 20 that there were no significant qualitative differences between the patterns derived from any of the remaining 4 embryos which gave interpretable patterns. Furthermore within each individual embryo, as with those shown in figs. 18 and 19, all blastomeres showed evidence of synthesis of some of the transcriptionally dependent bands suggesting that all had activated their genome.

6.3 Interpretation of results

The results show that separation of human embryos into their constituent blastomeres is possible with a high degree of success. The better rate of recovery of intact blastomeres from non–arrested embryos when compared to arrested embryos may be explained by the fact that some of the 'cells' in these arrested embryos observed with a low magnification stereo microscope may rather have been 'blebs' of cytoplasm and therefore produced an inflated presumptive cell number. Alternatively, although there is no published data to support the hypothesis, it could be proposed that the cell membranes in arrested embryos may have been less robust as a result of poor underlying cell metabolism. This would certainly be consistent with experience in our own laboratory where it is found routinely that cell lysis is more common when attempting disaggregation of cleavage arrested mouse embryos.

The marked difference in the proportion of interpretable tracks seen in

Figure 19 overleaf

Figure 19

Photograph of an autoradiogram showing the protein patterns from four blastomeres obtained by disaggregation an eight cell embryo which exhibited arrest of development at 72 hpi and was disaggregated 24 hours later.

Figure 19

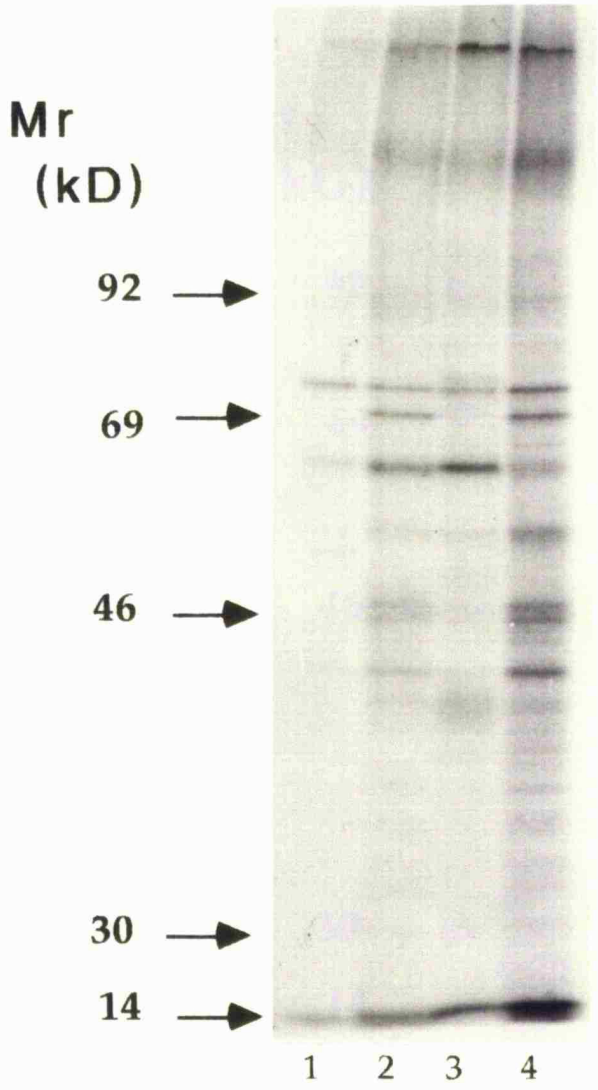
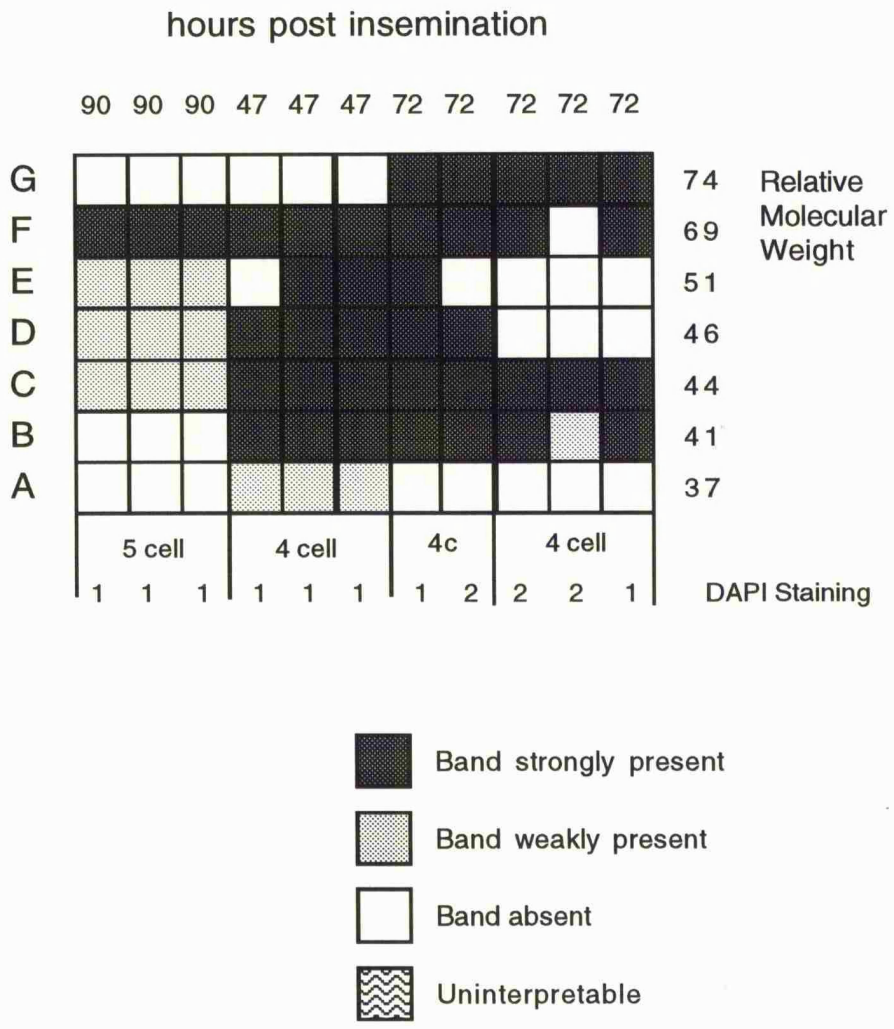


Figure 20 overleaf

Figure 20

Pictorial representation of protein bands A-G (Fig 7), as analysed using the vertical gel system, from individual blastomeres. Each column represents the data obtained from a single blastomere grouped with other blastomeres obtained from the same embryo. The number of cells in each embryo as assessed by light microscopy and the number of nuclear structures seen in each blastomere by fluorescence microscopy under U.V. illumination after DAPI staining is noted.

Figure 20


hours post insemination
90 90 90 47 47 47 72 72 72 72 72
G
F
E
D
C
B
A
74
69
51
46
44
41
37
Relative Molecular Weight
5 cell
4 cell
4c
4 cell
1 1 1 1 1 1 1 2 2 2 1
DAPI Staining
Band strongly present
Band weakly present
Band absent
Uninterpretable

arrested embryos (12 from 42) when compared to non–arrested embryos (22 from 30) could be a result of reduced protein synthesis in cleavage arrested embryos resulting in reduced uptake of radioactivity. Given the minute amount of protein within each blastomere, attempts to assess the amount of radioactivity incorporated were felt to be technically too difficult and unreliable to be feasible. There are data, however, from whole cleavage arrested mouse embryos (Goddard and Pratt 1983) which clearly support the hypothesis that uptake of radioactive precursors is diminished in cleavage arrested embryos.

In addition to the marked difference in the number of interpretable tracks between arrested and non–arrested embryos , it could be seen also that there was a marked variation in the density of the tracks between blastomeres that had been derived from the same embryo. Since embryos were radioactively labelled prior to disaggregation it could be expected that all blastomeres were exposed to equal concentrations of radioactivity and therefore that the variation in the density of tracks reflects a genuine reduction in the uptake of radioactive precursors rather than being the result of an experimental artefact. Furthermore the presence of some protein bands within many of these weaker tracks would argue against the variation in density being simply the result of failure of collection of the sample.

Possible explanations for this quantitative variation in protein synthesis between blastomeres include variation in the amount of protein synthesis at specific points in the cell cycle. Although there is evidence from the sheep (Crosby, Gandolfi et al. 1988) for a reduction in amino acid uptake as the fertilised oocytes begin cleavage, at present there are no data to support a cell cycle dependent variation in the quantity of protein synthesis. Alternatively, the overall reduced proportion of interpretable tracks from

cleavage arrested embryos would suggest a positive link between the amount of protein synthesis and continued embryonic development with progressive cell division. It may be argued therefore, that those blastomeres showing reduced or absent protein synthesis are more likely to be respectively in decline or dead. Furthermore it may be reasonable to postulate that, rather than cleavage arrest occurring as a result of failure of genome activation in a proportion of the blastomeres, cleavage arrest occurs as a result of a critical number of blastomeres being non–viable. Although on first inspection the only differences in pattern between blastomeres appeared to be quantitative, closer inspection revealed that within some of the the lighter tracks, (3, 4 and 8 in fig 18 and 1 and 3 from fig 19), a band migrating at the same molecular weight as band G (74kD) was present at least as strongly as in other blastomeres from the same embryo and also in some of these tracks (see 3, 4 and 8 in fig 18) a band migrating at the same molecular weight as the transcriptionally dependent band E was strongly present whilst being absent from the other overall more dense tracks. This would suggest that within all of these tracks, rather than the differences being simply the result of reduced incorporation of radioactive precursors, there was preferential synthesis of selected proteins in some blastomeres with a failure or reduction in the synthesis of other proteins being synthesised by other blastomeres from the same embryo. The findings from the arrested embryo might be questioned on the basis that track 3 (Fig 19) was derived from two blastomeres which it had not proved possible to separate and track 1 (Fig 19) had been derived from a blastomere which lysed during the act of collection. The consistency of these observations between this embryo and the non–arrested embryo in two separate experiments, analysed on separate gels would suggest that this is a genuine observation rather than

chance. The consistency would argue against these differences resulting from experimental artefact such as the loss of certain proteins in the course of processing the samples. This is further emphasised by some of the differences between tracks being the result of the presence of additional bands rather than the loss of bands.

