STUDIES ON THE EARLY DEVELOPMENT OF MAMMALIAN EMBRYOS BY NUCLEAR TRANSPLANTATION

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Thesis presented for a degree of

Doctor of Philosophy

University of Edinburgh

1989

To Cidinha, Michael and Olivia.

Declaration

I declare that this thesis is my own composition, and has not been accepted in any previous application for a degree. The work it describes and the ideas it contains are those of the author, unless otherwise stated. All help given by other people has been acknowledged.

Lawrence Charles Smith

Acknowledgements

I would like to thank Dr. I. Wilmut (A.F.R.C - Institute of Animal Physiology and Genetic Research - Edinburgh Research Station (IAPGR)) for supervising the work presented in this thesis, and for his supportive help and fruitful discussions during the preparation of this thesis. I also want to thank the support given by Dr. R.H.F. Hunter (formally at Edinburgh University; Department of Agriculture) and Dr. J.D. West (Edinburgh University; Department of Obstetrics and Gynaecology) for their support in discussions during the initial and final stages of these studies. I am also grateful to Dr. J.P. Simons for having initiated me on the techniques of embryonic micromanipulation. Experimental animals and laboratory facilities and materials were provided by the IAPGR.

It is a pleasure to thank everyone from the institute's mouse house, especially Mr. D. Parnham and Mrs. F. Thomson. Also at the Large Animal Unit, Miss M. Thomson, Mrs. M. Ritchie and Mr. W.A. Ritchie, for their help with the surgical and anaesthetic procedures. I also want to thank the farm staff (Mr. A.D. McGregor, Mr. W. Seawright and Mr. D. McGavin) for caring for the sheep before and after the experiments. Thanks to all the statistic and computer staff at the IAPGR for their advice during the analysis of the data and to the librarians for their help in tracing sources of reference. I am indebted to Mr. J. White and Mr. F.J.W. Borthwick for constructing the electrofusion apparatus. Last, I thank Mr. N.J. Russel and Mr. R.K. Field of the

Photography Department for producing all the photographic material presented in this thesis.

Finally, thanks to all the friends and colleagues I met in Edinburgh; especially to my wife for her continual and loving support during our stay in this city.

The work described in this thesis was supported initially by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and latterly by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil.

Contents

ABSTRACT

The early development of mammalian embryos has been investigated using a technique of nuclear transplantation. Studies concentrated on the interactions between donor nuclei and recipient cytoplasm of reconstituted sheep and mouse embryos by analysing their ability to support cell division and to develop further *in vitro* or *in vivo* to the blastocyst stage. Sheep experiments showed that some nuclei derived from 16-cell blastomeres (day-4) and cells from the ICM of early blastocysts (day-6) can support development to term and retain similar potential to support development *in vivo* to the morula-blastocyst stage when fused to 'enucleated' secondary oocytes. The highly beneficial effect of adding cytochalasin B for one hour to the medium immediately after fusion indicated an effect of microfilament-dependent cytoskeletal mechanisms on transplanted nuclei at the time of activation.

Cell cycle stage effects were detected for recipient cytoplasm and donor nuclei of reconstituted 1- and 2-cell mouse embryos. Synchronous transplantations enabled higher proportions of embryos to develop *in vitro* to blastocysts when fusing embryonic fragments from the same cell cycle. However, transplantations using nuclei and cytoplasm from different cell cycles produced more blastocysts when the recipient cytoplasm came from later stages of the either 1st or 2nd cell cycle. Karyoplasts from later stages of the cell cycle were also more able to support development in transplantations to 1- and 2-cell enucleated embryos.

Cleavage time was controlled by interactions between nucleus and cytoplasm already at the 1-cell stage with an indication that it may be necessary for DNA replication to be completed before mitosis and cytokinesis. Karyoplasts from transcriptionally inactive embryos were unable to support development when fused to cytoplasm after transcriptional activation suggesting a role for maternal cytoplasmic messages in activating transcriptionally inactive embryonic nuclei. Electrofusion parameters for strength and duration of the d.c. pulse were established for fusing mouse and sheep embryos. Preceding a.c. pulses were helpful in aligning 2-cell mouse embryos between the electrodes and improved fusion rates in sheep embryos when using ICM cells.

Together, these experiments have enabled a better understanding of the nucleocytoplasmic control mechanisms during early stages of mammalian development. The results with sheep embryos revealed potential applications for the production of genetically identical farm animals and, assuming that embryonic stem cells can be isolated from farm animals and that these have similar potential as ICM cells, nuclear transplantation may become the most efficient means of producing non-chimaeric transgenic offspring from transformed embryonic stem cells.

Chapter 1

Introduction

Recent improvements in micromanipulation and microscopical techniques have opened new possibilities for experimental research on mammalian embryology.

The use of the technique of nuclear transplantation to investigate the mechanisms by which early development is controlled, through genetic and epigenetic factors, will enhance our understanding of the genetic control of mammalian embryos during the preimplantation stage.

Due to viviparity, experimental research with mammalian embryos is largely limited to the period from final gametogenesis to late preimplantation for which the systems of *in vivo* or *in vitro* culture are adequate for some species. The main purpose of investigating this area is that of understanding the basic biology of the embryo at early stages of development and differentiation. Of particular interest are the interactions between the nucleus and the cytoplasm which affect either cleavage alone or the initial differentiation steps. Also important, are the stages in which the genome (nucleus) is modified to enable the transcription of specific messages and the ability of different cytoplasmic components to affect this orchestrated pathway of development.

There are several practical applications for research in this area. One concerns the medical and veterinary sciences where by

understanding the biology of embryonic abnormalities and asynchronies that lead to embryonic mortality we could reduce infertility in humans and increase the reproductive performance of animals. There could also be great advantages for animal breeding and production if the development of a technique of cloning laboratory and farm animals could be attained using nuclear transplantation. This would have both scientific and commercial applications.

Therefore, this literature review is concerned with describing some of our current knowledge on mammalian embryology drawing a background of events relevant to the later experimental chapters. Next, it provides information on experimental approaches concerned with the topic of embryonic development and cell cycle control. The review is followed by a general description of the materials and methods employed during the experiments. The experimental work is divided in five chapters of which one is related to the establishment of the technique of electrofusion of embryos. The other four chapters refer to experiments with a biological interest concerning both the investigation of developmental potentialities of nuclei and cytoplasms in sheep and mouse embryos and the nucleocytoplasmic control mechanisms of cleavage in mouse embryos. A final conclusion chapter describes some of the potential applications of the technique for scientific and commercial purposes.

1.1. Relevant events during development

This section is concerned with describing some of the cellular and molecular events taking place during early embryogenesis which are particularly related to the later experimental chapters. Most information about this subject has been derived from experiments on a small number of mammalian and non-mammalian laboratory species (mainly in mice) and care must be taken when extrapolating to other mammalian species.

1.1.1. Germ cells, meiosis and gametogenesis:

The germ cell is unipotent, since it can only differentiate in one direction, either into a spermatozoon or an egg, whereas the fusion product of both germ cells ,i.e. the fertilized egg, is totipotent and gives rise to all somatic cells as well as the new germ cells (Byskov, 1982). In most vertebrates, the germ line is established during early embryogenesis. However in mammals, the primordial germ cells can only be identified at the primitive streak stage. From the base of the allantois these cells migrate to the genital ridges during which period they proliferate at a steady rate. Soon after the first signs of sexual differentiation of the gonads have occurred, the germ cells in the ovary enter meiotic prophase and embark on a long period of oogenesis. At about the same time the germ cells in the testis cease proliferation and enter the G1 phase of mitotic interphase (McLaren,1985).

Four main biological significances have been considered for meiosis (Holliday, 1984). First the conventional view that it

generates, by recombination and sexual reproduction, the genetic diversity on which natural selection can act. Second, that recombination at meiosis plays an important role in the repair of genetic defects in germ line cells. Third, that it is essential in animals for the reprogramming of gametes which give rise to the fertilized egg. Fourth, that it helps to maintain the immortality of the germ line, possibly by a process of rejuvenation including the removal of faulty RNA and protein molecules, or by the elimination of defective meiocytes.

Female meiosis is arrested at diplotene soon after birth and remains at this stage until puberty. Although DNA synthesis does not occur during the arrested period, the chromosomes develop loops ('lampbrush') and become actively involved in the synthesis of RNA (messenger and ribosomal). At this point the oocyte embarks on a period of rapid growth increasing more than 300 times in volume. The fully grown oocyte contains about 200 times more RNA, 60 times more protein, 1000 times more ribosomes and 100 times more mitochondria than the typical somatic cell (Wassarman,1983). Some macromolecules are imported by endocytosis from granulosa cells. These cells also play a role in directing protein synthesis within the oocyte probably regulated by steroid signals (Moor and Osborn, 1983).

Pre-ovulatory hormonal alterations (LH surge) induce transformations in the follic le which activate meiosis and essential maturational processes in the oocyte. Meiotic maturation is characterized by the breakdown of the germinal vesicle (GVBD), condensation and separation of homologous chromosomes, with the emission of the first polar body and further arrest at metaphase

II. The GVBD releases proteins previously sequestered in the nucleus, which initiate a cascade reaction of morphological and molecular changes including 'pulsatile' movements of mitochondria between the cortex and the centre of the oocyte (Van Blerkom and Runner, 1984). Fusion of the sperm to the oocyte membrane reactivates meiosis which is completed by the emission of the second polar body.

In contrast to the oocyte, the spermatozoon is an extremely streamlined cell containing , apart from its nucleus, only elements for essential functions such as motility and penetration of the zona pellucida. Instead of histones, the DNA of the mature mammalian sperm is found tightly associated with protamine-like proteins. This renders the DNA replicatively and transcriptionally inactive at the time of fertilization. However, soon after fusion to the egg membrane, the protamines are released and oocyte cytoplasmic histones are added to the sperm DNA. In the mouse, the average number of mitochondrial genomes contributed by the sperm to the next generation is estimated to be no more than one per thousand maternal contributions and it has been suggested that all sperm organelles (mitochondria and centrioles) are destroyed within minutes of fusion (Gyllesten *et al.*, 1985).

1.1.2. Embryogenesis:

This section describes the molecular and cellular events which occur after fertilization during the early cleavage stages of mammalian development with special attention to the mouse embryo where most information is available.

1.1.2.1. Initial mitotic cleavages: Shortly after fertilization the sperm's nuclear membrane disintegrates and its chromatin decondenses due to oocyte cytoplasmic factor(s) synthesized after GVBD. A new nuclear envelope soon develops around the chromatin forming the male pronucleus (Thibault, 1977). Soon after the extrusion of the second polar body, the internal set of chromosomes gradually becomes surrounded by a membrane, resulting in the formation of the female pronucleus (Howlett and Bolton, 1985). During apposition, the two pronuclei enlarge significantly, move towards the centre of the ovum and come to lie in juxtaposition, terminating a long G1 phase of the first cell cycle. In mammals, the synthesis of DNA takes place during the final stages of pronuclear apposition and the association of maternal and paternal genomes (syngamy) occurs after the breakdown of pronuclear membranes and positioning of the two groups of chromosomes on the first spindle for the first cleavage.

All stages of embryogenesis up to the 2-cell stage occur independently of major activity of the embryonic genome in the mouse and are controlled at a post-transcriptional level by oocyte and follicular components assembled during oogenesis. The transition from maternal to embryonic control is characterized by two bursts of putative transcription just before and immediately after DNA replication, and the translation products are detected a few hours later. Contemporary with the expression of the embryonic genome there appears to be a selective inactivation or destruction of much of the pre-existing maternal m-RNA (Bolton *et al.*, 1984). It is also at this stage that the oocyte derived mitochondria transform from the atypical spherical and concentric

cristae shape to normal ovoid and transverse cristae shape (Piko, 1975).

The transition from maternal to embryonic control of development at the 2-cell stage is probably responsible for the extremely prolonged G2 phase of the second cell cycle. Certain strains of mice do not develop beyond the 2-cell stage *in vitro*. Reciprocal crosses between 'blocking' and 'non-blocking' strains have shown that the genotype of the egg alone determines whether the embryo blocks at the 2-cell stage. However, it is not clear whether the differences between the two types of egg arise from the characteristics induced by their respective genital tract environments or from differences in their capacities to adapt to *in vitro* conditions (Goddard and Pratt, 1983). The ability to overcome the 'block' by injecting very small volumes of 'non-blocking' cytoplasm (Muggleton-Harris *et al.*, 1982) suggests that the rescuing factor(s) is likely to be a modifying enzyme, co-factor, or self replicating molecule or organelle that is defective in 'blocking' strains after the exposure to known culture conditions.

Cleavage to the third cell cycle is not synchronous within embryos and it has been shown that the progeny from the earliest blastomere to cleave contribute more to inner cells of the morula and, therefore, to the embryonic tissues. This is because the first cell to cleave at the 2-cell stage generates more cell contacts than the last cell to divide and, therefore, is likely to contribute a disproportionate amount of cytoplasm and membrane to the central region of the developing 4- and 8-cell embryo (Graham and Lehtonen, 1979). However, this does not create any positional differences of a qualitative nature between blastomeres (Johnson,

1981a). The initial cell cycles do not produce any net growth of embryos as each blastomere halves approximately in size at each division.

1.1.2.2 Early morphological differentiation: The fourth cell cycle is characterized by the first morphological changes in the mouse embryo. After cleavage to the 8-cell stage a massive qualitative reorganization of the embryonic cytoplasm and membrane occurs, which develops around a radial axis. These changes lead to the polarization both of cell surface and cytoplasm. Cells change from a spherical to an epithelial morphology and microvilli and binding sites become located at the apical surface and the nucleus is displaced to a basal position. These changes minimize intercellular space and obscure cell boundaries leading to the flattening (compaction) of the cells. The following cleavage to the fifth cycle (16-cell) will create two recognizably different subpopulations of apolar and polar cells (Johnson and Ziomek, 1982). The differences observed between these cells arise as a result of a differential inheritance from polarized 8-cell blastomeres. It is suggested that these apolar and polar subpopulations will later become the ICM (inner-cell-mass) and trophectoderm, respectively (Johnson, 1981b).

The first macroscopically observable divergence of embryonic cells into two phenotypically distinct populations occurs at the blastocyst stage with the formation of ICM and trophectoderm. These two cell populations also differ immunologically and biochemically from each other. The trophectodermal cells, which arise from the outer layer of the morulae, elongate and form strong intercellular junctional complexes that provide the ultrastructural

basis for the accumulation of fluid in the blastocoel and expansion of the blastocyst. In comparison to trophectoderm cells, cells from the ICM are rounded and are not interconnected by means of junctional complexes. It is only after a few more cleavages that the normal nucleo:cytoplasm ic ratio is achieved and net growth is initiated.

It has been shown that the cells from the ICM and trophectoderm are committed to different pathways of development and, therefore, exhibit limited developmental capacities. Only extraembryonic structures (placental membranes) are derived from the trophectoderm, whereas ICM cells can give rise to all tissues of the later fetus and some extraembryonic membranes (including the allantois, amnion and yolk sac), but have lost their potential to form trophectoderm (Rossant, 1984). Some cells derived indirectly (embryo carcinoma) or directly (extraembryonic stem cells) from the ICM can be cultured and maintained undifferentiated *in vitro* and still retain pluripotential capacity to give rise to all tissues, including the germ line, when injected back into a blastocyst to produce a chimera (Rossant and Papaioannou, 1984).

1.1.2.3. Differentiation patterns during development: It is possible for fully grown oocytes of some laboratory species, to undergo spontaneous resumption of arrested meiosis during culture *in vitro*, to be fertilized *in vitro* and to develop normally through all stages of preimplantation *in vitro*. This provides evidence to suggest that early embryogenesis is regulated by the autonomous expression of an intrinsic programme with no requirements for specific exogenous signals or factors, such as conditions or

macromolecules unique to the oviduct or uterus (Van Blerkom, 1985).

This intrinsic programme is controlled by events which appear to give a periodicity to early mouse embryogenesis. These events are (1) fertilization, (2) destruction of most of the maternal m-RNA and switching on of the embryonic genome at the 2-cell stage, (3) the polarization of cells at mid 8-cell stage, which lays the foundation of ICM:trophectoderm divergence and (4) the beginning of blastocyst expansion and commitment to a restricted developmental fate at some time between 32- and 64-cell stage. Each of these events mark a fundamental change in the embryo and appear to coincide with the loss of developmental option (Johnson, 1981a).

The timetable for early development involves a minimum of three programmes: the 'oocyte programme' commencing with the reactivation of the oocyte and GVBD, an 'activation programme' commencing with fertilization, and an 'embryonic programme' commencing with the expression of the embryo's own genome (Johnson *et al.*, 1984). Recent evidence suggests that these programmes may be preceded by an 'oocyte growth programme' (Canipari *et al.*, 1984). Some of the molecular mechanisms that regulate these programmes have been examined. The oocyte programme is activated via the effect of LH on granulosa cells which, by reducing cAMP levels, leads to changes in phosphorylation of various phosphoproteins (Schultz *et al.*, 1983). The activation programme may be catalysed by changes in Ca++ and ionic fluxes induced by fertilization or parthenogenetic activation. The activation of the embryonic programme seems to

depend either on the first round of replication, or the first round of karyokinesis, or both (reviewed by Johnson *et al.*, 1984).

The first noticeable event to be controlled by the embryonic genome is the cleavage to 4-cell which relies on the translation products of both boosts of transcription around the second DNA replication cycle (Bolton *et al.*,1984). The transcripts alone are responsible for all events up to mid 8-cell stage when further transcripts are required. Therefore, there is some evidence to suggest that at least one DNA replication cycle , but probably two, are necessary to produce structural modifications to the DNA itself or to regulating proteins associated with DNA (Johnson *et al.*, 1984). If true, this would induce a periodical pattern to differentiation at every two rounds of DNA replication which, in the mouse, is of the order of 27 hours (Johnson, 1981b).

There is evidence to suggest that during DNA replication major changes might occur to the DNA and associated proteins (Johnson *et al.*, 1984). It has been suggested that reprogramming of the genome would take place during the chromatin modifications at replications. Reprogramming of the sperm derived chromatin during the 1-cell stage could occur during its decondensation and reconstitution with oocyte derived histones. The mechanisms of gene inactivation are correlated with a *de novo* methylation activity in the very early chick embryo (Groudine and Weintraub, 1982). This is supported by findings of heavily methylated repetitive DNA sequences from the two cell stage onwards while the same sequences in both spermatozoa and oocyte are relatively undermethylated (Chapman *et al.*, 1984). Presumably, during development and differentiation a class of early acting

embryonic genes would escape the inactivation process by the protective action of oocyte factors, by the presence of strong enhancer sequences that resist the inactivation process or react paradoxically to it, or by a combination of both (Tanaka *et al.*, 1983).

1.1.3. Commitment and Differentiation:

Commitment, and determination, of cells are states that precede differentiation in which cells are fixed to a specialized fate, but the manifestation of that fate has not yet become apparent (reviewed by Mclean and Hall, 1987). In almost all cases the state of being determined is only detectable by experimental intervention. Differentiated cells are specialized in function and structure. These cells acquire distinct morphology, distinct patterns of cell behaviour and at least some of the macromolecular constituents of the cell can be distinguished from those of other cells in the same organism that have become specialized to different functions. Each specialized cell population is not only similar at the level of protein content and function, but also at the DNA level by expressing precisely the same restricted subset of genes.

How can a single cell possibly contain the specificity and selectivity required to produce the vast range of differentiated cell types of the embryo and adult? Commitment of the cells in the early embryo could occur, theoretically, in one of two ways. Firstly, if cytoplasmic constituents, i.e. morphogenetic determinants, were segregated within the oocyte, so that some became localized within one cell and some within other cell at the first cleavage division,

then the fate of those cells would differ. With progressive segregation of such factors, the fate of cells would become more and more restricted. Embryos possessing such determinative molecules are said to display mosaic development and to have determinative patterns of cleavage (i.e., molluscs, nematoids, annelids and ctenophores).

The second type of commitment during development is also based on the existence of morphogenetic determinants that either are present in the oocyte, but not segregated to individual cells before cleavage commences, or arise within individual cells after cleavage has commenced. In either case, the fate of such cells will remain the same during early development. When determination occurs in these cases, it comes about because of interaction between individual cells of the embryo, and these interactions may result in the synthesis of new cytoplasmic determinants. Embryos displaying such control are said to have indeterminate or regulative development (i.e., echinoderms, amphibians and mammals).

Because determination occurs whether development is mosaic or regulative, one should not draw too rigid a distinction between the two types of development. Embryos with regulative development become mosaic at some stage, and embryos with mosaic development do make use of interactions, even quite early during their development. The critical issues are that cell fate becomes restricted through determination; that determination is progressive; and that determination occurs in a stepwise fashion, either very early during development (before cleavage commences) or somewhat later (during the cleavage process).

The first commitment that mammalian embryos make is determination of the inner-cell-mass from which the embryo will develop, or of the trophectoderm, the embryonic portion of the placenta. These determinations do not occur until after the 8-cell stage since destruction of individual cells up to the 8-cell stage does not lead to deficiencies in the resulting embryo (Seidel, 1952). Tarkowski and Wroblewska (1967) found that most individual blastomeres isolated at the 4- and 8-cell stages develop into trophoblast vesicles devoid of inner-cell-mass. They proposed that the differentiation in trophectoderm and inner-cell-mass was determined by the outer and inner positions of blastomeres at the morula stage (16 to 32 cells). This "inside-outside" hypothesis predicted that blastomeres would remain totipotent at least until they assume their position at the morula stage. A substantial body of work confirms this prediction, showing that mouse embryo blastomeres maintain their potency at the 2- to 16-cell stages (Kelly, 1977; Hillman *et al.*, 1972; Rossant and Vijh, 1980; Ziomec *et al.*, 1982). A similar degree of totipotency seems to exist in cleavage -stage rabbit, horse, cow and sheep embryos (Papaioannou and Ebert, 1986).

It has been noted that the development of junctions between cells is consistent with the creation of different microenvironments in the outer and inner portions of early mammalian embryos (reviewed by Johnson, 1985). There are no intercellular junctions between cells of 4-cell stage embryos, but by the 8-cell stage, tight junctions preferentially couple outer cells to one another but not to the inner cells. From the 8-cell to the blastocyst stage, gap junctions couple inner cells to one another

and to the outer cells. These gap junctions are lost at the same time as determination becomes irreversible, suggesting, but not proving, that cell-cell communication occurs via gap junctions. If this is so, and given that gap junctions are only 1.5 nm wide when fully open and allow passage of only ions and molecules up to 1500 daltons, then such molecules, or metabolic or ionic gradients are the most likely bases for commitment of the cells of early mammalian embryos (Pitts and Finbow, 1982).

1.1.4. Cell cycle regulation:

During early embryonic development cell differentiation must be regulated in time as well as in space. In some non-mammalian species it is clear that one important component of temporal regulation is the cell cycle (Satoh and Ikegami, 1981 a,b; Newport and Kirschner, 1982 a,b; Edgar *et al.*, 1986) and in the mouse embryo also there is evidence that at least some early steps in differentiation may relate to specific cell cycles or cell cycle phases (Smith and McLaren, 1977; Spindle *et al.*, 1985; Howlett, 1986; Garbutt *et al.*, 1987). These relationships of cell cycle with differentiation emphasize the importance for a better understanding of the mechanisms underlying cell cycle regulation. Although briefly, this section will describe some of the basic concepts in cell cycle compartmentalization and some of the few known factors which seem to be implicated with the different phases of the cell cycle in eukaryotes.

1.1.4.1. The mitotic cycle: Most cell components are made continuously throughout the interphase period between cell

divisions. It is, therefore, difficult to define distinct stages in the progression of the growing cell through interphase. One outstanding exception is DNA synthesis, since DNA is replicated only during a limited portion of interphase (S-phase). The other distinct stage is cell division (M-phase), which involves both nucleus division (mitosis) and cytoplasmic division (cytokinesis). This leaves two gap periods, namely G1 phase (before S-phase) and G2 (after S-phase). These G1, S and G2 phases (interphase) normally comprise 90% or more of the total cell cycle time. For example, in rapidly dividing cells of higher eukaryots, the successive cell divisions (M-phase) that interrupt interphase generally occur every 16 to 24 h, and each M-phase itself last only 1 to 2 h.

In multicellular organisms different cell types divide at different rates so that their numbers can be kept at a level that is optimal for the organism as a whole. In a mammal, some of the cell types have generation times of as little as 8 h while others of 100 days or more (Cheng and LeBlond, 1974). These differences in cell cycle times are due mainly to variations in the length of G1. Cell division in culture can be slowed or stopped by limiting the supply of essential nutrients, by depriving the cell of essential protein growth factors, by adding low levels of protein synthesis inhibitors, or by allowing the cells to become overcrowded. In every instance, the cell cycle is arrested in G1 phase. These findings imply that once the cell has passed out of G1, it is committed to completing the S, G2 and M phase. This no return point, or restriction point (R) has been shown to occur late in G1 (Pardee *et al.*, 1978).

Cell cycles in early embryos function simply to subdivide the embryo mass in the absence of growth (cleavage). Early cleavage cycles of the mouse embryo are longer than those of other embryos (12 to 24 h as opposed to 30 minutes in Xenopus, sea urchin and clam) and incorporate both G1 and G2 phases. The first and second cell cycles last for approximately 18 h each with prolonged G1 and G2 phases, respectively. The following cleavage cycles last 12 h with G1 and G2+M phases of 2 h each and S phase of 8 h (Bolton *et al.*, 1984; Howlett and Bolton, 1985; Smith and Johnson, 1986; Chisholm, 1988).

1.1.4.2. Cell cycle regulation: It is not known what determines whether a mammalian cell will go beyond the restriction point (R) in G1 and start a new cycle. One plausible hypothesis is based on the observation that cells behave as if they need to accumulate a threshold amount of some unstable "trigger protein" (also called U protein, for unstable protein) in order to pass through R and thereby be triggered to make DNA and divide. Because of its instability, this hypothetical protein would reach a concentration great enough to initiate a cycle of cell division only when synthesized relatively rapidly. In addition, its concentration would drop precipitously during the M phase, when protein synthesis is greatly reduced, and would build up toward the threshold level in G1 (Pardee, 1974).

A maturation or mitosis promoting factor (MPF), which induces dissolution of the nuclear membrane and condensation of chromosomes, has been described in oocytes and cleaving embryos of sea urchin, clam and Xenopus (Ford, 1985). MPF activity peaks immediately preceding mitosis and declines thereafter and when

microinjected *in vivo* it catalyses progress through mitosis (or meiosis) to the next interphase (Miake-Lye and Kirschner, 1985). Cycling cells also contain a factor(s) which antagonizes or inactivates MPF ('anti-MPF') (Murray, 1987) and in oocytes an activity responsible for arresting cells in metaphase ('cytostatic factor' or CSF) has been identified (Newport and Kirschner, 1984).

A careful study of protein synthesis following fertilization of germinal vesicle stage surf clam oocytes revealed that synthesis of several proteins ceased, while synthesis of three major proteins was switched on abruptly (Rosenthal *et al.*, 1980). Two of these proteins ('cyclins') exhibit an interesting periodic behaviour: they are destroyed specifically at the time of metaphase/ anaphase transitions of the first and second cleavage divisions. The function of the cyclins is unknown, but they are postulated to play a role in catalizing the onset of mitosis (Evans *et al.*, 1983). There are no obvious 'cyclin' proteins in the mouse embryo but an increase in protein phosphorylation and a cyclic post-translational modification of pre-synthesized polypeptydes occur during the second meiotic and the first two mitotic M phases (Howlett, 1986). The injection of cytoplasm of embryos at or around the first and second mitotic divisions into embryos undergoing the *in vitro* phenomenon of the '2-cell' block had a greater effect in rescuing 'blocked' embryos than did the cytoplasm of S phase embryos indicating the presence of MPF activity also in mammalian embryos (Pratt and Muggleton-Harris, 1988).

1.2. Experimental approaches to development:

This section will describe some of the experimental work using cell hybrids initially to investigate the control of cell cycle and later to examine other nucleo-cytoplasmic interactions. Finally, reports describing experiments using nuclear transplantation to reconstruct embryos will also be examined. Those reports concerning the developmental potential of nuclei of both amphibian and mammalian embryos will be described separately in a last section.

1.2.1. Cell cycle studies:

Several cell models have been used to study the control mechanisms of cell division and of the role of the nucleus in the event, and advantages and disadvantages are found in all of these. The most used model is that of Amphibian eggs (Rana and Xenopus). The cytoplasm of these eggs supports the replication of foreign DNA regardless of the tissue from which they came or of their specific origin (Briggs and King, 1952, 1957; King 1966; DiBerardino and King, 1967; Gurdon, 1964). The disadvantages found in this model are that this is a specialized cytoplasm which has gone through several prematuration events and that early divisions are very rapid and without an accompanying growth of cytoplasm. Although the volume of donor cytoplasm which enters the cell at fusion is small, the capacity of egg cytoplasm to translate foreign RNA must be taken into account (Gurdon *et al.*, 1971).

The use of fused tissue cells, particularly cells grown in culture, has increased awareness of nucleocytoplasmic interactions in the control of nucleic acid synthesis. With the availability of good synchronization methods it has been possible to combine cells from each of the cell cycle phases, M, G1, S, G2 (Johnson and Rao, 1970; Rao and Johnson, 1970), and in addition to study the changes which occur in initiation of DNA synthesis in G0 nucleus of blood cells (Johnson and Harris, 1969 a,b; Harris, 1974; Johnson and Mullinger, 1975). The disadvantages of this system are the stress conditions imposed upon the tissue culture cells during the experimental preparation (chemical synchronization, centrifugation, trypsinization, medium changes and Sendai virus fusion) which may produce some changes in the nucleus and its cytoplasmic environment. Another disadvantage is that the cytoplasm and membrane become part of the heterokaryon complex. Attempts have been made to overcome this drawback by spinning the donor nucleus into a small cytoplasmic envelope before fusing it to a host cell, but this may introduce yet another traumatic experience for the nuclear components (Ege and Ringertz, 1975).

Lower eukaryotic cells, such as Amoeba proteus and Physarum, have also been used to study cell cycle regulation. Although experimental results in DNA studies have in each case been confirmed in another test system, extrapolation of results to mammalian cells must be done with care. The advantage of the Amoeba model is that it is the only model in which the donor nucleus is transplanted into the host cell without accompanying donor cytoplasm and/or cell membrane. The long cell cycle (48 h) with non-stress selection has resulted in increased accuracy in the

timing of the homo- and heterophasic karyons. The disadvantage of Amoeba is that it lacks G1 phase and so the presynthetic activation for DNA replication cannot be separated from the premitotic events. Physarum has a single cytoplasm containing thousands of naturally synchronized nuclei whose synthetic activity can be studied biochemically as a group (Cummins and Rush, 1968). The major disadvantage of this system is that the resultant heterokaryon is composed of the nuclei, cytoplasm and cell membrane of both plasmidia and that individual nuclear and cytoplasmic interaction studies are impossible.

Fusion of cells at different stages of the cell cycle, using the four models described above, have been used to study the control of DNA synthesis and the conclusions will be summarized. Initiation of DNA synthesis can be induced by fusing G1 phase nuclei to S phase cytoplasm. Even a G0 nucleus which has opted out of the cell cycle can be triggered back to the cycle when fused to S phase cytoplasm. Although these experiments suggest that the cytoplasm is in a state which initiates synthesis of DNA, the nature of the DNA synthesis-inducing substances is unknown. Successful initiation of DNA synthesis requires RNA and protein synthesis, suggesting that de novo synthesis of protein ('initiation protein') may be important (for review see Klein and Bonhoffer, 1972). Furthermore, these proteins may have to penetrate into the nucleus to exert their influence since in heterokaryons made with nucleated erythrocytes (Ringertz *et al.*, 1971), the activation of the erythrocyte nucleus is preceded by a massive influx of proteins from the cytoplasm into the nucleus (Goldstein and Ron, 1969).

The inability of the G2 nucleus to undergo another round of DNA synthesis without an intervening mitosis appears to be a general phenomenon for eukaryotic cells. The unresponsiveness of the G2 nucleus may be accounted for by its DNA organization since significant differences in the degree of condensation of G1, S and G2 chromatin can be detected (Collins *et al.*, 1977). The maintenance of DNA synthesis seems to follow a different pattern. Once DNA synthesis has been initiated in the nucleus, the S nucleus continues to replicate its DNA regardless of the cytoplasmic environment. Thus, an S nucleus continues to incorporate Tdr-^{3}H in the presence of G1, S, or G2 cytoplasm and only mitotic cytoplasm causes inhibition, suggesting either a lack of activity of synthesizing enzymes or a change in the DNA template during this period.

In general, polykaryons formed by virus induced fusion have synchronized mitotic divisions. Rao and Johnson (1970) found almost perfect (99.5%) synchrony among nuclei in HeLa G1/S or HeLa G1/G2 heterokaryons. More than one factor seems to be involved in the synchronization of nuclei. Firstly, late G2 and mitotic cells seem to contain factors which induce G1 or S nuclei to enter mitosis more quickly. Also S phase cells contain potent factors which prevent or delay the entry of G2 nuclei into mitosis (Rao and Johnson, 1970) and mitosis itself may be prolonged so that lagging nuclei catch up with advanced nuclei during an extended metaphase (Oftebro and Wolf, 1967; Heneen *et al.*, 1970).

Fusion of a mitotic cell with a cell in interphase results in a precocious attempt of the interphase nucleus to enter mitosis. The

chromatin condenses into a chromosome-like structures, sometimes with a fragmented appearance, and the nuclear membrane disappears. This phenomenon has been termed premature chromosome condensation (PCC) (Johnson and Rao, 1970). This phenomenon has offered a new approach to the study of the organization of DNA replication units since the fragmented nature of S phase PC chromosomes has been connected to points of greatly extended chromosome fibres which are involved in the process of DNA synthesis (Röhme, 1974, 1975). The usefulness of being able to visualize interphase chromosomes by inducing PCC has also been appreciated in radiobiological work using X-irradiation, UV and alkylating agents (Hittelman and Rao, 1973, 1974 a,b).

1.2.2 Heterokaryons:

The term heterokaryons is used to describe multinucleate cells formed by the fusion between cells with genotypic or stable phenotypic differences. Gamete heterokaryons have been studied using Sendai virus to fuse spermatozoa with somatic cells (Brackett *et al.*, 1971; Sawiki and Koprowski, 1971; Gledhill *et al.*, 1972; Gabara *et al.*, 1973; Benedich *et al.*, 1974). After penetration of sperm into somatic cells, the sperm heads tend to retain their characteristic form and to remain in an inactive state in the cytoplasm; perhaps because they are confined to phagosomes (Benedich *et al.*, 1974). Some sperm heads remain visible in heterokaryons for as long as one month after fusion (Sawiki and Koprowski, 1971). In other cases, however, the chromatin of the spermatozoa has been dispersed (Zelenin *et al.*, 1974) and DNA

synthesis (Gledhill *et al.*, 1972) and transcription (Benedich *et al.*, 1974) has been initiated. There are indications, however, that haploid nuclei at a somewhat earlier stage of spermiogenesis may be easier to reactivate since proliferating hybrid cells could be produced by fusing rat spermatids with cells of an established mouse cell line (Nyormoi *et al.*, 1973). It has also been found that eggs fused with Sendai virus to uncapacitated sperm will form polar bodies and undergo cleavage (Ericsson *et al.*, 1971).

Attempts have also been made to fuse unfertilized and fertilized mouse eggs with somatic and embryonic cells (Graham, 1969; Baranska and Koprowski, 1970; Lin *et al.*, 1973; Tarkowski and Balakier, 1980; Czolowska *et al.*, 1984, 1986). Baranska and Koprowski (1970) noted that when unfertilized mouse eggs were fused with a variety of mouse, hamster, monkey or human cells, development proceeded to the early morula stage. Interphase nuclei behaved differently when fused to maturing, matured and activated oocytes (Tarkowski and Balakier, 1980). Two-cell blastomere nuclei fused to oocytes undergoing maturation underwent premature chromosome condensation (PCC), and two sets of chromosomes (meiotic and mitotic) came close to each other but the mitotic chromosomes were not fully incorporated into the meiotic spindle. When follicle cells and 2-cell blastomeres were fused with non-activated secondary oocytes, their chromosomes remained separated from the spindle of metaphase II. However, when the oocytes were activated the nuclei remained in interphase and the follicle cell nuclei increased their volume eight times. It has also been shown that thymocyte nuclei introduced before (10 to 30 minutes) or shortly after (up to 60

minutes) activation will grow larger than the female pronucleus. However, if fused long before activation (>30 minutes), these same nuclei will undergo PCC with subsequent reformation of nuclei that are sometimes deficient (as indicated by the presence of micronuclei), or of hybrid character (Czolowska *et al.*, 1984).

Ultrastructural studies of oocyte-thymocyte heterokaryons have shown that complete nuclear remodeling, initiated by nuclear envelope breakdown and chromosome condensation (which is followed by formation of pronucleus-like nucleus) is possible only during a short time gap between metaphase II and telophase of meiotic division (Szollosi *et al.*, 1988). This indicates that the thymocyte nucleus can follow the sequence of morphological changes in concert with the development of the native nucleus only after exposure of the chromatin to the ooplasm. If hybridization was effected with pronuclear oocytes, the thymocyte nucleus retained its interphase character but showed particular modifications in nucleolar morphology (identical to changes observed during reactivation of the nucleolus in stimulated lymphocytes) and in the activity of the nuclear envelope (blebbing). This suggests that the nucleus not exposed to MPF activity would be influenced by both the programme specific for the oocyte (blebbing) and the programme inherent in the introduced somatic cell nucleus (Szollosi *et al.*, 1988).

Mouse blastomeres from 2- to 8-cell embryos have also been fused with established mouse cell lines (Bernstein and Mukherjee, 1972). At the 2-cell stage mouse A9 nuclei in A9+blastomere heterokaryons showed a reduction in the rate of RNA synthesis. On the other hand, the blastomere nuclei, which normally show a low

level of transcription at this stage, had an accelerated RNA synthesis. In heterokaryons using blastomeres from the 4-cell stage, a stage where blastomere nuclei normally synthesize RNA 5 to 10 times more rapidly than at the 2-cell stage, no depression in A9 RNA synthesis could be detected. It appears, therefore, that the RNA synthesis of the A9 nuclei is controlled by the state of the blastomere cytoplasm.

1.2.3. Reconstruction of mammalian embryos:

McGrath and Solter (1983) developed a non-invasive technique for nuclear transplantation in mouse embryos which has been largely used in early developmental studies to fuse blastomere karyoplasts and single embryonic cells to enucleated embryos. Reports have concentrated on investigations concerning the role of maternal and paternal genomes in development and whether maternal traits were inherited through the nucleus or cytoplasm. Other reports have dealt with the potential of mammalian nuclei and these will be described later together with those using amphibian eggs (Section 2.2.4)

The advantages of using uniparental derived mammals, of either haploid or diploid genome, to understand factors that influence early development have been largely recognized (see reviews by Graham, 1974 and Kaufman,1981). These studies have concentrated mostly on the development of unfertilized, but artificially activated, eggs (parthenogenesis) where differentiation is exclusively controlled by the maternal genes. This allows investigations of the role of the male genome in early development,

differentiation and also of haploid and diploid gene expression in embryogenesis. Although normal development of parthenogenetically activated embryos has been achieved in the mouse to the forelimb bud stage (Kaufman *et al.*,1977), most parthenogenones die at or soon after implantation. It was suggested that their death is caused by the absence of a genetic or extra genetic contribution from the sperm or by the expression of recessive lethal mutations caused by excessive homozygosity of the genome.

To investigate these assumptions, nuclear transplantation techniques were used to produce gynogenetic and androgenetic embryos by removing the male or female pronucleus soon after fertilization and diploidizing the genome with cytochalasin B. Initial reports described normal development *in vitro* to blastocyst (Markert and Petters,1977) and later it was reported the birth of homozygote diploid uniparental females of gynogenetic and androgenetic origin (Hoppe and Illmensee,1977). The main difference between gynogenetic and parthenogenetic embryos is the form of activation (sperm or artificial) which suggested that the death of parthenogenones is caused by the lack of some extra genetic factor.

After continuous attempts by other laboratories to reproduce these results without success, it was suggested that enucleation had been incomplete in Hoppe and Illmensee's experiments which involved the technique of puncture of the egg's plasma membrane (McGrath and Solter,1984c). Later experiments, using a noninvasive technique of nuclear transplantation, would suggest that failure of both parthenogenetic and gynogenetic embryos to

develop beyond the 25-somite stage is not due to the lack of extragenetic contribution from the sperm but probably due to excessive homozygosity (Surani and Barton,1983). These authors showed that triploid embryos (one male and two female pronuclei) where diploidy had been restored by removing the female pronucleus, would also only develop to the 25-somite stage.

Experiments to follow would show that neither homozygosity alone nor sperm extragenetic contribution are the cause of death of embryos with two female pronuclei (parthenogenones and gynogenones). McGrath and Solter(1984b) produced biparental gynogenones and androgenones by replacing male and female pronuclei, respectively, for a pronucleus of the opposite sex. Results showed that , although heterozygotes, neither gynogenones nor androgenones would develop to term suggesting that maternal and paternal contributions to the embryonic genome are not equivalent and that both parental sexes are necessary for development. Surani *et al.* (1984) came to the same conclusion by using parthenogenetic eggs as recipients and reconstructing heterozygosity by introducing a male or female pronucleus. Only eggs in which male pronucleus had been introduced developed to term while those with two female pronuclei developed only poorly after implantation, confirming that homozygosity is not alone the cause of embryonic inviability. The development to term of reconstituted embryos produced from the recipient cytoplasm of diploid parthenogenones shows that the sperm had no essential or additional role, apart from restoring a complete genome at fertilization (Mann and Lovell-Badge,1984).

It has been proposed that the phenomenon of specific imprinting of the genome, which occurs at some stage during gametogenesis, may render the paternal genome essential for some stages of embryogenesis (Barton *et al.*,1984). Differential activity of maternally and paternally derived chromosome regions in mice has been reported, suggesting a differential functioning of gene loci within these regions (Cattanach and Kirk,1985). The cause for the death of parthenogenetic and androgenetic embryos may be due to non-complementation mechanisms, as reported for regions of some chromosomes in mice (Searle and Beechey, 1978), and a few embryos that reach post-implantation development fail to undergo X-inactivation correctly and die (Mann and Lovell-Badge 1987).

The technique of nuclear transplantation has also been used for testing whether events that take place during embryonic development, of either paternal or maternal origin, are inherited through the nuclei or cytoplasm. A classic example is the nuclear transfer experiments on the mexican Axolotl where it was reported that a protein synthesized during oogenesis interacts with the nuclei at the blastula stage, activating genes that are essential for gastrulation and organogenesis (Brothers,1976). In mammals, these techniques have been particularly useful for investigating the inheritance of mutant genes with maternal effects. The first model to be used was the T^{hp} mutation, an allele of the mouse T/t complex, which is invariably lethal when inherited from the female parent (Johnson, 1974). Nuclear transplantations between one-cell embryos from T^{hp}/+ and +/+ females showed that the defect responsible for the maternal pattern of inheritance is transmitted through the female

pronucleus (embryonic defect) and not through the cytoplasm of the ovum (oogenic defect) (McGrath and Solter, 1984a).

The other maternally inherited trait to be tested by nuclear transplantation was the OM mutation in DDK mice. When females of this strain are mated to males from another strain (non-DDK), many embryos die between morulae and implantation. However, this does not occur in the reciprocal cross (Wakasugi *et al.*, 1967). The effect has been shown to involve the embryos alone and not the uterine environment of the mother (Wakasugi, 1973). Nuclear transplantation experiments in which the incompatible strain's pronuclei were introduced into non-DDK enucleated cytoplasm did not reduce the number of viable embryos when compared to non-DDK controls. It was concluded that the effect is not due to incompatibility between pronuclei, but probably between components of the DDK cytoplasm ooplasm membrane and the foreign spermatozoa (Mann, 1986). Further experiments using enucleated DDK as host embryos for the pronuclear donor groups and cytoplasmic transfers between these groups could bring more evidence for these conclusions. Nuclear transplantation has also been used as a mean of determining that the stage specific embryonic antigen (SSEA-3) is a cytoplasmic contribution of the unfertilized ovum (McGrath and Solter, 1983).

1.2.4. Developmental potential of nuclei:

Embryogenesis involves the orderly formation of specific and determined regions, followed by the emergence of organ primordia and eventually the differentiation of specific cell types within the

developing organ system. The high degree of stability in development suggested that differences in cell types and functions are irreversible and result from the partitioning of different genetic determinants into different cell nuclei (Weismann, 1915). However, this proposal was refuted for nuclei from the earlier stages of development by the primitive cloning experiments of Loeb (1894) in the sea urchin and by Spemann (1914) in salamander. Through constriction experiments in eggs, these authors showed that nuclei of the early cleavage stages were equivalent to the zygote and suggested that cytoplasmic localizations in the egg were the principal agents for differentiation.

Four decades later, Briggs and King (1952) developed the technique of nuclear transplantation which opened the way to determine whether the stability of the differentiated state involves irreversible genetic changes, or whether the gene pool of differentiated somatic cells remains the same as that of the zygote nucleus. Although cell hybridization and other cellular and molecular biology studies have helped to test the theory of nuclear equivalence of somatic cells, nuclear transplantation is the only technique able to test the genome as a whole from the initial stages of development and, therefore, remains the most rigorous test of the genetic potentialities of living nuclei.

This section describes initially the nuclear transplantation experiments using amphibian eggs to investigate the potentiality of embryonic and somatic cells. The final part concentrates on the more recent approaches using mammalian embryos from the laboratory and farm species.

1.2.4.1. Nuclear potential studies in amphibian: The technology of nuclear transplantation in amphibia has remained virtually unchanged since its beginning (Briggs and King, 1952). The egg is activated by pricking with a glass microneedle so that rotation occurs and the dark animal hemisphere comes to lie uppermost. The black dot can be extirpated surgically, or can be physiologically ablated with laser or ultraviolet irradiation. Nuclear transplantation is performed by inserting a glass micropipette with donor cell which is injected into the ovum. The type of development that ensues is then a reflection of the introduced nucleus. Transplanted nuclei that lead to the formation of fertile adult frogs are interpreted to be totipotent. If a nucleus can support only limited development, perhaps to a post-neurula embryo which has functional nerve and muscle cells, it is considered to be genetically pluripotent.

Tests of several embryonic cell types by various investigators revealed that there is a decrease in the number of individuals that develop normally, as donor nuclei are tested from progressively older stages of embryogenesis (reviews by: King, 1966; DiBerardino and Hoffner, 1970; Gurdon, 1974; Briggs, 1977; McKinnel, 1978). Many, if not all, blastula nuclei are totipotent when fused to enucleated eggs (Briggs and King, 1952; McKinnel, 1962). However, only some early gastrula stage nuclei have been shown to be totipotent and a very small percentage at any later stage (Briggs and King 1960). These results suggest that the development capacity of most embryonic nuclei becomes progressively restricted during the process of cell type determination.

Once the answer was obtained that nuclei from undetermined regions of the embryo were totipotent, studies were then carried out on nuclei from determined regions of advanced embryos. The advanced development of nuclear transplants from larval cells is summarized below. Three original somatic nuclei derived from cells of young Xenopus laevis have promoted eggs to develop into fertile adult frogs. One female and one male originated from nuclei of cells from the intestine (Gurdon, 1962; Gurdon and Vehlinger, 1966) and the third frog, a female, was derived from the nucleus of a non-ciliated epidermal cell (Kobel *et al.*, 1973). Thus, 0.2% to 0.3% of the total number of larval nuclei tested in these two studies were genetically totipotent. In addition, 0.1% to 1.9% of the original somatic nuclei tested from various cell types have led to the formation of apparently normal larvae, and 0.2% to 2.3% to abnormal larvae, demonstrating genetic pluripotency (Gurdon, 1962; Gurdon and Vehlinger, 1966; Gurdon and Laskey, 1970; Kobel *et al.*, 1973; Marshall and Dixon, 1977). In contrast to somatic nuclei, primordial germ cell nuclei from young feeding larvae of R. pipiens promoted a higher percentage of normal development; 7.6% of the injected eggs reached the feeding larval stage (Smith, 1965).

Several investigators have tested the developmental capacity of nuclei from adult cells. The most advanced nuclear transplant reported has been a feeding larva obtained from spermatogonial cells of R. pipiens (DiBerardino and Hoffner, 1971). Among adult somatic nuclei tested in Xenopus laevis, there are 33 cases of early larvae obtained in support of nuclear pluripotency (Brun, 1978; Gurdon *et al.*, 1975; Laskey and Gurdon, 1970; McAvoy *et al.*,

1975; Wabl *et al.*, 1975). One of these larvae was reported to be normal and originated from a crest cell nucleus of the intestine, but this apparent normal larva died during an early larval stage (McAvoy *et al.*, 1975). The remaining nuclear transplant larvae were morphologically abnormal and the percentage success obtained ranged from 0.3% to 6.0%. No adult frog has yet developed from a transplanted adult nucleus. There has, however, been one claim that metamorphosis was attained by nuclear transfers of organ cultured adult lens cell (Muggleton-Harris and Pazzella, 1972), but in this study there is no documentation that the nucleus was derived from a specialized lens cell, nor is there evidence presented that the transplanted nucleus and not the egg nucleus was responsible for the development.

The studies involving nuclei of adult cultured skin cells and lymphocytes have provided the most critical attempts to test nuclei from specialized somatic cells. Over 99.9% of the donor skin cells displayed immunoreactive keratin, and four heartbeat larvae (3.1%) were obtained that had the nucleolar marker (1nu) of the transplanted nucleus (Gurdon *et al.*, 1975). In the case of lymphocytes, 96.1% to 98.7% of the donor cells were shown to be immunoglobulin-bearing cells and six (6%) abnormal larvae resulted. The main conclusion from those studies of normal and adult cells is that sufficient genetic information is present and functional to direct an egg throughout the course of embryogenesis and to specify the formation of cell types, tissues and organ systems normally found in a larva.

In order to determine the nature of the developmental restrictions displayed by nuclei from advanced cell types, an

extensive series of studies was performed on abnormal embryos and larvae of Rana nuclear transplants (reviewed by DiBerardino, 1979). Most abnormal nuclear transplants examined exhibited chromosomal abnormalities in number and/or structure, which in most cases arise during the first cell cycle of the egg (DiBerardino and Hoffner, 1970). These abnormalities are now known to be the cause of developmental arrest. The most severe chromosomal alterations cause developmental arrest of blastula embryos, whereas relatively minor karyotypic alterations permit development to early larval stages. Evidence has been presented that most of these numerical and structural changes in the chromosomes (a) involve genetic loss, (b) are a reflection of chromosomal differentiation acquired progressively through embryogenesis, and (c) are not a result mainly of technical damage (DiBerardino, 1979; DiBerardino, 1980; DiBerardino and Hoffner, 1980). It appears that the cytoplasmic cell cycle of the egg, which is much faster than the nuclear cycle of advanced cell types, induces premature changes in transplanted nuclei, resulting in incomplete DNA replication, chromosome breaks and rearrangements, and hypoaneuploidy in the most severely affected embryos.

An attempt was made to lengthen the cell cycle in Rana eggs to test whether the chromosomal abnormalities were merely a result of insufficient time to reverse changes to the genome during cell differentiation. This attempt did improve development, but so far this success was limited to embryonic nuclei of tailbud endodermal cells (Hennen, 1970). It has also been suggested that the chromatin may require remodeling of its proteins that is not possible in the egg cytoplasm. This hypothesis is tenable, especially

when one considers the replacement of chromatin proteins by more basic proteins that occurs during spermiogenesis (Dixon, 1972). Also, after fertilization in the sea urchin, histone specific to sperm chromatin, is replaced by embryonic histone, and additional protein replacements and modification occur in the chromatin of male pronuclei (Poccia *et al.*, 1981). Thus, it is hypothesized that conditioning of test nuclei in oocyte cytoplasm might lead to enhanced genetic potential (DiBerardino, 1980).

To test whether nuclei conditioned in oocytes had enhanced genetic potential, blastula nuclei from undetermined cells and tailbud nuclei from determined endothelial cells were injected singly into maturing oocytes at first metaphase. Approximately 24 h later when the oocytes completed maturity, they were activated and the host nuclei were removed surgically. Nuclei from both cell types promoted development through embryogenesis and microscopic studies showed the transplanted nuclei had transformed into metaphase chromosomes aligned on spindles soon after injection into first meiotic metaphase hosts (Hoffner and DiBerardino, 1980)

Studies with erythrocyte nuclei from adult R. pipiens have shown that when they are transplanted into mature eggs, they show little or no response (DiBerardino and Hoffner, 1983) but, when they are conditioned in diplotene oocytes which are then induced to mature with progesterone and then activated, the erythrocyte nuclei enlarge and incorporate ^{3}H-thymidine triphosphate (Leonard *et al.*, 1983) and can support development to swimming larvae stage (DiBerardino and Hoffner, 1983). These results with non-cycling and terminally differentiated erythrocytes

demonstrate: (a) erythrocyte nuclei contain the genes to specify larval development; (b) dormant genes can be reactivated by conditioning in the cytoplasm of oocytes; (c) the means whereby genes are reactivated involve in some way the preparation of the chromosomes by the oocyte cytoplasm to synthesize DNA in the cytoplasm of activated eggs; and (d) the formation of larvae attests to the fact that the DNA is replicated many times with a high degree of fidelity, and that the DNA is capable of widespread RNA synthesis required for protein synthesis in order to specify the cell, tissue and organ types present in larvae (Etkin and DiBerardino, 1983).

1.2.4.2. Developmental potential in mammals: The first report of mice born from nuclear transplantation came from Illmensee and Hoppe (1981). They described a method which requires a single puncture of the membrane. Trophectodermal (TE) and inner-cell-mass (ICM) cells were injected into fertilized eggs and the pronuclei were immediately sucked out using the same pipette producing a survival rate after microsurgery of 38 and 39 percent, respectively. Most embryos injected with TE nuclei were not capable of initiating nor supporting development beyond the preimplantation stage. However, 48 of the 142 embryos (34%) injected with ICM cells reached the morula-blastocyst stage and 19% (3/16) of the embryos transferred to synchronized recipients developed to term showing that mouse ICM nuclei remain developmentally equivalent to the totipotent zygote nucleus. However, other laboratories have not been able to reproduce these results and it has been suggested that enucleation may have been incomplete in these experiments (McGrath and Solter, 1984c).

In contrast, McGrath and Solter (1983) developed a technique that can have a success rate of virtually 100 percent, which has been used by several laboratories. These authors performed very thorough experiments to investigate the developmental potential of cleavage stage embryos in mice and found a loss in developmental potential at much earlier stages (McGrath and Solter, 1984c). They reported that, although 95% (20/21) of enucleated zygotes fused with zygote pronuclei developed to blastocyst, only 13% (19/151) developed to blastocyst when two-cell nuclei were fused and none developed when 4-, 8-, 16-cell and ICM nuclei were fused to enucleated zygotes indicating a rapid loss of totipotency of the transferred nucleus already by the 2-cell stage. By performing cytoplast transplantations they also showed that the difference to Illmensee and Hoppe's results (1981) was not due to the cytoplasm and plasma membrane which are fused together with the nucleus during transplantation. Other authors were also unable to obtain development to blastocyst when fusing enucleated zygotes to karyoplasts derived from embryos beyond the 4-cell stage (Surani *et al.*, 1987; Robl *et al.*, 1986). Since the mouse embryo becomes transcriptionally active at the 2-cell stage (Flach *et al.*, 1982), it was assumed that the developmental restriction was related either to genome differentiation or to the toxicity of stage-specific factors secreted by the transplanted nuclei (McGrath and Solter, 1984c; Solter *et al.*, 1986; Solter, 1987).

Later it was shown that some of these problems could be avoided if later stage enucleated embryos were used as recipient cytoplasms for karyoplasts derived from stages beyond the

activation of the embryonic genome. Robl *et al.* (1986), enucleated both blastomeres of two-cell mouse embryos and fused two- and eight-cell nuclei to one of the blastomeres. They reported the development of 93% (40/43) and 58% (14/24) development to blastocyst, respectively, showing that the two-cell cytoplasm was more suitable for the transfer of later embryonic nuclei. However, when the reconstituted 2-cell embryos were transferred to synchronized recipients, only those derived from 2-cell karyoplasts developed normally while none of the 8-cell reconstituted group developed beyond the twelfth day of gestation. A similar experiment was later performed by a different group (Tsunoda *et al.*, 1987) showing that not only the 8-cell reconstituted 2-cell embryos (8%) but also those derived from 4-cell karyoplasts (25%) were able to develop to term suggesting that indeed, cytoplasmic factors were essential for the development of nuclei from advanced cells.

In farm animals the activation of transcription is, in general, initiated at later stages than in mice. For instance, cattle, sheep and rabbit embryos activate the genome at the 8- to 16-cell stage (Cotton *et al.*, 1980; Camous *et al.*, 1986; Crosby *et al.*, 1988) while pig embryos do it at the 4-cell stage (Chartrain et al., 1986). It has been reported that 'enucleated' secondary oocytes fused to single eight and sixteen cell blastomeres from each of these species will develop to blastocysts *in vitro* and to term when transferred to a sychronized secondary recipient (Willadsen,1986; Prather *et al.*, 1987; Stice and Robl, 1988). These results support the interpretation that nuclei from transcriptionally inactive blastomeres remain totipotent. Another interpretation for the

difference with the mouse experiments is that, although these nuclei may have suffered some differentiation, they would have had at least three DNA replication cycles to de-differentiate before being required to initiate transcription. It is also possible that the cytoplasm from secondary oocytes is better able to support nuclei from the later embryonic stages as was shown for cattle where 4-cell stage nuclei were unable to support development when fused to enucleated zygotes (Robl *et al.*, 1987).

GENERAL MATERIALS AND METHODS

The basic equipment and materials used during the experimental procedures in the chapters to follow and the methodology common to most experiments will be described in this section. Information will be provided on the methods for obtaining embryos from mice and sheep, the basic micromanipulation procedures and the techniques used for producing fusion during nuclear transplantation. More detailed and specific Materials and Methods will be provided for each of the experimental chapters.

2.1. **Production of embryos and basic manipulations:**

This section describes the methods by which female mice and sheep were obtained and/or handled in order to produce embryos for experimentation and the methods used for the collection, *in vivo* and *in vitro* culture and, for sheep embryos, the transfer of manipulated embryos to recipients. Each species will be dealt with separately since the methodology differed significantly.

2.1.1. Harvesting and culture of mouse embryos:

To initiate the experiments with nuclear transplantation it was necessary to set-up matings between two inbred strains, CBA/Ca and C57BL/6, to produce donor F1 females which, from previous

experience, were known for their homogeneity, and for their ability to produce embryos with a clear cytoplasm which eased the visualization of nuclei. These embryos were found to be able to develop *in vitro* to the blastocyst stage when cultured from the 1-cell stage.

A weekly supply of 20 to 25 four-week old F1 females was calculated as being required for daily experimentation. This was based on observation that on average 60% of superovulated females will plug (12-15 mated females) giving 3-4 females on each of 4 days. As each superovulated mouse produces on average 20 fertilized eggs, there would be 60-80 embryos on each experimental day. To breed such females, a standard 56-cage rack was allocated and managed in the following way. Management of the breeding stock was performed once a week when females from mating rows were transferred to a pregnancy row and replaced either by females having their litters weaned at 3 weeks after birth, or by females in the pregnancy row which had not become pregnant. Pregnant females were transferred to individual cages in a maternity row where they were kept until weaning. Females which had lost their litters or that did not become pregnant for two cycles were culled. In this way it was possible to select for females with higher reproductive ability.

Records for the number of females mated, number littering, number that were culled for having lost their litters, average litter size, weaning rates and proportion of female pups were kept for 25 weeks to assess the performance and efficiency of the breeding system (Table 2.1). C57BL/6 females mated to CBA/Ca had a significantly higher littering rates ($P<0.01$), weaned pups more efficiently ($P<0.01$) and produced more female pups ($P<0.01$) when

Table 2.1. Reproductive performance of C57 BL/6 and CBA/Ca inbred female mice bred to males of the opposite strain to produce F1 hybrids for experimentation recorded during a period of 25 weeks..

Dam strain	Mated females	Percentage littered	Percentage killed litter	Litter size	Percentage weaned	Percentage females
CBA/Ca	117	41.0%[a] (48)*	3.4%[a] (4)	6.9 ± 1.9[a] (333)	85.6%[a] (285)	45.3%[a] (129)
C57BL/6	159	64.8%[b] (103)	5.7%[a] (9)	8.4 ± 2.1[a] (868)	93.5%[b] (812)	52.2%[b] (424)
Total	276	54.7% (151)	4.7% (13)	7.95 ± 2.0 (1201)	91.3% (1097)	50.4% (553)

Within each trait, a≠b (P<0.01).

* numbers from which the percentages were derived.

compared to CBA/Ca females mated to C57BL/6 males. However, there was no significant difference in the size of their litters ($P>0.05$) nor in killing or abandoning their pups immediately after birth ($P>0.05$). *In vitro* comparisons showed no difference between the ability of embryos derived from F1 hybrids originated from C57BL/6 or CBA/Ca dams to develop to the blastocyst stage ($P>0.05$). These results indicated that a higher production of F1 derived embryos could be obtained from the limited space available by using only C57BL/6 females as dams due to their better maternal and reproductive performance. These management methods were used for the remaining experimental period.

Hybrid F1 females were induced to superovulate by an intraperitoneal injection of 5 i.u. of pregnant mares serum gonadotrophin (PMSG; Folligon, Intervet Ltd., U.K.) diluted in 0.9% saline. After a period varying from 44 to 48 hours after the PMSG injection, ovulation was induced by intraperitoneal injection of human chorionic gonadotrophin (hCG; Chorulon, Intervet Ltd, U.K.) diluted in 0.9% saline. Injections were performed at different times during the day and night to enable the harvesting of embryos at different stages of the initial cell cycles at approximately 13:00 hours. Donor females were used at a young age (4 to 6 weeks) to avoid the influence of photoperiods on the endogenous luteinizing hormone (LH) surges. Males used for the matings with F1 hybrid females were also F1 hybrids bred from matings between MF1/Olac outbred albino females and SWR inbred albino males. Apart from MF1/Olac that was imported (Olac, U.K.) all other stock were available at the institute's mouse house. The F1 females were caged

with F1 males immediately after the hCG injection and checked the following morning for the appearance of a vaginal plug.

The medium used for the culture *in vitro* of mouse embryos is based on Whitten's bicarbonate buffered medium (N°16; Whittingham, 1971). Medium used for the handling was a modified Krebs-Ringer solution in which some of the bicarbonate is substituted with HEPES buffer (M2; Quinn *et al.*, 1982). All chemicals used for the preparation of medium were of a Analar grade. Concentrated stock solutions were prepared to enable a more prolonged period of storage and to avoid weekly preparation using all chemical ingredients (Table 2.2). Stocks B and C were renewed every 3 weeks and stocks A, D and E at 3 month intervals. The stock solutions were diluted in double-distiled water, added to the bovine serum albumin (BSA; code 81001, Pentex, Miles Laboratories Ltd., U.K.) and then filtered with 0.2 μm pore filter. Cultures were done in 40 μl drops of medium covered with sterile mineral oil (Heavy Mineral oil, Sigma, Poole, U.K.) that had previously been equilibrated with medium in the following way. After autoclave sterilization, the oil was cooled to room temperature and a 10% volume of N°16 without BSA was added and vigorously mixed to allow equilibration. After a few hours, when medium and oil had separated into different phases, clear equilibrated oil was extracted for use in culture dishes. The culture dishes were prepared several hours before use and placed in a humidified incubator with an atmosphere of 5% CO_2 in air.

Embryos at different cleavage stages were recovered from the oviduct of females within a few minutes of cervical dislocation. The oviducts were flushed by introducing a blunt 30 gauge needle into

Table 2.2. Chemical composition of concentrated stocks and method for the preparation of medium No16 and M2.

Stock A	Component	g/100 ml
(10 x concentrated)	NaCl	5.534
	KCl	0.356
	KH_2PO_4	0.162
	$MgSO_4.7H_2O$	0.293
	sodium lactate	2.610
		or 4.349 g of 60% syrup
	glucose	1.000
	penicillin	0.060
	streptomycin	0.050

Stock B	Component	g/100 ml
(10 x concentrated)	$NaHCO_3$	2.101
	phenol red	0.010

Stock C	Component	g/10 ml
(100 x concentrated)	sodium pyruvate	0.036

Stock D	Component	g/ 10 ml
(100 x concentrated)	$CaCl_2.2H_2O$	0.252

Stock E	Component	g/100 ml
(10 x concentrated)	HEPES	5.958
	phenol red	0.010

Stock	No16	M2
A (10x)	1.00 ml	1.00 ml
B (10x)	1.00 ml	0.16 ml
C (100x)	0.10 ml	0.10 ml
D (100x)	0.10 ml	0,10 ml
E (10x)		0.84 ml
H_2O	7.80 ml	7.80 ml
BSA	40 mg	40 mg
	10.0 ml	10.0 ml

the ampulla, and passing approximately 0.1 ml of M2 through the oviduct and into a glass dish. When cumulus cells were attached to the zona pellucida, embryos were flushed directly into a 150 i.u/ml hyaluronidase solution (Sigma, U.K.) diluted in M2. After the dispersal of cumulus cells, embryos were rinsed in M2 alone. As soon as possible embryos were transferred to equilibrated N°16 drops in culture dishes and replaced in the incubator until further manipulation. Micromanipulated and control embryos were cultured for periods varying between 3 and 4 days after which they were assessed to characterize their developmental stage.

2.1.2. Harvesting, culture and transplantation of sheep embryos:

Harvesting unfertilized oocytes and preimplantation embryos

Ewes to be used for oocyte or embryo collection were either from the Scottish Blackface or Welsh Mountain breeds. They were managed on a outdoors regime, with a small supplementation of hay and turnip in the winter. Oestrus cycle synchronization of donor ewes was achieved by intravaginal positioning of progestagen sponges for 12 to 16 days (Veramix, Upjohn, U.K.). Ewes were induced to superovulate with single injections of 3 mg/day (total 6 mg) of equine follicle stimulating hormone (FSH; reference for method; Stockwell Hartree *et al.*, 1968) on 2 successive days. Ovulation was induced with an 8 mg single dose of gonadotrophin releasing hormone equivalent (GNRH; Receptal, Hoechst, U.K.) 24 hours after the second injection of FSH.

To obtain fertilized embryos, ewes were artificially inseminated by laparoscopy using a technique described by Salamon (1987).

Ewes are held in a recumbent position and a local anaesthetic is injected subcutaneously in three point of the abdomen immediately cranial to the mammary gland. Using a surgical blade, three holes are inflicted to the skin where the laparoscope, a grasping forceps and a blunted paravertebral needle with the semen are introduced. An injection of 0.2 ml of fresh semen diluted in PBS (2:1) is injected into the tip of each uterine horn pointing towards the oviduct. The incisions are closed with surgical clips which are removed a few days later.

Ewes were fasted for 24 hours prior to surgery. Anaesthesia was induced by single intra-venous injection of 8-20 ml 10% w:v sodium thiopentone (Intraval, May and Backer Ltd., U.K.). An endotracheal tube was inserted and anaesthesia maintained by a mixture of oxygen, nitrous oxide and halothane (Fluothane, ICI plc., U.K.) in a semi-closed system. Ewes were placed in a recumbent position and the ventral abdominal area shaved and disinfected. Under aseptic conditions, an incision was made parallel with, and 1 cm lateral to, the mid-line. Fascia and peritonium were incised at the mid-line, and the reproductive tract exteriorized. Once the manipulations to the reproductive tract had ended, 10 ml of sterile isotonic saline were introduced into the pelvic cavity to minimize adhesions. Fascia and peritoneum were closed with continuous stitches of a resorbable suture and skin surgical clips were applied on the incision.

To recover unfertilized oocytes from the oviducts, a plastic canula was introduced in the ampulla and 20 ml of a phosphate buffered medium (OCM; Ovum Culture Medium, Flow, U.K.) was flushed through the oviduct into a glass dish. Recovery of later

embryonic stages was performed by flushing a similar amount of OCM through the uterine horn and oviduct using a 2-way foley catheter (180003; Rush-gold ballon catheter, Rommelshausen, F.R.G.). Embryos were located using a dissecting microscope (Wild-M8 Zoom Stereomicroscope, Switzerland) and placed into fresh OCM medium until further manipulation.

Culture of embryos *in vivo*

The embedding of embryos in agar layers was performed following a procedure similar to that described by Willadsen (1982). Two sterile agar solutions at 1.0% and 1.2% in 0.9% saline were kept at 45°C on a heated block. Manipulated embryos were initially placed in a solution of foetal calf serum (FCS; Flow, U.K.) at 50% in OCM. After 2 minutes, they were introduced into the 1.0% agar solution cooled to 37°C and immediately aspirated into the tip of a 200 µm diameter glass pipette for the first embedding. The tip of the pipette was withdrawn from the agar solution and placed in a dish with OCM for a few seconds before expelling the small agar cylinder containing the embryos. Small sections of the cylinder containing one or two embryos were cut with hypodermic-needles fitted on 1-ml syringes. These sections were placed in the 1.2% agar solution after it had cooled to 37°C. The second embedding was produced by aspirating these small cylinder containing embryos into the tip of a 700 µm diameter glass pipette which was withdrawn from the agar solution and placed in OCM. The agar was cut into small chips as mentioned before.

Agar embedded embryos were transferred to oviducts of sheep at the luteal phase of the cell cycle. These temporary recipients were not synchronized to the embryos because this had

been shown not to be necessary (Willadsen, 1980). The reproductive tract was exteriorized as described above and the oviduct was ligated in two positions close to the utero-tubal junction. The agar chips were placed in a 1 mm diameter glass pipette and introduced into the ampullary region of the oviduct to a depth of between 3 to 5 cm. When the passage of the transfer pipette was assisted with a small forceps to pass the bends in the oviduct, there was a significant increase in the proportion of embryos recovered after 4 to 5 days (43/83 = 52% vs. 99/140 = 71%, $P<0.01$). At recovery the ligatures were cut loose and the oviducts were flushed with OCM into glass dishes where the 'chips' could be located.

Transplantation of morulae and blastocysts to the uterus

Embryos which developed into compact morulae or blastocysts during the *in vivo* culture period, were dissected as small blades from the agar 'chips' using 27-gauge hypodermic-needles and placed in OCM + 20% FCS until their transfer to second recipients. Progestagen sponges were withdrawn from the recipient ewes and oocyte donors at the same time to attempt synchronization of oestrus cycles. Anaesthesia and surgery were performed as before to expose the reproductive tract. A small perforation was produced at the tip of each of the uterine horns where a pipette was introduced to place the embryos. Recipient ewes were checked for return to oestrus and the ewes that did not return were ultrasound scanned at approximately 70 days of gestation to confirm establishment of pregnancy. Pregnant ewes were allowed to carry gestation to term.