A number of explanations may be offered for these qualitative differences in pattern between blastomeres.

Blastomeres may synthesise different proteins at differing points in the cell cycle. Evidence for cell cycle dependent variations in protein synthetic pattern does exist in the mouse (Howlett 1986a). Thus the differences in the protein synthetic pattern may simply reflect blastomeres being at different points in the cell cycle when sampled. It is difficult to see, however, how this could account for the findings in the cleavage arrested embryo unless it is postulated that blastomeres arrest at these specific points in the cell cycle. Confirmation of a cell cycle dependent variation in protein synthetic pattern would require sequential observation of embryos so that cells could be taken at known points in the cell cycle. Alternatively it might be possible to treat embryos with agents such as nocodazole to block cytokinesis and than examine their protein synthetic patterns.

Alternatively, the absence of many of the usual protein bands in these lighter tracks might suggest that protein synthesis is reduced within these blastomeres and may indicate that these blastomeres are in decline. Thus the proteins seen might represent those being synthesised on the longest lived mRNA.

In summary it would appear from these results that there are genuine qualitative and quantitative differences in the protein synthetic pattern between blastomeres within an embryo. Despite these differences, however, all the blastomeres gave interpretable patterns showed evidence of synthesis of some of the transcriptionally dependent bands. This

suggests that within an individual embryo, variation in the state of genome activation between blastomeres is not a common occurrence.

Chapter Seven

Protein synthetic patterns in parthenogenetically activated oocytes

7.1 Introduction

In certain species, such as the frog and particularly the sea urchin, activated oocytes are used regularly (Tarkowski, Witowska et al. 1970; Kaufman 1983; Whittingham 1980) as material to study aspects of pre–implantation development. Parthenogenetically activated oocytes provide large numbers of 'embryos' which can be relied upon to be synchronous in their development in a way in vivo or in vitro fertilised embryos cannot, due to the variation in the precise time of sperm penetration. The availability of large numbers of synchronous samples means that samples can be pooled, overcoming insignificant variations between individual embryos and allowing analyses to be made which require larger amounts of material such as two dimensional gel electrophoresis, southern blotting and many others. The precise synchrony of the samples is also of particular advantage when studying temporally dependent processes such as protein synthesis during the cell cycle. Notwithstanding these experimental advantages, the use of parthenogenetically activated human oocytes would have additional practical and ethical advantages since it would enable oocytes which failed to fertilise following insemination, and which are normally discarded, to be used for helpful scientific research (Winston, Braude et al. 1993). It would also overcome some of the ethical and moral objections to the use of donated embryos or embryos specifically created for experimentation.

In mammals parthenogenesis has been most extensively studied in the mouse where parthenogenetically activated oocytes can reach the 25 somite stage before pregnancy fails (Kaufman 1973). These studies have

shown that the method used to activate the oocyte influences the 'type' of parthenote formed and hence its potential for development. For instance electric shock, or other physical methods such as heat treatment, tend to lead to extrusion of the second polar body followed by cell division, resulting in a uniform haploid parthenote. However, treatment with calcium ionophore, particularly in association with culture in medium low in magnesium and calcium, tends to cause either immediate cleavage before extrusion of the second polar body, resulting in a mosaic haploid parthenote, or suppression of second polar body extrusion, resulting in a homozygous diploid parthenote. This latter course of development produces a genetically more balanced organism and produces parthenotes which are capable of reaching advanced embryological stages (Kaufman 1978).

Although spontaneous parthenogenetic activation of human oocytes has been reported (Edwards, Donahue et al. 1966; Johnson, Pickering et al. 1990) until recently there has been no reliable method to induce such development. Using the calcium ionophore A 23187, Winston et al (Winston, Johnson et al. 1991) were able to induce parthenogenetic activation in up to 60% of human oocytes, a figure which compares favorably with the overall fertilisation rates achieved when fertilizing human oocytes in vitro. Thus it is now possible to create large numbers of activated human oocytes as required. However before parthenogenetically activated oocytes can be used as a model for the study of pre-implantation development it must be demonstrated that they have a similar pattern of development to normally fertilised oocytes. The ability to synthesise proteins appropriate to the developmental stage of the embryo is one parameter by which the comparative usefulness of parthenotes could be judged. A series of experiments were therefore carried out to radioactively

label the proteins being synthesised by parthenotes at various stages of development and to compare the patterns produced following one dimensional SDS–PAGE with the patterns obtained previously from normally fertilised non–arrested embryos. The protein synthetic patterns obtained from a number of oocytes which had been subject to the activation protocol but failed to activate were also analysed.

7.2 Results

7.2.1 Activation rate

Activation with A23187 was attempted in a total of 89 oocytes between 18 and 24 hours following unsuccessful insemination. 33 (48%) oocytes had one pronucleus on initial inspection, 9 (13%) had two pronuclei and 13 (19%) had undergone immediate cleavage. Although 30 oocytes failed to demonstrate criteria of activation (presence of pronuclei or cellular division) at the time of the initial inspection, 10 of these underwent cell division subsequently. Thus the overall activation rate was 73%. Both activated and non activated oocytes, were subject to radioactive labelling for analysis of the protein synthetic patterns.

7.2.2 Development

Only 6 parthenotes progressed beyond the 4 cell stage before developmental arrest, 4 arresting at the 5–cell stage, 1 at the 6 cell stage and 1 at the 7–cell stage. None progressed beyond the third cleavage division.

7.2.3.Protein synthetic patterns

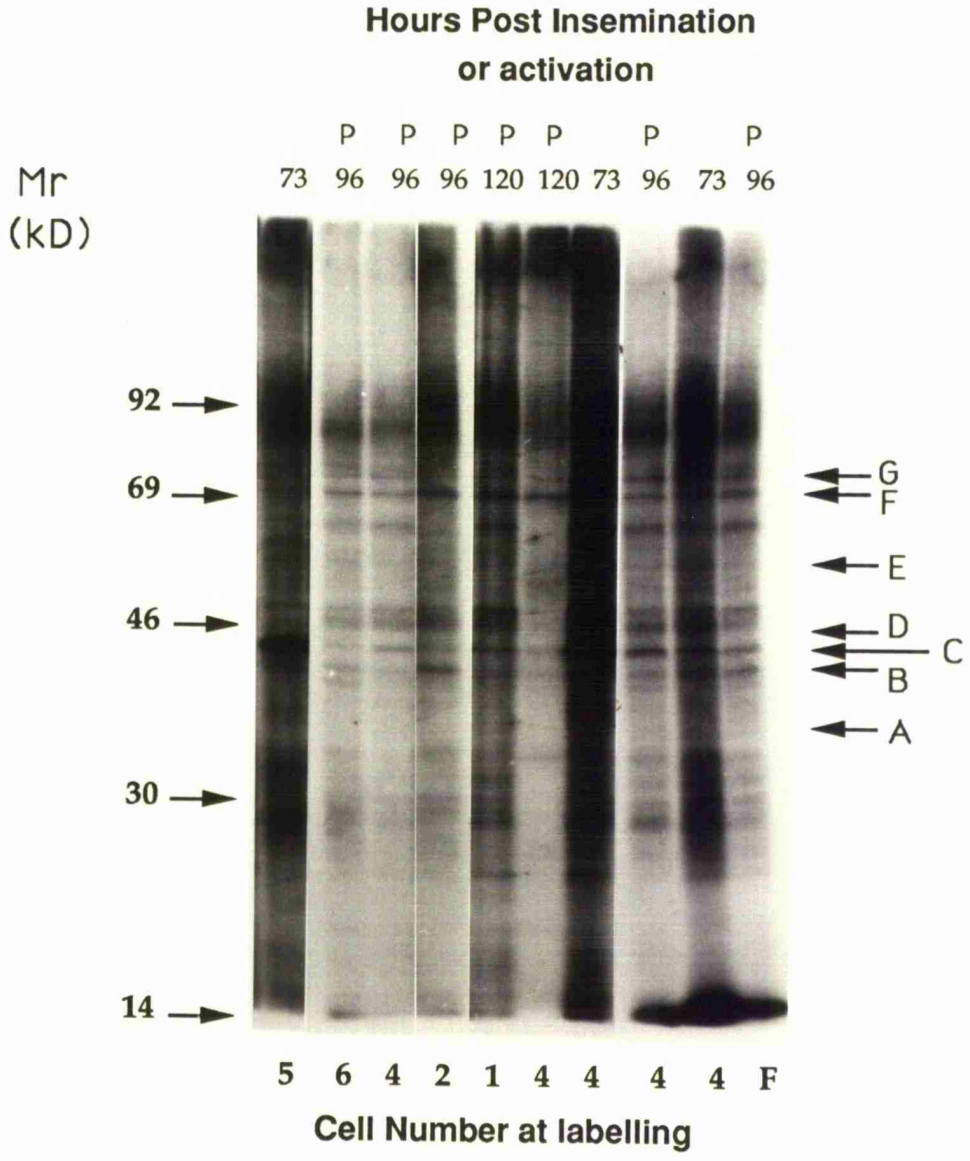
34 oocytes provided interpretable gel patterns after labelling with ^{35}S–methionine. This included 25 activated oocytes and 9 non–activated oocytes. Fig 21 is an autoradiogram of a gel on which some of the patterns from parthenogenetically activated oocytes are shown.

Figure 21 overleaf

Figure 21

Photograph of an autoradiogram showing the protein patterns from seven parthenogenetically activated human oocytes (labelled P). A typical pre–gene activation pattern (extreme left track) and post activation pattern (second from right track) obtained from cleavage arrested embryos have been included for comparison.

Figure 21


Hours Post Insemination
or activation
P P P P P P P
73 96 96 96 120 120 73 96 73 96
Mr
(kD)
92
69
46
30
14
G
F
E
D
C
B
A
5 6 4 2 1 4 4 4 4 F
Cell Number at labelling

The patterns obtained were analysed for the presence of the seven protein bands (A to F) used for analysis in embryos. The relative density of each of these bands is shown diagrammatically in fig 22. Band A (37kD) was present in the majority of oocytes which had failed to activate but was never strongly present in those that did. Band B (41kD) was strongly present in the majority of activated and failed activated oocytes as well as early cleavage stages, but appeared to weaken in intensity in those parthenotes that had progressed to the 4–cell stage or beyond. Bands C and F, (44kD and 69kD) were seen predominantly following activation although they were occasionally present in failed activated oocytes. Bands D and G (46kD and 74kD) were present infrequently in any oocytes but where they occurred tended to be from the 4–cell stage onwards. Band E was strongly present in the majority of failed activated oocytes and was rarely present following activation.

7.3 Interpretation of results

The high level of success achieved, 73% in this series rates confirms that the protocol described by Winston is effective at parthenogenetically activating human oocytes. The proportions of the 'types' of parthenote created, namely one pronucleate, two pronucleate or immediate cleavage are also consistent with those in the original study. As in Winstons study there was a predominance of one pronucleate oocytes (48%) although the frequency was not as high as the 68% amongst the aged oocytes in Winston's study.

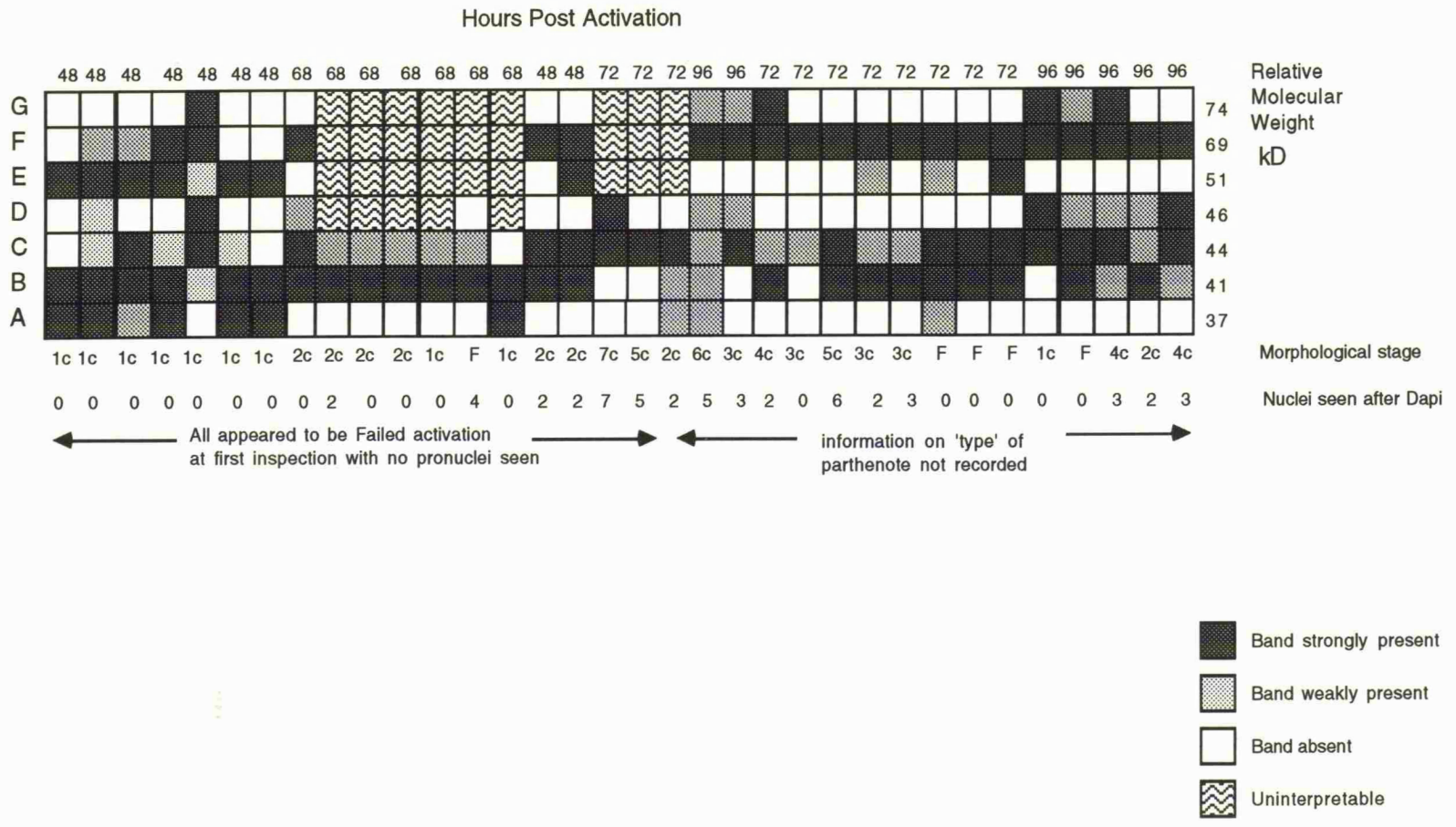
There was a considerable variation in the pattern of development of oocytes following exposure to the calcium ionophore when compared to fertilised oocytes. Some oocytes underwent cell division despite the absence of pronuclei on initial inspection, others showed immediate

Figure 22 overleaf

Figure 22.

Pictorial representation of protein bands A-G (Fig 7), as analysed using the vertical gel system, from individual parthenogenetically activated oocytes and oocytes where activation failed. Each column represents the data obtained from a single oocyte. The number of cells, number of pronuclei seen at initial inspection and the number of nuclear structures seen by fluorescence microscopy under U.V. illumination after DAPI staining is noted for each embryo.

Figure 22


Hours Post Activation
48 48 48 48 48 48 48 68 68 68 68 68 68 68 48 48 72 72 72 96 96 72 72 72 72 72 72 72 96 96 96 96 96
G
F
E
D
C
B
A
Relative Molecular Weight kD
74
69
51
46
44
41
37
1c 1c 1c 1c 1c 1c 1c 2c 2c 2c 2c 1c F 1c 2c 2c 7c 5c 2c 6c 3c 4c 3c 5c 3c 3c F F F 1c F 4c 2c 4c
Morphological stage
0 0 0 0 0 0 0 0 2 0 0 0 4 0 2 2 7 5 2 5 3 2 0 6 2 3 0 0 0 0 0 3 2 3
Nuclei seen after Dapi
All appeared to be Failed activation at first inspection with no pronuclei seen
information on 'type' of parthenote not recorded
Band strongly present
Band weakly present
Band absent
Uninterpretable

cleavage.

There are a number of possible explanations for this variation. Firstly, studies in the mouse (Kaufman 1978) have shown that as oocytes age, the second meiotic spindle 'migrates' towards the centre of the oocyte. When these aged oocytes are activated the central position of the spindle means that formation of the second polar body may be associated with an equal, rather than unequal division of the cytoplasm leading to the formation of two equal size haploid cells, instead of the secondary oocyte and the second polar body. Thus the appearance is that the oocyte has undergone immediate cleavage. The proportion of parthenotes undergoing immediate cleavage might be influenced by variations in the interval between oocyte recovery and exposure to the ionophore between batches of oocytes. In practice, however, oocytes were activated in groups which were subject to the activation protocol at the same time following recovery. Despite this there was still variation in the type of parthenote created within each group.

Secondly, since parthenogenetic activation bypasses some of the processes involved in normal fertilisation such as sperm penetration, fusion with the oolemma and the entry of the paternal DNA into the cell, it is likely that for some of the successfully activated oocytes the processes of pronuclear formation and breakdown took place earlier than is usual in fertilised oocytes. Thus, since the activated oocytes were routinely inspected at 19–22 hpa for signs of activation, in some, pronuclear formation and breakdown may already have taken place in those parthenotes which did not appear to have pronuclei on initial inspection but later went on to divide.

In common with Winston's original study, none of the parthenotes developed beyond the third cleavage division. Two conclusions may be

drawn. The ability of some parthenotes to reach the 7 cell stage confirms that, up until this stage at least, cleavage is independent of paternal genetic information. This is the first time this has been demonstrated in the human and concords with previous evidence from other species regarding the importance of maternally inherited information in directing development in the cleavage stage embryo. Furthermore, the lack of reliance on sperm for successful cleavage refutes previous theories suggesting that the meiotic spindle was introduced to the oocyte by the sperm (Sathananthan, Kola et al. 1991).

However the failure of parthenotes to reach later stages of development might suggest that beyond the 7–cell stage development is dependent on the presence of paternal factors. Alternatively, parthenotes might be subject to the same problems of cleavage arrest as normally fertilised embryos such as inadequate culture conditions.

Analysis of the nucleus to cytoplasm ratio of parthenotes shows that they are subject to the same variability as that observed in embryos, some having more nuclear structures than cells and others fewer. It is of interest that a substantial proportion of the parthenotes examined (18 out of 34) failed to stain adequately with DAPI. This had not been seen in non–arrested embryos (0 out of 13). Since failure of DAPI staining tended to occur more often in failed activated oocytes, and in activated oocytes which had totally fragmented, it is likely that in these, the nuclei, and hence nuclear proteins, had become so disrupted and diffuse as to make visualisation of a discrete spot of DAPI stain impossible. Thus little can be inferred from a number of parthenotes which failed to stain and it is only possible to conclude that irregularity of nuclear to cytoplasmic ratios as seen in embryo also occur in parthenotes.

Comparison of the protein synthetic patterns from parthenogenetically activated oocytes with those from non–arrested embryos demonstrated a

number of common features. The loss of band A following activation is directly comparable to the loss of this band in embryos shortly after fertilisation. The consistent strong synthesis of band F in activated oocytes is also comparable with the post–fertilisation appearance of band F in embryos. Bands D and G which were shown to be transcriptionally dependent in the examination of cleavage stage embryos and usually present after the 8–cell stage, were occasionally present in parthenotes despite the general failure of the parthenotes to develop much beyond the second cleavage division. Band C, which in the embryo appeared as a transitional band between the pre–gene activation and the post–gene activation pattern, was always present in activated oocytes but was also commonly seen in failed activated oocytes. A surprising finding was that Band E was strongly present in those oocytes which had failed to activate but was rarely present in successfully activated ones. This is in contrast to the studies in oocytes and embryos where band E was usually absent in oocytes, appearing only at later stages of embryonic development, and was demonstrated to be transcriptionally dependent. Possible explanations for the appearance of band E in failed activated parthenotes might include that band E represents a protein that is expressed when oocytes age. However since Band E was not found commonly in aged oocytes which had not been subject to the activation protocol (see chapter 3) this would seem unlikely. Alternatively it is possible that band E represents a protein in parthenotes that migrates at the same molecular weight as the protein or proteins forming band E in embryos. Further examination would require characterisation of the protein which would be technically extremely difficult if at all possible given the minute amounts of material available. In summary it appeared that parthenogenetically activated oocytes did synthesise similar proteins to those synthesised by embryos and that, with

the exception of band E, the protein bands A to G followed a similar pattern to that seen in normal embryos. Furthermore although the findings with regard to band E were surprising, it appeared that some parthenotes were capable of synthesising some proteins that had been shown to be transcriptionally dependent in cleavage stage embryos (see Chapter 4). However, before it could be concluded that parthenotes were capable of transcriptional activity further experiments using transcriptional inhibitors would be necessary.