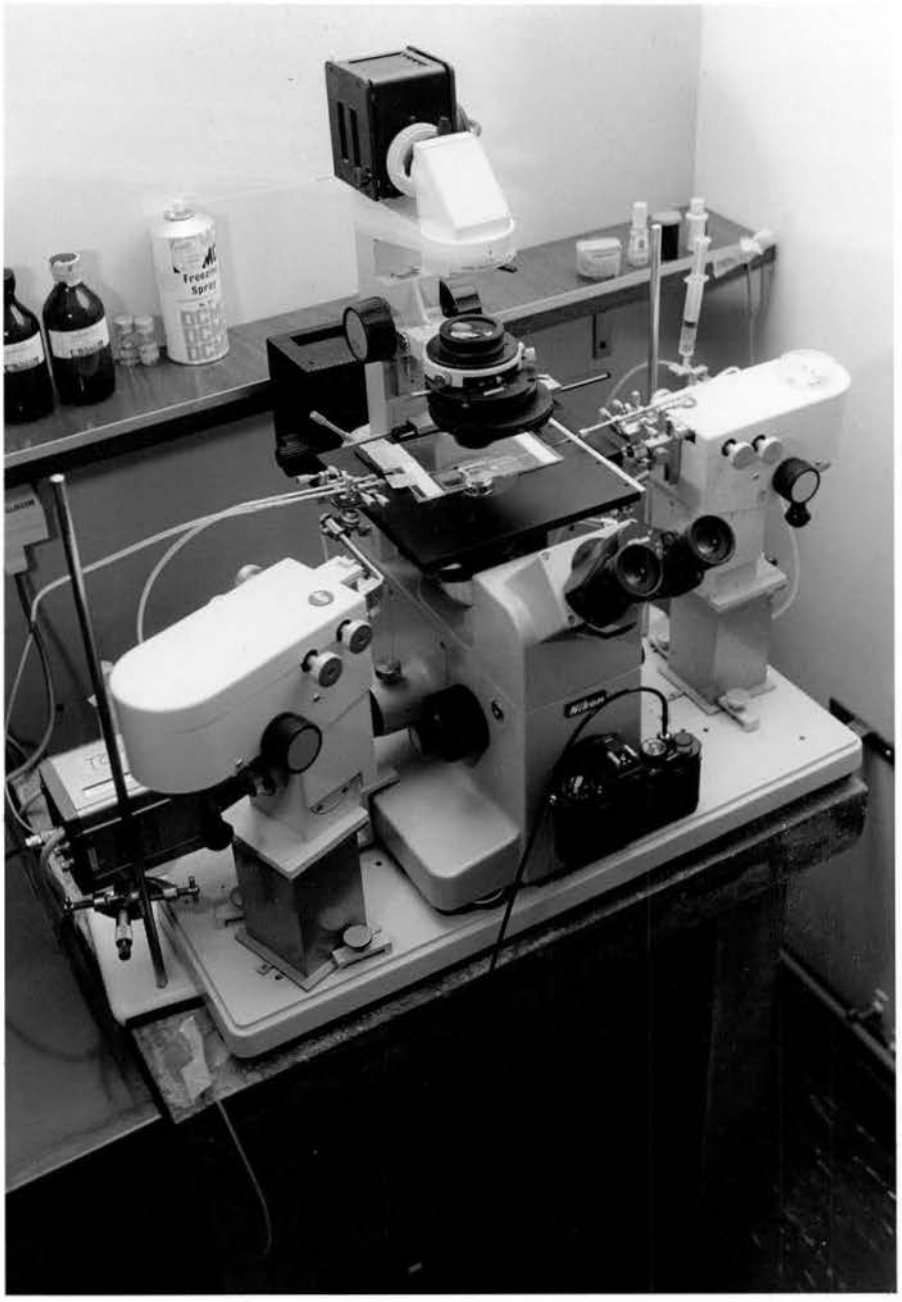
2.2. **Micromanipulation of embryos: equipment and techniques.**

This section describes the basic equipment required for micromanipulation techniques and the methods for manufacturing the various microtools necessary for positioning and performing microsurgery in mammalian embryos. It also describes the micromanipulation methodology for nuclear transplantation based on a non-invasive technique which was first described by McGrath and Solter (1983).

2.2.1. Basic materials for micromanipulation:

The equipment used for micromanipulation is shown in Figure 2.1. The optical equipment consisted of an inverted microscope (Diaphot-TMD; Nikon U.K. Ltd., U.K.) with condenser and objectives for differential interference contrast (Nomarski optics) producing magnifications up to 400 times. The microscope was equipped with a 35 mm camera for still photomicroscopy and with a video camera, recorder and monitor. There were two micromanipulators (Micromanipulators M; E. Leitz Ltd., U.K.) with coaxial coarse and fine adjustment, tilting top and control for transverse and sagittal movement. These manipulators were raised from the base to the level of the microscope stage and had double or single unit instrument holders which held instrument tubes for the micropipettes.The hydraulic system was controlled by three micrometer syringes (Agla Micrometer Syringe Kit, Wellcome Reagents Ltd, U.K.) which were connected through flexible

Figure 2.1. Equipment used for the micromanipulation of mammalian embryos. Comprises a microscope with differential interference contrast optics with magnification up to 400X; 2 micromanipulators (left and right); Three sets of microsyringes and micrometers for fine pressure control attached through plastic tubing to pipette holders with micropipettes.



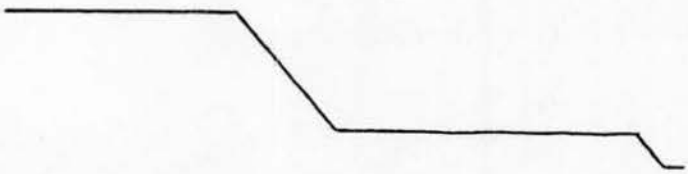


Figure 2.1a. Sketch of holding pipette.
The pipette is bent in 4 positions in the same plane to provide a very short final segment. If the final segment was too long and bent upwards, so that the tip was higher than the last bend, it would be impossible to pick up an oocyte that was resting on the microscope slide.

polythene tubing (mod.21.852-0062; Bel-art Products, U.K) to the instrument tubes. The whole of the hydraulic system was filled with an inert mineral oil (Fluorinert 77, Sigma, U.K.) with the exception of a small air bubble located in the microsyringe which improved pressure control during micromanipulation.

Two kinds of microtools were required for micromanipulations; one positions and holds the embryos so that the second may perform the microsurgery. The holding pipettes were prepared immediately before use from 1.0 mm diameter glass capillaries (GC100-10, Clark Eletromedical Instruments, Reading, U.K.). The capillaries were pulled by heating with a small Bunsen burner flame to a diameter of 100 to 150 μm. The pulled capillary was positioned on a microforge (MF1-microforge, Research Instruments Ltd., U.K.) and bent in four positions at angles of 45°. Fig. 2.1a. The pipettes were broken close to the last bend and polished with the heated filament until the hole on the tip had a 20 to 25 μm diameter.

The microsurgical pipettes were prepared in advance of use since they were more laborious to manufacture. They were prepared from 1.0 mm diameter glass capillaries (GC100F-15, Clark Electromedicals Inst., U.K.) which were pulled using a horizontal puller (753 Micropipette Puller, Campden Instruments Ltd., U.K.). These pipettes were broken at a right angle to a diameter varying from 18 to 35 μm according to the embryonic stage and species concerned. They were then positioned on a beveller, built in our workshop with 1 micron Aluminium Oxide grading surface, and were bevelled to a 45° angle. Once bevelled, the pipettes were rinsed in a 20% solution of hydrofluoric acid

which polished the outer surface. A final tip was pulled on the pipette on the microforge to ease penetration through the zona pellucida. To provide a 'siliconizing' effect, the pipettes were rinsed in a 1.25% solution of detergent (Tween-80, Sigma, U.K.) and placed overnight in a dry incubator for drying. This fine coat of detergent on the internal and external surfaces reduced superficial tension so that cellular fragments and debris no longer stuck to the tip of the pipettes during micromanipulation.

2.2.2. Micromanipulation techniques:

Prior to microsurgery, mouse embryos were incubated at 37°C for 30 minutes in N°16 containing 1.0 µg of cytochalasin D/ml and 0.03 µg of Nocadazole/ml (CDN: Sigma, U.K.). Sheep embryos were incubated for 60 minutes at room temperature in OCM containing 7.5 µg of Cytochalasin-B (CB; Sigma, U.K.). Embryos were micromanipulated in a chamber as shown in Figure 2.2. These microsurgical chambers were built from a siliconized microsurgical slide to which two glass supports were fixed with silicone grease (BDH, U.K.) to avoid leakage. Medium was added to the centre of the slide (350 µl) and a coverslip was positioned over and fixed to the glass supports with silicone grease. Finally, both sides of the chamber were filled with fine mineral oil (Dow Corning Fluid, Dow, U.K.). The medium used in the microsurgical chamber during manipulations also contained the cytoskeletal inhibitors at identical concentrations.

The method used for the enucleation of mouse embryos is shown in Figure 2.3. Embryos were held in position with the

Figure 2.2. Micromanipulation chamber built from a histological slide, two glass supports and a coverslip. Sealing is produced with a fine silicone grease. The internal chamber contains medium with cytoskeletal inhibitors which is separated from the atmosphere by two layers of fine mineral oil, one on each side of the chamber. The holding (left) and microsurgical pipettes are introduced into the chamber through these oil layers and have access to the embryos in the chamber from a horizontal position.

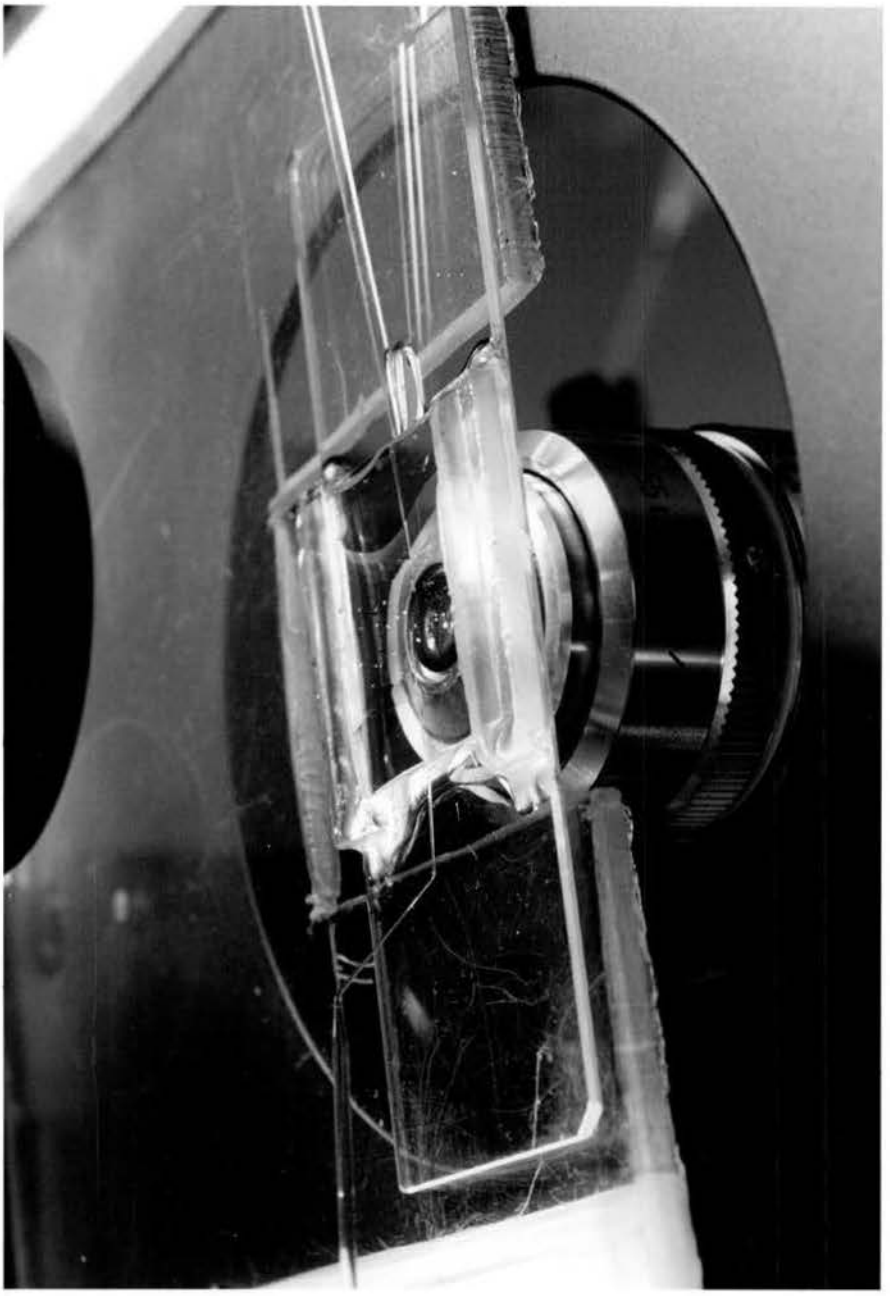
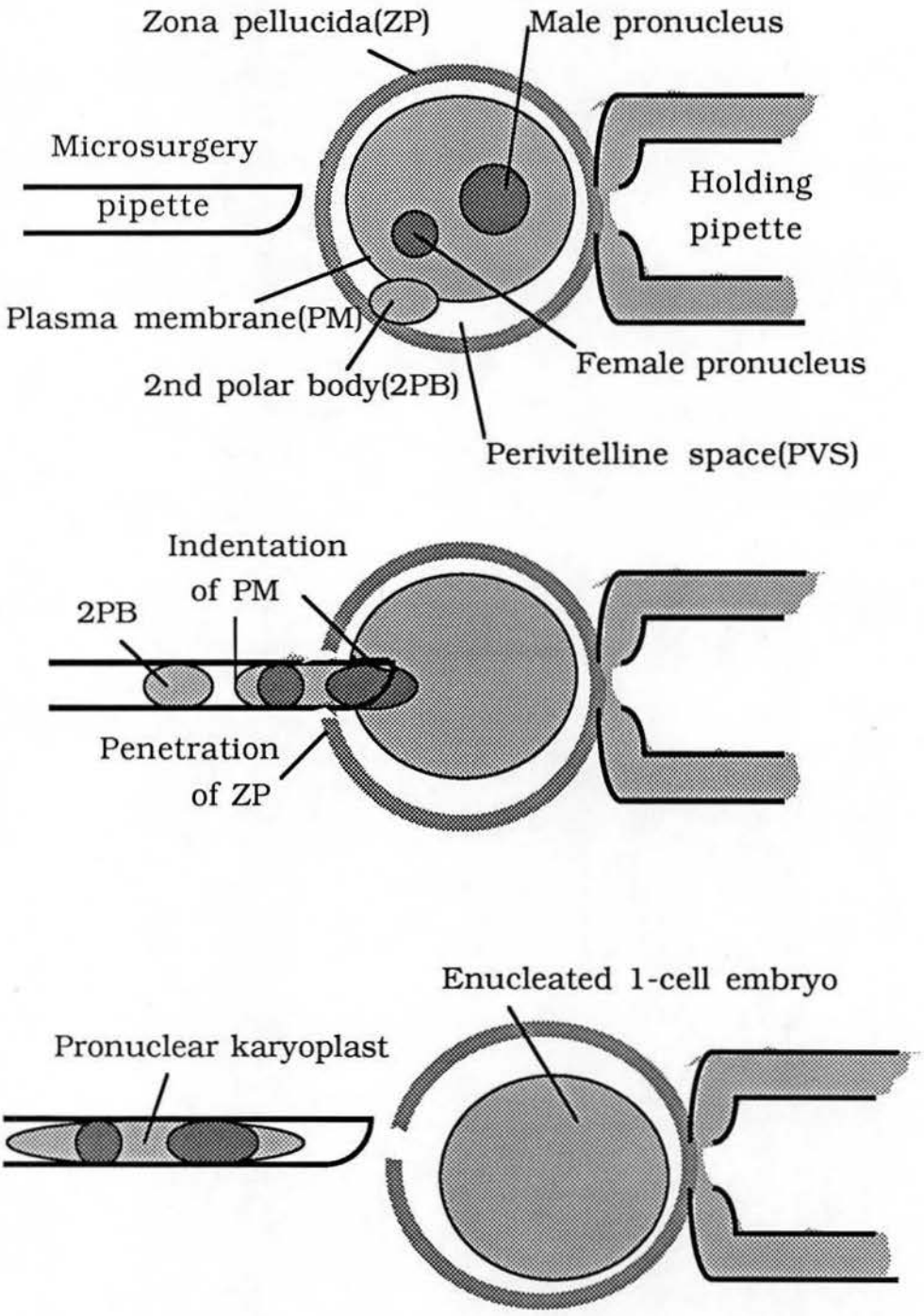


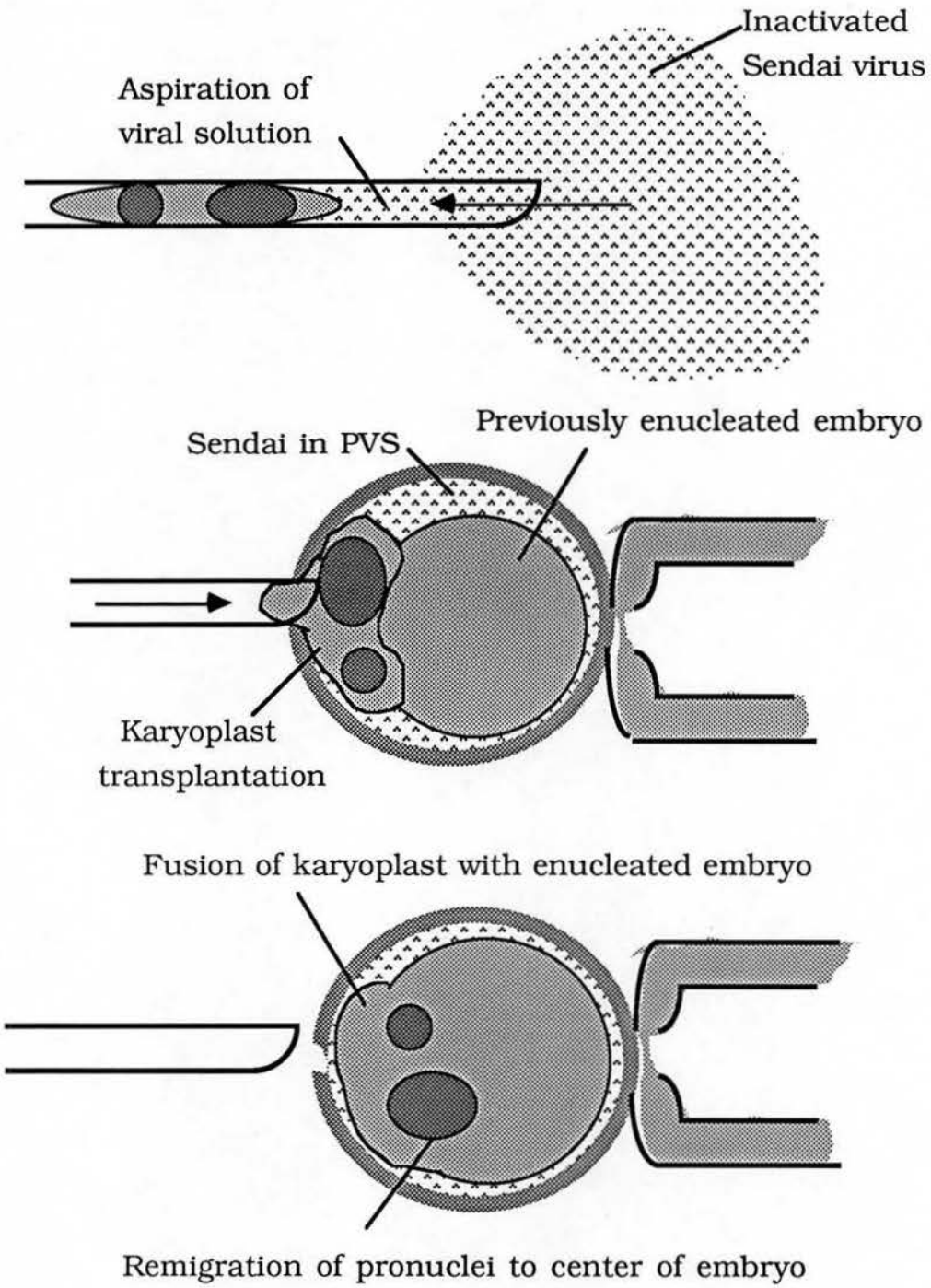
Figure 2.3. Technique for enucleating 1-cell embryos placed in cytoskeletal inhibitors by withdrawing pronuclei into a karyoplast.



holding pipette and the zona pellucida was penetrated with the microsurgical pipette into the perivitelline space, avoiding penetration of the plasma membrane. The pipette was advanced to a point adjacent to the nucleus or pronucleus which was aspirated into the pipette. The nuclei were always overlaid by the plasma membrane and a small amount of cytoplasm. As the pipette was withdrawn from the embryo there was the formation of a cytoplasmic bridge extending between the karyoplast within the pipette and the enucleated embryo within the zona pellucida. This bridge would stretch to a fine thread, pinch off and reseal. In the case of sheep oocytes, nuclei were not present and enucleation was performed by aspirating one quarter of the cytoplasm immediately surrounding the first polar body in an attempt to remove the metaphase II chromosomes. This technique is described in detail in Chapter 4.

Transplantation of mouse karyoplasts was done immediately after blastomere enucleation (Figure 2.4). The pipette was moved to a 5 µl drop in the micromanipulation chamber which contained inactivated Sendai virus. A volume similar to that of a pronuclear karyoplast was aspirated into the tip of the pipette. The pipette was then moved back into the micromanipulation chamber with the enucleated embryo and advanced through the hole in the zona pellucida. Inactivated virus and karyoplast were sequentially injected into the perivitelline space. The manipulated embryo was removed from the chamber, rinsed in M2 lacking cytoskeletal inhibitors and incubated in N°16 at 37°C for the culture period. Fusion would occur typically within one hour of manipulation. With sheep embryos, the whole blastomere was injected into the perivitelline space of an

Figure 2.4. Technique for introducing nuclei from a karyoplast into an enucleated 1-cell embryo by inactivated Sendai virus-mediated fusion.



enucleated oocyte with no viral solution since fusion was obtained with electrical pulses (Figure 2.5; see Chapter 3).

2.3. **Fusion methodology:**

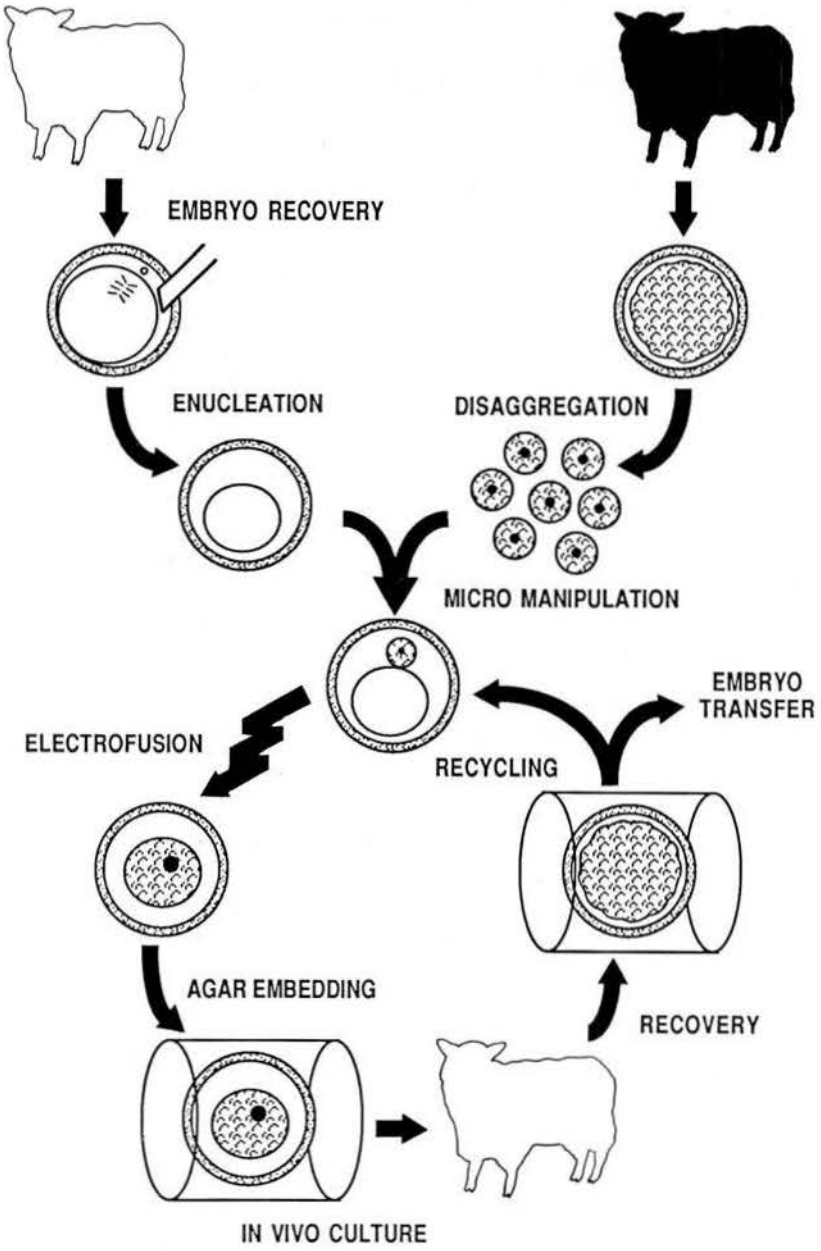
The method used for fusing karyoplasts to enucleated embryos was either using an inactivated Sendai virus solution (mice) or by electrofusion (mice and sheep). Different methods were used because higher fusion rates were obtained with Sendai for mice and with electrofusion for sheep embryos. This section will describe the methods used for obtaining an inactivated Sendai virus solution with a suitable fusogenic activity and some information on the assembling of the electrofusion apparatus.

2.3.1. Preparation of the inactivated Sendai virus solution:

The viral stock which produced growth in embryonated eggs was obtained from the virology department of the Royal Veterinary Faculty, University of Edinburgh. The results obtained at this attempt are described below together with the methodology which was based on Giles and Ruddle (1973).

Thirty 9-day embryonated chicken eggs were candled and the air space was marked with an arrow indicating the position of the embryo. Twenty-six were fertilized and these were grouped in batches of 5 for inoculation into the allantoic cavity opposite to the embryo. Four groups were injected with 0.2 ml of a 10^{-3} dilution of the viral allantoic fluid in Hank's Balanced Salt solution (HBSS,

Figure 2.5. Diagrammatic representation of the technique of nuclear transplantation in farm animals as described by Willadsen (1986). The recycling of embryonic cells has already been shown to be possible (Marx, 1988).


EMBRYO RECOVERY
ENUCLEATION
DISAGGREGATION
MICRO MANIPULATION
EMBRYO TRANSFER
ELECTROFUSION
RECYCLING
AGAR EMBEDDING
RECOVERY
IN VIVO CULTURE

Sigma, U.K.). The fifth group was used as a control and injected with HBSS alone. The site of injection was sealed with drops of sterile melted paraffin. Eggs were incubated at 35.5°C in a moist chamber for 72 hours and turned three times a day.

At the end of the incubation period, the eggs were placed in a refrigerator at 4°C for at least 2 hours to reduce the bleeding during the harvesting procedure. The eggs were surface sterilized and aseptically opened by cracking the shell with a sterile scapel handle. The pieces of shell were gently removed with sterile forceps to expose the inner shell membrane. Allantoic fluid (5 to 10 ml per egg) was withdrawn using a 10 ml sterile syringe with 1.5 inch 18-gauge needle by inserting the needle through the inner shell membrane into the allantois.

The titre of the fluid was measured in a haemoagglutination assay. A packed cell volume of 0.5 ml of chick red blood cells (rbc) were washed twice in HBSS and resuspended in 100 ml of HBSS which was used throughout as the diluent. Serially increasing 2-fold dilutions were made in a tray (96-U-WS-Trays, Linbro Chem. Co., U.K.) from an initial 1:10 dilution of the allantoic fluid. To each 0.5 ml of each dilution, 0.5 ml of rbc suspension was added and mixed in each well. The tray was covered with Saran wrap and incubated at 4°C for 2 hours. A negative agglutination reaction was indicated by a 'button pattern' of rbc collected in a tight disk at the bottom of the well, while a positive agglutinin reaction was indicated by a 'shield pattern', a diffuse, or continuous sheet of rbc covering most of the well. The virus titre was read at the highest dilution giving a positive reaction. The harvested allantoic fluid produced an average titre of 4094 HAU while the control had no titre.

The allantoic fluid from the injected eggs was centrifuged at 2000 x g for 20 min. The supernatant fluid was decanted and saved, and the pellet discarded. If it was desired to replenish infectious virus stock, a sample of supernatant fluid was removed, dispersed in 1 ml amounts, and stored in liquid nitrogen in sealed ampoules. The supernatant was centrifuged at 16000 x g for 1 hour. The remaining supernatant fluid was completely removed and discarded. The pellet at the bottom of the tube was resuspended in 1% BSA in HBSS without glucose (HBSSG-) the volume approximately one tenth the original volume taking care not to atomize the virus and contaminate the environment. The virus suspension was centrifuged at 2000 x g for 20 minutes and transferred to a sterile container. At this the average titre of the solution was 26,027 HAU.

A chemical method of inactivation was chosen, using beta propiolactone (BPL; Sigma, U.K.) which destroys the viral RNA. A 0.5% solution of BPL was diluted in a saline bicarbonate solution (1.68 g $NaHCO_3$ + 0.85 g NaCl + 0.2 ml of 0.5% phenol red + 100 ml double distilled water). One ml of 0.5% BPL in saline bicarbonate was added to each 9 ml of Sendai virus suspension and agitated for 10 min. The solution was incubated at 37°C for 2 hours with agitation and statically at 4°C overnight to inactivate the remaining BPL. A sample was removed for HAU assay and for infectivity determination. At this stage the titre had fallen to 16,670 HAU. The infectivity of the inactivated Sendai virus was determined by inoculating 9-day embryonated eggs with 0.2 ml of 1:4 and 1:20 dilution of the inactivated material for 72 hours for a first egg passage. Samples (0.2 ml) of undiluted allantoic fluid harvested for each series (1:4 and 1:20) of the first egg passage were used to initiate a second 72 hours

egg passage. There was no HAA in either egg passages indicating that the virus had been completely inactivated.

3.3.2. Electrofusion techniques:

This section includes a description of the electrofusion apparatus and the way in which embryos were prepared for electrofusion. The equipment used for electrofusion is shown in Figure 2.6. Detailed information of the technique will be provided in Chapter 3.

Fusion was performed using an apparatus assembled by Mr. J. White and F.J.W. Borthwick from the institute's workshop. The parameters were set from the models described by Kubiac and Tarkowski (1985) and from information obtained from J-P. Renard of the Pasteur Institute, Paris, France. The apparatus was used in 2 different forms. Initially, the pulse generator was designed to produce single direct-current (d.c) pulses which extended from 0 to 60 volts and of duration varying from 5 to 200 μsec. Later, an extra device was included which preceded the d.c pulse with a 500 kHz alternating current (a.c) for 0 to 25 sec with an intensity of 1 to 8 volts.

The pulse generator was connected to the electrofusion chamber which consisted of parallel 100 μm diameter platinum electrodes attached to the surface of a 5 cm diameter glass Petri dish (Figure 2.7). Dishes were manufactured with distances between electrodes of either 100 or 200 μm. Electric d.c and a.c pulses were monitored on an oscilloscope which showed width and height of every pulse produced.

Figure 2.6. Equipment used for the electrofusion of mammalian embryos. General view of the pulse generator, oscilloscope, dissecting microscope and electrofusion chamber.

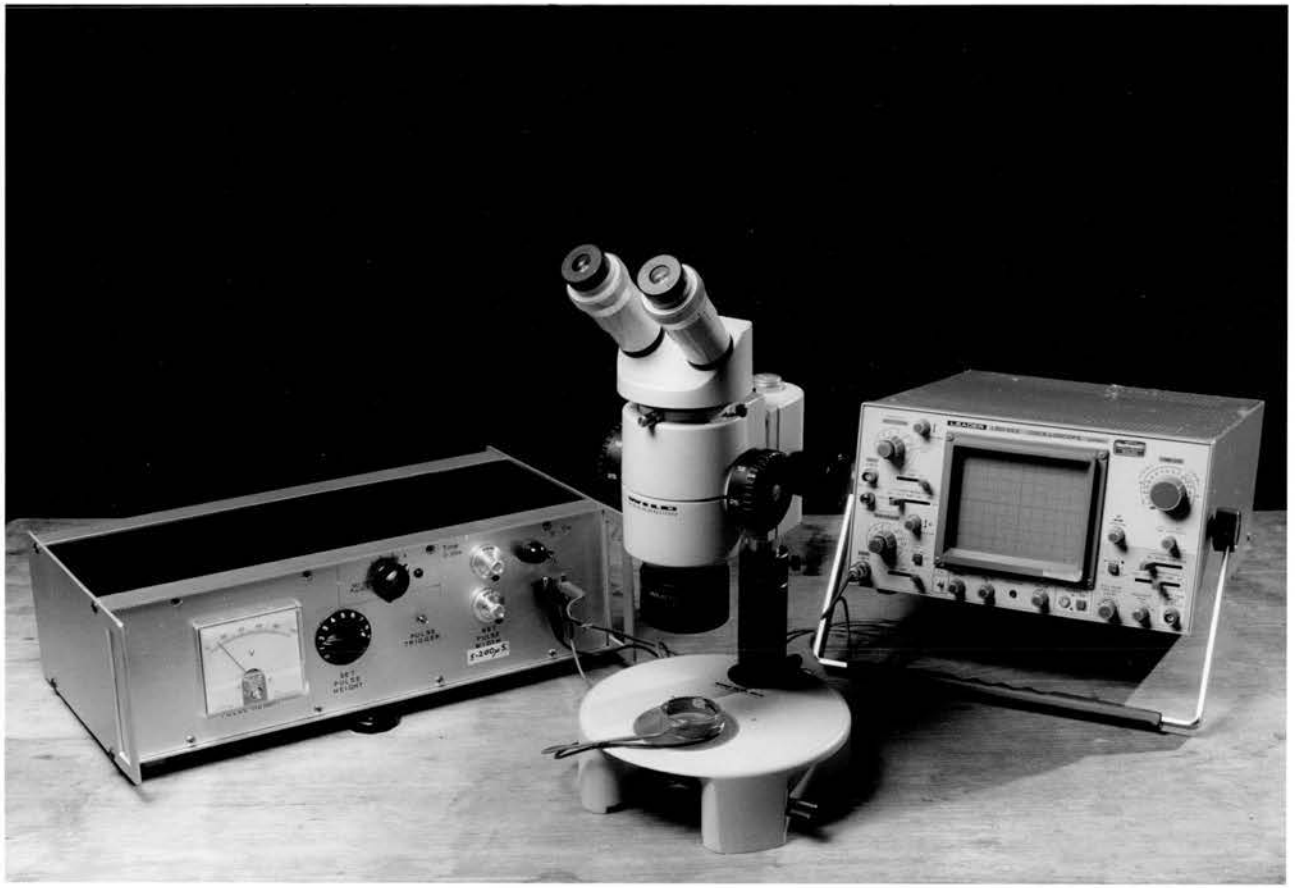
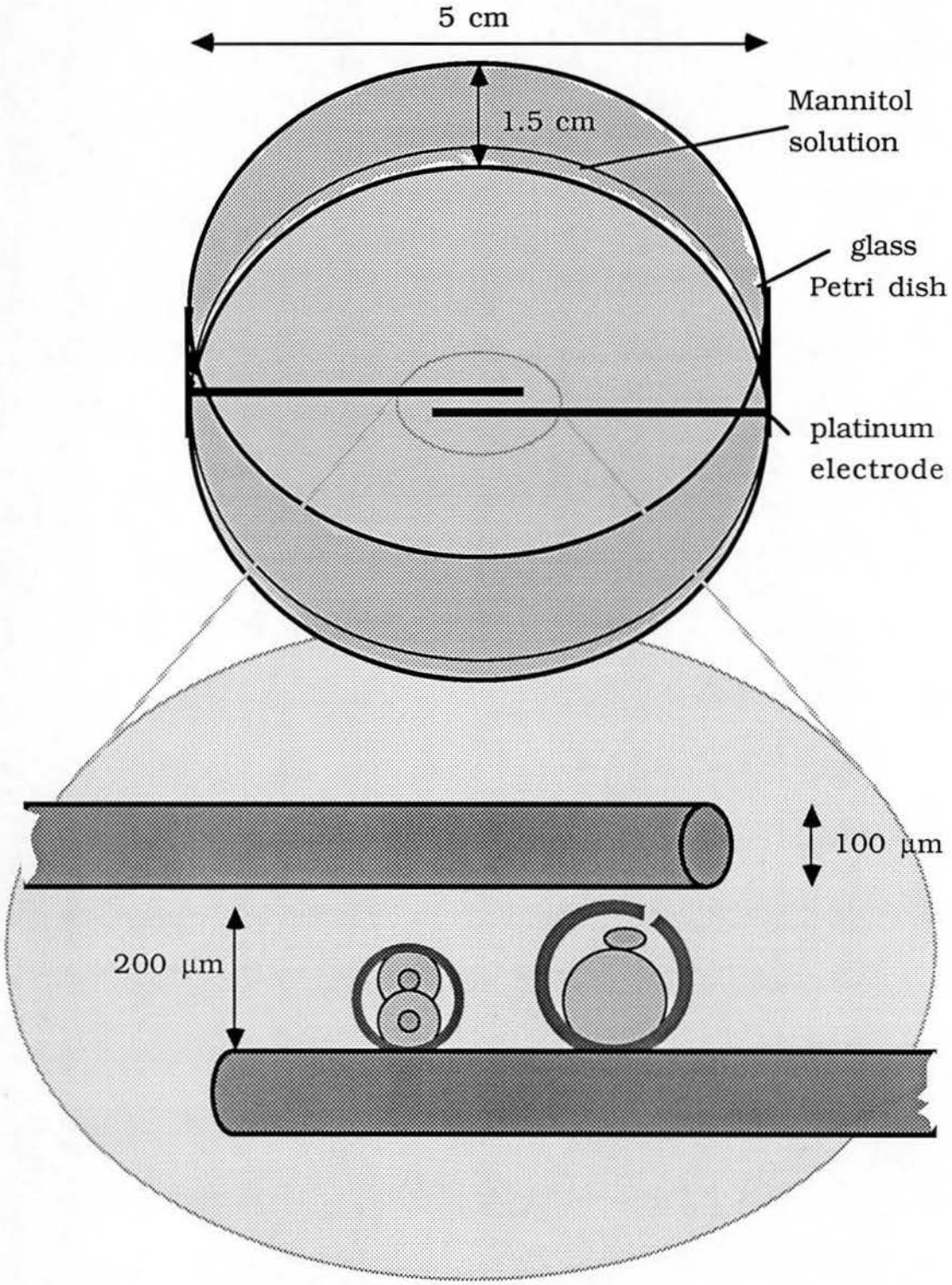


Figure 2.7. Diagram of electrofusion chamber built from two 100 μm platinum wires stuck to the bottom of a glass Petri dish.



Medium used for electrofusion was either an electrolyte and highly conductive medium (M2) or an isotonic and non-conductive solution composed of 0.3 M mannitol, 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ as described by Willadsen (1986). The mannitol solution was used only when applying a.c pulses since a.c causes turbulence and heating in electrolyte solutions. In this case embryos were equilibrated in mannitol for 15 min before electrofusion. Immediately after fusion, embryos were placed in manipulation medium and incubated at 37^{o}C for a period of 1 hour when fusion was assessed.

2.4. **Analysis of data:**

Statistical analysis was done in most cases using Fisher's exact test for two-way contingency tables. This test was available as a software program developed by D.I. Sales (IAPGR-1988). The electrofusion data was analysed with a GENSTAT software package that transforms binomial data into logit values and performs a regression analysis. This enabled the inclusion of the variations between batches that were very high in the electrofusion experiments. Individual treatments could also be tested for significance using a t-test table.

ELECTROFUSION OF MOUSE AND SHEEP EMBRYOS

3.1. **Introduction**:

The technique for fusion of cells using electrical pulses has become an important tool for studying the development of mammalian embryos. The reason for this is that it provides an easy and rapid way for fusing together embryonic cells to produce polyploidy, or to fuse together specific components of embryonic cells such as membrane bound nuclei to enucleated embryos for the technique of nuclear transplantation. Other methods available for cell fusion are either unreliable and toxic (i.e, polyethylene glycol and lysolecithin) or, as for inactivated Sendai virus, laborious to obtain and show species and batch variability (McGrath and Solter, 1983a; Willadsen, 1986)

Electrofusion has been successfully used to fuse blastomeres together and for nuclear transplantation experiments in mammalian species as diverse as mice (Kubiak and Tarkowski, 1985; Tsunoda *et al.*, 1987; Kono and Tsunoda, 1988; Barra and Renard, 1988; Clement *et al.*, 1988), rabbit (Ozil and Modlinski, 1986; Stice and Robl, 1988; Clement *et al.*, 1988), sheep (Willadsen, 1986), cattle (Robl *et al.*, 1987; Prather *et al.*, 1987) and pigs (Robl and First, 1985b; Clement *et al*, 1988). Although some of these reports focused specifically on the technique of

electrofusion, the results obtained vary considerably in different laboratories and much research is required to define optimal conditions for fusion.

This chapter, therefore, focuses on defining the effects of the duration and strength of direct- and alternating-current pulses and their influence not only upon fusion and alignment rates, but also on the further viability of electrofused embryos to develop. Although mouse 2-cell embryos were used as a model in most experiments, the technique was also tested in nuclear transplantations to mouse enucleated 1-cell embryos and 'enucleated' sheep secondary oocytes.

3.2. **Materials and methods:**

The electrofusion apparatus and the way in which it was assembled has been described in detail in Chapter 2 (General Materials and Methods). This section provides information on the methods used for the alignment, fusion and further culture of embryos exposed to alternating (a.c.) and direct current (d.c.) pulses. Experiments were designed to investigate the effects of field strength and pulse duration of a.c. and d.c. fields and to assess the viability of the embryos after each treatment. There were also variations in the fusion medium utilized during these procedures and in the stage of development of mouse and sheep embryos used for experimentation.

Pronuclear and 2-cell mouse embryos were fused initially by exposure to a single d.c. pulse of varying lengths and voltages using

an electrolyte solution (M2) as a fusion medium. In this case the electrofusion chamber employed had electrodes 100 μm apart and embryos were positioned individually and manually so that the surface of contact between the cellular membranes to be fused were as parallel as possible to the platinum electrodes. The alignment of embryos was performed under a dissecting microscope at 50 x magnification using a mouth controlled micropipette.

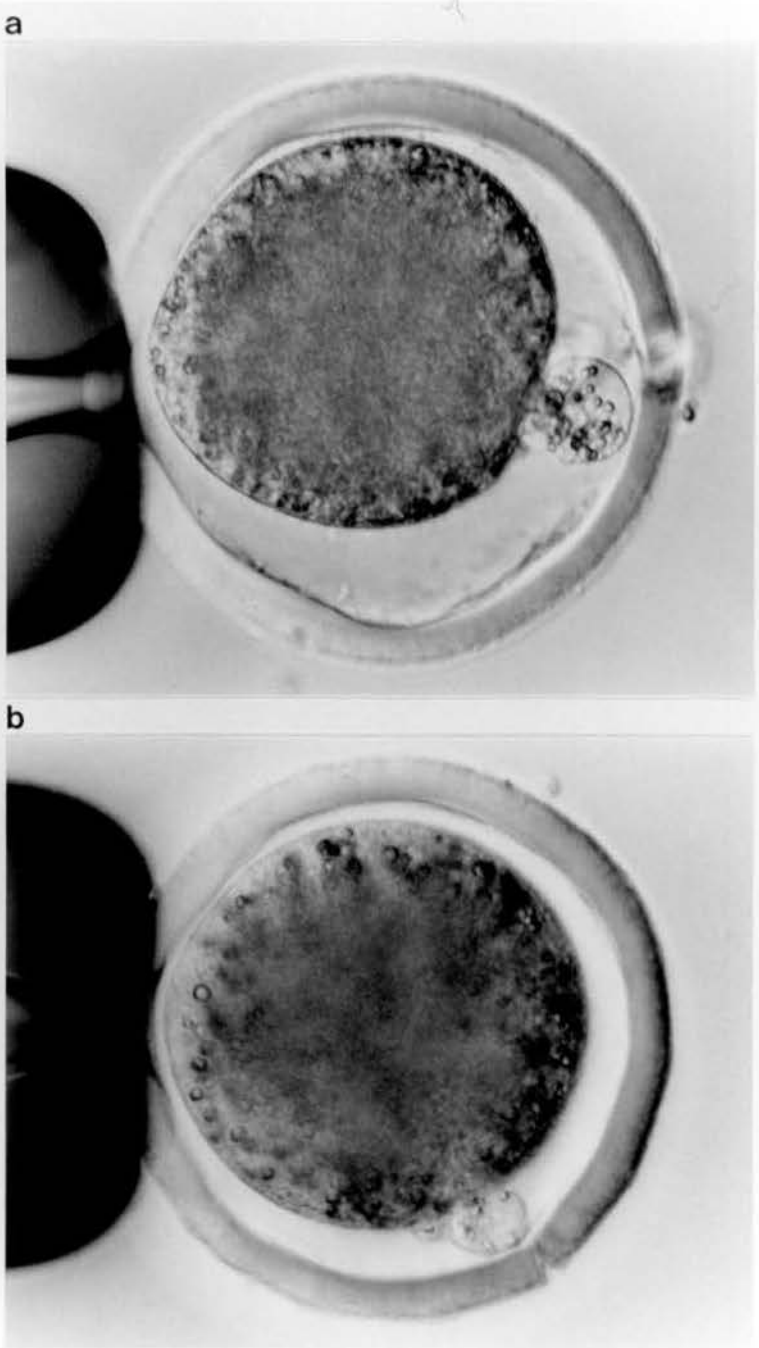
In an attempt to simplify and improve the rates of fusion, the electrofusion apparatus was later adapted with an alternating-current device which produced 500 kHz pulses of varying lengths and voltages immediately before the d.c. fusing pulse. To enable the free rotation of the embryos in the a.c. electric field, the electrofusion chamber with electrodes 100 μm apart was replaced by one with electrodes positioned 200 μm apart and the electrolyte fusion medium was replaced by a solution of 0.3 M mannitol with low conductivity, since it has been shown that a.c causes temperature rises in electrolyte solutions (Zimmerman and Vienken, 1982). Embryos to be fused using the preceding a.c. field were allowed to equilibrate in the mannitol solution for 10 to 15 minutes before placing in the electrofusion chamber. Once the optimal pulse length and intensities had been determined for aligning 2-cell embryos in the a.c. field, the effects of intensity and duration of d.c. pulses on fusion, cell lysis and subsequent *in vitro* development was determined. To investigate the effects of fusion on further development, mouse embryos were placed in medium N°16 immediately after the electrofusion pulse and incubated at 37°C in a 5% CO_2 humidified atmosphere. These embryos were

assessed for lysis one hour later and replaced in the incubator for a further 3 to 4 days to examine their ability to develop into normal blastocysts *in vitro*.

Experiments with sheep embryos were performed using micromanipulated secondary oocytes from which approximately a quarter of the cytoplasm surrounding the first polar body had been removed. A single cell from either a 16-cell embryo or the inner-cell-mass of an early blastocyst was introduced into the perivitelline space of the oocyte (Figure 3.1). Electrofusion techniques in sheep were performed using the mannitol solution in the fusion chamber with electrodes 200 μm apart. Micromanipulated embryos were initially exposed to three 20 volt, 100 μsec d.c. pulses given at approximately 0.3 sec apart (Proc.1). Later this procedure was modified so that the embryos were exposed to a preceding 2 volt, 500 kHz a.c. pulse for 5 sec followed by a single 25 volt, 80 μsec d.c pulse (Proc.2). Immediately after the d.c. pulse, the embryos were placed in OCM at 37°C for one hour at which stage they were assessed for fusion.

Due to the great variability of replicates within similar treatment groups, the data on fusion, lysis and development were transformed into logits and analysed as a normal distribution by fitting a regression analysis. This enabled the correction for the great variation within groups which could not be accounted for when using a simple chi-square or Fisher's test analysis of the pooled rates. Analysis was done using a statistical package (Genstat Reference Manual, 1986). This program allows the determination of interactions between different parameters, effects caused by

Figure 3.1. Micromanipulated sheep embryos prepared for electrofusion in which single cell from (a) 16-cell stage (day 4) and (b) ICM of early blastocysts (day 6) were placed into the perivitelline space of 'enucleated' secondary oocytes.


a
b

either parameters and of comparisons of individual cells in a two-way table.

3.3. **Results**:

This section presents the results concerning fusion, alignment, lysis and development rate of mouse and sheep embryos. Experiments in which an a.c. pulse preceded a d.c. pulse (non-conductive medium) were performed at a later stage and therefore not concurrent with those using d.c. pulses alone (conductive medium). Although this poses a problem when trying to compare the two procedures, this comparison was still informative since major differences were detected and there are no reasons to believe that changes other than those introduced by the experimental procedure would have influenced these results.

3.3.1. Electrofusion of mouse embryos in an electrolyte solution:

Mouse 2-cell embryos were positioned between electrodes in M2 medium and exposed to d.c pulses of various field strengths and durations. Fusion rates of the different groups and the number of embryos exposed in each group are presented in Table 3.1. There was no significant interaction between the electric field and the duration of the d.c. pulse ($P>0.05$) neither did the strength of the electric field alone have a significant effect on the fusion rates ($P>0.05$). Although the effect of pulse duration was also not significant ($P>0.05$) the chi-square value was very close to the 5%

Analysis was performed on expected and not observed values and, therefore, the significancy information on the tables refers to the expected values.

Table 3.1. Rates of fusion between blastomeres of 2-cell mouse embryos aligned manually between electrodes 100μm apart and exposed to single direct current pulses in an electrolyte solution (M2).

Field strength (kV/cm)	Pulse duration (μsec)					Total
	60	80	100	120	140	
1.0	51.9% (27)*	84.0% (25)	55.6% (27)	83.3% (24)	80.8% (26)	70.5% (129)
1.5	55.6% (27)	73.1% (26)	67.9% (28)	77.9% (26)	72.4% (29)	69.1% (136)
2.0	61.5% (26)	72.0% (25)	46.2% (26)	44.0% (25)	70.4% (27)	58.9% (129)
Totals	56.3% (80)	76.3% (76)	56.8% (81)	68.0% (75)	74.4% (82)	66.2% (394)

* Number of embryos in each group

level of significance. Individual comparisons indicated higher fusion rates at pulse durations of 80, 120 and 140 μsec and at field intensities of 1.0 and 1.5 kV/cm ($P<0.01$).

Since there was no obvious effect of the field intensities examined with 2-cell embryos, a single intensity of 1.5 kV/cm was used to examine the effects of different pulse durations on fusion rates between pronuclear karyoplasts and enucleated 1-cell embryos. After having manually aligned the manipulated embryos between the electrodes, these were exposed to single pulses with lengths varying from 50 to 200 μsec. Fusion rates for the different pulse lengths are shown in Table 3.2. Although there seems to have been a trend towards longer periods producing greater fusion rates, there was no significant effect of these pulse durations on fusion rates ($P>0.05$). The ability of the fused reconstituted embryos to develop further to the blastocyst stage *in vitro* after 94 hours was recorded and is presented as percentages in Table 3.3. No significant effect of pulse duration could be detected concerning the ability of these embryos to develop to blastocysts ($P>0.05$). However, there was a slight loss in viability during the electrofusion procedure determined by the significant difference of the rates of blastocyst formation in the pooled electrofused groups as compared to the controls ($P<0.05$). This indicates that the technique alone has a small, but real, deleterious effect on the viability of the reconstituted embryos shown by the inability of some embryos to develop to the blastocyst stage *in vitro*.

Table 3.2. Rates of fusion between pronuclear karyoplasts and enucleated 1-cell embryos aligned manually between electrodes 100μm apart and exposed to a single direct current pulse in a electrolyte solution (M2). Manipulated embryos were exposed to pulses of different durations (μsec), and at a constant 1.5 kV/cm field strength.

50μsec	75μsec	100μsec	125μsec	150μsec	175μsec	200μsec
55.1% (49)*	70.0% (50)	64.0% (50)	73.5% (49)	70.0% (50)	64.0% (50)	81.6% (49)

* Number of manipulated embryos in each group.

Table 3.3. Effect of variation in pulse length on the proportion of embryos developing to blastocysts after a 94 h period *in vitro* . Reconstituted embryos were derived by the electrofusion of pronuclear karyoplasts to enucleated 1-cell embryos in an electrolyte solution (M2) at constant 1.5 kV/cm field strength.

Control	50µsec	75µsec	100µsec	125µsec	150µsec	175µsec	200µsec
81.8%	52.6%	64.3%	66.7%	85.2%	70.0%	59.1%	60.7%
(44)*	(19)	(28)	(21)	(27)	(20)	(22)	(28)

* Number of manipulated embryos in each group.

3.3.2. Alignment of 2-cell mouse embryos in an a.c. field:

These experiments were designed to investigate the effects of a.c pulses of varying lengths and intensities on the alignment and further viability of 2-cell embryos. Alignment rates were determined by microscopical observation of the orientation of the 2-cell embryos in the electric field. The effects of electric field strength and duration on alignment rates of 2-cell embryos randomly positioned between electrodes 200 μm apart in mannitol solution are shown in Table 3.4. There was not a significant interaction between the duration of the pulse and field strength ($P>0.05$). However, the effect of pulse intensity was highly significant ($P<0.001$) with a clear indication of higher alignment rates at higher a.c. field intensities. Pulse duration was also significant at a 5% level ($P>0.025$), but this parameter was less effective than the intensity of the pulse. The most effective alignment settings were those at field intensities of 0.025 kV/cm or higher for periods of 10 sec or longer.

The viability of treated 2-cell embryos after their exposure to the mannitol solution and the a.c. pulse was examined by culture in medium N°16 for 3 days. Ninety-nine percent (333/336) of the embryos exposed to the various a.c. pulse treatments developed to the blastocyst stage regardless of whether derived from fused or non-fused embryos. There was no significant difference between the a.c. treated embryos and control 2-cell embryos, placed in mannitol for similar periods, concerning their ability to develop into blastocysts *in vitro* (73/76; $P>0.05$). These results indicate that the technique of cellular alignment using these levels of field intensity and pulse duration has no significant effect on the

Table 3.4. The effect of pulse length and voltage upon alignment rates of 2-cell mouse embryos positioned between electrodes 200 μm apart and exposed to 500 kHz alternating-current.

Pulse duration	Alternating-current pulse intensity				Totals
	0.05kV/cm (1.0V)	0.125kV/cm (2.5V)	0.25kV/cm (5.0V)	0.375kV/cm (7.5V)	
5 sec.	36.7% (30)	73.3% (30)	93.3% (30)	96.0%* (26)	74.1% (116)
10 sec.	50.0% (30)	96.8%* (31)	96.7%** (30)	100%** (26)	85.5% (117)
20 sec.	38.5% (26)	84.6% (26)	96.2%* (26)	100%** (25)	79.6% (103)
Totals	41.9% (86)	85.1% (87)	95.3% (86)	98.7% (77)	79.8% (336)

** ($P<0.01$); * ($P<0.05$) differ significantly from the average alignment rate;

viability of 2-cell mouse embryos. All the control 2-cell embryos not placed in mannitol developed into blastocysts (40/40). These results also indicate that after placing 2-cell embryos in the mannitol solution there is no significant decrease in their ability to develop to blastocyst *in vitro*. In all the embryos examined, there was not a single case of lysis which further supports the conclusion that these alignment treatments are harmless. Approximately 3% of the treated embryos fused within one hour from treatment indicating that an a.c. pulse at these levels of intensity can, in a small proportion of cases, cause fusion between 2-cell blastomeres.

3.3.3. Electrofusion 2-cell embryos in non-conductive medium:

In this experiment mouse 2-cell embryos were placed between electrodes 200μm apart in mannitol and exposed to a fixed 0.25 kV/cm a.c. pulse for 5 seconds followed immediately by a single d.c. pulse. After treatment, data were recorded on rates of fusion, lysis and development to blastocysts. The effects of field strength and pulse duration of the d.c. pulse on fusion rates are shown in Table 3.5. There was not a significant interaction between the effects of field strength and pulse duration on the proportion of embryos fusing after treatment ($P>0.05$). Although no significant effect could be detected by the duration of pulse($P>0.05$), there was a trend towards higher fusing rates at the lowest pulse durations. However, the effect caused by the strength of the pulse was highly significant ($P<0.001$) with the highest rates of fusion at the lower field intensities. The best rates of fusion were recorded

Table 3.5. Rates of fusion of blastomeres of 2-cell mouse embryos placed between electrodes 200μm apart in a non-conductive medium (mannitol), aligned with an 500kHz a.c. pulse and exposed to a single d.c. pulse of varying intensities and durations.

Field Strength (kV/cm)	Pulse duration (μsec)					Totals
	60	80	100	120	140	
1.0	80.0%** (40)^	70.0%** (40)	40.0% (40)	70.0% (40)	52.5% (40)	62.5% (200)
1.5	52.5% (40)	50.0% (40)	32.5% (40)	20.0% (40)	22.5% (40)	35.5% (200)
2.0	22.0% (41)	27.5% (40)	17.1% (41)	12.5% (40)	22.5% (40)	20.3% (202)
Totals	51.2% (121)	49.2% (120)	29.8% (121)	34.2% (120)	32.5% (120)	39.4% (602)

^ Number of embryos in each group;
** Differ significantly from the average fusion rate ($P<0.01$).

at intensities of 1.0 kV/cm and at pulse durations of 80 μsec or less.

The effect of pulse duration and field strength on the proportion of embryos lysing after treatment are shown in Table 3.6. There was no significant interaction between the intensity and the duration of the pulse ($P>0.05$). The effects of pulse duration alone were also not significant ($P>0.05$) although there was a trend towards higher rates of lysis with longer pulses. However, the effect of the strength of the d.c. pulse was highly significant ($P<0.001$) indicating that stronger pulses cause lysis at much higher rates. Lysis did not occur only when the strength of the pulse was 1.0 kV/cm and with pulses of 120 μsec or less. It is interesting to note that two different kinds of lysed embryos were observed; whilst one third (27/80) of the lysed embryos had both blastomeres lysed, the other two thirds (53/80) had only one lysed blastomere. The fact that double lysed embryos were only observed in the group exposed to 2.0 kV/cm field strength indicates that this kind of lysing is related to the intensity of the d.c. pulse.

The ability of the non-lysed fused and non-fused 2-cell embryos to develop further *in vitro* to the blastocyst stage was assessed by placing the embryos in N°16 medium and culturing for 3 days in a 5% CO_2 incubator at 37°C. The rates of development to blastocyst of each of the experimental groups are presented in Table 3.7. There was not a significant interaction between the effects of field strength and pulse duration on the ability of treated embryos to develop into blastocysts ($P>0.05$). There was also not a significant effect of the duration of the pulse

Table 3.6. Rates of lysis of blastomeres in 2-cell mouse embryos placed between electrodes 200μm apart in a non-conductive medium (mannitol), aligned with an 500kHz a.c. pulse and exposed to a single d.c pulse of varying intensities and durations.

Field Strength (kV/cm)	Pulse duration (μsec)					Totals
	60	80	100	120	140	
1.0	none (40)^	none (40)	none (40)	none (40)	5.0%** (40)	1.0% (200)
1.5	2.5% (40)	2.5% (40)	2.5%** (40)	10.0%** (40)	17.5%** (40)	7.0% (200)
2.0	15.0%** (41)	25.0%** (40)	31.7%** (41)	57.5%** (40)	32.5%** (40)	32.2% (202)
Totals	5.8% (121)	9.2% (120)	11.6% (121)	22.5% (120)	18.3% (120)	13.5% (602)

^ Number of embryos in each group;
** Differ significantly from the average lysing rate ($P<0.01$).

Table 3.7. Rates of development *in vitro* to blastocysts of non-lysed 2-cell embryos assessed after 3 days. embryos were placed between electrodes 200μm apart in a non-conductive medium (mannitol), aligned with an 500kHz a.c. pulse and exposed to a single d.c. pulse of different intensity and duration.

Field Strength (kV/cm)	Pulse duration (μsec)					Totals
	60	80	100	120	140	
1.0	90.0%** (40)^	97.5%** (40)	100%** (40)	92.5% (40)	89.5% (38)	94.0% (198)
1.5	84.6% (39)	87.1% (31)	59.0% (39)	55.0% (36)	33.3% (33)	64.0% (178)
2.0	42.9% (35)	26.7% (30)	39.3% (28)	5.9% (17)	7.4% (27)	27.0% (137)
Totals	73.7% (114)	73.3% (101)	69.2% (107)	62.4% (93)	48.0% (98)	65.7% (513)

^ Number of embryos in each group; ** $P<0.01$.

(P>0.05) although there was a trend towards a higher development when shorter pulses were used. However, there was a highly significant effect of the intensity of the pulse (P<0.001) indicating a developmental advantage of embryos exposed to the lower field intensities. Best developmental rates were reported for embryos exposed to d.c pulses of intensity of 1.0 kV/cm and for durations from 60 to 100 μsec.

Developmental rates of fused and not-fused embryos were considered jointly since there was no indication of a developmental advantage of the diploid group over the group that may have been tetraploid. Actually, when considering the total population of embryos exposed to electric pulses, the proportion developing to blastocysts is higher in the group derived from fused embryos (182/237=76.8%) than in the group derived from non-fused embryos (145/284=51.1%) (P<0.01). However, the majority of the embryos that did not fuse were derived from groups which showed lower developmental rates while those that did fuse were mostly derived from groups with higher developmental rates. To avoid this bias, fused and non-fused were compared within the groups where lysis had not occurred (1.0 kV/cm and pulse duration of 120 μsec or less). In this population the electrofusion technique was less likely to have caused any deleterious effect on further development of both fused and non-fused groups. This assumption was supported by the results which showed that the proportion of embryos developing to blastocyst in the fused group was not significantly different from the non-fused group, nor from the control 2-cell embryos placed in mannitol (94.2% vs. 96.4% vs. 96.6%, P>0.05).

3.3.3. Electrofusion of sheep embryos:

Fusion rates between 'enucleated' secondary oocytes and single disaggregated embryonic cells from either 16-cell or inner-cell-mass (ICM) of early blastocyst stage embryos are shown in Table 3.8. Data is presented for each of two electrofusion procedures. Although the two groups were not tested concurrently, comparisons between electrofusion procedures indicated a significant improvement in the efficiency of fusion when using Proc.2 for both 16-cell ($P<0.01$) and ICM ($P<0.01$) groups. Comparisons within nuclear donor stage were performed concurrently and therefore were less likely to be influenced by their exposure to unidentified variation. Using Proc.1, 16-cell blastomeres fused significantly more efficiently to 'enucleated' oocytes than ICM cells ($P<0.01$). This was possibly due to to the larger surface area of contact between the fusing membranes since the diameter of an early blastomere ICM cell (20 μm) is approximately two thirds that of 16-cell embryo blastomere (30 μm) (Figure 3.1, a and b). However, when comparing groups within Proc.2, There was no significant difference between groups suggesting that the diameter of the 16-cell blastomere or ICM cell was no longer a critical factor for fusion ($P>0.05$).

3.4. **Discussion:**

The data presented above shows that both mouse and sheep embryos can be fused by exposure to a short direct-current (d.c.) which may be preceded by an alternating-current (a.c.) pulse.

Table 3.8. Rates of fusion between 'enucleated' secondary oocytes and single cells derived from either 16-cell embryos or the inner-cell-mass of early blastocysts in sheep after exposure to electrofusion direct current (d.c.) with and without a preceding alternating current (a.c.) polarization pulse.

Electrofusion Procedure	16-cell	ICM	Total
d.c	65% (49)*	28% (46)	47% (95)
a.c + d.c	90% (109)	81% (26)	88% (135)

* number of manipulated embryos exposed to the specific treatment.

Using a single d.c pulse in an electrolyte solution, fusion rates of over 80% can be achieved between sister blastomeres of 2-cell mouse embryos and between pronuclear karyoplasts and enucleated 1-cell mouse embryos. However, by eliminating the need for a precise manual alignment of embryos between the electrodes, the a.c. field enables many embryos to be fused during each procedure and, therefore, simplifies the technique for use with larger number of embryos. In sheep, rates of fusion are improved by preceding the fusion pulse with an a.c. when cells with small diameter are used for fusions to 'enucleated' oocytes.

When fusing together the blastomeres of mouse 2-cell stage embryos, Kubiak and Tarkowski (1985) showed that a double d.c pulse at 1.0 kV/cm field strength produced higher fusion rates than did intensities of 0.8 and 0.6 kV/cm. However, due to limitations of their pulse generator these authors were unable to examine the effects of higher field strengths. The results presented in Table 3.1 show a highly variable pattern of fusion at the intensities and durations examined, which reflects the large variation in fusion rates in different batches of the same treatment. To account for this variation, all data were transformed into logits and analysed by a regression analysis. It is suggested that this variation may have been caused either by differences in the degree of contact between sister blastomeres and/or by differences in the precision of alignment between the electrodes, since the positioning was done manually using a mouth controlled pipette under a dissecting microscope at 50X magnification. Although there was not a significant effect of field strength in electrolyte fusion medium, there was a trend towards higher

fusion rates at the lower intensities, suggesting that field strengths around 1.0 kV/cm may be optimal for these conditions. There was also no effect of varying the duration of the pulses between 60 and 140 μsec, a 2.3 fold range but, when pulse duration was examined at a 12.5 fold range (20 to 250 μsec) other laboratories found beneficial effects of prolonging the pulse (Kubiak and Tarkowski, 1985). The same authors reported large variation between batches within treatments, further supporting the need to use a method of statistical analysis which accounts for this variation.

Although fusion rates were not improved by replacing the fusion medium with a non-conductive mannitol solution and preceding the d.c. pulse with an a.c. pulse in 2-cell mouse embryos, there was a reduction in the variability within treatments which enabled the detection of field strength effects. The precision and reliability of the alignment and adhesion induced by the polarization of cells in the a.c. field were most probably responsible for the homogeneity of these results. Although recordings were not made of the rates of alignment in their experiments, Kono and Tsunoda (1988) found good levels of orientation at field intensities as low as 0.02 kV/cm. However, their experiments were performed in a free floating system while in these experiments embryos were resting on the surface of the dish and, therefore, may have needed a stronger a.c. field for orientation. The finding that viability is maintained even at the highest field intensities is reassuring since these may be required for the alignment of cells of different diameters, ie. karyoplasts and enucleated eggs (Kono and Tsunoda, 1988).

Optimal levels for the strength and duration of the d.c pulse on rates of fusion, lysis and development *in vitro* were at 1.0 kV/cm for periods between 60 and 100 μsec, respectively. These results are similar to those reported by Clement *et al.* (1988) where higher fusion rates were achieved using 70 μsec pulses at field strengths varying from 0.52 to 0.94 kV/cm. These authors also examined the effects of multiple pulses showing no significant effect of 1 to 4 pulses, but a negative effect on fusion and viability after more than 4 pulses. Contrary to the results reported here and those mentioned above, Kubiak and Tarkowski (1985) found higher fusion rates with pulses of longer duration (250-1000 μsec). Although there was no information on lysis rates and further viability of treated embryos, it was reported that pulses of 2000 μsec duration caused immediate lysis. Therefore, one may suspect that these authors also found higher lysing rates and possibly lower viability after treatments using prolonged pulses.

The experience gained from the experiments with mouse embryos enabled a better understanding of the parameters affecting fusion rates and further viability which allowed a more effective utilization of the methodology when experimenting with sheep embryos. Although the two procedures applied to fuse sheep embryos were not compared concurrently, the data indicate that manual alignment can be used with d.c. pulses to obtain fusion between large blastomeres and 'enucleated' secondary oocytes, but that the efficiency of fusion is severely decreased when using cell with diameters less than the width of the perivitelline space. However, blastomere size seems to be less

limiting when an alternating current precedes the d.c. pulse, possibly due to the proper alignment and adhesion caused by the polarization of cells in a.c. fields leading to a closer contact between the fusing membranes at the time of the d.c. fusing pulse (Zimmermann and Vienken, 1982).

Fusion rates for 16-cell and ICM cells was similar to those reported previously in sheep when approximately 90% of the cell fragments fused to 'halved' secondary oocytes after exposure to three 0.75 kV/cm 100 μsec d.c. pulses at 0,1 sec intervals given between two o.3 kV/cm a.c. pulses lasting for several seconds (Willadsen, 1986). These levels of fusion have also been reported when fusing rabbit (84%) and pig (92%) blastomeres to 'enucleated' oocytes (Stice and Robl, 1988; Prather *et al.*, 1988). However, the results with cattle embryos have been less successful (67%) (Prather *et al.*, 1987), suggesting that there may be variations between mammals in relation to the optimal electrofusion parameters. This variation is present when using inactivated Sendai virus since the few attempts using this fusogenic agent with sheep embryos were unsuccessful in this laboratory. However, there seems to be differences in the activity of the viral solutions in different laboratories since fusion rates of 50% have been reported previously in sheep (Willadsen, 1986). The advantage of the Sendai method when used for mouse embryos is that, apart from the high fusion rates, it provides a single step procedure while using the electrofusion technique the microsurgery must be performed separately from the fusion step. For these reasons, the following experiments using mouse

embryos were entirely performed using an inactivated Sendai virus solution as the fusogenic agent.

DEVELOPMENTAL POTENTIAL OF SHEEP BLASTOMERES FUSED TO 'ENUCLEATED' SECONDARY OOCYTES

4.1. **Introduction:**

Mammalian species show differences regarding the embryonic stage at which nuclei can successfully support normal development when fused to an enucleated egg. Research using mouse embryos has indicated that nuclear totipotency is lost soon after the time of embryonic genome activation (Solter, 1987; Surani *et al.*, 1987). These suggestions are based on observations that mouse embryos initiate transcription at the early 2-cell stage (Flach *et al.*, 1982) and karyoplasts derived from embryos beyond the 2-cell stage are unable to support development after fusion to enucleated zygotes (McGrath and Solter, 1984c; Surani *et al.*, 1987).

Sheep, cattle and rabbit embryos initiate transcription at the 8- to 16-cell stage (Cotton *et al.*, 1980; Camous *et al.*, 1986; Crosby *et al.*, 1988) and blastomere nuclei from these embryonic stages fused to enucleated oocytes have been able to support full term development (Willadsen, 1986; Prather *et al.*, 1987; Stice and Robl, 1988). These reports show that the developmental clock of blastomere nuclei can be reset to the time of fertilization after fusion to the cytoplasm of a secondary oocyte since blastocyst formation occurred after a period similar to that of a fertilized egg. However, since little or no transcriptional activity would have

occurred at that stage of development, no reprogramming may have been necessary to dedifferentiate the genome's developmental program back to a totipotent state.

This study was undertaken to investigate whether nuclei derived from sheep embryos beyond the stage of transcriptional activation were able to support development when fused to enucleated secondary oocytes. It was also intended to investigate whether there was a decline in potential from the 16-cell to the ICM of the blastocyst stage.The effects of the microfilament dependent cytoskeletal mechanisms which operate during oocyte activation on the transferred nucleus were also investigated.