Chapter Eight

Determination of the significance of the protein synthetic patterns of parthenogenetically activated oocytes.

8.1 Introduction

In the previous chapter it was shown that activated oocytes were capable of synthesising some of the same proteins as embryos at similar developmental stages. However the behaviour of the proteins migrating at the same molecular weight as band E raised the possibility that some proteins synthesised by activated oocytes might differ from those synthesised by embryos. To demonstrate that activated oocytes were capable of transcriptional activity, and thus further validate them as a model for the study of pre–implantation embryonic development, it was necessary to further examine the character of the changes in the protein synthetic pattern in parthenotes.

A series of experiments was therefore carried out to examine the effects of exposure to the transcriptional inhibitor α–amanitin on activation and protein synthesis at various stages of development. The use of parthenotes for these experiments had the added advantage that the effects of transcriptional inhibitors could be tested at earlier stages of development than were possible in embryos because of ethical constraints (see introduction)

8.2 Results

8.2.1 α–amanitin exposure prior to activation

8.2.1.1 Development and morphology

In 1 experiment, 16 oocytes were exposed to α–amanitin for 4 hours prior to attempted activation. 11 of these subsequently activated successfully.

The activated oocytes were then cultured continuously in the presence of α–amanitin until 71 hpa before radioactive labelling. Although 5 cleaved successfully, none completed the second cleavage division. Cleavage was totally inhibited in 6 and only 2 reached the 3 cell stage despite the prolonged culture period. DAPI staining was unsuccessful in 7 oocytes which included all those which failed to cleave. None of those which did stain had appropriate nuclear to cytoplasmic ratios.

8.2.1.2 Protein synthetic patterns

Ten of the oocytes successfully activated after exposure to α–amanitin gave interpretable patterns when analysed by one dimensional PAGE (Fig 23). The relative density of the 7 protein bands A–F are shown in a diagrammatic format in Fig 24. All of the parthenotes and failed activated oocytes showed strong synthesis of bands B and E. Nine also showed evidence of synthesis of band A. Five showed weak synthesis of band C but only 1 of the parthenotes showed reduced synthesis of band F and none showed synthesis of bands D and G.

8.2.2.α–amanitin exposure 24 hours post–activation

8.2.2.1 Development and morphology

In 3 experiments, 14 oocytes deemed to have successfully activated were exposed to α–amanitin at 24 hpa. 6 were left in culture until 48 hpa and 8 until 72 hpa before radioactive labelling. Cleavage was inhibited in 4 of the 14, 4 successfully completed the second cleavage division before developmental arrest, the maximum cell number reached being 6. DAPI staining was unsuccessful in 6 of the 14 parthenotes, which included all those that failed to cleave. The nuclear cytoplasmic ratio was found to be appropriate in 2. The remainder, with one exception, had fewer nuclear

Figure 23 overleaf

Figure 23

Photograph of an autoradiogram showing the protein patterns from 16 oocytes parthenogenetically activated following 4 hours pre–incubation with α– amanitin prior to attempted activation and maintained in culture for 72 hours post–activation. Tracks 1 to 5 derive from oocytes which failed to activate, tracks 6 to 11 are from oocytes which showed evidence of pronuclear formation but which failed to cleave and tracks 12 to 16 are oocytes which cleaved after activation. The maximum cell number achieved and the number of nuclear structures seen after DAPI staining is noted for those that successfully cleaved.

Figure 23

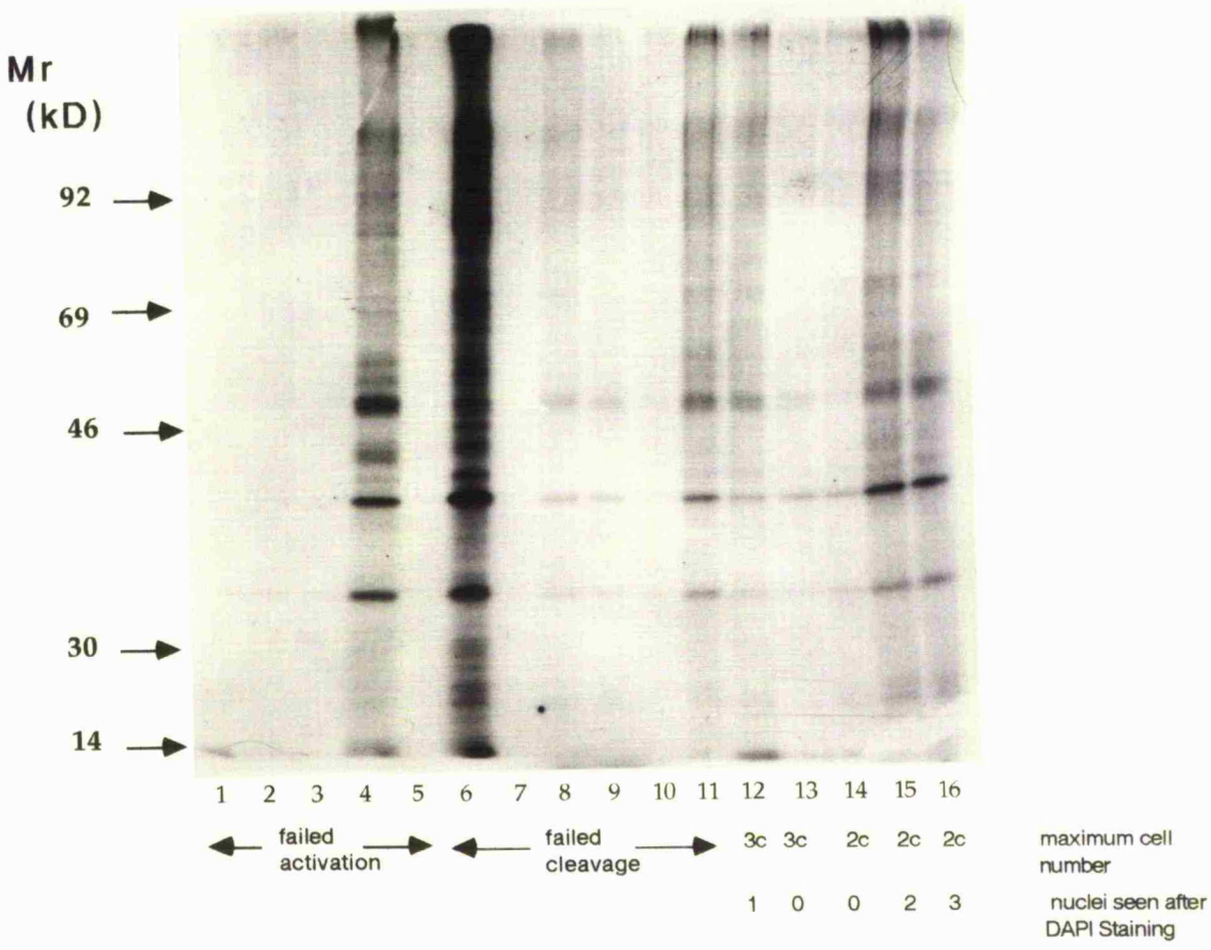

Mr
(kD)
92
69
46
30
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
failed activation
failed cleavage
3c 3c 2c 2c 2c
maximum cell number
1 0 0 2 3
nuclei seen after DAPI Staining

Figure 24 overleaf

Figure 24

Pictorial representation of protein bands A-G (Fig 7), as analysed using the vertical gel system, from individual oocytes which failed to activate, shown in the the three left hand columns, 10 parthenogenetically activated oocytes treated with α– amanitin prior to attempted activation and 14 parthenogenetically activated oocytes treated with α– amanitin 24 hours following activation. Each column represents the data obtained from a single oocyte. The number of cells, number of pronuclei seen at initial inspection and the number of nuclear structures seen by fluorescence microscopy under U.V. illumination after DAPI staining is noted for each embryo