4.2. **Materials and methods:**

4.2.1. Manipulation of embryos and oocytes:

Technical procedures for superovulation and the flushing of embryos and secondary oocytes from the oviducts of donor ewes have been described earlier in Chapter 2 (General Materials and Methods). Sixteen-cell and early blastocyst stage embryos were recovered from inseminated Welsh Mountain ewes at approximately 96 h (day-4) or 144 h (day-6) after GnRH injection at 8:00 A.M. Disaggregation of 16-cell embryos into single blastomeres was performed by tearing the zona pellucida with a glass needle, removing the embryo and gently pipetting the cells with a flame polished pipette with an internal diameter of approximately 50 μm. Once separated, these blastomeres were transferred to drops of OCM containing 20% FCS until further use in micromanipulation.

After having split the zona pellucida with a glass needle, early blastocysts were treated by an immunosurgical technique in order to selectively obtain cells from the inner-cell-mass (ICM). The blastocysts were placed in a dish with a heat-inactivated bovine J104 serum diluted 1:10 in OCM at 35°C. J104 is an allogenic antiserum raised in a cow by a skin graft from its calf and found to be active against sheep lymphocytes (Dr. R.L. Spooner, personal communication). After a 30 minute period in the serum, the blastocysts were washed several times in OCM in order to remove all traces of the serum. After this wash, blastocysts were placed in guinea-pig serum diluted 1:5 at 37°C. This served as a source of complement to lyse the outer cells to which the antibodies had attached. After a period of 60 minutes all blastocysts had collapsed and these clumps of cells were removed from the guinea-pig serum and placed into fresh OCM. Lysed outer cell layers were removed using a fine bore pipette with a internal diameter of approximately 50 μm. ICM disaggregation was induced by the exposure to a 0.25% w/v trypsin solution and fine bore pipetting with a 30 μm diameter pipette.

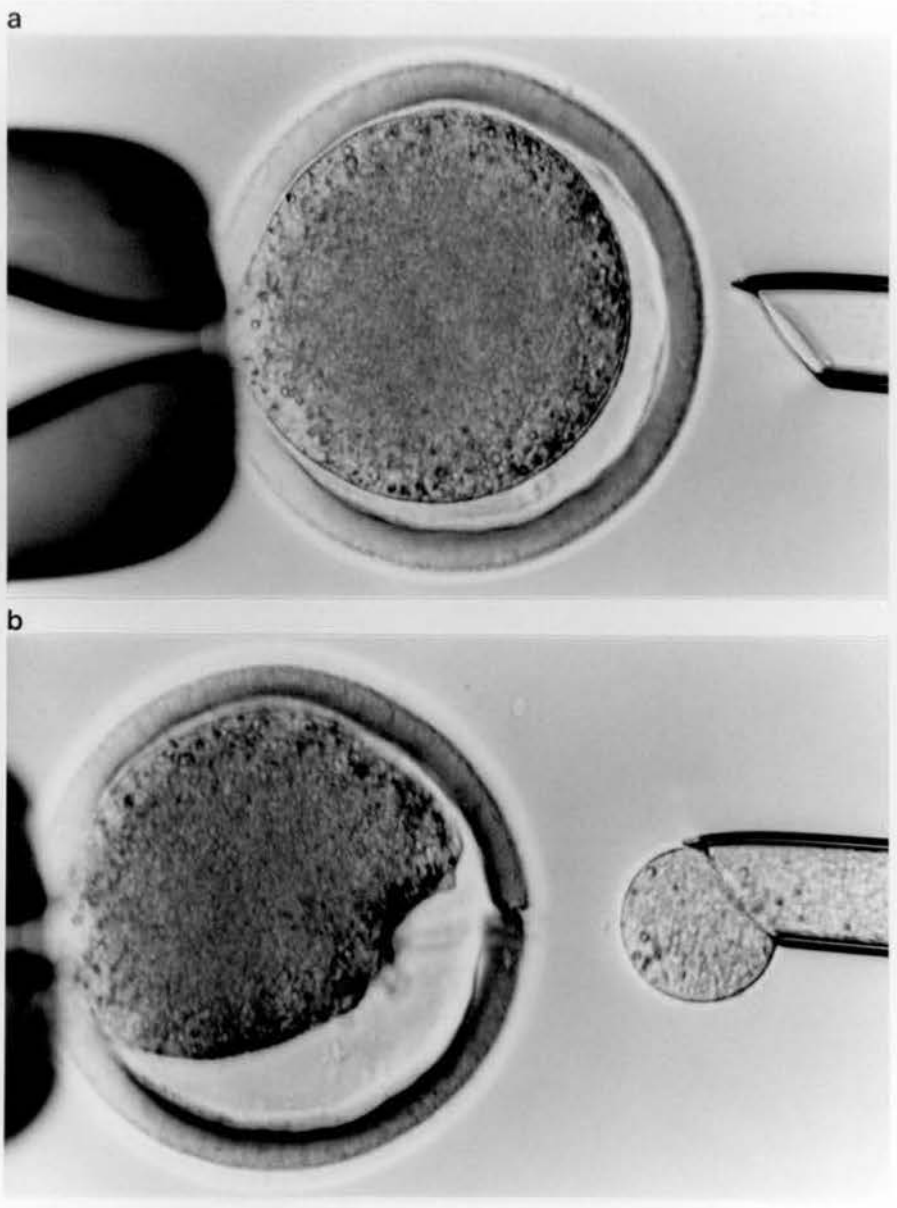
Unfertilized secondary oocytes were obtained by flushing the oviducts of Scottish Blackface ewes at approximately 33 to 35 h after GnRH injection. Oocytes derived from several donors were pooled into a single dish with OCM and randomly drawn in groups of 7 to 10 and transferred into a dish with 7.5 μg/ml of CB in OCM where they were kept for a period lasting one hour. After this period of preparation for 'enucleation', the oocytes were placed in the micromanipulation chamber with the same CB medium together

with a group of disaggregated cells from a single donor embryo for further micromanipulation.

4.2.2. Micromanipulation and embryo transplantation:

Secondary oocytes were positioned with a holding pipette so that the first polar body was facing the microsurgery pipette. When a polar body could not be localized, a smooth patch of cytoplasm normally surrounding the area of polar body extrusion was used to position the oocyte for enucleation (Figure 4.1; a and b). The relationship of this smooth cytoplasmic patch with the position of the second metaphase plate had been previously established using a DNA-specific fluorescent stain (H-33342; 10 μg/ml Hoechst 33342, Sigma, Poole, U.K). A 40 μm pipette was passed through the zona pellucida into the perivitelline space and approximately a quarter of the cytoplasm adjacent to the first polar body was aspirated and removed in a membrane bound fragment. These cell fragments were later pooled together and placed in H33342 for one hour. After staining, the fragments were observed under fluorescence to ascertain whether the metaphase chromosomes were present and access the proportion of oocytes that had been correctly enucleated (Figure 4.2).

Once the secondary oocytes had been 'enucleated', a single blastomere was aspirated into the tip of the microsurgical pipette and, using the same hole in the zona pellucida, injected into the perivitelline space of the 'enucleated' oocyte. These micromanipulated embryos were removed from the micromanipulation chamber and placed into a dish with OCM and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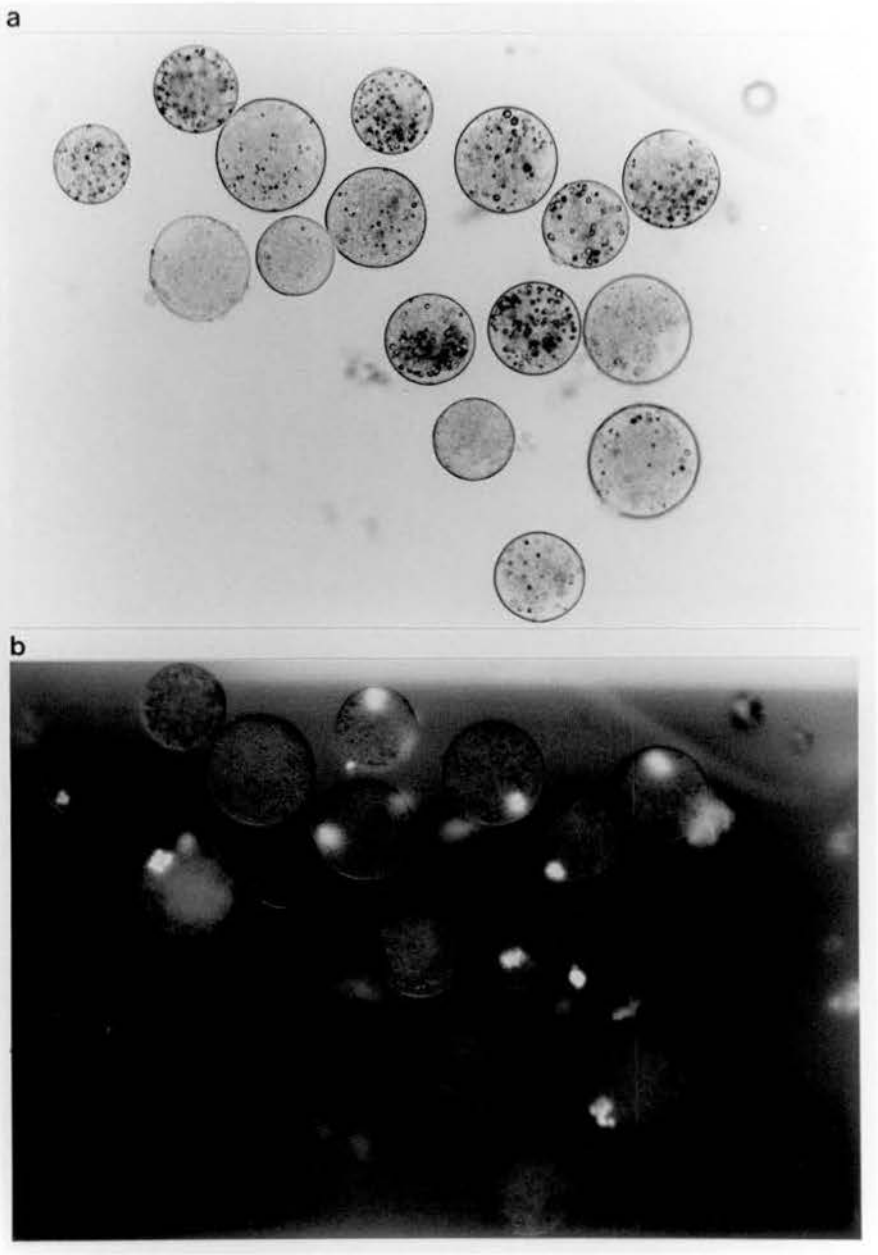
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remained in this medium until the electrofusion procedure. Electrofusion was performed in a mannitol solution as described in detail in Chapter 3. At the end of the electrofusion procedure, embryos were initially washed in OCM and placed in fresh OCM for a further one hour at 37°C after which they were assessed for fusion. Later, the effects of microfilament-dependent cytoskeletal mechanisms on the potential of transplanted nuclei was examined by adding CB to the post-fusion medium. This drug effectively disrupts the microfilament component of the cytoskeleton and prevents cytokinesis (Siracusa *et al.*, 1980).

Fused embryos were embedded in agar 'chips' and transferred to the oviducts of temporary recipient ewes as described in Chapter 2. At recovery after 4 to 5 days, those embryos which had not developed to the morula-blastocyst stage were stained with H33342 to enable the visualization of nuclei. Embryos that had developed normally to the morula-blastocyst stage were transferred to the uteri of definitive recipient ewes to access their ability to implant and develop to term. Recipient ewes that did not return to oestrus were ultrasound scanned at around day 70 of gestation. Pregnant ewes were allowed to follow gestation to term. Total proportions for each specific treatment were obtained by pooling the results from different experimental days (replicates) and data analysis was performed using Fisher's exact test for two-way contingency tables.

4.3. **Results:**

4.3.1. Embryo collection and micromanipulation:

Fifty-nine Welsh Mountain ewes were induced to superovulate, inseminated and flushed during a total of 16 experimental days producing an average of 3.7 16-cell embryos per ewe at day 4 after oestrus and 2.1 early blastocysts per ewe at day 6 after oestrus. Embryos at other developmental stages were recovered, but were not used in experimentation. During the same experimental period, 62 Scottish Blackface ewes were superovulated and their oviducts were flushed producing an average of 7.0 secondary oocytes per ewe of which 81.9% (353) had normal appearance and were successfully micromanipulated. However, by checking the portion of cytoplasm removed during 'enucleation' with a DNA specific stain (H33342), it was noted that metaphase II chromosomes were removed from only 67% (153/233) of the cell fragments analysed.

4.3.2. *In vivo* development in temporary recipients:

Although these groups were not examined concurrently, the development to morula-blastocyst stage of reconstituted embryos placed in ligated oviducts for 4 to 5 days was influenced by placing treated embryos in cytoskeletal inhibitor medium (7.5 µg/ml of OCM) for one hour immediately after electrofusion. Twenty-six out of 65 (40%) embryos recovered from temporary recipients had developed to the morula-blastocyst stage when placed in CB after fusion, while only 5 out of 52 (10%) of recovered embryos had

developed to the same stages in the group placed in OCM alone ($P<0.01$).

More detailed information on the developmental stage of recovered reconstituted embryos derived from the fusion of 16-cell blastomeres to 'enucleated' oocytes and the effects of adding CB to the post-fusion medium are presented in Table 4.1. Although groups were not examined concurrently, a retrospective comparison showed that embryos placed in CB differed from those placed in OCM alone in most developmental groups. When no CB was added, most embryos arrested after a few cleavages, while fewer arrested at similar stages in the CB group (78% vs. 47%, $P<0.01$) (Figure 4.3a). A few embryos had a larger number of blastomeres which showed no sign of compaction (Figure 4.3b). The staining of these non-compact embryos with H-33342 showed a core with a small number of nucleated cells surrounded by anucleate fragments (fragmentation). There were no significant differences in the rates of fragmentation of embryos from groups placed in OCM alone or in OCM containing CB after fusion ($P>0.05$). However, embryos placed in CB medium after fusion had a significantly higher rate of development to the morula-blastocyst stage when compared to those placed in OCM alone (35% vs. 11%, $P<0.01$) (Figure 4.3c and d).

The development *in vivo* of reconstituted embryos derived from the fusion of ICM cells to 'enucleated' secondary oocytes and the effects of adding CB to the post fusion medium are presented in Table 4.2. On average, 20 viable ICM cells were recovered from each blastocyst manipulated, of which approximately 15 could be disaggregated for transplantation. Although OCM and OCM+CB groups were not compared concurrently, a retrospective analysis of

Table 4.1. Development during 4 to 5 days in temporary recipients of reconstituted embryos derived from the fusion of 'enucleated' secondary oocytes to 16-cell (day-4) single blastomeres.

post fusion medium	number of embryos recovered	embryonic stage at recovery				
		1-2 cell	3-16 cell	>16-cell non-compacted	compact morula	blastocysts
OCM	46 [6]***	21 (46)*	15 (33)	5 (11)	4 (9)	1 (2)
OCM + CB**	49 [4]	12 (24)	11 (22)	9 (18)	4 (8)	13 (27)

* number (percentage) developing to each embryonic stage.

** cytoskeletal inhibitor medium (7.5 μg CB/ml).

*** number of days on which experiment was performed

Table 4 2: Development during 4 to 5 days in a temporary recipient of reconstituted embryos derived from the fusion of 'enucleated' secondary oocytes to early blastocyst ICM (day-6) single cells.

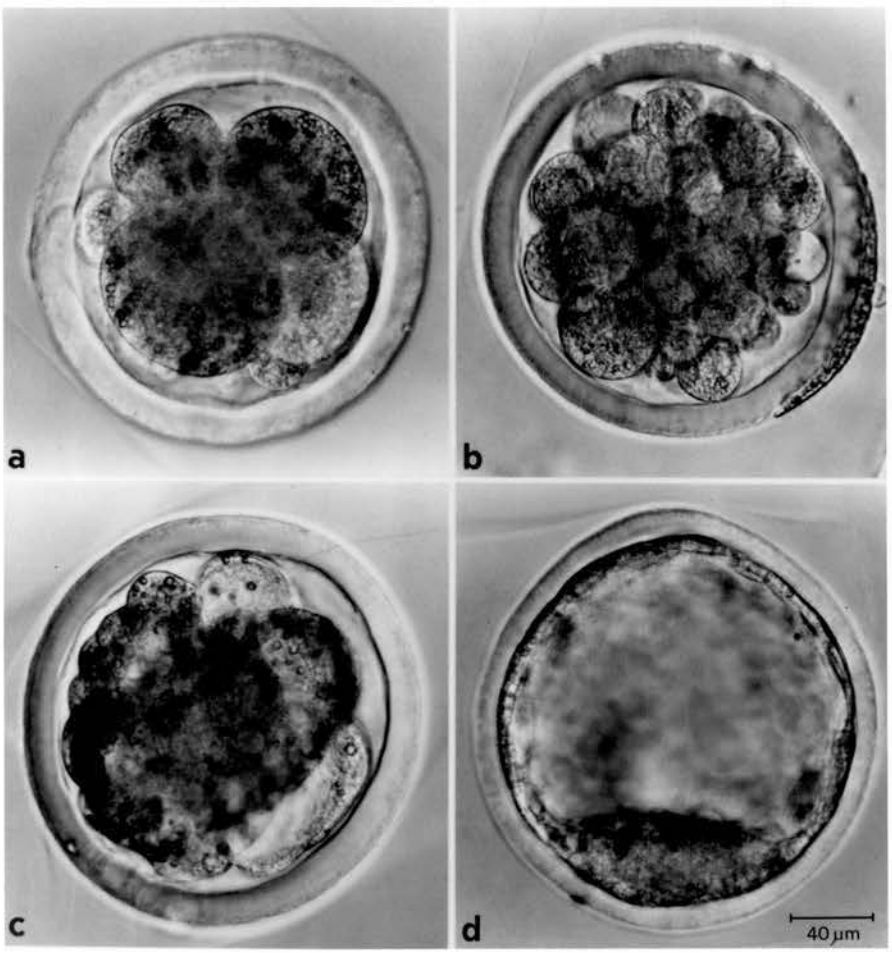
post fusion media	number of embryos recovered	embryonic stage at recovery				
		1-2 cell	3-16 cell	>16-cell non-compacted	compact morula	blastocyst
OCM	6 [2]***	3 (50)*	1 (17)	2 (33)	none (0)	none (0)
OCM + CB**	16 [2]	2 (13)	2 (13)	3 (19)	3 (19)	6 (38)

* number (percentage) developing to each embryonic stage

** cytoskeletal inhibitor medium (7.5 µg CB/ml).

*** number of days on which experiment was performed

Figure 4.3. Developmental stage attained by agar-embedded reconstituted embryos derived from 16-cell blastomeres or ICM cells after 4 to 5 days of culture in ligated sheep oviducts. (a) Two-cell embryo with fragments; (b) non-compact embryo with anucleate outer blastomeres; (c) compact morula; (d) expanded blastocyst. Only (c) and (d) were used for transplantations to second recipients.


a
b
c
d
40 µm

their performances showed that there was a significant increase in the rate of development to the morula-blastocyst stage when embryos were placed in CB medium for one hour after electrofusion (0% vs. 56%, $P<0.01$) and more embryos stopped mitosis after a few cleavages when no CB was added to the post-fusion medium (67% vs. 25%, $P<0.01$). The morphological appearance of embryos recovered from the ICM and 16-cell groups was similar (Figure 4.3).

A few morulae and blastocysts were transferred to the uteri of second recipient ewes and their individual ability to develop is shown in chronological order of transfer in Table 4.3. One to three embryos were transferred to each recipient ewe of which 40% (4/10) were diagnosed pregnant at scanning (70 days). As the embryo transfers were performed in late-February, near to the end of the reproductive season, this discrepancy may reflect fetal loss or the ewes' having become anoestrus. The proximity to the end of the season also interfered with the synchronization of ewes used as secondary recipients leading to asynchronies with oocyte donors of up to 2 days. Pregnancy was only established in recipients within one day of synchrony with oocyte donor and receiving more than one embryo. One pregnant ewe died at 130 days gestation due to a clostridial infection. A single and fully developed Welsh Mountain lamb was recovered from the uterus at post-mortem examination. Three other pregnant ewes delivered Welsh Mountain lambs at approximately 145 days gestation (Figure 4.4). The lamb derived from the ICM blastomere group died during parturition due to distocia (Figure 4.4b). At post-mortem examination it was found to be fully developed with no external or internal abnormalities.

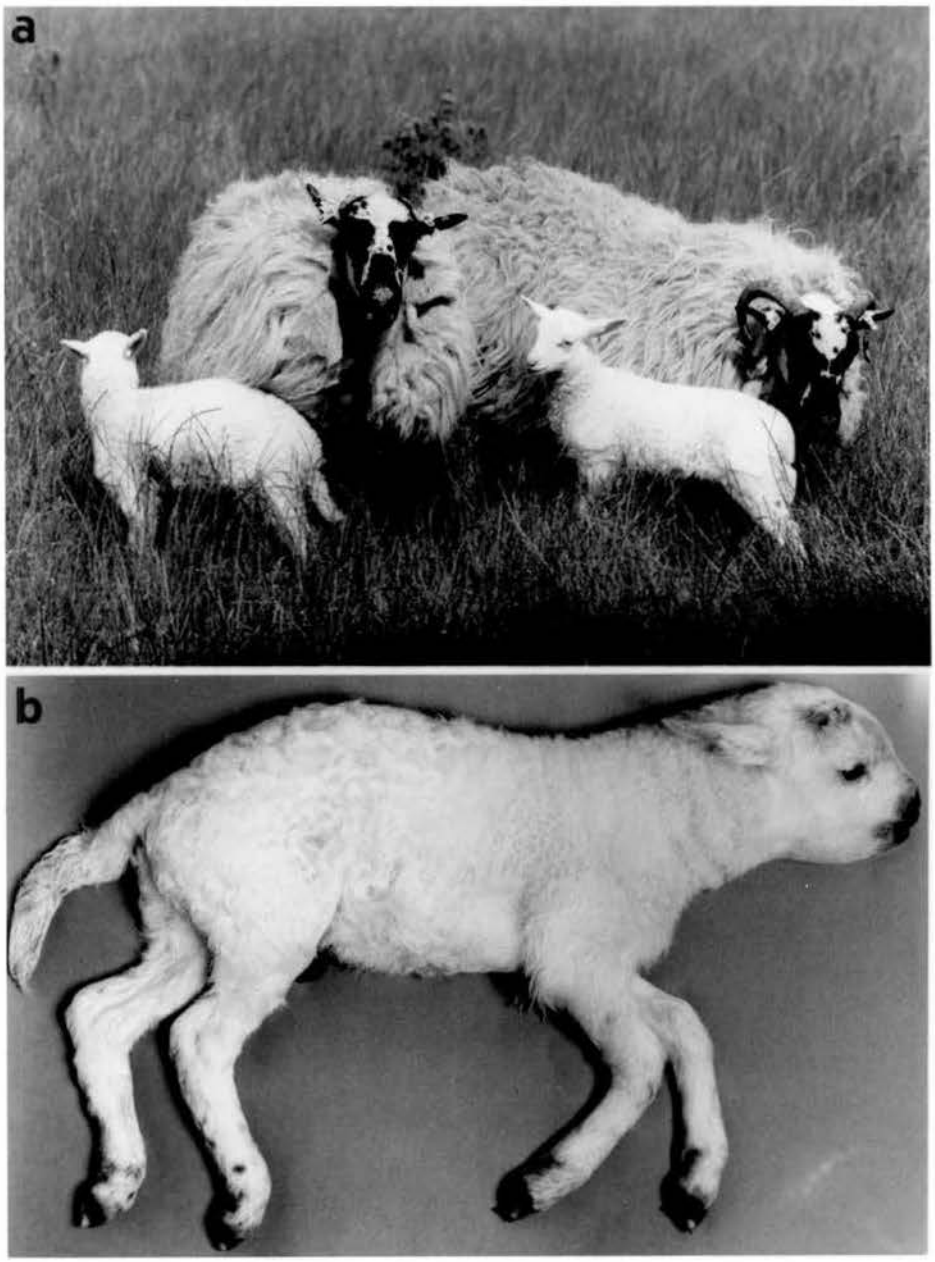
Table 4.3: Development *in-vivo* of reconstituted embryos recovered as morulae or blastocysts from temporary recipients and transferred to the uteri of definitive recipients.

recipient number	degree of** synchrony	nuclear donor	embryos* transferred	returned to estrus	day 70 diagnosis	gestation period
7E23	-2 days	16-cell	1 blast.	21 days	-----	-----
7E40	synchron.	16-cell	3 blast.	15 days	-----	-----
7E26	synchron.	16-cell	1 blast,2 mor.	-----	positive	died 130
7E209	-1 day	ICM	2 blast.	17 days	-----	-----
7E62	-2 days	ICM	3 blast.	17 days	-----	-----
7E174	-2 days	16-cell	1 blast.	-----	negative	-----
7E86	-1 day	16-cell	2 blast.	-----	positive	143 days
7E41	+1 day	16-cell	3 blast.	-----	positive	145 days
7E122	synchron.	16-cell	1 blast.	-----	negative	-----
7E141	synchron.	ICM	1 blast,2 mor.	-----	positive	148 days

* blast. = blastocyst(s) ; mor. = morula(e).

** difference between the definitive recipient and the oocyte donor.

Figure 4.4. Welsh Mountain lambs derived from the fusion of blastomeres from Welsh Mountain embryos to Scottish Blackface unfertilized and 'enucleated' oocytes. (a) Two of the lambs derived from 16-cell blastomere group; (b) lamb derived from the ICM cell group showing complete development to term. The head edema is a characteristic result of the dystocia.


a
b

As confirmed by the staining of the extirpated cytoplasm with H33342, the real enucleation rate of the 'enucleated' oocytes used for fusion to 16-cell and ICM cells was not significantly different (100/148, 68% vs. 56/85%, 66%, P>0.05). This, plus the fact that these groups were examined concurrently during the same experimental period, allowed a comparison to be made between the 16-cell and the ICM groups when placed in CB medium after fusion. No significant difference could be detected between these two groups regarding their potential to support development to compact morulae or blastocysts in ligated oviducts (17/49, 35% vs. 9/16, 56%, P>0.05). Three of the 14 embryos (21%) transferred to definitive recipients developed into normal fetuses in the 16-cell group and one out of nine (11%) in the ICM group. Although the number of embryos transferred to secondary recipients is too small for statistical comparisons, they do not indicate major differences between the groups. However, pregnancy rates in both groups may have been affected by asynchronous transfers and seasonal effects.

5.4. **Discussion:**

The absence of Scottish Blackface characteristic in the lambs confirms that the donor nuclei dictated their phenotypes. All recipient eggs were unfertilized oocytes and, therefore, not capable of development which further supports the conclusion that the donor nuclei were successfully incorporated. These results show that in sheep, nuclei from embryos beyond the stage of genomic activation are still able to support normal development to birth when transplanted into the cytoplasm of an

enucleated secondary oocyte. Moreover, 16-cell and early blastocyst ICM nuclei were able to support development to blastocyst at similar rates. These findings show that genomic activation does not completely restrict the potential of blastomere nuclei and that nuclei of some cells can be reprogrammed to the time of fertilization, revealing considerable opportunities in animal breeding and production.

Willadsen (1986) reported full-term development in sheep after transfer of nuclei derived from 8-cell and possibly 16-cell stage embryos fused to enucleated oocytes. Although the reconstituted embryos reset development to the time of fertilization, the donor nuclei may not have been transcriptionally active since the transition from maternal to embryonic control of transcription has been reported to occur around the 4th to 5th cell cycle in sheep. This is inferred from the fact that embryos collected at the 4-cell stage will consistently cleave beyond the 5th cell cycle *in vitro*, but stop cleaving between the 3rd and 5th cell cycle (5- to 12-cell) when transcription is blocked with alpha-amanitin (Crosby *et al.*, 1988). As the embryos used in these studies were similar to blastocysts shown to have 153 cells (Witenberger-Torres and Sevellec, 1987), the cells were probably at the 7th to 8th cell cycle and expected to be beyond the stage of transition to embryonic control over cleavage and development.

It has been proposed that transcriptionally-active nuclei cannot successfully interact with cytoplasm which is still operating on a maternally inherited programme (Crosby *et al.*, 1988). There are three experimental observations that are

incompatible with this hypothesis. In cattle, where the genome is activated by the 8-cell stage (Camous *et al.*, 1986), it has been shown that normal development to term can be achieved from the fusion of cells from 32-cell stage morulae to enucleated oocytes (Marx, 1988). Similarly, in the present experiments, nuclei from sheep ICM cells have supported normal development to term. Finally, in mice, where the genome is activated at the initial stages of the 2nd cell cycle (Flach *et al.*, 1982), enucleated zygotes fused to karyoplasts derived from 2-cell embryos at the late stages of the cycle (post-activation) were able to support preimplantation development at higher rates than those derived from embryos soon after cleavage and, therefore, before or at the time of genomic activation (Chapter 5).

One other factor that influences the development of reconstituted embryos is the ability of the cytoplasm to reprogram transferred nuclei. Szollosi *et al.* (1989) reported that the changes in nuclei after fusion to unfertilized oocytes depend upon the time of transfer in relation to activation. When thymocyte nuclei were transferred up to three hours after activation, the nuclear membrane broke down and then reformed. By contrast, this remodeling did not take place following transfer at a longer interval after activation. This indicates that whereas chromatin of nuclei fused to oocytes soon after activation or fertilization would be exposed to the cytoplasm immediately, after later fusion this would not occur until the first mitotic division. There are experimental observations suggesting that cytoplasm derived from unfertilized oocytes have a greater potential to reprogram nuclei than does the cytoplasm from

pronuclear stage embryos. In cattle, nuclei derived from the 2-cell or later stage embryos were unable to support cleavage beyond the 4-cell stage *in vivo* when fused to enucleated zygotes (Robl *et al.*, 1987). However, when fused to enucleated oocytes, the same group showed that development to blastocyst could be achieved from nuclei of embryos up to the 17- to 32-cell stage (Prather *et al.*, 1987). In mice, 3% of the reconstituted embryos derived from the fusion of 8-cell karyoplasts to unfertilized oocytes 3 hr after activation developed to the 8-cell to blastocyst stages (McGrath and Solter, 1986), while none of those fused to pronuclear cytoplast cleaved beyond the 4-cell stage (McGrath and Solter, 1984c). These results may reflect two reasons for the differences between the cytoplasm stages. Firstly, that the specific factors causing reprogramming are only present in the cytoplasm of oocytes for a short period after activation and that the chromatin requires a direct exposure to these factors to bring about reprogramming which will only occur after nuclear membrane breakdown. Secondly, that these factors are present throughout the first cell cycle, but become sequestered in or around the pronuclei and are removed during the process of enucleation. There is a need for further research to define the mechanisms concerned with reprogramming.

It has been reported that electrical pulses will induce the activation of oocytes in several mammalian species (Gulyas, 1976). In mice, secondary oocytes activated in cytochalasin B medium do not extrude the second polar body (Balakier and Tarkowski, 1976) while activation in the absence of inhibitors causes haploidization and irreversible chromosome condensation

in most interphase nuclei fused to enucleated secondary oocytes (Czolowska *et al.*, 1986). The improved development of reconstituted embryos placed in CB medium for one hour immediately after the fusing pulse suggests that when eggs were not placed with inhibitors the exogenous nucleus may have been affected by microfilament-dependent cytoskeletal mechanisms operating at the time of oocyte activation, possibly causing haploidization or other aneuploidy defects to the genome.

If genomic activation does not cause irreversible differentiation and reprogramming can be induced by fusion to the correct cytoplasmic stage, it would seem possible that one may develop methods for producing large numbers of genetically identical offspring in several mammalian species. Further research is required to investigate the factors influencing the decrease in the levels of development both to blastocyst and to term of reconstituted embryos. It is possible that the stage of the cell cycle of nuclear donor and recipient cells must be synchronized to enable compatible nucleocytoplasmic interactions and further development to term. Although mouse embryos are unique for showing a low potential for further development after nuclear transfer already at the 2-cell stage, they should provide an useful model for studying the effects of cell cycle stage on the development of nuclear transplanted embryos.

CELL CYCLE EFFECTS IN RECONSTITUTED 1-CELL EMBRYOS

5.1. **Introduction:**

Contrary to what is found with embryos from other mammalian species, mouse blastomere nuclei seem to lose their potential to support development at early stages of development. Previous reports have shown that karyoplasts from embryos at the 2-cell stage or later have only a limited potential to support further development when fused to enucleated zygotes (McGrath and Solter, 1984c). These observations indicate that in mice, either the 1-cell embryo is unsuitable as a recipient cytoplasm for nuclei of more advanced embryonic stages or that the nuclei differentiate irreversibly after first cleavage making nuclear transplantations impossible in this species. The latter explanation has been mostly accepted since the embryonic genome initiates transcription at the early 2-cell stage (Flach *et al.*, 1982) while in other mammals this occurs at a slightly later stage (Cotton *et al.*, 1982; Camous *et al.*, 1986; Crosby *et al.*, 1988).

The chapter describes experiments to investigate the effects of embryonic stage, both between and within the cell cycle, of karyoplasts and recipient enucleated embryos on the development

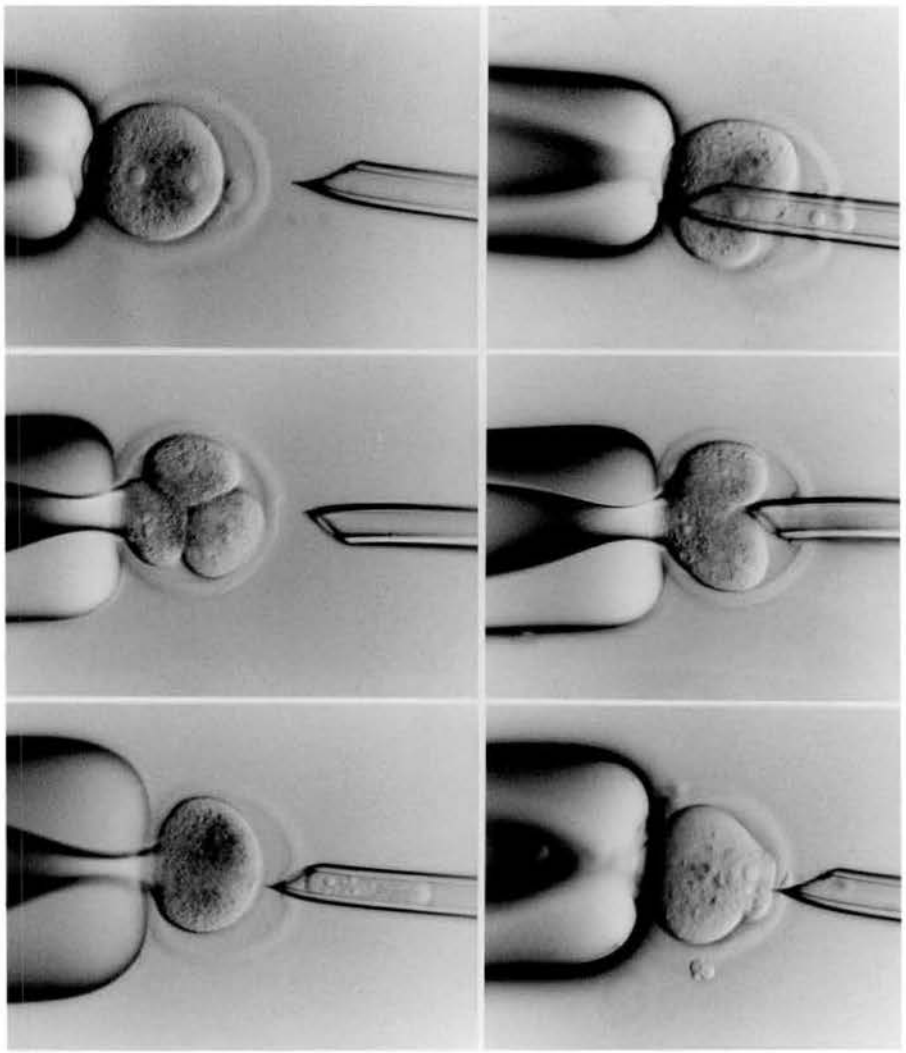
in vitro after transplantation of blastomere nuclei to enucleated zygotes in mice.

5.2. **Materials and Methods:**

One- and 2-cell embryos were obtained as described in Chapter 2 (General Materials and Methods). In some experiments, the superovulation procedure was timed to produce three embryonic stages for each of the 1st and 2nd cell cycles. Zygotes were classified as early (16-20 h after hCG), mid- (20-24 h after hCG) or late (24-28 h after hCG) according to the time of hCG injection. Two-cell embryos were also classified as early (0.5-1 h after an observed cleavage), mid- (40-44 h after hCG) or late (44-48 h after hCG) for similar reasons. After hCG injection, females were paired with F1 males and inspected the following morning for copulation plugs. Embryos were flushed from the oviduct at approximately 1.5 h before microsurgery.

Embryos were placed in N°16 with cytoskeletal inhibitors (1 μg/ml cytochalasin D, Sigma and 0.3 μg/ml nocodazole,Sigma) for 30 min before being placed in M2 with cytoskeletal inhibitors for another 30 min during microsurgery. Nuclear transplantation was carried out as described in Chapter 2 using the technique developed by McGrath and Solter (1983b). Typically, fusion occurred within 1 h of the positioning of karyoplasts and inactivated Sendai virus solution within the perivitelline space of the enucleated 1-cell embryo. Figure 5.1 shows the technical procedures used to enucleate pronuclear embryos and for karyoplast transplantation.

Figure 5.1. Technical procedures employed in the enucleation of 1-cell embryos and for the collection and transplantation of 4-cell karyoplasts.



Only those blastocysts with a distinguishable inner-cell-mass were considered having developed normally and, therefore, used for statistical comparisons.

After nuclear transplantation the embryos were washed several times and placed in N°16 under paraffin oil and incubated at 37.5°C in a humidified atmosphere of 5% CO_2 in air. The embryos were assessed for fusion the following day and then placed in fresh N°16 for a further 3 days when their stage of development was recorded. Embryos were classified 94 h after manipulation as developing into normal blastocysts (successful), or as embryos arrested at a previous stage of development (failure). Data on the proportion developing to blastocysts were compared using Fisher's exact test for two-way contingency tables.

5.3. **Results:**

5.3.1. Developmental potential of blastomere nuclei:

Reconstituted embryos were produced from the fusion to enucleated zygotes of karyoplasts from the first 4 embryonic cell cycles (Table 5.1). The proportion of embryos developing into blastocysts *in vitro* when pronuclei were transferred was not significantly different from that in the control group, suggesting that the technique had little or no effect on the viability of the embryos (96.1% vs. 93.9% , $P>0.05$). However, there was a significant reduction in viability when karyoplasts from 2-cell embryos were fused to enucleated zygotes when only 45.2% ($P<0.01$) developed into normal blastocysts. Moreover, a further reduction in developmental potential was found when karyoplasts from 4- and 8-cell embryos were fused to enucleated zygotes when only 2% ($P<0.01$) and none ($P<0.01$) of the respective

Table 5.1. Effects of embryonic stage on the development *in vitro* to blastocyst after 90 h of enucleated 1-cell embryos fused to karyoplasts derived from the first four cell cycles.

Controls	Karyoplast embryonic stage			
	1-cell	2-cell	4-cell	8-cell
96.1%[a] (76)*	93.9%[a] (49)	45.2%[b] (31)	2.0%[c] (50)	none[c] (24)

a≠b≠c (P<0.01);

*number of fused embryos in each group.

reconstituted embryos developed into blastocysts. Most 4-cell derived embryos stopped development at pre-compaction stages, although a few embryos compacted and developed several vacuoles, but very rarely formed normal blastocysts. The development of 8-cell derived embryos was very limited; most embryos cleaved only a single time and stopped at the 2-cell stage.

5.3.2. Within cell-cycle synchrony effects:

The effect of within-cell cycle-stage synchrony on the viability of reconstructed embryos was examined through the exchange of pronuclei between embryos at similar ('synchronous') and different ('asynchronous') times after hCG injections to obtain early and late stages of the first cycle. Their development to blastocysts is summarized in Table 5.2. 'Synchronous' transfers between embryos at early and late stages of the cycle developed similarly (94% vs. 97%, $P>0.05$), and not significantly differently from the controls (96%, $P>0.05$). Likewise, the asynchronously transferred groups did not differ significantly from each other (74% vs. 78%, $P>0.05$). However, when treatments of both groups were pooled and compared to one another, the 'asynchronous' group showed a lower developmental potential than the 'synchronous' group (76% vs. 95%, $P<0.01$).

5.3.3. Across cell cycle transplantations:

The effect of cycle stage when transferring nuclei from embryos at different cell cycles was investigated by transferring karyoplasts from 2-cell embryos to enucleated zygotes. Embryos were

Table 5.2. Cell cycle stage synchrony effects within the first cell cycle on the proportion of recontituted embryos developing *in vitro* to blastocysts 90 h after fusion of pronuclear karyoplasts to enucleated 1-cell fertilized embryos.

Cytoplast stage	Manipulated controls	Synchronous transfers	Asynchronous transfers
Early zygote (16-20 h after hCG)	91.9%[a] (35)*	96.7%[a] (30)	73.9%[b] (23)
Late zygote (24-28 h after hCG)	98.7%[a] (75)	93.9%[a] (49)	77.8%[b] (27)
Totals	96.4% (110)	94.9% (79)	76.0% (50)

a≠b (P<0.01)

*number of fused embryos after microsurgery.

reconstructed by the fusion of karyoplasts and cytoplasts at early, mid-, and late stages of the 2nd and 1st cell cycle, respectively. An indication of the relationship of these timed intervals after hCG and known biochemical events in the 1st and 2nd cell cycle are shown in Figure 5.2. The donor females and the superovulation regime were the same in the present experiments and in the biochemical studies, although the males were of a different origin. Confirmation that the time of development was similar to that in earlier reports is provided by the observation that most embryos collected for the E2 stage cleaved between 30 and 34 h after hCG which, assuming that ovulation and fertilization occur at around 12 h after hCG in mice, is consistent with the results reported by Howlett and Bolton (1985) when 90% of the embryos cleaved between 18 and 22 h after *in vitro* fertilization.

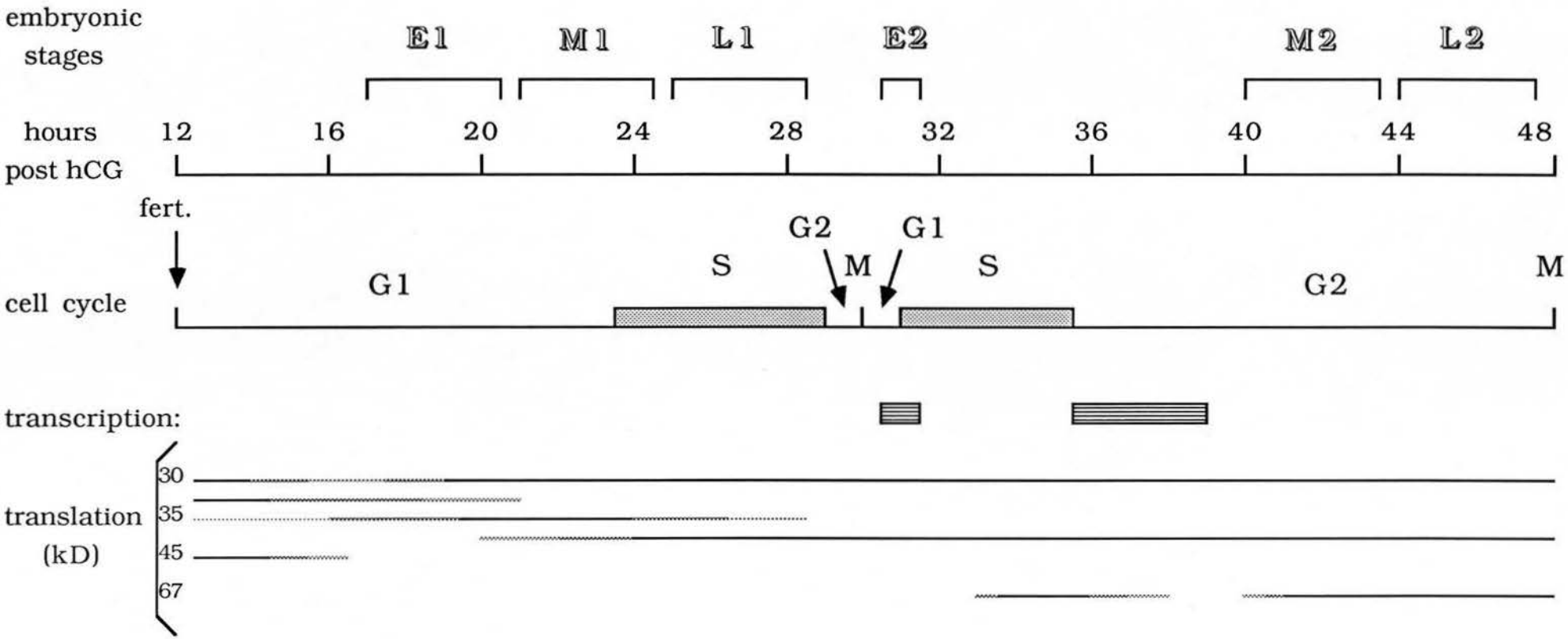
The development of the reconstituted embryos to blastocysts *in vitro* is summarized in Table 5.3. There was a significant effect of the stage of the cycle of the cytoplast and a smaller effect of the cell cycle stage of the karyoplast. The average effect caused by the stage of the enucleated embryo indicated a greater developmental potential when these were derived from later zygotes ($P<0.01$). This effect was consistent for all three karyoplast stages to which they were fused. Although the average effect caused by varying the karyoplast stages also indicated an increasing potential of nuclei derived from blastomeres obtained and manipulated at later stages of the 2nd cell cycle ($P>0.05$), this effect was not consistent for all three stages of enucleated 1-cell embryos. Recipient enucleated embryos from early- and mid-stage zygotes showed a greater potential to develop into blastocysts when fused to karyoplasts

Table 5.3. Effects of the cell cycle stage of karyoplast and enucleated 1-cell embryos on the proportion of reconstituted embryos developing *in vitro* to blastocyst 90 h after manipulation.

Cytoplast stage	Early 2-cell (30-32 h)	Mid 2-cell (40-33 h)	Late 2-cell (44-48 h)	Cytoplast totals
Early 1-cell (16-20 h)	2.3% (44)*	18.9% (37)	20.0% (25)	12.3% (106)
Mid 1-cell (20-24 h)	12.5% (40)	43.6% (39)	29.4% (34)	28.3% (113)
Late 1-cell (24-28 h)	66.0% (47)	48.8% (41)	63.5% (52)	59.6% (141)
Karyoplast totals	28.2% (131)	37.6% (117)	42.9% (112)	35.8% (360)

*No. of fused embryos after microsurgery.

Figure 5.2. Molecular and cellular events during the first and second cell cycles of mouse embryos and their relationship to the time in which 1- and 2-cell embryos were manipulated (adapted from Bolton et al., 1984; Howlett and Bolton, 1985).



from the final (mid and late) rather than initial (early) stages of the second cell cycle ($P<0.01$). However, there was no apparent effect of karyoplast stage in the recipient cytoplasm group derived from late stage zygotes ($P>0.05$).

5.4. **Discussion:**

These results confirm that the interactions between nucleus and cytoplasm are of critical importance at these early embryonic stages in mice. The development of embryos after nuclear transplantation was influenced by both the cleavage stage of development of the donor embryo, and differences in stage within the cell cycle of karyoplasts and enucleated embryos. When transferring pronuclei between zygotes, maximum development was obtained if donor and recipient embryo were at the same stage of development. By contrast, such 'synchronous' transfers did not lead to the greatest proportion of embryos developing to blastocysts when nuclei were transferred from 2-cell embryos to zygotes. Karyoplasts and cytoplasts derived from embryos at later stages of the cell cycle had a greater potential to develop *in vitro*. These results contrast with those of Howlett *et al.* (1987) who found that karyoplasts from early 2-cell embryos had a greater potential than did the late 2-cell karyoplasts. These differences may be due to the source of recipient enucleated embryos used in each experiment since Howlett *et al.* (1987) fused the karyoplasts to ethanol activated oocytes. These were activated 17 h after hCG, before enucleation and manipulation 5 h later (22 h after hCG). These observations suggest that, within the same cell cycle,

karyoplasts should be transferred to enucleated embryos at similar stages of the cell cycle (ie. same stage after hCG injection). However, this is not necessarily true when transferring between cell cycles where several other factors may influence subsequent development of reconstituted embryos.

The viability of the nuclear transplanted embryos was greatly influenced by the embryonic stage from which the karyoplasts were obtained with a marked reduction when taken beyond the 4-cell stage. This result is similar to one reported previously (McGrath and Solter,1984c), for which no development to blastocysts was achieved. These results contrast with those found with amphibian nuclear transplantations in which nuclei from embryos at the blastula stage could support development to adult frogs (Briggs and King, 1952) and nuclei from intestinal cells could support the development of embryos to the tadpole stage (Gurdon, 1962). Moreover, the nuclei in other mammalian species seem to retain totipotency for longer than do those of mice as shown in the reports by Willadsen (1986), Prather *et al.* (1987), Stice and Robl (1988) and that presented in the previous chapter (Chapter 4) where cells from the ICM of sheep early blastocysts were able to support development to term.

The most striking difference concerning the developmental mechanisms in these species concerns the timing of transition from maternal to embryonic control of development. While the mouse genome is activated at the 2-cell stage (Flach *et al.*,1982), in sheep, rabbit and cattle it is activated at around the 8- to 16-cell stage (Cotton *et al.*, 1980; Camous *et al.*,1986; Crosby *et al.*,1988) and at the mid-blastula stage in amphibians (Newport and

Kirschner, 1982b). There are different ways by which development may be abnormal following nuclear transfer. First, post-activation nuclei fused to mouse enucleated zygotes may not have enough time to be reprogrammed and initiate *de novo* transcription at the 2-cell stage. Second, the active nuclei may continue transcribing messengers which are transported into the cytoplast (Ege and Ringertz, 1975) and directly or indirectly affect subsequent development of the reconstituted embryo. It remains to be confirmed whether either of these mechanisms account for the species differences.

In the absence of other effects, it might be imagined that transplantation was most likely to be successful between embryos at the same stage of the cell cycle. Such an effect of cell cycle stage 'synchrony' between donor karyoplast and recipient enucleated embryo were shown by transplantation within the first cell cycle. The lower development after 'asynchronous' transfers may have been due to responses of the nuclei to regulatory signals from the surrounding cytoplasm controlling cell division (Ege, Zeuthen and Ringertz 1975) causing aneuploidy or other chromosomal defects. By contrast, the developmentally beneficial effects of synchrony were not observed when 2-cell karyoplasts were transferred to zygote cytoplasts at similar stages of the cell cycle. This indicates that other factors may be affecting the viability of the reconstituted embryos when karyoplast and enucleated embryo are derived from different cell cycles. The greater potential of the transfers to embryos enucleated at late stages of the cell cycle could be a result of cytoplasmic differentiation since it has been shown that

polypeptide patterns do vary during the first cell cycle interphase (Howlett and Bolton, 1985).

The karyoplasts derived from embryos collected at later stages of the 2nd cell cycle also showed a greater potential to support blastocyst development. This effect was particularly marked when the nuclei were transferred to cytoplasts at the early and mid stage of the cell cycle. Since the early 2-cell karyoplasts were obtained from embryos collected during or soon after cytokinesis, and therefore before or during the activation of the embryonic genome (Flach *et al.*, 1982), greater development occurred following the transfer of nuclei recovered after the activation of the embryonic genome. These results are contrary to the suggestion that the genomic activation irreversibly restricts nuclei and is responsible for the decreased viability of the reconstituted embryos (Howlett *et al.*, 1987). However, another possible interpretation of these results is that the early karyoplast had still to embark on a prolonged period of nuclear activity while the later karyoplasts had already resumed transcription, at around 41 h after hCG injection (Flach *et al.*, 1982), and may have no longer been releasing substantial amounts of stage-specific messengers into the cytoplasm.

These observations reveal the importance of controlling cell cycle stage when transferring nuclei between embryos. As the optimum stage of the cycle varies with the stage of the embryo, it will be important to define the ideal combination of cell cycle stages for each stage of donor and recipient embryo. Transfer at different stages of the cell cycle creates an opportunity to investigate the influence on subsequent development of factors

such as genome differentiation, the influence of stage specific factors and breakdown of the nuclear membrane. As most effects should be present during the first cell cycle immediately after transfer, it would be useful to investigate these cell cycle stage effects on the timing of mitosis and cleavage.

CLEAVAGE CONTROL IN RECONSTITUTED 1-CELL MOUSE EMBRYOS

6.1. **Introduction:**

Nuclear transplantation experiments with amphibian oocytes have indicated that the cytoplasm is dominant over the transplanted nucleus which assumes the activity characteristic of the host cell immediately after incorporation (Gurdon, 1968; Subtelny, 1968). Once activated, amphibian eggs proceed into a rapid, nearly synchronous series of several cell divisions lasting only a few minutes each, with no G1 or G2 phases and without measurable RNA synthesis (Laskey *et al.*, 1977; Forbes *et al.*,1983; Karsenti *et al.*, 1984; Newport and Kirschner, 1982b). During this period the embryos rely entirely on components stored in the cytoplasm and, although enucleated activated eggs fail to divide, they undergo periodic surface contraction waves closely timed with the cell cycle (Hara *et al.*, 1980; Sakai and Kubota, 1981; Sawai, 1979). From these experiments, it appears that during early cleavage in these species the cytoplasmic cell-cycle oscillatory mechanism operates independently from the nucleus on which it acts.

In mice, the early morphological and molecular events that take place following fertilization have also been shown to be under a post-transcriptionally controlled programme, maternally inherited through the oocyte's cytoplasm (reviewed by Johnson *et*

al., 1984). Neither physical nor chemical enucleation affects any of the molecular changes that have been detected before the early 2-cell stage (Petzholdt *et al.*, 1980; Braude *et al.*, 1979; Flach *et al.*, 1982; Bolton *et al.*, 1984). At this stage, two bursts of transcriptional activity can be detected, immediately before and after DNA synthesis, which provide most messages for the following two cell cycles (Johnson and Pratt, 1983; McLachlin *et al.*, 1983). As for amphibia, these reports indicate that the mouse embryo is controlled by maternally inherited cytoplasmic cell-cycle regulators up to the beginning of the 2-cell stage and that, after the activation of the embryonic genome, the nucleus starts providing the cell-cycle regulatory factors for the following stages of development.

Studies using cell hybrids derived by the fusion of cells at different stages of the cell cycle have provided information which has led to the identification of some of the biochemical mechanisms underlying the cell-cycle oscillatory apparatus (reviewed by Ringertz and Savage, 1976; Ford, 1985). A maturation- or mitosis-promoting factor (MPF) which appears during G2-phase, is at maximum concentration just before mitosis and disappears thereafter, has been shown to initiate mitosis by inducing nuclear membrane breakdown and chromosome condensation (Sunkara *et al.*, 1979; Miake-Lye *et al.*, 1983; Newport and Kirschner, 1984). Cycling cells also contain a factor(s) which inactivates MPF (IMF: inhibitor of mitotic factor) and in oocytes an activity responsible for arresting cells in metaphase ('cytostatic factor' CSF) has been identified (Newport and Kirschner, 1984; Ford, 1985; Murray, 1987). These inhibiting

factors are active during the G2 phase and disappear during the transition to S-phase when MPF can first be detected.

In this study, karyoplasts and cytoplasts derived from 1- and 2-cell embryos at either the beginning or final stages of interphase were fused to enucleated 1-cell embryos at two different stages of the first cell cycle. This has enabled the investigation of nucleo-cytoplasmic interactions involved in the control of cleavage time in reconstituted mouse embryos and their relationship to the transcriptional activity of the nucleus.

6.2. **Materials and Methods:**

Animal and embryo sources: Hybrid F1 females (C57BL/6 x CBA/Ca) were superovulated and mated to produce fertilized embryos for manipulation and culture as described in Chapter 3. The hCG injections were timed in order to provide embryos at early (E1: 20-22 h after hCG) or late (L1: 27-29 h after hCG) stages of the first cell cycle or at early (E2: 1-1.5 h after cleavage) or late (L2: 43-45 h after hCG) stages of the second cell cycle.

Micromanipulations: Enucleated embryos and donor karyoplasts were prepared as described in Chapter 2 (General Materials and Methods). The cytoplasmic transplantations were performed by removing a membrane bound portion of cytoplasm from the donor embryo, at a volume similar to that of a karyoplast, and expelling this cytoplast into the perivitelline space of the recipient embryo together with the fusogenic viral suspension. As in karyoplast

transplantations, fusion would occur within one hour from manipulation.

Cleavage assessment: Micromanipulated and control embryos exposed to cytochalasin B were placed in culture immediately after completion of manipulation. After periods of 8, 16, and 24 hours from manipulations, the embryos were removed from the incubator and placed on a dissecting microscope pre-heated stage at 37°C to assess cleavage rates. These periods for assessment were performed as quickly as possible to avoid large temperature fluctuations. In some experiments embryos were assessed at hourly intervals for periods lasting between 8 to 10 hours. Every experiment had controls of two kinds. One was composed of embryos that experienced periods of exposure to manipulation medium exactly as treated embryos (manipulated control) and another which was placed in culture immediately after their removal from mouse oviducts (non-manipulated control). Treated and control groups were compared using Fisher's exact test for two-way contingency tables.

Karyotypes: Control and reconstituted embryos which had developed to blastocysts after approximately 90 hours in culture were karyotyped according to Dyban (1983). Blastocysts were placed in a 3 mg/ml solution of Colcemid (Gibco, U.K.) diluted in medium N°16 for 4 h in a 5% CO_2 incubator at 37°C. Hypotonic treatment was produced by placing the embryos in a 0.9% solution of sodium citrate for approximately 5 minutes. After this period the blastocysts were placed in a fixative solution of glacial acetic acid and methanol (1:3) for approximately 3 minutes. After placing the embryos on a slide with a minimal amount of liquid, a drop of

softener of glacial acid and methanol(1:1) was added and then washed off with the fixative solution. Once dry, the slides were placed in a staining solution (5% Giemsa; Gurr, U.K.) and observed under 400x magnification to count metaphase chromosomes of the mitotic cells.

6.3. **Results**:

An indication of the relationship between the developmental stages at which embryos were manipulated for karyoplast and cytoplast transplantations and the known molecular and cellular events in the first and second cell cycle are shown in Figure 6.1. Although the activity of factors promoting (MPF) or inhibiting (IMF) mitosis have not been characterized in the early mouse embryo, their expected period of activity based on studies using somatic cells is also presented.

6.3.1. Transfers within the cell cycle:

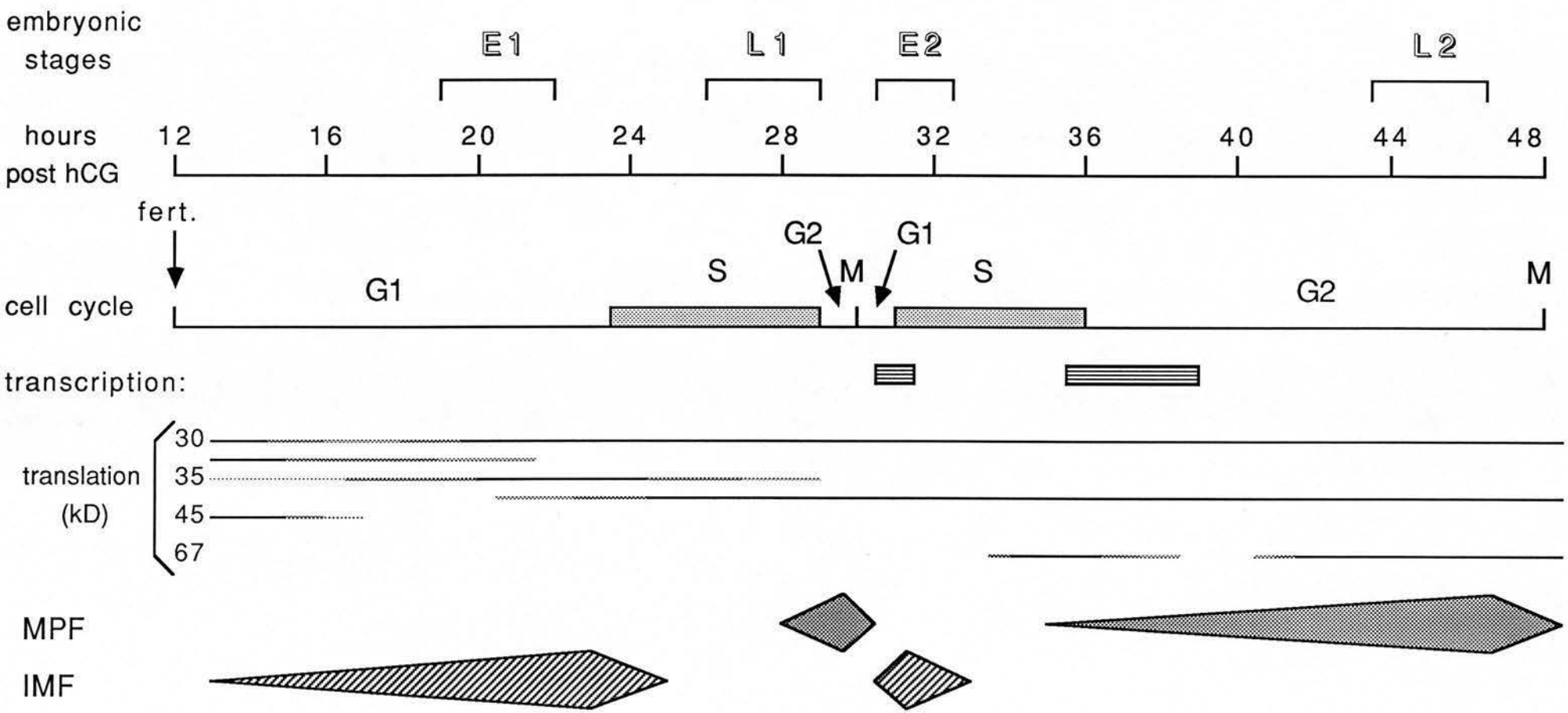
Cleavage rates for reconstituted embryos derived from the fusion of enucleated 1-cell embryos to pronuclear karyoplasts at the beginning or final stages of interphase are shown in Table 6.1. Rates are shown for periods of 8, 16 and 24 hours after manipulation. Cleavage rates for manipulated and non-manipulated controls were similar at all periods analysed after manipulation indicating that the exposure of embryos to the manipulation medium had no effect on the timing of cleavage. There was also no difference, at any period after manipulation, between the cleavage

Table 6.1. Rates of cleavage of control and reconstituted embryos derived from nuclear transplantations between early (E1) and late (L1) 1-cell embryos assessed at 8 h intervals from manipulations.

stages of		number of	cleavage rate after manipulation		
nucleus	cytoplasm	embryos	8 hours	16 hours	24 hours
1) **control**	**E1**	84	4.8 %	100 %	100 %
2) **E1**	**E1**	45	2.2 %	100 %	100 %
3) **L1**	**E1**	34	2.9 %	82.4 %*	97.1 %
4) **control**	**L1**	43	88.4 %	100 %	100 %
5) **L1**	**L1**	41	97.6 %	100 %	100 %
6) **E1**	**L1**	35	22.9 %*	100 %	100 %

* 'Asynchronous' group differs significantly from the respective 'synchronous' group ($P<0.01$).

Table 6.1. Molecular and cellular events during the first and second cell cycles of mouse embryos and their relationship to the time in which 1- and 2-cell embryos were manipulated. The hypothetical locations of the mitotic promoting factor (MPF) and of the inhibitor of mitotic activity (IMF) have been suggested based on information from other cells.



rates of control and reconstituted embryos derived from transfers between similar stages of interphase ($P>0.05$). These results indicate that the technique of nuclear transplantation had no effect on the time of cleavage of embryos in the reconstituted groups.

Embryos derived from fusions between karyoplasts and enucleated embryos at opposite extremes of interphase showed significant changes in cleavage time after manipulation when compared to the controls with the same type of cytoplasm. E1 karyoplasts fused to L1 cytoplasts (group 6) had significantly higher rates of cleavage at 8 h after manipulation when compared to the E1 controls (group 1) ($P<0.01$) and significantly lower cleavage rates when compared to the L1 controls (group 4) at the same period after manipulation ($P<0.01$). There were no differences at any further period after manipulation ($P>0.05$). These results show that the interphase stage of the reconstituted embryo was intermediate between the expected cleavage times for either karyoplast or cytoplast alone indicating not a dominance from any structure, but an interaction between the two.

Although there were no significant differences at 8 h after manipulation ($P>0.05$), reconstituted embryos derived from the fusion of L1 karyoplasts to E1 cytoplasts (group 3) had a significantly lower cleavage rate at 16 h after manipulation when compared to the 'synchronous' group (2) ($P<0.01$). Some of this effect was caused by a small proportion of reconstituted embryos (2.9%) that had not cleaved by 24 h after manipulation and did not cleave at any time thereafter. However, the cleavage rate at 16 h after manipulation relative to the embryos that did eventually cleave was also significantly lower for the reconstituted embryos

when compared to either controls (P<0.05). These results indicate that the late karyoplast was not only unable to shorten the interphase of the reconstituted embryo but that the interaction between karyoplast and cytoplast caused an extension beyond the expected cleavage time for the early recipient cytoplast. Moreover, this interactive effect was able in some cases to inhibit mitosis completely.

6.3.2. Transfers across the cell cycle:

The effects on time of cleavage of transfers between embryos from different cell cycles were tested by fusing early (E2) and late (L2) 2-cell karyoplasts to enucleated 1-cell embryos. Cleavage rates of reconstituted embryos derived from fusions to L1 enucleated embryos and for karyoplast and cytoplasm controls at 8, 16 and 24 h after manipulation are presented in Table 6.2. Reconstituted embryos using L2 karyoplasts (group 10) did not have significantly different cleavage rates from either L1 (group 9) or L2 (group 11) controls at any time after manipulation (P>0.05). However, when E2 karyoplasts were fused to L1 enucleated embryos (group 8), there was significantly lower cleavage rate at 8 h after manipulation as compared to L1 controls (group 9) (P<0.01). With the exception of a small group (5.4%) of reconstituted embryos that did not cleave at any time, this group had similar cleavage rates at 16 and 24 h after manipulations (P>0.05). This reconstituted group (8) also had significantly higher cleavage rates when compared to the donor karyoplast controls (group 7) at 8, 16 and 24 h after manipulation (P<0.01). These results indicate an effect of the E2 karyoplast in extending the interphase of the

Table 6.2. Rates of cleavage of control and reconstituted embryos derived from the transplantation of karyoplast of early (E2) and late (L2) 2-cell embryos to enucleated late (L1) 1-cell embryos. Cleavage rates were assessed at 8 h intervals from manipulations.

stages of		number of	cleavage rate after manipulation		
nucleus	cytoplasm	embryos	8 hours	16 hours	24 hours
7) **E2**	**control**	25	none	none	32.0 %
8) **E2**	**L1**	37	24.3 %**	94.6 %	94.6 %*
9) **control**	**L1**	100	73.0 %	100 %	100 %
10) **L2**	**L1**	40	75.0 %	100 %	100 %
11) **L2**	**control**	44	65.9 %	100 %	100 %

* differs significantly from L1 controls ($P<0.01$).

reconstituted embryo when compared to its control L1 recipient cytoplasm expected time of cleavage. However, this is substantially less than the expected donor karyoplast cleavage time, since E2 controls only cleaved approximately 12 h later. This same effect was also shown when recording the cleavage rates at hourly intervals after manipulation (Figure 6.2).

Cleavage rates for reconstituted embryos derived from the fusion of 2-cell karyoplasts to E1 enucleated embryos are presented in Table 6.3. There was no significant difference between the times of cleavage of embryos derived from fusions using E2 karyoplast (group 13) and the E1 controls (group 14) at any period after manipulation ($P>0.05$). However, the cleavage rate of reconstituted embryos differ significantly from the E2 controls (group 12) at 16 and 24 h after manipulation ($P<0.01$) which indicates that the karyoplast was unable to extend interphase beyond the 8 h assessment. This interpretation was confirmed by hourly observations of cleavage rates (Figure 6.3). Reconstituted embryos derived from the fusion of L2 karyoplasts to E1 enucleated embryos (group 15) did not cleave up to 8 h after manipulation producing a cleavage rate not significantly different from the recipient cytoplasm controls ($P>0.05$) but significantly different from the L2 donor karyoplast controls (group 16) of which more than two thirds had already cleaved ($P<0.01$). These results shows that the L2 karyoplast had no effect in shortening the interphase of the reconstituted embryos when compared to the E1 controls. However, the cleavage rate of the reconstituted embryos at 16 and 24 h after manipulations was significantly lower than the E1 cytoplasm controls ($P<0.01$). Most of this effect seems

Figure 6.2. Rates of cleavage of reconstituted embryos derived from the transplantation of karyoplasts of early (E2) and late (L2) 2-cell embryos fused to late 1-cell (L1) enucleated embryos assessed at hourly intervals immediately after manipulation.