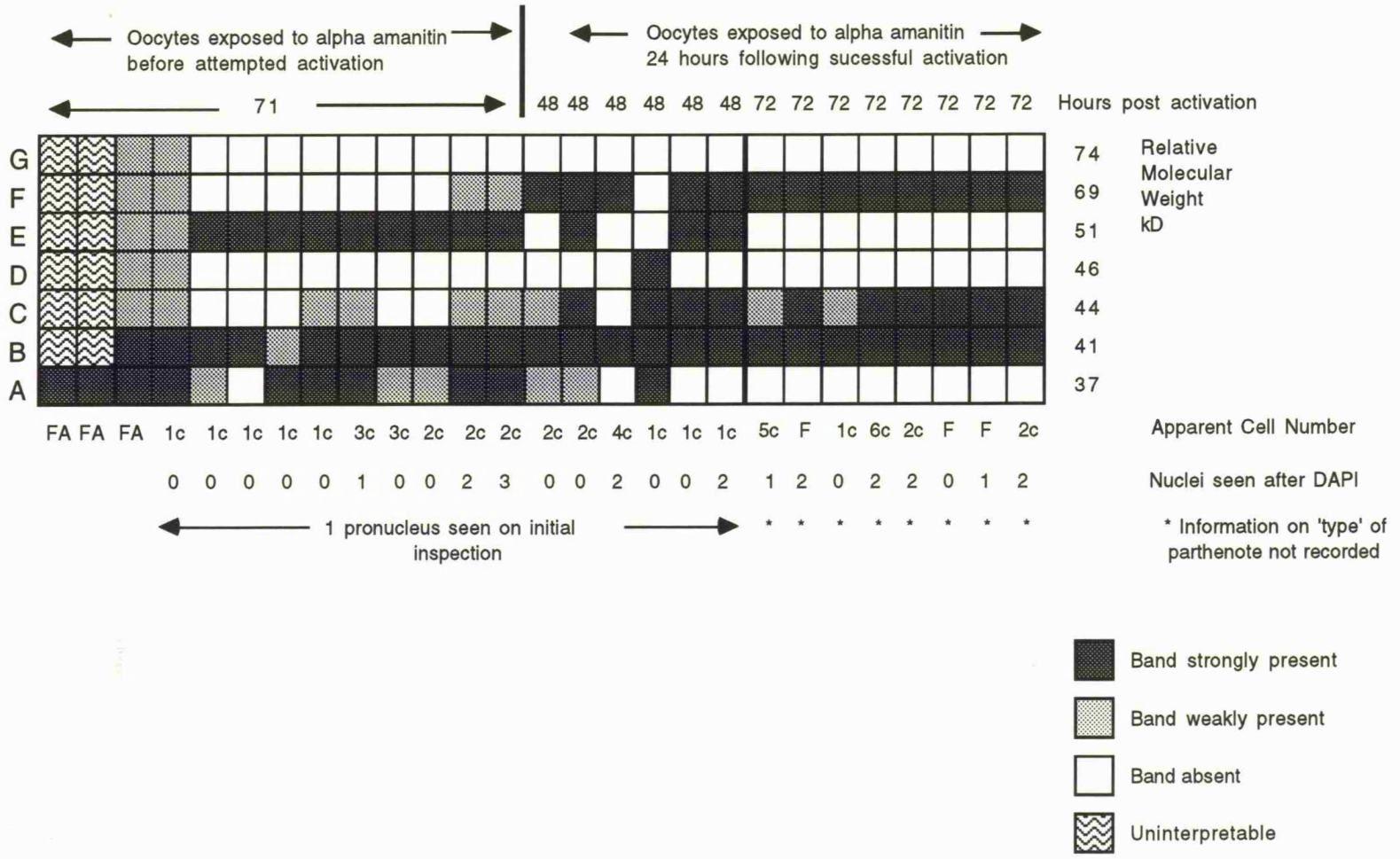

Oocytes exposed to alpha amanitin before attempted activation
71
Oocytes exposed to alpha amanitin 24 hours following sucessful activation
48 48 48 48 48 48 72 72 72 72 72 72 72 72
Hours post activation
G
F
E
D
C
B
A
74
69
51
46
44
41
37
Relative Molecular Weight kD
FA FA FA 1c 1c 1c 1c 1c 3c 3c 2c 2c 2c 2c 2c 4c 1c 1c 1c 5c F 1c 6c 2c F F 2c
Apparent Cell Number
0 0 0 0 0 1 0 0 2 3 0 0 2 0 0 2 1 2 0 2 2 0 1 2
Nuclei seen after DAPI
1 pronucleus seen on initial inspection
* * * * * * * *
* Information on 'type' of parthenote not recorded
Band strongly present
Band weakly present
Band absent
Uninterpretable

Figure 24

structures than cells.

8.2.2.2 Protein Synthetic Patterns

All 14 of the parthenotes gave interpretable patterns. An autoradiogram of patterns from 8 are shown in Fig 25. The data from all 14 are shown diagrammatically in Fig 24. Overall it can be seen Band A was present in 3 oocytes, strongly only in 1. Band B was strongly present in all. Band C was present in 13, strongly in 10 ; band D was only present in 1 and band G in none. Only 3 showed evidence of synthesis of band E whereas 13 showed strong synthesis of band F.

8.3 Interpretation of results

The results show that exposure of parthenotes to α–amanitin both before and after activation has distinct effects on their development and protein synthetic patterns. Examining these in turn.

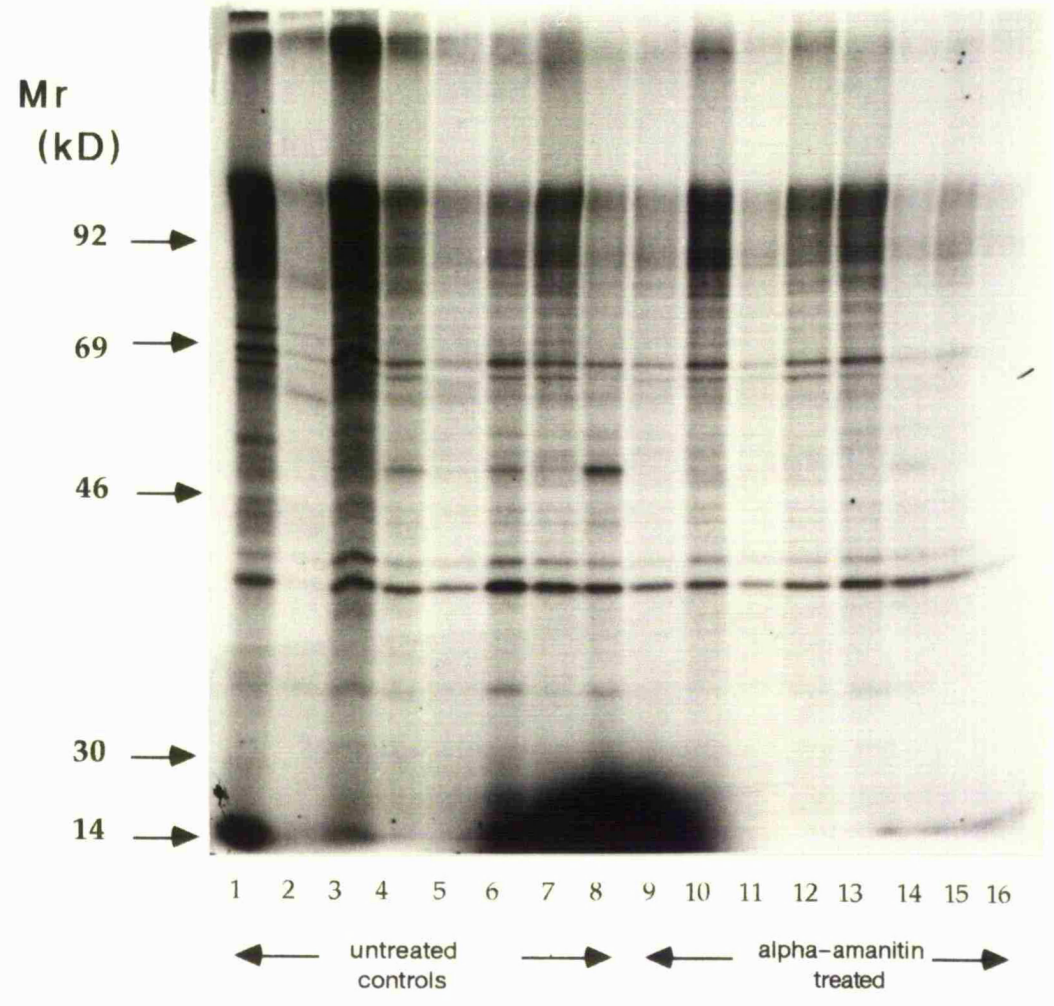
Exposing oocytes to α–amanitin before attempted activation or shortly after activation had a marked effect on the subsequent cleavage rate with 6 of the 11 parthenotes exposed to α–amanitin before activation failing to cleave and 4 of the 14 exposed 24 hpa. Comparison of this high rate of cleavage failure with the low rate seen in untreated parthenotes (3 from 27) might suggest that α–amanitin treatment has a specific effect on the early cleavage divisions. This effect might be due to a non–specific effect of α–amanitin on cell processes other than transcription such as translation. However, as has been discussed previously (see Chapter 4), although the original work defining a specific role for α–amanitin as an inhibitor of RNA Polymerase II used isolated nuclear preparations(Lindell, Weinburg et al. 1970) there has been little evidence from experiments on whole cells and embryos in other mammalian species to suggest that α–amanitin might exert any other effects. It could be postulated that it was in fact the

Figure 25 overleaf

Figure 25

Photograph of an autoradiogram showing the protein patterns from 16 parthenogenetically activated oocytes. All showed evidence of successful activation. The morphology at the time of labelling and the number of nuclear structures seen after DAPI staining is noted. Tracks 1–8 derive from untreated control parthenogenetically activated oocytes. Tracks 9–16 derive from oocytes which were incubated in the presence of α– amanitin from 24 hours post–activation. All were maintained in culture for 72 hours post–activation before labelling.

Figure 25


Mr
(kD)
92
69
46
30
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
untreated controls
alpha-amanitin treated

specific action of α–amanitin in inhibiting transcription that resulted in the high rate of cleavage failure. In doing so, however, it would also have to be postulated that transcription takes place within the first 24 hours following activation.

To date there is no evidence to suggest that transcription takes place at this early stage in the human. Indeed the only evidence so far from Braude et al's original paper (Braude, Bolton et al. 1988) indicates that gene expression in embryos does not take place until at least the 4–cell stage. Closer inspection of this paper reveals however that their conclusions were based on analysis of 2 oocytes only, which were fertilised in the presence of α–amanitin.

Further evidence indicating transcriptional activity within 24 hpa can be deduced from some of the differences observed between the protein synthetic patterns of those parthenotes created after exposure to α–amanitin and those exposed 24 hpa. Of particular relevance is the general absence or low level of synthesis of band F in those parthenotes created following α–amanitin treatment in contrast to its almost universal strong presence in those treated 24 hpa. In embryos and untreated parthenotes band F appeared almost immediately following fertilisation and activation respectively. Its synthesis also appeared to be insensitive to treatment with transcriptional inhibitors, as indeed it also appeared to be in those parthenotes treated with α–amanitin 24 hpa. Thus its absence in the parthenotes treated before activation is surprising. The most obvious explanation is that band F represents a protein whose synthesis is transcriptionally dependent in the first 24 hpa but that after this time it ceases to be so, possibly due to the mRNA responsible being particularly long lived and stable. Although a change from transcriptional dependence to independence would seem unlikely, there is supporting evidence for

such a situation from the mouse (Flach, Johnson et al. 1982) where gene activation appears to take place in two phases. A low level of transcriptionally dependent protein synthesis has been detected between 18 and 21 hpi, followed by more generalised gene activation at 26–29 hpi. Furthermore the first transcriptionally dependent protein detected in these experiments had a molecular weight of 67 kD and, although sensitive to α–amanitin if the embryo was treated before 21 hpi, its synthesis appeared to be insensitive to exposure after this time. The similarity between this protein in the mouse and band F in these human parthenotes, which had been estimated to have a relative molecular weight of 69kD, is striking and it is not unreasonable to suggest that they might represent the same protein, the apparent difference in molecular weight being a result of error in estimating the value.