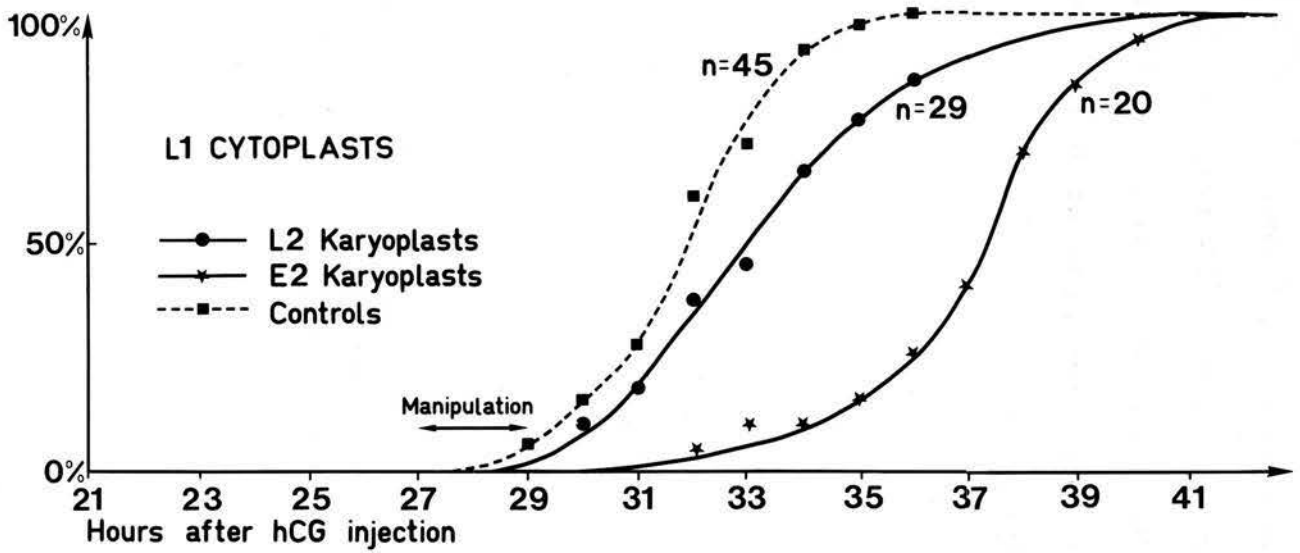
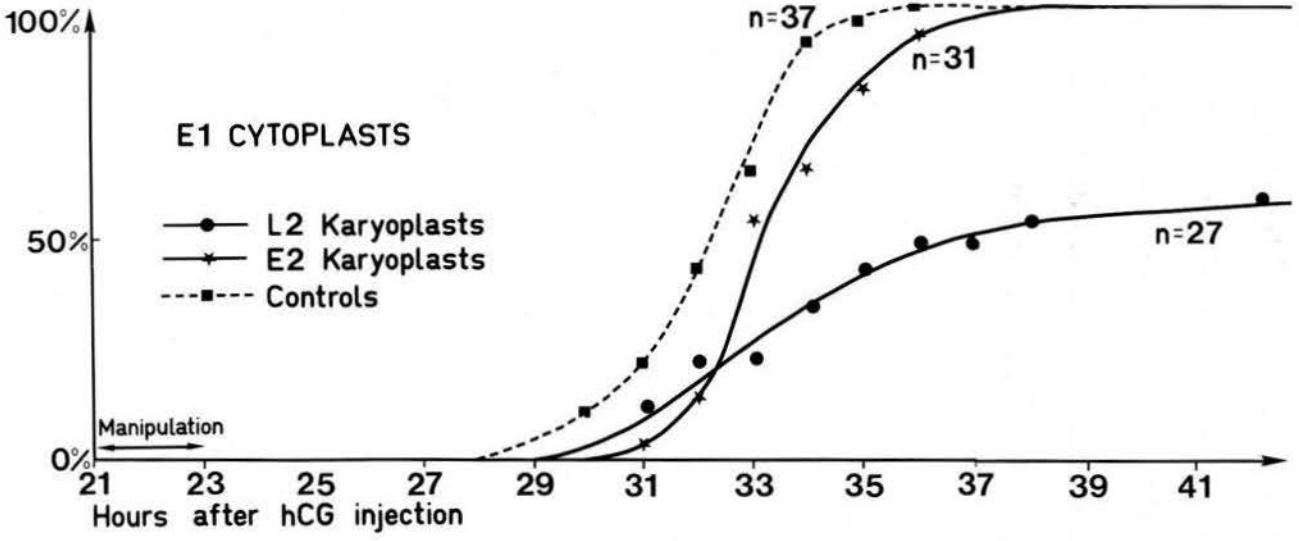

100%
50%
0%
L1 CYTOPLASTS
L2 Karyoplasts
E2 Karyoplasts
Controls
n=45
n=29
n=20
Manipulation
21
23
25
27
29
31
33
35
37
39
41
Hours after hCG injection

Table 6.3. Rates of cleavage of control and reconstituted embryos derived from karyoplast transplantations between early (E2) and late (L2) 2-cell embryos to enucleated early 1-cell (E1) embryos. Cleavage was assessed at 8 h intervals from manipulations.

stages of		number of	cleavage rate after manipulation		
nucleus	cytoplasm	embryos	8 hours	16 hours	24 hours
12) **E2**	**control**	40	none	none	35.0 %
13) **E2**	**E1**	39	none	100 %	100 %
14) **control**	**E1**	52	2.0 %	100 %	100 %
15) **L2**	**E1**	40	none	27.5%*	45.0 %**
16) **L2**	**control**	34	67.6 %	100 %	100 %

* Group differs significantly from E1 controls ($P<0.01$).

Figure 6.3. Rates of cleavage of reconstituted embryos derived from the transplantation of karyoplasts of early (E2) and late (L2) 2-cell embryos fused to early 1-cell (E1) enucleated embryos assessed at hourly intervals beginning 6 h after manipulation.


100%
50%
0%
E1 CYTOPLASTS
L2 Karyoplasts
E2 Karyoplasts
Controls
n=37
n=31
n=27
Manipulation
21
23
25
27
29
31
33
35
37
39
41
Hours after hCG injection

to have been caused by a large proportion (55%) of the reconstituted embryos not cleaving at any time after manipulation. Of those embryos that did cleave, a smaller proportion had cleaved by 16 h after manipulation (61 %), a significantly lower proportion than in the E1 control group which had all cleaved by that time ($P<0.01$). Similar results were present at hourly cleavage assessments of reconstituted embryos (Figure 6.3).These results indicate that the L2 karyoplast was not only unable to shorten the interphase of the reconstituted embryos, but that it lengthened the interphase and completely blocked further cleavage in some embryos.

6.3.3. Cytoplast transplantations:

Fusion of small cytoplasts from karyoplast donor embryos to control embryos was performed to test whether the effects of the karyoplasts on cleavage was due to the nucleus alone or to the small amount of cytoplasm and plasma membrane which is added to the reconstituted embryo at the time of karyoplast fusion. Cleavage rates of the cytoplast transplanted embryos are presented in Table 6.4. The fusion of either L1 (group 18) or L2 (group 19) cytoplasts to E1 embryos caused no significant effect on their cleavage rate when compared to the E1 controls (group 17) at either 8, 16 or 24 h after manipulation($P>0.05$). Similarly, there was no significant effect on cleavage time after fusing cytoplasts derived from E1 (group 20) and E2 (group 21) embryos to L1 embryos and comparing these to the L1 controls (group 22) at 8 16 and 24 h after manipulation ($P>0.05$). These results show that the cytoplasm and plasma membrane of donor karyoplasts had no

Table 6.4. Rates of cleavage of control and reconstituted embryos derived from the transplantation of cytoplasts from 1- an 2-cell early (E1, E2) and late (L1, L2) embryos to early and late 1-cell embryos

stages of		number of	cleavage rate after manipulation		
cytoplast	embryo	embryos	8 hours	16 hours	24 hours
17) **control**	**E1**	104	1.0 %	100 %	100 %
18) **L1**	**E1**	36	2.8 %	100 %	100 %
19) **L2**	**E1**	37	2.7 %	100 %	100 %
20) **E1**	**L1**	28	100 %	100 %	100 %
21) **E2**	**L1**	24	91.7 %	100 %	100 %
22) **control**	**L1**	43	88.4 %	100 %	100 %

effect on the cleavage rates of the reconstituted embryos indicating that the effects observed earlier were caused by the interaction between the donor nucleus alone and the cytoplasm of the recipient enucleated embryo.

6.3.4. Karyotype analysis:

A few reconstituted embryos were allowed to develop to blastocysts *in vitro* for karyotyping. All reconstituted blastocysts derived from the fusion of early karyoplasts to late cytoplasts from either within or across cell cycle transplantations showed diploid karyotypes. Although only the reconstituted embryos which developed normally were analysed, these results indicate that nuclei transferred either before or during the S-phase complete DNA replication before mitosis proceeds. Although reconstituted blastocysts derived from within and across cell-cycle transplantations of late karyoplasts (G2) to early cytoplasts (G1) were also found to have a diploid number of chromosomes, this does not eliminate the possibility of these being endoreplicated chromosomes which could not be determined by these karyotyping techniques.

6.4. **Discussion:**

These findings indicate that the cell-cycle regulatory apparatus operating during the first cell cycle of mouse embryos can be altered by replacing the pronuclei by a nucleus derived from an embryo at a different stage of interphase, regardless of whether or

not the embryonic genome has been activated. This suggests that an interaction between nucleus and cytoplasm is required for control of the first mitotic cleavage. Although the cytoplasm from advanced stages of interphase is able to change the original timing of a nucleus so that it will enter mitosis earlier than expected, there also seems to be a requirement for a few hours of delay before cleavage may occur. However, the nucleus derived from an embryo at advanced stages of interphase is not only unable to shorten the cell cycle of an early enucleated embryo but there seems to be an incompatibility between nucleus and cytoplasm which causes either an extension of the cell cycle beyond the expected cytoplasmic timing or, when using 2-cell nuclei, an occasional complete blockage of development at the 1-cell stage. In the latter, the nucleus seems to loose the ability to reset its oscillatory cell-cycle apparatus with that of the cytoplasm.

Although it has been shown that G2 and mitotic somatic cells contain mitotic inducing activity (MPF) which, when fused to G1 and S nuclei, induces mitosis more quickly, it has been observed that the entry of these hybrid cells into mitosis is delayed until the G1- or S-nuclei have completed DNA synthesis, and subsequently, both nuclei have entered mitosis synchronously (Rao and Johnson, 1970; Rao *et al*, 1975). This need for completing DNA synthesis before mitosis may proceed would explain the slightly extended cell cycles of reconstituted embryos derived from the fusion of both E1 and E2 karyoplasts to enucleated L1 embryos, since both karyoplast donor stages were either in G1 or at the very early stages of the S-phase of their respective cell cycle (Figure 6.1). The diploid karyotype of the reconstituted embryos analysed is

also indicative that DNA replication did occur before mitosis, at least in those embryos developing to blastocyst *in vitro*. Previous experiments (Chapter 5) have indicated that most embryos from both reconstituted groups are capable of developing to blastocysts *in vitro*, which suggests that the accomplishment of synchrony between the nucleus and cytoplasm results in little harm for further development.

Rao and Johnson (1970) also found in their hybridization experiments that S phase cells contained potent factors which prevented or delayed the entry of G2 nuclei into mitosis. These findings were later supported by investigations on the effects of different cell cycle stage extracts on the maturation promoting activity (MPA) of X. laevis oocytes (Adlakha *et al.*, 1983). The experiments indicated the existence of mitotic inhibitor factors (IMF) which are activated at the end of mitosis, peak during G1 and early S phase and are less effective during mid- and late S phase. Extracts from early, mid- and late G2 phase cells had no IMF activity which is not surprising since MPF are known to accumulate during the G2 phase (Sunkara *et al.*, 1979). This same mitotic inhibitory effect seems to have been present in the cytoplasm of E1 enucleated embryos which, allowing for the possible asynchrony of *in vivo* fertilized embryos, would be at G1 or early stages of S phase (Figure 6.1). Karyoplasts derived from L1 embryos were probably in late S phase or G2 and, supposedly under the influence of mitotic promoting activity which would have been inactivated by the IMF in the E1 cytoplasm. In this case, the nucleus may have required some reprogramming to initiate the cell-cycle clock which would have caused the delay beyond the E1

controls. However, this delaying effect was even stronger when fusing L2 karyoplasts to the E1 enucleated embryos since more than half of the reconstituted embryos did not cleave at any stage later.

One possible cause for this effect, is that these nuclei were derived from embryos from a further cell cycle which, due to embryonic genome activation, may have differentiated to an extent that they were no longer able to interact with the recipient cytoplasm. Another possibility is that, unlike the L1 karyoplasts, these L2 nuclei were in late G2 and had already synthesized DNA. When exposed to the G1/S phase cytoplasm of the E1 embryo, some nuclei endoreplicated their DNA causing damage to their mitotic apparatus. The karyotype analysis performed on the reconstituted blastocysts would not have identified a duplication in the DNA content of the chromosomes. Morphological abnormalities such as 'pulverization' and chromatid breakage have been shown to occur at the first mitotic cleavage to 8-cell blastomere chromosomes fused to fertilized and parthenogenetically activated embryos (Dyban *et al.*, 1988). It is possible that these same abnormalities caused the arrest of reconstituted embryos before cleavage.

The presence of cytoplasmic factors that promote mitosis in cultured mouse embryos 'blocked' at the 2-cell stage has also been reported (Muggleton-Harris, 1982). As for MPF, these promoting factors have shown to be peak in activity during the transition between G2 and M phase and to decline there after (Pratt and Muggleton-Harris, 1988). However, it remains to be shown whether these factors would have any effect on the cell-cycle

timing of 'non-blocking' embryos. Although these cycling activities may explain some of the effects found in these reconstituted and cytoplasm injected embryos, biochemical and cytological studies are still required to characterize these mitotic controlling factors in mouse embryos. This would provide a understanding of the cell-cycle control mechanism which operates during early embryonic development in mammals and may also improve the efficiency of nuclear transplantations at these early stages.

CLEAVAGE AND DEVELOPMENT IN RECONSTITUTED 2-CELL EMBRYOS

7.1. **Introduction:**

During early cleavage stages, the mouse embryo experiences major changes in the biochemical components of both nucleus and cytoplasm. Transcriptional activation by the embryonic genome at the initial stages of the second cell cycle and the appearance of translation products in the cytoplasm are known to be necessary for controlling the developmental pathway which will eventually produce the first differentiation steps in the morphology of the 8-cell blastomere (reviewed by Johnson *et al.*, 1984). This programmed interaction between the nucleus and cytoplasm has been examined by nuclear transplantation experiments with mouse embryos. Studies have shown that the ability of a blastomere nucleus to support development depends on the embryo cytoplasmic stage to which it is fused (Robl *et al.*, 1986; Howlett *et al.*, 1987; Tsunoda *et al.*, 1987). These authors used recipient cytoplasm from the first two cell cycles and showed that nuclei from 4- and 8-cell stages were capable of development *in vitro* to blastocysts (Robl *et al.*, 1986) and to term (Tsunoda *et al.*, 1987) after transfer to 2-cell cytoplasm, but not to 1-cell enucleated embryos.

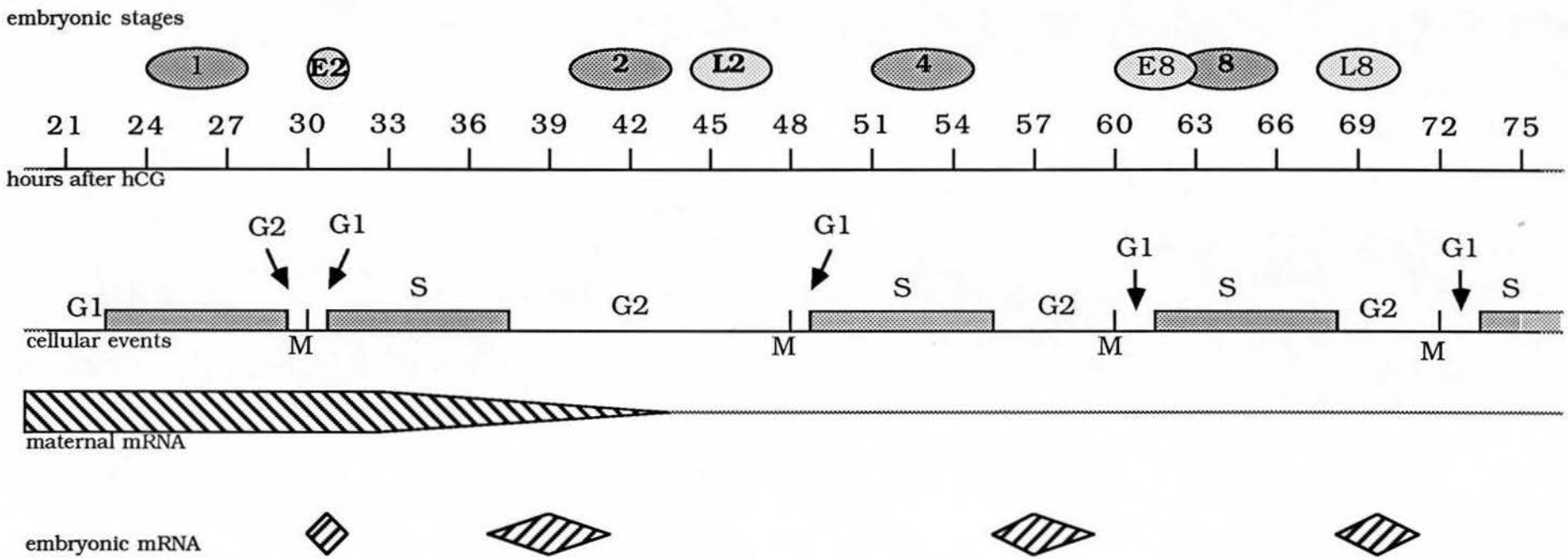
This chapter intends to examine the timing of the developmental steps in both nucleus and cytoplasm by analysing

their effects in the control of cleavage and to support blastocyst formation *in vitro*. Experiments were performed to investigate cell cycle stage effects of 2-cell recipient blastomeres fused to nuclei from 2- to 8-cell stage blastomeres at different stages of the cell cycle.

7.2. **Materials and methods:**

Female F1 hybrids were superovulated to provide embryos as described in Chapter 2 (General Materials and Methods). The hCG injections were timed differently for each embryonic stage. An indication of the relationship of these timed intervals after hCG and known biochemical events during the initial cleavage stages is shown in Figure 7.1. In the first experiment, pronuclear embryos were harvested and manipulated between 24 and 28 h after hCG, 2-cell embryos between 38 and 42 h after hCG, 4-cell embryos between 51 and 55 h after hCG and 8-cell embryos between 62 and 66 h after hCG injections. In the second experiment, early 2-cell embryos (E2) were harvested at 30 h after hCG and manipulated between 1 and 1.5 h after first cleavage and the late 2-cell stage embryos (L2) were harvested and manipulated between 44 and 48 h after hCG. For the third experiment, E2 and L2 control and recipient enucleated embryos were obtained and manipulated as described previously. Early 8-cell embryos (E8) were harvested and manipulated between 61 and 64 h after hCG and the late 8-cell stages (L8) between 67 and 70 h after hCG injections.

Figure 7.1. Molecular and cellular events during the first,second, third and fourth cleavage cell cycles. Relationship with the time (embryonic stages) of transplantation to enucleated 2-cell blastomeres (adapted from: Bolton et al., 1984; Smith & Johnson, 1986).



Embryonic stages from the pronuclear to the 4-cell stage were obtained by flushing the oviducts as described previously in Chapter 3. Eight-cell embryos used in the first experiment and E8 stage embryos of the third experiment were only used at a pre-compaction stage while the L8 stage embryos were all compacted (Figure 7.2). Eight cell embryos were obtained either from the oviduct or from uterine flushings. The harvesting from the uterus was performed by passing a 30 gauge needle through the cervical orifice and injecting M2 medium into the uterine cavity to collect the flushings from the tip of each uterine horn into a glass dish. Embryos were washed in fresh M2 and placed in N°16 in an incubator until further manipulations.

Nuclear transplantations were performed by enucleating both blastomeres of the recipient 2-cell embryo and transferring a single karyoplast and inactivated Sendai solution into the perivitelline space. Karyoplasts were positioned in such way to provide membrane contact with a single blastomere. Only those embryos in which the karyoplast had fused to a single blastomere were used for experimentation. Two kinds of controls were provided in each experimental batch. One where the 2-cell embryo was placed in the manipulation medium with cytoskeletal inhibitors for a period similar to the nuclear transplanted group (non-manipulated control) and another where, apart from this, the nucleus of one of the blastomeres was removed leaving a single nucleated blastomere (manipulated control) (Figure 7.3).

Assessment of number of embryos that reached the blastocyst stage in treated and control groups was performed at 72 h after manipulations in the first experiment and at 72 and 86 h

Figure 7.2. Eight-cell stage embryos positioned for enucleation of blastomeres in order to obtain karyoplasts for transplantation to 2-cell enucleated blastomeres. (a) Non-compact 8-cell embryo collected and manipulated between 61 and 64 h after hCG, and (b) compacted late 8-cell embryo collected and manipulated between 67 and 71 h after hCG injection.

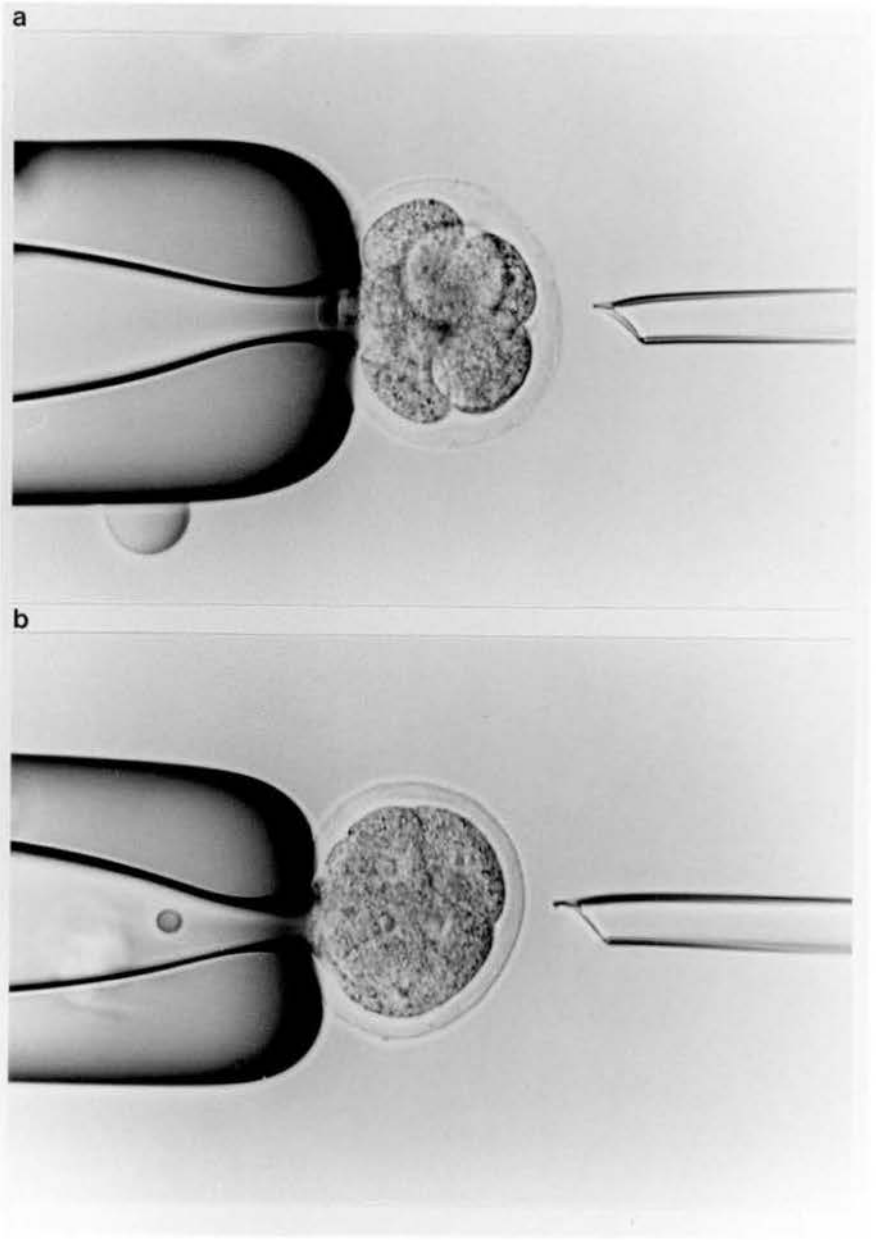
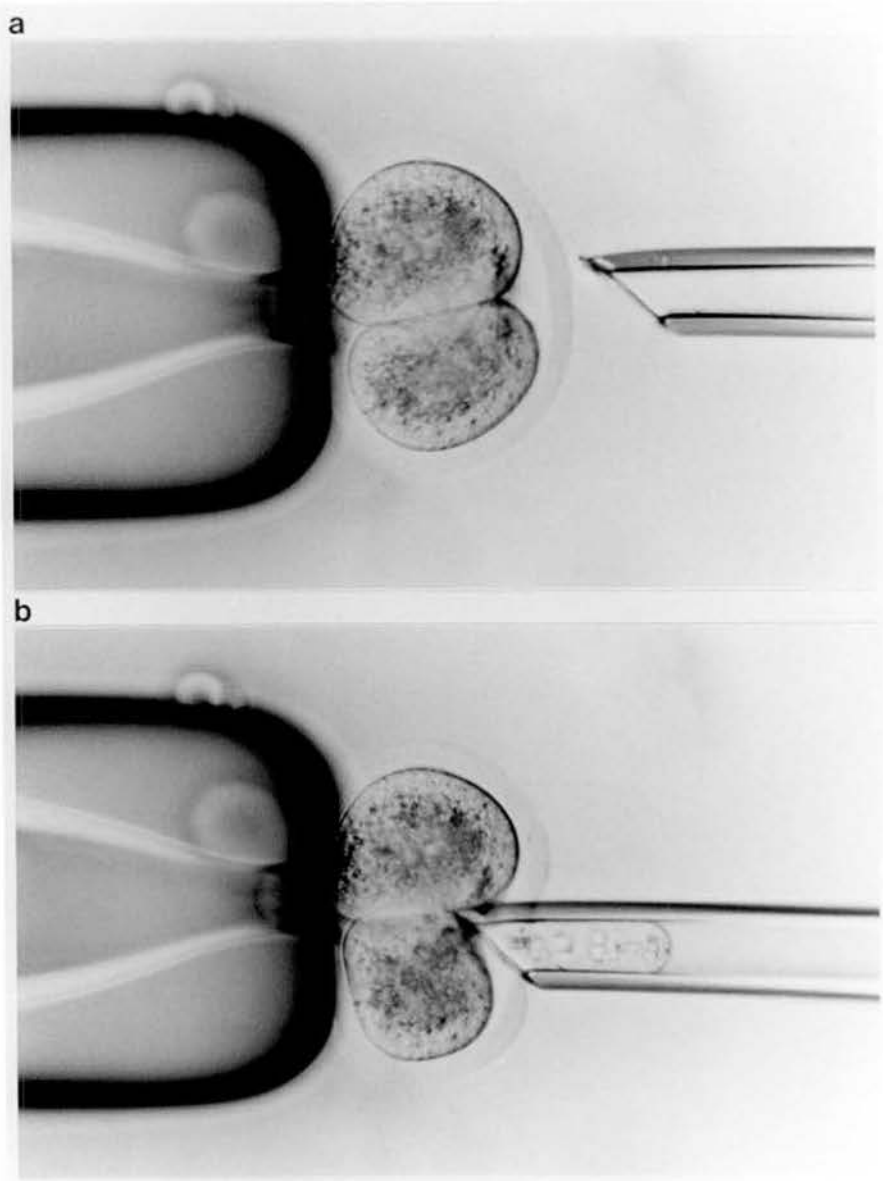

a
b

Figure 7.3. Preparation of manipulated 2-cell controls for experiments. (a) Positioning of the embryo for microsurgery and (b) enucleation of a single blastomere.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b

after manipulations in the second and third experiment when using L2 and E2 stage recipient embryos, respectively. For the later two experiments, reconstituted and control embryos were observed at approximately 8, 16, 24 and 40 h after manipulations in order to assess the time of cleavage. This assessment was performed as quickly as possible to avoid major fluctuations in the temperature. Non-manipulated 2-cell controls were identified as cleaved when at least one of the blastomeres had cleaved (3- or 4-cell stage). Data on the number of control and treated embryos developing to blastocyst stage and on their cleavage rates were analysed using Fisher's exact test for 2-way contingency tables.

7.3. **Results:**

Experiment 1: This experiment was designed to assess the potential of blastomere nuclei at different embryonic stages after transfer to single enucleated 2-cell blastomeres. Results are given in percentage reaching the blastocyst stage *in vitro* at 72 h after manipulation (Table 7.1). The ability of control embryos to develop into blastocysts was slightly decreased by removing the nuclei from one of the blastomeres ($P<0.05$). This result showed that reconstituted embryos should be compared only to manipulated controls since both rely on a single nucleated blastomere to support development. The proportion of embryos reconstituted with 2-cell karyoplasts that developed to blastocysts was similar to that of manipulated controls, indicating that the technique of nuclear transplantation had no effect on the viability of reconstituted embryos to develop *in vitro* ($P>0.05$). Reconstituted

Table 7.1. Development rates *in vitro* of control and reconstituted 2-cell single blastomeres to the blastocyst stage 70 h after micromanipulation.

2-cell controls	manipulated controls	Stage of Karyoplasts			
		1-cell	2-cell	4-cell	8-cell
97.5%[a]	89.4%[a]	none[c]	93.3%[a]	90.0%[a]	50.0%[b]
(121)*	(85)	(20)	(60)	(40)	(20)

a≠b≠c (P<0.01)

* Number of fused embryos in each group.

embryos derived from the fusion of single enucleated 2-cell blastomeres to 4-cell karyoplasts developed similarly as to those derived from 2-cell karyoplasts and to the manipulated controls ($P>0.05$). This indicates that there are no noticeable changes in nuclear potential and/or in nucleo-cytoplasmic compatibility between the second and the third cell cycle. However, when using 8-cell karyoplasts, there was a substantial decline in the percentage reaching the blastocyst stage indicating changes in the potential of nuclei between the third and fourth cycle ($P<0.01$). Embryos reconstructed from fusions between pronuclear karyoplasts and 2-cell blastomeres were not only unable to develop into blastocysts, but arrested mitotic division completely. These reconstituted embryos remained as 2-cell blastomeres until fragmentation occurred due to degeneration a few days later. It was also noted that the pronuclei remained apart in the blastomere as if the mechanism for pronuclear apposition was not present in the 2-cell cytoplasm.

Experiment 2: In this experiment, controls and reconstituted embryos derived from nuclear transplantations between 2-cell embryos at similar ('synchronous') and opposite ('asynchronous') extremes of the second cell cycle were compared to investigate the effects of nucleo-cytoplasmic asynchrony on the control of cleavage timing and developmental potential. An indication of the appearance of a 'sychronously' transferred group at 16 and 72 h after manipulation is shown in Figure 7.4 a and b, respectively. Although most reconstituted blastocysts were of normal size, some were slightly smaller than the non-manipulated controls. Rates of blastocyst formation *in vitro* are shown in Table 7.2. Although

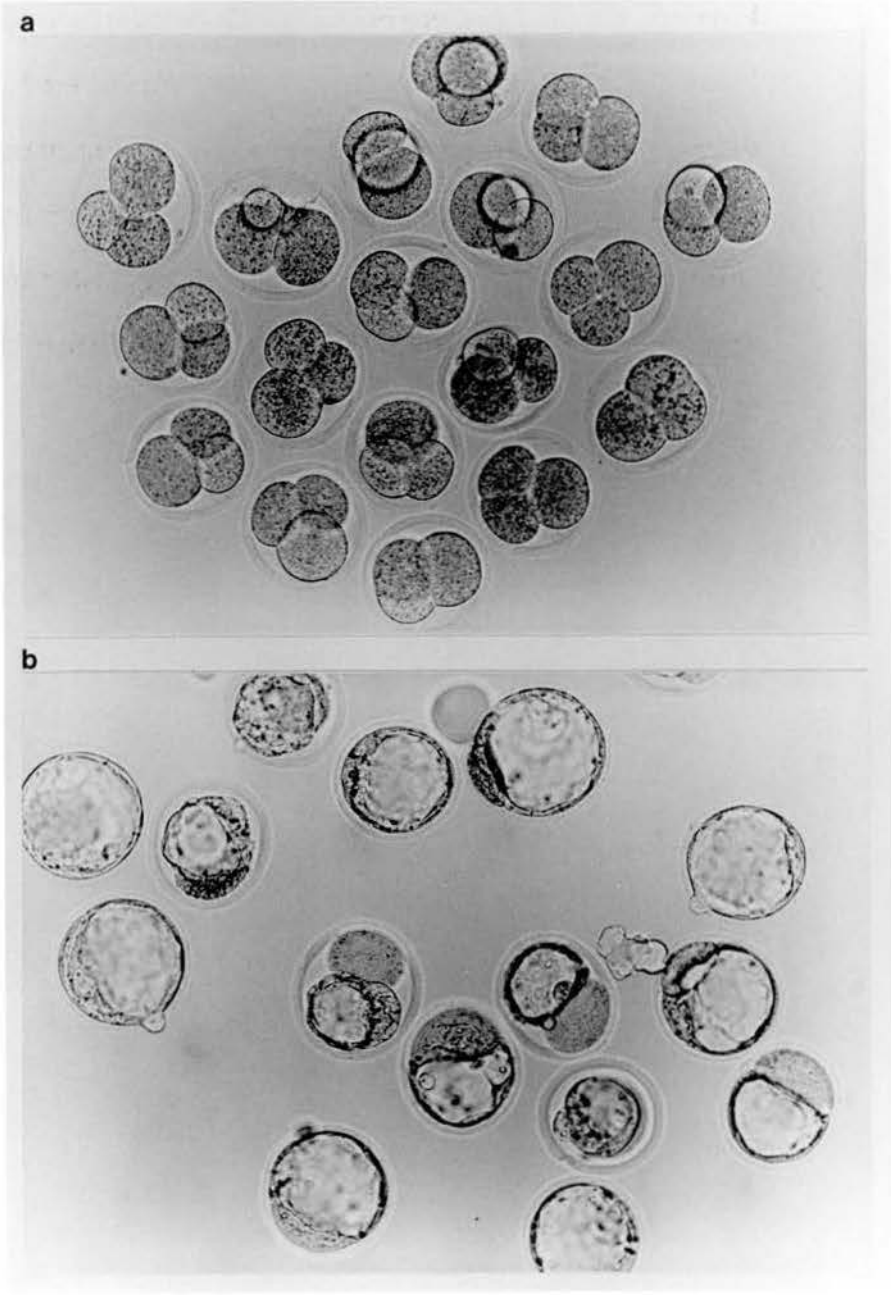
Table 7.2.- Effect of synchrony between karyoplasts and enucleated single 2-cell blastomeres on the *in vitro* development to blastocyst of reconstituted embryos 70 h after transplantation.

Cytoplast Stage	Non-manipul. control	Manipul. control	Synchronous transfers	Asynchronous transfers
Early 2-cell	96.1% (413)	86.2%[a] (65)*	92.0%[a] (50)	64.9%[b] (37)
Late 2-cell	94.4% (323)	94.9%[a] (78)	92.2%[a] (42)	19.5%[c] (41)

* number of reconstituted embryos in each group

a≠b≠c (P<0.01)

Figure 7.4. Reconstituted 2-cell embryos derived from the fusion of a 2-cell karyoplast to a single blastomere of an enucleated 2-cell embryo. (a) At 16 h after manipulation only the blastomeres which have fused to karyoplasts have cleaved (3- or 5-cell embryos), and (b) at 72 h after manipulation most embryos have developed into blastocysts. Blastomere expansion causes expulsion of the enucleated blastomere from the zona pellucida in many cases. Blastocyst size is on average slightly smaller than the non-manipulated controls.


a
b

there was no difference in potential between manipulated and non-manipulated controls in the late 2-cell group ($P>0.05$), in the early 2-cell groups, these controls differed significantly from one another ($P<0.01$). Comparing these results with what was found in the previous experiment, it is noted that single nucleated 2-cell blastomeres are increasingly more capable of developing into blastocyst when recovered and manipulated at later stages of the second cell cycle. Also supporting the findings of the first experiment, the proportion of reconstituted embryos reaching the blastocyst stage when transplanted at similar stages of the cell cycle ('synchronous') was not different from the manipulated controls ($P>0.05$) indicating that the technique had no effect on their *in vitro* viability. However, the potential of embryos derived from transplantations between embryos at opposite extremes of the cell cycle ('asynchronous') was greatly reduced when compared to the 'synchronous' groups and controls ($P<0.01$). Moreover, the reciprocal 'asynchronous' groups were significantly different from each other showing that an early 2-cell nucleus fused to a late cytoplasm is less able to support development to blastocyst *in vitro* than a late nucleus fused to an early cytoplasm ($P<0.01$).

During the initial period of *in vitro* culture both reconstituted and control embryos were observed at 8, 16, 24 and 40 h after manipulation to assess the proportions having cleaved at each period. Cleavage rates for control and reconstituted embryos derived from fusions of 'synchronous' and 'asynchronous' karyoplasts to enucleated early 2-cell (E2) stages are shown in Table 7.3. Although there were no differences in cleavage rates of

Table 7.3. Rates of cleavage of control and reconstituted embryos derived from the fusion of early (E2) and late (L2) 2-cell karyoplasts to single enucleated E2 blastomeres assessed at several intervals after nuclear transplantation.