The prolonged synthesis of band A in those parthenotes created following α–amanitin treatment in contrast to its absence in those treated 24 hpa is also of interest. In embryos and untreated parthenotes synthesis of band A usually declines following activation or fertilisation and ceases entirely by the 2–cell stage. Its continued synthesis by the parthenotes created after α–amanitin treatment might be due to the the fact that few of these reached advanced stages of development, only 2 reaching the 3 cell stage. Continued synthesis of band A might therefore reflect synthesis of appropriate proteins for the developmental stage the parthenotes had reached. However few of the parthenotes exposed to α–amanitin after activation reached advanced stages of development. Despite this few showed synthesis of band A. This might suggest a more specific effect of α–amanitin treatment in the first 24 hours of development which has preserved the synthesis of band A. Since it has already been proposed that transcriptional activity might take place in the first 24 hpa, it could be that

the reduction in synthesis of band A normally seen after activation or fertilisation is also an early transcriptionally dependent process. Once again studies in the mouse (Flach, Johnson et al. 1982) support this possibility. These suggested that fertilisation appeared to accelerate the loss of some proteins and that early treatment with transcriptional inhibitors appeared to reduce the rate of this loss. However, it was also found that even in the presence of transcriptional inhibitors, synthesis of many of the proteins declined simply as a function of time spent in culture. In view of the later stage of genome activation in the human when compared to the mouse and therefore the possible greater stability of human maternal mRNA, determining whether band A in human parthenotes represented a similar protein to those seen in the mouse would require analysis of the protein synthetic patterns of parthenotes which had been created after α–amanitin treatment and kept in culture for longer than 71 hpa as was the case in these experiments. To allow definite conclusions to be drawn on genome activity within the first 24 hpa in human parthenotes requires analysis of greater numbers than is presented here. Definitive evidence to support genome activation in the human embryo in the first 24 hours of development can only come from analysis of the protein synthetic patterns of oocytes which are fertilised after exposure to transcriptional inhibitors. However such experiments are unlikely to be performed in the near future given the ethical difficulties associated with obtaining large numbers of 'fresh' oocytes.

The general absence of bands D and G in parthenotes, whether exposed to α–amanitin before activation or 24 hpa, suggests that these bands demonstrated to be transcriptionally dependent in embryos are also transcriptionally dependent in parthenotes. Since these bands are also occasionally seen in later stage untreated parthenotes, it would tend to confirm that some parthenotes are capable of transcriptionally dependent

protein synthesis.

The pattern of synthesis of the other transcriptionally dependent band in embryos, band E, is surprising. Although, this was felt to be transcriptionally dependent in embryos, its pattern of synthesis and response to exposure to α–amanitin was the least reliable of the 3 bands felt to be transcriptionally dependent, bands D, E and G. It was synthesised rarely by parthenotes, but did appear with some consistency in failed activated oocytes, the possible reasons for which were discussed in the previous chapter. The results presented here show that it was synthesised rarely by parthenotes exposed to α–amanitin 24 hpa, which is consistent with its pattern of synthesis in untreated parthenotes. It was present strongly, however, in parthenotes which were created after α–amanitin treatment. Once again this would suggest a specific role for early stage α–amanitin treatment in protecting the synthesis of band E as well as band A. This possibility could be investigated in the same way as those required to investigate the nature of the band A.

Relating the pattern of synthesis of band E in parthenotes to that seen in embryos is however difficult. It might be that fertilisation and activation result in differing proteins which migrate at the same molecular weight as band E. Investigating this possibility has been discussed in the previous chapter. Less likely although possible is that activation itself or the agent used has a specific effect on band E, possibly along with other proteins which do not form part of our analysis. Determining if activation itself has an effect would be difficult but determining if the agent used, in this case calcium ionophore, had an effect could be investigated by examining the protein synthetic patterns of parthenotes created by other methods such as ethanol exposure or electric shock. Unfortunately the reduced rate of activation these agents induced would require relatively large numbers of

oocytes which are not always easy to obtain in such large cohorts.
Lastly the results appear to show that the effects of α–amanitin exposure on the protein synthetic pattern of parthenotes are more marked than those seen in non–arrested embryos, as indicated by the definite loss or indeed preservation of bands seen after α–amanitin treatment of parthenotes when compared to the more subtle reduction in level of synthesis seen following α–amanitin treatment of embryos. There are several reasons why this may be so. Firstly the use of parthenotes provided sufficient quantities of synchronous material to allow these data to be collected from only 3 experiments with analysis on 3 gels so allowing greater uniformity of experimental conditions. Secondly the use of parthenotes allowed exposure to α–amanitin at earlier stages in larger numbers than had been possible with embryonic material that was surplus to therapeutic requirements. Since gene activation takes place between the 4 and 8 cell stages and embryos were only available for exposure to α–amanitin following embryo transfer, which in general takes place at the 4–cell stage, it is possible that some embryonic mRNA transcription might have occurred before the embryos were available for α–amanitin treatment. Thus synthesis of a transcriptionally dependent proteins might still be detectable in an embryo exposed at the 4–cell stage, albeit at a reduced level. The marked effects observed emphasise the advantages of being able to use parthenotes as an experimental model in preference to donated embryos.
To summarise, although definite conclusions can only be drawn from more experiments on a greater number of parthenotes, the results of these experiments appear to show that parthenotes are capable of transcriptionally dependent protein synthesis. This would suggest that in terms of protein synthesis parthenotes would be a suitable experimental

model for the study of early human development. The results are also consistent with transcriptional activity in parthenotes in the first 24 hours following activation. This contrasts with the reported findings from embryos which suggest no evidence of gene activity until at least the 4 cell stage. However, the findings from embryos were based on relatively small numbers when compared to the number of parthenotes studied here and, in view of the supporting evidence for early genome activity in the mouse, further studies need to be undertaken into the possibility that gene activity might take place at this early stage in embryos.

Chapter Nine

Discussion and Conclusions

The possible interpretation of each set of results and some of the methodological difficulties encountered during the course of this study have been discussed individually in each of the preceding chapters. The aim of this chapter is to draw together each of these individual items in the hope of shedding some light on the mechanisms of human pre–implantation development and also to discuss how the methods used might have influenced the results and what impact this might have upon their interpretation.

9.1 Materials and Methods

9.1.1. Material

The main source of material for study is to use embryos and oocytes donated as surplus to the therapeutic needs of patients undergoing assisted conception procedures. Personal moral objections, cryopreservation of surplus embryos and the relatively small numbers of assisted conception procedures performed in units with ongoing research programmes all serve to restrict the numbers of embryos available for analysis from this source. The small amount of experimental material available means that strategies commonly used in the study of domestic and laboratory species such as the pooling of samples to provide quicker results and to overcome individual embryonic variation, are not possible in the human. Thus experimental design in the human must be limited to those experiments which are capable of yielding reliable results using only single embryos (or oocytes). It is also the case that conclusions have to be drawn from the study of smaller amounts of material in the human than would be

considered necessary for a valid study of laboratory and domestic species where material is more plentiful.

In addition to restricting the absolute volume of material available for study, the reliance on material surplus to therapeutic procedures has further specific restraints. Very early stage human material, fresh oocytes and pronucleate stage embryos are difficult to obtain since normally all of the oocytes should be placed with the sperm and all resulting embryos must remain at the patient's disposal until embryo transfer has been completed, usually 48 hpi. Thus relatively little material has been available for study at these early stages. Thus the analysis of early stage parthenogenetically activated oocytes, as presented here, probably represents the most comprehensive study of protein synthesis and gene activity in the human at these early stages, albeit at present of uncertain relevance to the development of normally fertilised oocytes.

The reliance on material surplus to therapeutic requirements, i.e. once the best 2 or 3 embryos are selected for transfer, implies that those remaining may be of inferior quality. Thus the results of studies might not necessarily represent the 'norm' as the population could be biased towards abnormal embryos.

The problems of access to material are compounded by the variation in the rate of development of human embryos *in vitro* (Bolton and Braude 1987), and the marked tendency for cleavage to stop entirely (Bolton, Hawes et al. 1988). Both of these make it difficult to define a 'normal' pattern against which to compare the development of embryos which have been subject to experimentation such as embryo biopsy or, in the case of the work presented here, exposure to transcriptional inhibitors.

Given these problems with access to and the development of human embryos *in vitro* it is clear that the conclusions that are drawn are

tentative and may be refuted or confirmed as more information accrues.

9.1.2 Methods

Since embryos and oocytes were available only in small amounts and often irregularly, the material had to be analysed as it became available. On occasions this meant that only one or two embryos were analysed at any one time. Adherence to strict laboratory procedures was essential to ensure uniform experimental conditions so as to allow results from experiments carried out at different times to be combined. The use of the more consistent vertical SDS–PAGE system meant that, on the whole, a high level of uniformity was maintained throughout the experiments. The introduction and subsequent abandonment of the horizontal gel system did, however, lead to the loss of some useful data. In retrospect committing so many embryos to analysis using this system was a mistake. Although the system was first tested on mouse embryos and felt to be satisfactory based on the clear presence of several protein bands which had been well characterised by ourselves and others previously, it was not until the first human samples were ready for analysis that it was appreciated how difficult use of this system would be when there was no similar body of data for the human on which to rely.

Furthermore, ultrathin rapid gel systems are usually used to identify gross differences in the electrophoretic mobility of one or two proteins during the course of laboratory procedures such as the identification of abnormal subtypes of haemoglobin in the investigation of clinical disorders such as haemoglobinopathies. The use of the ultrathin system can provide a result rapidly and cheaply, but is not appropriate for the analysis of subtle qualitative changes in protein synthesis where there are a large number of proteins present.

Since analysis of the autoradiograms from the horizontal system was based

on the objective assessment using a gel scanner, rather than subjective visual assessment as used with the vertical system, the failure to detect clear transcriptional dependence of some the proteins associated with later stage non–arrested embryos analysed using the horizontal system, might be considered a criticism of the work in general. However in the vertical gel system, the effects of α–amanitin treatment on the protein synthetic pattern were so obvious as to make the result clear. Furthermore few embryos which had been exposed to α–amanitin were analysed using the horizontal system. Thus, given the variable nature of the material available, it may have been the case that had efforts persisted with the horizontal system, transcriptional dependence would have been detectable.

The difficulty in applying objective analysis using a gel scanner to the vertical gel system was not entirely surprising, given the tracks on the autoradiograms from this system were rarely straight and the bands were relatively broad. The difficulties encountered in attempting to produce tracks of equal density by assessing the amount of radioactivity incorporated have also been encountered by others (Braude 1979). Indeed it is interesting to note that the only references to the successful use of this technique have been in domestic and laboratory species where samples have been pooled. Pooling of samples provides a much larger amount of radioactivity in the sample so reducing the scope for error in assessing the amount incorporated and allowing the amount loaded on the gel to be adjusted accordingly. The small amounts of radioactivity present in the single embryo samples assessed here could have introduced errors of assessment. The wide range of values obtained made appropriate adjustment of the volume to be loaded onto the gel impossible for the weaker samples as more volume was required than was available, and for

the better labelled ones, the small volume of sample required made accurate loading of the sample onto the gel very difficult.

Despite the problems encountered with the use of ^{35}S–methionine radiolabelling and PAGE, at present there does not seem to be a clearly superior alternative method of analysing gene activity. Although Tesarik's (Tesarik, Kopecny et al. 1986) use of uridine incorporation is appropriate for demonstrating RNA synthesis, it does not appear to be technically any easier to perform nor does it allow for more objective analysis of results than was possible using our system.

Alternatively the activity of specific genes has been assessed in other systems such as drosophila and yeast by analysing the synthesised RNA. However, the small amount of RNA present in human embryos would make direct analysis almost impossible, although it might be possible to use the polymerase chain reaction to amplify the initial RNA from its cDNA. Specific probes could then be used to detect different RNA populations. The major difficulty with such an approach would be distinguishing between those cDNA's that had been derived from embryonic RNA and those derived from maternal RNA. Thus despite its apparent limitations, I feel radiolabelling and PAGE still represents the least complicated and most informative technology available.

9.2 Results

9.2.1 Introduction

Studies of a number of mammalian species have suggested that the mechanisms operative during pre–implantation development are remarkably consistent across species and indeed may be similar across genera although the timing and rate of development may differ. Thus despite the relative lack of specific knowledge, it has been assumed that the

mechanism of pre–implantation development in the human would be consistent with these previous studies. Although the aim of these series of experiments was to investigate specifically the role of gene expression in cleavage arrest in the human embryo, the experiments required to validate the techniques used, including confirmation of the original work by Braude et al (Braude, Bolton et al. 1988) and the study of the development and protein synthesis of parthenogenetically activated oocytes mean that this work forms one of the most systematic studies of the biochemistry and development of the pre–implantation human embryo carried out to date. I would therefore like to review the results of these experiments to examine the degree to which the human follows the same mechanisms of development as seen in other species.

In most species mRNA's are synthesised by the oocyte prior to ovulation (Davidson, Crippa et al. 1966), some of which are utilised immediately to synthesise proteins required by the oocyte, whilst others remain dormant as stored or 'masked' mRNA to be activated only if the oocyte is fertilised or activated (Braude and Pelham 1979). At a point in development specific to the species (Frei, Schultz et al. 1989; Davis 1985; Crosby, Gandolfi et al. 1988; Goddard and Pratt 1983) the embryonic genome activates and embryonic mRNA becomes the template for protein synthesis. This activation of the genome may be coincidental with active degradation of the maternal mRNA's (Flach, Johnson et al. 1982) despite the fact that most of the mRNA's synthesised by the embryo appear to be the same type as the maternal mRNA's they replace since few new proteins are synthesised. In the most extensively studied mammalian species, the mouse, a biphasic genome activation has been demonstrated (Goddard and Pratt 1983) with a low level genome activity detectable within the first 24 hours following fertilisation, followed by a more generalised genome activation during the 2–cell stage (24–36 hpi).

Although cleavage arrest is observed in all species at all stages of pre–implantation development, there seems to be a distinct tendency for development to arrest at about the same stage as genome activation, leading to the hypothesis that failure of genome activation may be one of the underlying causes of cleavage arrest. However, the predisposition to developmental arrest varies between species, and between strains within species (Goddard and Pratt 1983). In most species the tendency to arrested development can be ameliorated or overcome by the manipulation of culture conditions (Nasr-Esfahani, Johnson et al. 1990: Crosby, 1988) which might suggest that whilst failure of genome activity per se may not be a common cause of cleavage arrest, it may be associated with a change in the culture requirements of the developing embryo.

9.2.2 Non arrested and α–amanitin treated embryos

9.2.2.1 The oocyte and early cleavage stages

The protein synthetic patterns of oocytes and early cleavage stage embryos were shown to be very similar, which would suggest that the active mRNA templates in the early cleavage stage embryo are similar to those which are used in the oocyte. The inability of transcriptional blockade to affect the pattern in the early cleavage stage embryo significantly would further indicate that these templates are not synthesised de novo by the early embryo. The results are therefore consistent with the hypothesis that protein synthesis and development in the early cleavage stage human embryo are directed by mRNA's inherited in the oocyte in a similar way to that seen in other species. Specific evidence for the existence of these mRNA's could be obtained by extracting them from the oocyte and expressing them in a cell free system, as was performed in the mouse (Braude and Pelham 1979). The requirements of the extraction and

translation process for a large quantity of synchronous oocytes, makes this work almost impossible to repeat in the human given the paucity of material available.

Notwithstanding the lack of this specific evidence, the consistency of the results makes it highly likely that protein synthesis and development in the early cleavage stages in the human, as in other species, are directed by mRNA's which are maternal in origin.

Several further conclusions flow from this hypothesis.

Firstly, the ability of early stage embryos to continue to cleave despite the presence of transcriptional inhibitors, suggests that the maternal mRNA's are capable of fulfilling most if not all of the developmental requirements of the early cleavage stage embryo without a significant contribution from the embryonic genome. Thus gross features of development such as cell number and cell division are likely to be poor indicators of embryonic genetic competence and ultimately viability at least up until the 4–cell stage. The likely lack of correlation between these gross morphological features and embryo viability has unfortunate implications for assisted conception procedures as these are the only criteria available on which to base the choice of embryos for transfer in IVF–ET.

Secondly, the relatively small number of proteins which could be demonstrated to be transcriptionally dependent at the 4 to 8–cell stage is consistent with most of the maternal mRNA's being relatively long lived and stable and perhaps more surprisingly their persistence for some time after genome activation. The continuing role for maternal mRNA's in the human after genome activation contrasts with the relatively brief contribution maternal mRNA's make in the mouse where it can be demonstrated that most of the maternal mRNA's are actively destroyed almost immediately following genome activation (see below).

Further information regarding the longevity of maternal mRNA might be gained by radioactive labelling the mRNA's as they are synthesised by the oocyte. In laboratory species this has been achieved by injecting radioactive precursors of RNA beneath the ovarian capsule in intact animals (Bacharova and DeLeon 1980). The persistence of the maternal message was then calculated by measuring the rate of decay of radioactivity in harvested oocytes. Although corresponding experiments in vivo would not be feasible in the human, it might be possible to perform similar experiments using immature oocytes allowed to mature *in vitro* after being released from ovaries obtained from subjects undergoing operations to remove the uterus and ovaries (Racowsky, Kaufman et al. 1992), although the high rate of abnormality observed amongst such oocytes (Lopata and Leung 1988) would cause problems with the interpretation of results.

9.2.2.2 The 4 to 8–cell stage

The suppression by transcriptional inhibitors of the qualitative change in the protein synthetic pattern seen usually in human embryos between the 4 and 8 cell stages is consistent with the change in pattern being the result of transcriptional activity, thus indicating that the first detectable activity of the human genome takes place at the 4 cell stage. This finding is consistent with previous studies aimed specifically at defining the time of human genome activation (Braude, Bolton et al. 1988; Tesarik, Kopecny et al. 1986).

A specific stage at which genome activation occurs and the high rate of cleavage arrest coincident with this stage (Bolton, Hawes et al. 1988) is consistent with developmental events seen in other species. In the mouse the switch from maternally inherited control to embryonically coded information is abrupt, associated with a rapid degradation of maternal

mRNA's (Flach, Johnson et al. 1982), and has also been shown to be associated with a reduction in the overall rate of protein synthesis as the transition occurs. However, in the human the relatively small number of proteins which could be demonstrated to be transcriptionally dependent at the 4 to 8–cell stage and for some time afterwards, suggests that in the human the change over in control is gradual and that either the mRNA's are long lived or that further important transitions occurs later, for instance after the blastocyst stage. The difficulty in distinguishing maternal from embryonic proteins has been put forward as the reason for the failure to detect changes in enzyme activity during early embryonic stages and makes the use of micro–enzyme assay as a technique for pre–implantation diagnosis of metabolic disorders such as Tay–Sachs disease in the human unattractive (Braude, Monk et al. 1989). The rate of this transition in the human might therefore be investigated further by assessing the rate of protein synthesis in human embryos at various developmental stages.

9.2.2.3 Late cleavage stage embryos.

Few obvious changes in the protein synthetic pattern were detectable after genome activation. This contrasts with the mouse (Goddard and Pratt 1983) which appears to have 2 main groups of transcriptionally dependent proteins, one of which can be detected at the 2–cell stage and the other at the morula stage. Since relatively few later stage embryos were available for analysis, it is possible that a similar phenomenon occurs but was not detected in these experiments. A study aimed specifically at examining later stage embryos would provide more useful information.

All later stage embryos showed evidence of transcriptionally dependent protein synthesis. This along with the inhibition of cleavage in later stage embryos by transcriptional inhibitors would be consistent with genomic

activity being essential to allow progressive development, possibly in order to replace the gradually declining store of maternal mRNA. Thus it might be reasonable to suspect failure of genome activation as a significant cause of cleavage arrest.

9.2.3 Cleavage arrested embryos

The continued synthesis by cleavage arrested embryos of many of the proteins synthesised normally by non–arrested embryos indicates that cleavage arrested embryos are not 'dead' but metabolically active whilst being unable to pass on to the next stage of development. This synthesis may be on maternal mRNA templates, on newly synthesised embryonic mRNA's or synthesis using mRNA derived from mitochondrial DNA. Although synthesis of some proteins on mRNA derived from mitochondrial DNA may be possible, the diversity and number of proteins synthesised strongly suggest that an additional source must be active. The continued synthesis of proteins by oocytes and embryos exposed to transcriptional inhibitors strongly suggests an active, prolonged, role for maternal mRNA templates. This probable contribution of stable maternal mRNA to protein synthesis to embryos which are nevertheless developmentally compromised has important implications for efforts to devise specific tests for the health of an embryo prior to transfer in therapeutic IVF cycles, since it will be difficult to distinguish between the embryonic contribution, which will most likely reflect the potential viability of the embryo, and the maternal contribution, which would continue at a detectable level regardless of the condition of the embryo. Thus pre–implantation diagnostic tests of embryo health could not be based simply on the presence or absence of a particular embryonic metabolite unless that product could be shown to be uniquely embryonic

rather than maternal.

The synthesis by some of the spontaneously arrested embryos of specific proteins whose synthesis was usually suppressed by exposure to transcriptional inhibitors is consistent with the hypothesis that despite the arrest of cleavage some embryos are still capable of gene expression, and that cleavage arrest might still occur even when genome activation has taken place.

Inhibition of genome expression using transcriptional inhibitors, however, invariably resulted in cleavage arrest. Indeed half of the cleavage arrested embryos analysed showed no evidence for the protein changes associated with gene expression. It could therefore be argued that gene expression in those embryos showing evidence of it may not be complete and that cleavage arrest might result from the failure of certain key genes to activate. Given the small amounts of material available and the limitation on technology this imposes, it is difficult to see how such a subtle point might be investigated further. It is possible that once the mechanisms responsible for controlling mitosis in cells are better understood specific proteins such as the cdc proteins (Hunt 1989) might be identified whose synthesis in adequate amounts is essential for the initiation and completion of cell division. Once such proteins are identified it might be possible to probe protein gels derived from arrested embryos to determine whether they are being expressed and in what sort of quantity.

The arrest of cell division before the 4–cell stage in those embryos in which there was no evidence of the protein changes associated with genome activity would be consistent with the hypothesis that development to later stages requires genome activation. However, the presence of detectable genome activity in some embryos that had arrested at the 2–cell stage or before cleavage had even commenced might suggest a

permissive role for genome activation in progressive development rather than a causative role in cleavage arrest.

One possibility would be that DNA replication had occurred but cytokinesis had arrested, attempts to investigate this possibility using DAPI staining of nuclei did not seem to indicate that this was the case. However, simple visualisation and enumeration of spots DAPI stain is too insensitive a technique to determine accurately the amount of DNA present in a cell. Multiple spots of stain may represent nuclear fragments rather than whole nuclei, and apparent absence of staining could be due to a degeneration of the cell with the DNA having become too diffuse to visualise easily.

There are two strategies which could be used to indicate more accurately the amount of DNA in the cell and to allow the recognition of karyokinesis in the absence of cytokinesis. One method would involve accurate assessment of the amount of DAPI stain incorporated using fluorimetry and computer assisted image storage and calculation of the DNA content from the fluorescence. Another approach would be to use Fluorescent In–Situ Hybridisation (FISH) to probe for one or multiple chromosomes. Extra rounds of karyokinesis would then be recognised as extra fluorescent signals. Both techniques are laborious but are currently available.

9.2.4 Single blastomeres

The concurrent presence in the protein synthetic pattern of some embryos of both transcriptionally dependent proteins, and proteins which were insensitive to transcriptional blockade raises the possibility that the blastomeres within an embryo might be heterogeneous with respect to their status of genome activity. The possibility of this type of mosaicism is

supported by Tesarik's (Tesarik, Kopecny et al. 1986) finding of differential uridine incorporation between blastomeres within an embryo.
Technically the investigation of the single blastomeres was the most demanding of the experiments presented and required a change in technique for the work to proceed, namely the switch to radiolabelling before disaggregation rather than after. The relative ease of recovery of intact blastomeres from non–cleavage arrested embryos when compared to cleavage arrested embryos and the greater number of resulting interpretable tracks from them I believe may reflect the underlying low rate of metabolism and poor condition of embryos which have undergone cleavage arrest. Although there is little direct evidence at present to support this assumption, studies of glucose and pyruvate utilisation by pre–implantation embryos (Hardy, Hooper et al. 1989) are consistent with reduced metabolism by those showing abnormal development. Further information could be gained from quantification of the amount of protein synthesised by embryos and comparing the results from cleavage arrested and non–arrested embryos.
Qualitative comparison of the patterns produced by single blastomeres showed that the blastomeres from any individual embryo were more likely to be synchronous,i.e. with all either synthesising some transcriptionally dependent proteins or all failing to do so. However, 2 embryos did show evidence of selective, strong synthesis of a small number of proteins in some blastomeres with little or no detectable synthesis of most of the protein bands commonly seen in the embryonic protein pattern demonstrating that asynchrony does occur.
Selective protein synthesis might be the result of altered protein synthesis at differing points in the cell cycle. This possibility could be investigated further by analysis of blastomeres at regular intervals after mitosis had

been observed. The most practical way of achieving this might be to disaggregate the embryo and then observe the individual blastomeres for mitosis. The delicacy of human embryos and their sensitivity to manipulation would require a relatively large number of embryos to be analysed in order to be fortunate enough to obtain a reasonable number blastomeres that would still go on a divide after the dissagregation. Such an effort would be worthwhile, as it could reveal other useful information about proteins that alter during the cell cycle, as has been observed in the mouse (Howlett 1986b).

Alternatively the reduction in the number of proteins seen might simply reflect synthesis on the most long lived of the maternal mRNA in cells which are otherwise in decline. This possibility might be investigated by sequential analysis of blastomeres at regular intervals after exposure to transcriptional inhibitors to determine which are the last proteins to disappear.

Whatever the underlying reason for the differences observed between blastomeres it seems possible that an individual blastomere within an embryo might not be representative of the whole embryo. Further investigation of this phenomenon and the extent to which blastomeres can show varied levels of viability and still result in a viable embryo is crucial to our appreciation of what represents a normal pattern of pre–implantation development and must represent a high priority for future investigation.

9.2.5. Parthenogenetically activated oocytes

In many ways the studies of the human parthenotes were some of the most rewarding of these series of experiments.

Successful cleavage of parthenogenetically activated oocytes until the 8–cell stage leads to a number of conclusions concerning the genetic

control of early cleavage stages in the human.

Firstly it suggests that paternal genetic information is not essential or is relatively non–contributory in the early stages of cleavage.

Secondly, it appears to confirm that the oocyte provides all the necessary information to direct development at least up until the 8–cell stage.

Third, the failure of parthenotes to develop beyond the 8–cell stage would suggest that like embryos some important event relevant to further development takes place at this stage.

All of these points have been suspected or suggested by previous experiments using embryos, the parthenote however provides the physical evidence to corroborate these theories.

From our previous knowledge about the time of genome activation in human embryos the failure of parthenotes to progress beyond the 8–cell stage might be the result of genetic incompetence with parthenotes being capable of development until such a point as an intact functioning genome was required.

The synthesis by some parthenotes of proteins that appeared to be transcriptionally sensitive in embryos was strong evidence, however, that parthenotes were capable of genome expression. The sensitivity of some of these proteins to transcriptional inhibition was further evidence that parthenotes were capable of synthesising the same, or similar, transcriptionally dependent proteins to those synthesised by embryos.

The effects of exposure to a transcriptional inhibitor before or shortly after activation were surprising. The intention of exposing the oocytes before activation was to avoid any possibility of genome activity contributing to the mRNA pool, thus providing a 'pure' maternal mRNA pattern. It was not anticipated that such a small difference in the time of exposure would result in such a profound difference in the protein synthetic pattern.

As has been discussed previously the results of these experiments where parthenotes were exposed to transcriptional inhibitors were consistent with transcriptionally dependent activity occurring within the first 24 hpa. A low level of genome expression at such an early developmental stage is consistent with the pattern of genome activation in the mouse embryo (Flach, Johnson et al. 1982) where a low level genome activity, detectable within the first 24 hours following fertilisation, is followed by a more generalised gene activation. The possibility that the human also follows this pattern has been examined inadequately previously due to the ethical difficulties associated with gaining access to stages earlier than 4–cells. Time restraints did not allow further experiments to confirm these findings in parthenotes, but these ought to be done. If these experiments also suggest genome activation in the first 24 hours of development, I feel this would form a very strong case for the use of human fertilised embryos to investigate this possibility in normally fertilised oocytes which had been exposed to transcriptional inhibitors. Oocytes surplus to therapeutic requirements in GIFT procedures could be used for these experiments.

9.3 Future directions

Throughout the course of the above discussion I have tried to indicate where I feel further work should be directed. It is absolutely right that human embryos should only be used where no other alternative will do, and that we should at all times have to justify the use of human embryos by reference to the information we hope to gain

It has become clear during the course of this work and my background reading that we have, with the best of intentions, attempted to use our knowledge gained from other mammalian systems to give us a head start with human investigation so as to make the best use of a scarce resource.

Unfortunately entering investigation of the human at a relatively advanced level of knowledge of mammalian systems does mean that we have little by the way of core information on which to base or interpret our findings. Thus, although we might be able to point to findings from other species, often the mouse, which are consistent with findings in the human, it is impossible to be sure that these would apply equally to the human. Furthermore some findings have been found to have no precedent in other species. There is ample evidence from the failure of human embryos to behave in a similar way to embryos from other species to suggest that in many ways the human species is different in its embryonic physiology.

For these reasons I am convinced that it is essential to investigate some of the more fundamental aspects of human embryonic physiology which are already well documented in other species, if for no other reason than to document how closely our development does follow that of other species at a fundamental level. I would propose that these investigations should involve determining the rate of protein synthesis in oocytes and embryos with reference to their stage of development to test the hypothesis that abnormally developing embryos or those which are ultimately doomed to failure of development would show altered or reduced protein synthesis when compared to 'normal' controls. If the hypothesis were confirmed then these measurements would provide a useful guide for strategies aimed at improving embryonic development such as the manipulation of culture conditions.

Another area which I feel needs urgent evaluation is the role and contribution of maternal mRNA. Whilst there is considerable evidence that, as in other species, the early stages of development in the human are directed by information inherited via the oocyte, there is also evidence that in the human this information is utilised in a different manner to

that seen in other species. The means of performing this evaluation are, as yet, unclear and probably require refinement of *in vitro* human oocyte maturation techniques which would allow access to oocytes as they synthesise their mRNA.

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