Treatment	number of embryos	percentage of embryos cleaved after manipulation			
		8 hr	16 h	24 h	40 h
1) non-manip.E2 control	224	0.5%	5.8%	72.3%	100%
2) manipul.E2 control	40	none	none	35.0%	100%
3) E2 karyoplast	50	none	none	40.0%	100%
4) L2 karyoplast	37	none	43.2%*	75.7%*	100%
5) manipul. L2 control	34	67.6%	100%	100%	100%
6) non-manip.L2 control	177	68.9%	100%	100%	100%

*differs significantly from the other reconstituted group ($P<0.01$).

manipulated (group 5) and non-manipulated controls (group 6) from the late 2-cell stage ($P>0.05$), the non-manipulated controls from early 2-cell stage (group 1) cleaved significantly earlier than the manipulated control (group 2) when assessed at 24 h after manipulation ($P<0.01$). This difference may reflect the asynchrony already present between sister blastomeres at the time of second cleavage or be due to the method of assessing non-manipulated controls that where recorded as cleaved at either the 3- or 4-cell stages. The asynchrony between sister blastomeres may have been accentuated by temperature fluctuations during manipulation of the early 2-cell group throughout the first mitotic cleavage. Manipulated controls (group 2) and the 'synchronous' reconstituted group (group 3) cleaved at similar times after manipulation indicating that the nuclear transplantation technique itself had no effect on the timing of cleavage. However, the fusion of late 2-cell karyoplast to the early cytoplasm produced cleavage at an earlier stage than the reconstituted and control early 2-cells as shown by the greater proportion cleaved at 16 and 24 h after manipulation ($P<0.01$). However, the cleavage time of this 'asynchronous' reconstituted group (group 4) was not similar to the late 2-cell control (group 5) at either 8, 16 or 24 h after manipulation ($P<0.01$) which indicates that the interaction between nucleus and cytoplasm produced a cleavage time intermediate between the expected cleavage times for the nucleus and cytoplasm.

Rates of cleavage of control and reconstituted embryos derived from the fusion of early and late 2-cell karyoplasts to single enucleated late 2-cell enucleated blastomeres are shown in

Table 7.4. The cleavage time of the non-manipulated controls (group 12) was significantly earlier than that of the manipulated controls (group 11) as shown by higher cleavage rates at 24 h after manipulation ($P<0.01$). Again, this may either reflect the method of assessment of non-manipulated controls or the susceptibility of sister blastomere asynchrony caused by manipulating the early 2-cell group during the first mitotic cleavage. The 'synchronous' reconstituted group (group 9) had similar cleavage rates to the controls (group 8) at every recorded interval after manipulation indicating no effect of the technique of nuclear transplantation on the time of cleavage of this reconstituted group ($P>0.05$). However, rates of cleavage of the 'asynchronous' reconstituted group (group 10) was different from the "synchronous' group (group 9) at every recorded interval after manipulation ($P<0.01$). This result indicates not only a substantial delay in cleavage time when an early karyoplast was fused to a late enucleated blastomere, but also that the interaction between nucleus and cytoplasm was incompatible with development for the majority of embryos. This was shown by the proportion of embryos which stopped developing as 68.3% (28/41) of these 'asynchronously' transplanted embryos did not cleave at any time, but fragmented. However, the few embryos that cleaved and that developed further into normal blastocysts *in vitro*, did it earlier than the expected time for early 2-cell controls shown by the higher cleavage rates at 16 h after manipulation ($P<0.01$). This indicates that the nuclei that were compatible with the late cytoplasm influenced the cleavage time of these reconstituted embryos causing them to enter mitosis earlier than the expected for the stage of the cytoplasm.

Table 7.4. Rates of cleavage of control and reconstituted embryos derived from the fusion of early (E2) and late (L2) 2-cell karyoplasts to single enucleated L2 blastomeres assessed at several intervals after nuclear transplantation.

Treatment	number of embryos	percentage of embryos cleaved after transplantation			
		8 hr	16 h	24 h	40 h
7) non-manip.L2 control	146	71.2%	100%	100%	100%
8) manipul.L2 control	44	65.9%	100%	100%	100%
9) L2 karyoplast	51	60.8%	100%	100%	100%
10) E2 karyoplast	41	4.9%*	24.4%*	31.7%*	31.7*%
11) manipul. E2 control	25	none	none	32.0%	100%
12) non-manip.E2 control	189	none	1.1%	59.3%	100%

* differs significantly from the other reconstituted group ($P<0.01$).

Experiment 3: This experiment investigated the cell cycle stage effects of nuclear transplantations across cell cycles using 2-cell enucleated blastomeres and 8-cell karyoplasts at different stages of their respective cell cycle. Rates of *in vitro* development to blastocyst of control and reconstituted embryos are presented in Table 7.5. As shown in experiment 2, the potential of single 2-cell control blastomeres to develop to blastocyst was similar for E2 and L2 stages ($P>0.05$). The ability of reconstituted embryos to support development to blastocysts was substantially smaller than the manipulated controls ($P<0.01$) as already determined in Experiment 1. Moreover, there was a significant difference between the reconstituted groups ($P<0.05$) indicating cell cycle stage effects. This effect was partially derived from the recipient enucleated blastomere stage as shown from lower developmental rates when using E2 enucleated blastomeres ($P<0.05$). There was also a smaller effect caused by the donor karyoplast stage indicating lower developmental potential of L8 karyoplasts as compared to those derived from E8 blastomeres ($P<0.05$). However, this effect was not significant when comparing within each cytoplasmic stage ($P>0.05$) possibly due to the reduced number of reconstituted embryos in each group.

These cell cycle effects were also investigated by assessing the time of cleavage of control and reconstituted embryos. The cleavage rates at 8, 16, 24 and 40 h after manipulation are shown in Table 7.6. There were differences in cleavage rates between control and reconstituted groups fused to E2 and L2 enucleated blastomeres indicating that nucleo-cytoplasmic interaction can have an effect at the time of first cleavage after reconstitution. The

Table 7.5.- Effect of cell cycle stage of 8-cell karyoplasts fused to enucleated single 2-cell blastomeres on the *in vitro* development to blastocyst of reconstituted embryos 70 h after transplantation.

Enucleated blastomere stage	Manipul. Controls	Stage of karyoplast		Enucleat. blast. totals
		Early 8-cell	Late 8-cell	
Early 2-cell	84.0%[a] (25)*	none[b] (17)	none[b] (21)	none (38)
Late 2-cell	97.8%[a] (44)	26.5%[c] (34)	5.6%[b,c] (18)	19.2% (52)
Karyoplast totals	92.8% (69)	17.6% (51)	2.6% (39)	11.1% (90)

* number of reconstituted embryos in each group; a≠b≠c (P<0.05)

Table 7.6. Rates of cleavage of control and reconstituted embryos derived from the fusion of early (E8) and late (L8) 8-cell karyoplasts to single enucleated early (E2) and late (L2) 2-cell enucleated blastomeres assessed at different intervals after nuclear transplantation.

Treatment		number of embryos	percentage of embryos cleaved after manipulation			
nucleus	cytoplasm		8 h	16 h	24 h	40 h
13) manipul.E2	control	25	none	none	32.0%	100%
14) E8 ----	E2	17	none	5.9%	5.9%	11.8%
15) L8 ----	E2	21	none	none	none	14.3%
16) E8 ----	L2	34	32.4%	88.2%	100%	100%
17) L8 ----	L2	18	27.8%	44.4%	55.6%	55.6%
18) manipul. L2	control	44	65.9%	100%	100%	100%

fusion of E2 enucleated blastomeres to both E8 (group 14) and L8 (group 15) karyoplast groups delayed cleavage time as compared to E2 manipulated controls (group 13) as shown by lower cleavage rates at 24 and 40 h after manipulation. Moreover, in most cases these reconstituted embryos did not cleave at all indicating a complete incompatibility of the E2 cytoplasm to nuclei of both compact and non-compact stages of the fourth cell cycle. These results also indicate that different nuclear donor stages had no effect on the time of cleavage when fused to E2 enucleated blastomeres ($P>0.05$).

When comparing L2 reconstituted groups to their respective manipulated controls, fewer reconstituted embryos had cleaved at most periods after manipulation indicating a delay of cleavage caused by these cross-cycle nucleo-cytoplasmic interactions ($P<0.05$). There were also differences between the reconstituted groups indicating a later cleavage time for those derived from L8 karyoplasts ($P<0.05$). However, this effect seems to have been caused by the complete developmental arrest of approximately half the reconstituted embryos in the L8 group (group 17) since cleavages rates relative to those embryos that did eventually cleave were similar to the L2 controls (group 18) at every stage after manipulation ($P>0.05$). These results indicate that, at least at the first cleavage, there was no effect on cleavage time, but a significant difference in compatibilities between the recipient cytoplasm when donor nuclei were derived from different stages of the fourth cell cycle.

7.4. **Discussion:**

These results have demonstrated that the ability of reconstituted single 2-cell blastomeres to proceed into cleavage and to develop further to the blastocyst stage *in vitro*, depends on the stage of the cell cycle at which nuclei and cytoplasm were at the time of fusion. They have also shown that the compatibility of nuclei from the first four cleavage stages to the 2-cell cytoplasm differs markedly at each embryonic stage as shown by the varying ability of reconstituted groups to support development to blastocyst.

These differences may reflect changes in transcription. When nuclei were transferred across embryonic stages in the first experiment, 4-cell karyoplasts showed similar potential in supporting development to blastocyst as those reconstituted embryos derived from 2-cell karyoplasts. This suggests that there may be little differentiation during the 2- to 4-cell stages. Support is provided by previous molecular studies showing that, although transcriptional activity initiates at the early 2-cell stage and continues throughout the cleavage stages, its inhibition from the late 2-cell stage with alpha-amanitin is compatible with many developmental events up to the late 8-cell stage (Johnson and Pratt, 1983; McLachlin *et al.*, 1983). Changes in transcription may also explain the decreased nucleo-cytoplasmic compatibility observed when fusing enucleated 2-cell blastomeres to 8-cell karyoplasts which, by then, may have already experienced stage-specific transformations required for further differentiation. Similar blastocyst formation rates have been reported previously for the transplantation of 8- and 4-cell karyoplasts to enucleated 2-

cell embryos (Robl *et al.*, 1986; Tsunoda *et al.*, 1987). However, the lack of appropriate controls did not allow a comparison of the developmental potential of their 4-cell reconstituted group (72%) to the transplantations using 2-cell karyoplast (Tsunoda *et al.*, 1987).

The large difference between the developmental potential of reciprocal 'asynchronous' groups, after transplantation within the 2-cell stage, provides some clues towards the mechanisms of transcriptional activation and of the timing of nuclear and cytoplasmic modifications during the second cell cycle. Nuclei from late 2-cell blastomeres were still able to support development to blastocyst at a reasonably high rate when fused to enucleated blastomeres from early 2-cell embryos. This occurred regardless of the fact that, at the time of enucleation (E2) the cytoplasm would probably not have been exposed to stage specific messages shown to be essential for further development (Johnson and Pratt, 1983). This suggests that the transplanted nuclei were able to re-initiate the transcription of these essential messages enabling cleavage within 24 h and further development to blastocysts of most reconstituted embryos. However, donor nuclei at the E2 stage that had probably only just initiated transcription and DNA synthesis , were unable to support cleavage and development to blastocyst in most cases when fused to the cytoplasm of late 2-cell blastomeres. This arrest in development occurred despite the fact that the recipient cytoplasm would have already been exposed to the stage-specific messages required for most morphological events up to the 8-cell stage. This suggests that the cytoplasm of a late 2-cell embryo lacks factors either to

terminate the second round of DNA replication or to initiate the transcriptional activity of the embryonic genome. There is evidence that maternally derived messages stored in the cytoplasm are responsible for the transcriptional activation of the embryonic genome during the early 2-cell stage and that most, if not all, maternal mRNA is destroyed by the late 2-cell stage (Flach *et al.*, 1982; Barton *et al.*, 1984). This information is compatible with the findings in this experiment in which nuclei that had not been through the period of transcriptional activation were unable to support further development when exposed to the cytoplasm of embryos already depleted of maternally inherited messages. This same incompatibility may have arisen from the transplantation of pronuclei to 2-cell cytoplasm where neither blastocysts nor cleavage could be achieved by the reconstituted embryos.

In general, the development to blastocyst of embryos reconstituted with 8-cell karyoplasts was very limited. The 11% found contrasts with the 50% developing in the first experiment. This difference may be partially explained by differences in the methodology used in each case. Reconstituted embryos derived from 8-cell karyoplasts may be more susceptible to temperature fluctuations and only those embryos from the third experiment were removed from the incubator for cleavage assessment. None the less, there was a clear effect of the recipient cytoplasm stage (late was best) particularly when using early 8-cell karyoplasts. Similarly, the cytoplasm of the late 1-cell embryo was shown to have better ability to support development in 'across' cell-cycle transplantations (Chapter 5). In both cases, cytoplasm from embryos late in the cell cycle may be able to cope with nuclei from

advanced embryonic stages by promoting a faster breakdown of the nuclear membrane and, therefore, allowing a quicker interaction between the genome and cytoplasmic reprogramming factors (see Chapter 5; Szollosi et al., 1989).

The cell cycle control mechanisms do not seem to be under the independent control of either the nucleus or cytoplasm alone. An interactive control mechanism seems to be more likely, since the 'asynchronous' reconstituted embryos cleaved after a period intermediate between each of the 'synchronous' or control groups. However, the interaction between the E2 nucleus and the L2 cytoplasm appears to have been more traumatic since most embryos arrested development before cleavage. This was also observed in 'asynchronous' transplantations to enucleated 1-cell embryos (Chapter 5) but, contrary to what occurred in experiment 3, arrested embryos were found only with across cell cycle transplantations using L2 karyoplasts. This suggests that when nuclei and cytoplasm are derived from incompatible stages most reconstituted embryos are unable to proceed into the first mitotic cleavage. This nucleo-cytoplasmic incompatibility was also apparent with fusions of 8-cell karyoplasts to E2 enucleated blastomeres that only cleaved in exceptional cases.

Together, these findings indicate major nuclear and cytoplasmic modifications during these early cleavage stages as shown by different degrees of compatibility between the several embryonic stages investigated. The cell cycle stage of the recipient 2-cell cytoplasm seems able to determine the time of cleavage more reliably than the stage of the donor nucleus, while the latter will in most cases determine whether the cell is able to progress

into mitosis or stop cleaving completely. This interactive effect between nucleus and cytoplasm is even more evident in their potential to develop to blastocyst with effects in transplantations 'across' and 'within' the second cell cycle. Further research into the biochemical activity in these reconstituted embryos would allow a better understanding of the effects of these interactions on DNA synthesis and transcription.

FINAL CONCLUSIONS

The results presented in this thesis have provided new information on the early development of mammalian embryos. Studies have concentrated on the interactions between the nucleus and cytoplasm during the initial cleavage stages and on the effects of nuclear transplantation on cell division and further development *in vitro* or *in vivo* to the blastocyst stage and to term after transfer to synchronized recipients. More specifically, these experiments have enabled a better understanding of electrofusion, cell cycle control mechanisms and the developmental potential of embryonic nuclei.

Although experiments were performed on embryos from only two mammalian species, the comparison between sheep and mouse may have also enabled a better understanding of the embryonic development in other mammals. If we consider the time in when the embryonic genome initiates transcription as an important factor affecting development in reconstituted embryos, these species seem to represent two extremes in mammalian early developmental mechanisms. No other mammal initiates transcription earlier than the 2-cell stage, as for the mouse (Flach *et al.*, 1982) or later than the 8- to 16-cell stage as for the sheep embryo (Crosby *et al.*, 1988). Other mammalian embryos analyzed have shown transcription initiating at the 2-cell (goats; Chartrain *et al.*, 1987), 4-cell (pigs and humans; Norberg, 1970; Braude *et al.*, 1988) or between the 8- to 16-cell stage (rabbit and cattle;

Cotton *et al.*, 1985; Camous *et al.*, 1986). The results obtained in these experiments may be extrapolated to other mammalian species, with care.

Two findings contradict a previous concept that once transcription has been activated the nuclei from mammalian embryos lose their ability to support development. First, nuclei from late 2-cell blastomeres (post-transcription) supported development to blastocyst at similar rates to early 2-cell karyoplasts (pre-transcription) after fusion to enucleated zygotes (Chapter 5). Second, in sheep the potential of nuclei from early blastocyst ICM (post-transcription) to support development to blastocyst was similar to 16-cell blastomeres (peri-transcription) when fused to 'enucleated' secondary oocytes (Chapter 4). Further research is still required to investigate the ability of reconstituted blastocysts to produce normal gestation. Although ICM and 16-cell nuclei were able to support full term development, a larger number of transfers of treated and control blastocysts to appropriately synchronized recipients would enable a better assessment and comparison of their potentialities. Another way to examine the potentialities of ICM nuclei would be to assess later embryo ICM cells (day 7 or 8 after oestrus) or by comparing them to contemporary trophectoderm nuclei.

Mouse embryos reconstituted at different stages of the cell cycle have increased the understanding of nuclear and cytoplasmic control over cell division during the first cell cycles. Nucleus and cytoplasm seem to interact to control cleavage from the earliest stages after fertilization. In general, it seems that the cytoplasm must await for a signal from the nucleus indicating that DNA

replication has been completed so that it may engage the mitotic and cytokinetic apparatus for cleavage. In some instances, the incompatibility between the cell compartments was too pronounced to enable this interaction and cleavage was completely blocked. This incompatibility occurred not only when nuclei from late stages were transferred back to an earlier cytoplasm, but also when early nuclei were transferred to later cytoplasm (Chapter 7). This reinforces the existence of developmental 'domains' and a need for maternally derived signals to reactivate transcription in dormant embryonic nuclei. Further cellular and molecular investigations are required to examine the chromatin and its activity in arrested reconstituted embryos derived from fusion of donor cells at different stages of the cell cycle. Cell cycle stage effects of recipient enucleated eggs fused to 8-cell blastomeres have already been examined and shown to induce different chromosomal behaviour after fusion to fertilized or unfertilized embryos (Dyban *et al.*, 1988).

The electrofusion experiments have shown that parameters of field strength and pulse duration should be set individually according to the species and embryonic stage to be fused. However, direct-current field intensities of approximately 1.0 kV/cm and pulse durations between 60 and 100 µsec are effective in most cases and provide higher fusion rates, less lysis and better viability after treatment. A preceding alternating-current pulse was shown to be effective by increasing fusion rates when cell contact was limited by fusing small cells to 'enucleated' eggs, and by providing the alignment of cells in the electric field without the need for manual positioning between the electrodes. Further

experiments on the effects of multiple d.c. pulses may prove useful in improving fusion rates. The variability found between individual batches of similar treatments highlights the need for fairly large groups and for using a statistical method of data analysis which includes the variation between different batches in the same treatment.

While embryonic stem cells have not yet been isolated from livestock (Handyside *et al.*, 1987), the successful transfer of nuclei from ICM cells in sheep suggests that in the future it may be possible to transfer nuclei from embryonic stem cells in livestock, as stem cells in mice are similar to ICM cells (Evans and Kaufman,1983). In this case stem cells would provide an ideal source of nuclei for transfer to produce genetically identical 'clones' and non-chimaeric transgenic offspring from transformed embryonic stem cells (reviewed by Wilmut and Smith, 1988). Groups of 'clones' of cattle have already been produced by nuclear transfer (Marx, 1988). The recycling of transferred nuclei and the use of nuclei from frozen embryos will enable the production of an unlimited number of 'clones' of different ages.

The potential applications of mammalian 'clones' both for research and commerce are very large. As genetically identical animals are more uniform than random-bred animals fewer animals are needed to achieve greater statistical validity in experiments. Clone families also enable studies of interactions between genotype and non-genetic factors such as treatments or environmental effects such as climate and disease. 'Cloning' also allows the multiplication of genetically superior individuals in a population resulting in rapid genetic progress (Nicholas and Smith, 1983).

Smith (1989) has predicted considerable advantages of using cloning in beef cattle. Two types of cattle clones are envisaged, terminal clones chosen for economic merit in production traits, such as growth and carcass traits, and maternal clones chosen for reproduction and maternal traits such as short calving intervals, ease of calving, milking and mothering ability and small mature size. In the short term, benefits will come from testing a large number of clones and gains in economic merit of 15 to 30 percent of the mean can be achieved. Further improvements will come from rebreeding the best clones and testing and selecting a new set of clones, and annual genetic response rates of 2 to 3 percent per year are possible (Smith, 1989).

However, Wooliams (1989) pointed out that with fixed resources (defined by the number of embryo transfers) a conflict arises between the size of the clone families and the selection pressure that can be applied without exacerbating inbreeding rates. It also must be noted that many factors may contribute to phenotypic differences among genetically identical animals. Cytoplasmic inheritance (mitochondria and other organelles), epigenetic phenomena (random X-inactivation, mutations and random cell migration patterns), recipient uterine environment (size, age, parity and disease) and all the post-natal environmental effects (nutrition, season, location) are some of the factors that may influence the phenotype of each individual clone (Seidel, 1983).

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J. Reprod. Fert. (1988) **84,** 619–624

Printed in Great Britain

Influence of cell cycle stage at nuclear transplantation on the development *in vitro* of mouse embryos

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Summary. Nuclei were transplanted from embryos of mice at different stages of the 1st and 2nd cell cycle to oocytes enucleated at various times after fertilization. After transfer of pronuclei, a greater proportion of embryos developed to blastocysts if donor and recipient embryos were at the same stage of the cell cycle (synchronous transfer = 94%, asynchronous transfer = 76%). By contrast, when 2-cell blastomere nuclei were fused to the cytoplasm of enucleated zygotes, there was a significant effect of both cytoplast and karyoplast cell cycle stage on the development of the reconstituted embryos. Karyoplasts and cytoplasts derived from embryos at later stages of the cell cycle had greater potential to support development to blastocysts *in vitro*. It is suggested that the secretion of stage-specific messengers and the timing of nuclear membrane breakdown are the main factors causing the karyoplast and cytoplast effects, respectively.

Keywords: embryo; nuclear transfer; cell cycle; mouse

Introduction

Given the right environment, a fertilized mammalian egg is able to differentiate into the many cell types of the adult animal. During embryogenesis, the genome and epigenetic factors interact and, through mitosis, commit daughter cells to different pathways of differentiation. By investigating the relationship between nucleus and cytoplasm at pre-commitment stages of development, the initial mechanisms of embryonic differentiation may be studied.

These early nucleo-cytoplasmic interactions can be examined using a non-invasive technique of nuclear transplantation (McGrath & Solter, 1983). Karyoplasts from embryos at the 4- and 8-cell stage have only a limited potential to support further development when fused to enucleated zygotes (McGrath & Solter, 1984). However, when fused to enucleated 2-cell blastomeres they will develop to blastocysts *in vitro* (Robl *et al.*, 1986) and to term after transfer to a synchronized recipient (Tsunoda *et al.*, 1987). These observations emphasize the need for a close nucleocytoplasmic relationship at the early stages of mammalian embryonic development.

The present report describes experiments to investigate the effects of embryonic stage, both between and within the cell cycle, of karyoplasts and cytoplasts on the development *in vitro* after transplantation of blastomere nuclei to enucleated zygotes in mice.

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Materials and Methods

Source of embryos. Embryos were obtained from 4- to 7-week-old F1 (C57BL/6 × CBA/Ca) mice that had been induced to superovulate by intraperitoneal injections of 5 i.u. PMSG (Folligon: Intervet, Cambridge, U.K.) and 5 i.u. hCG (Sigma, Poole, Dorset, U.K.) given 48 h apart. In some experiments, these injections were timed to produce three embryonic stages for each of the 1st and 2nd cell cycles. Zygotes were classified as early (16–20 h after hCG), mid- (20–24 h after hCG) or late (24–28 h after hCG) according to the time of injection. Two-cell embryos were also classified as early (0·5–1 h after an observed cleavage), mid- (40–44 h after hCG) or late (44–48 h after hCG) for similar reasons.

After hCG injection, females were paired with F1 males (MF1/Olac × SWR) and inspected the following morning for copulation plugs. Embryos were flushed from the oviduct at approximately 1·5 h before microsurgery using Hepes-buffered medium (M2; Quinn *et al.*, 1982) and placed in a bicarbonate-buffered medium 16 + 0·4% (w/v) BSA (M16 + BSA; Whittingham, 1971) until microsurgery.

Microsurgery and culture. Embryos were placed in Medium M16 + BSA with cytoskeleton inhibitors (1 μg cytochalasin D/ml (Sigma) and 0·3 μg nocodazole/ml (Sigma)) for 30 min before being placed in medium M2 with cytoskeleton inhibitors for another 30 min during microsurgery. Nuclear transplantation was carried out by the technique described by McGrath & Solter (1983). This method involves removing nuclei surrounded by a small portion of cytoplasm and plasma membrane (karyoplast) and placing this karyoplast together with a fusogenic agent (inactivated Sendai virus) into the perivitelline space of an enucleated embryo (cytoplast). Typically, fusion occurs within 1 h of manipulation.

After nuclear transplantation the embryos were washed several times and placed in Medium M16 + BSA under paraffin oil (Heavy Mineral Oil, Sigma) equilibrated in Medium M16 and incubated at 37·5°C in an humified atmosphere of 5% CO_2 in air. The embryos were assessed for fusion the following day and then placed in fresh Medium M16 + BSA for a further 3 days when their stage of development was recorded. Embryos were classified at ~94 h after manipulation as developing into normal blastocysts (successful), or as embryos arrested at a previous stage of development (failure). Data on the proportion developing to blastocysts were compared by χ^2 analysis.

Results

Experiment 1

Reconstituted embryos were produced from the fusion of enucleated zygotes to karyoplasts from the first 4 embryonic cell cycles. The proportion of embryos developing into blastocysts *in vitro* when pronuclei were transferred was not significantly different from that in the control group, suggesting that the technique had little or no effect on the viability of the embryos (73/76 = 96·1% *vs* 46/49 = 93·9%, $P > 0{\cdot}05$). However, there was a significant reduction in viability when karyoplasts from 2-cell embryos were fused to enucleated zygotes with only 45·2% (14/31, $P < 0{\cdot}01$) developing into normal blastocysts. Moreover, a further reduction in viability was found when karyoplasts from 4- and 8-cell embryos were fused to enucleated zygotes, with only 2% (1/50, $P < 0{\cdot}01$) and none (0/24, $P < 0{\cdot}01$) of the respective reconstituted embryos developing into blastocysts. Most 4-cell-derived embryos stopped development at pre-compaction stages although a few embryos compacted and developed several vacuoles, but very rarely formed normal blastocysts. The development of 8-cell-derived embryos was very limited; most embryos cleaved only a single time and stopped at the 2-cell stage.

Experiment 2

The effect of within-cell cycle-stage synchrony on the viability of reconstructed embryos was examined through the exchange of pronuclei between synchronous and asynchronous zygotes collected at early and late stages of the first cycle. Their development to blastocysts is summarized in Table 1. Synchronous transfers between embryos at the early and late stages of the cycle developed similarly ($P > 0{\cdot}05$), and not significantly differently from the controls ($P > 0{\cdot}05$). Likewise, the asynchronously transferred groups did not differ significantly from each other ($P > 0{\cdot}05$). However, when treatments of both groups were pooled and compared with one another, the asynchronous group showed a lower viability than did the synchronous group ($P < 0{\cdot}01$).

Table 1. Stage synchrony effects within the first cell cycle on the development *in vitro* of blastocysts 90 h after fusion of pronuclear karyoplasts to enucleated mouse zygotes

Cytopolast stage	Manipulated controls	Synchronous transfers	Asynchronous transfers
Early zygote (16–20 h after hCG)	91·9* (35)†	96·7 (30)	73·9 (23)
Late zygote (24–28 h after hCG)	98·7 (75)	93·9 (49)	77·8 (27)
Totals	96·4 (110)	94·9 (79)	76·0 (50)

*Percentage of blastocysts 90 h after microsurgery.
†No. of fused embryos after microsurgery.

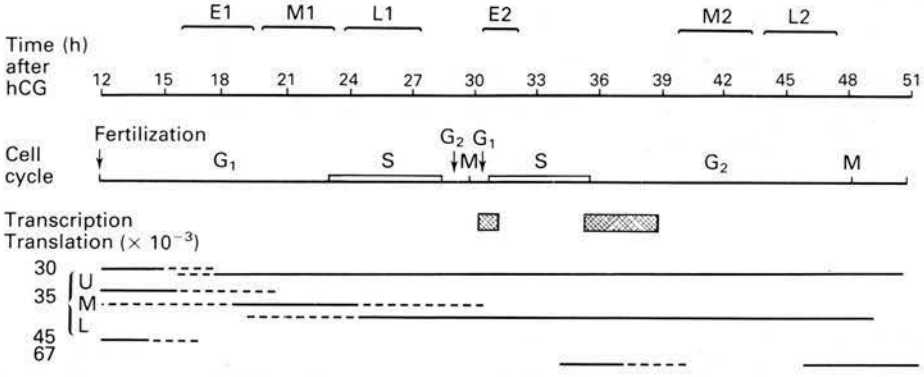

Fig. 1. Molecular and cellular events during the first two embryonic cell cycles and their relationship to the time of karyoplast and cytoplast transplantations. The diagram shows, in relation to time after fertilization, periods of DNA transcription and synthesis of peptides of different molecular weight at higher (——) and lower (– – –) levels (adapted from Bolton *et al.*, 1984; Howlett & Bolton, 1985). The stages at manipulation of donor and recipient embryos were: E1, M1 and L1 = recipient cytoplasts derived from zygotes at early, mid- and late stages of the first cell cycle; E2, M2 and L2 = donor karyoplasts derived from 2-cell embryos at early, mid- and late stages of the second cell cycle.

Experiment 3

The effect of cycle stage when transferring nuclei from embryos at different cell cycles was investigated by transferring karyoplasts from 2-cell embryos to enucleated zygotes. Embryos were reconstructed by the fusion of karyoplasts and cytoplasts at early, mid-, and late stages of the 2nd and 1st cell cycle, respectively. An indication of the relationship of these timed intervals after hCG and known biochemical events in the 1st and 2nd cell cycle is shown in Fig. 1. Although the males were of a different origin, the donor females and the superovulation regimen were the same in the present experiments and in the biochemical studies. Confirmation that the time of development was similar to earlier reports is provided by the observation that most embryos collected for the E2 stage cleaved between 30 and 34 h after hCG which, assuming that ovulation and fertilization occur at around 12 h after hCG in mice, is consistent with the results reported by Howlett & Bolton (1985) in which 90% of the embryos cleaved between 18 and 22 h after in-vitro fertilization.

Table 2. Karyoplast and cytoplast cell cycle stage effects on the development of reconstructed mouse embryos 90 h after the fusion of 2-cell karyoplasts to enucleated zygotes

Cytoplast stage	Early 2-cell (30–32 h)	Mid 2-cell (40–44 h)	Late 2-cell (44–48 h)	Cytoplast totals
Early zygote (16–20 h)	2·3* (44)†	18·9 (37)	20·0 (25)	12·3 (106)
Mid zygote (20–24 h)	12·5 (40)	43·6 (39)	29·4 (34)	28·3 (113)
Late zygote (24–28 h)	66·0 (47)	48·8 (41)	63·5 (52)	59·6 (141)
Karyoplast totals	28·2 (131)	37·6 (117)	42·9 (112)	35·8 (360)

*Percentage of blastomeres 90 h after microsurgery.
†No. of fused embryos after microsurgery.

The development of the reconstituted embryos to blastocysts *in vitro* is summarized in Table 2. There was a significant effect of the stage of the cycle of the cytoplast and a smaller effect of the cell cycle stage of the karyoplast. The average effect caused by the cytoplast stages indicated a greater potential of cytoplasts derived from later zygotes ($P < 0{\cdot}01$). This effect was consistent with all three karyoplast stages to which they were fused. Although the average effect caused by varying the karyoplast stages also indicated an increasing potential of nuclei derived from blastomeres obtained and manipulated at later stages of the 2nd cell cycle ($P > 0{\cdot}05$), this effect was not consistent with all three cytoplast stages. Cytoplasts from early- and mid-stage zygotes showed a greater potential to develop into blastocysts when fused to karyoplasts from the final (mid- and late) rather than initial (early) stages of the second cell cycle ($P < 0{\cdot}01$). However, there was not an evident karyoplast effect in the cytoplast group derived from late stage zygotes ($P > 0{\cdot}05$).

Discussion

These results confirm that the interactions between nucleus and cytoplasm are of critical importance at these early embryonic stages. The development of embryos after nuclear transplantation was influenced by both the cleavage stage of development of the donor embryo, and differences in stage within the cell cycle of karyoplasts and cytoplasts. When transferring pronuclei between zygotes, maximum development was obtained if donor and recipient embryo were at the same stage of development. By contrast, such 'synchronous' transfer did not lead to the greatest proportion of embryos developing to blastocysts when nuclei were transferred from 2-cell embryos to zygotes. Karyoplasts and cytoplasts derived from embryos at later stages of the cell cycle had a greater potential to develop *in vitro*. These results contrast with those of Howlett *et al.* (1987) who found that karyoplasts from early 2-cell embryos had a greater potential than did the late 2-cell karyoplasts. These differences may be due to the source of cytoplast used in each experiment since Howlett *et al.* (1987) fused the karyoplasts to ethanol-activated oocytes. These were activated 17 h after hCG, before enucleation and manipulation 5 h later (22 h after hCG). Together, our observations suggest that, within the same cell cycle, karyoplasts should be transferred to cytoplasts at similar stages of the cell cycle (i.e. same stage after hCG injection). However, this is not necessarily true when transferring between cell cycles when several other factors may influence subsequent development of reconstituted embryos.

The viability of the nuclear transplanted embryos was greatly influenced by the embryonic stage from which the karyoplasts were obtained with a marked reduction when taken beyond the 4-cell stage. This result is similar to one reported previously (McGrath & Solter, 1984), for which no development to blastocysts was achieved. These results contrast with those found with amphibian nuclear transplantations in which nuclei from embryos at the blastula stage could support development to adult frogs (Briggs & King, 1952) and nuclei from intestinal cells could support the development of embryos to the tadpole stage (Gurdon, 1962). Moreover, the nuclei in other mammalian species seem to retain totipotency for longer than do those of mice, since lambs and calves have been born from the fusion of 8- and 16-cell blastomeres to enucleated oocytes (Willadsen, 1986; Prather *et al.*, 1987). The most striking difference concerning the developmental mechanisms in these species concerns the timing of transition from maternal to embryonic control of development. While the mouse genome is activated at the 2-cell stage (Flash *et al.*, 1982), in sheep and cattle it is activated at around the 8- to 16-cell stage (Crosby *et al.*, 1988; Camous *et al.*, 1986) and at the mid-blastula stage in amphibians (Newport & Kirschner, 1982). There are two ways by which development may be abnormal following transfer of nuclei after the activation of the embryonic genome. First, post-activation nuclei fused to mouse enucleated zygotes may not have enough time to be reprogrammed and initiate de-novo transcription at the 2-cell stage. Second, the active nuclei may continue transcribing messengers which are transported into the cytoplast (Ege & Ringertz, 1975) and directly or indirectly affect subsequent development of the reconstituted embryo. It remains to be confirmed whether either of these mechanisms account for the species differences.

The effects of cell-cycle stage synchrony between karyoplast and cytoplast were shown by transplantations within the first cell cycle. The slower development after asynchronous transfers may have been due to responses of the nuclei to regulatory signals from the surrounding cytoplasm (Ege *et al.*, 1975) causing diploidy or other genomic defects. By contrast, the developmentally beneficial effects of synchrony were not observed when 2-cell karyoplasts were transferred to zygote cytoplasts at similar stages of the cell cycle. This indicates that other factors may be affecting the viability of the reconstituted embryos when karyoplast and cytoplast are derived from different cell cycles. The greater potential of the transfers to late cytoplasts could be a result of cytoplasmic differentiation since it has been shown that polypeptide patterns do vary during the first cell cycle interphase (Howlett & Bolton, 1985). However, this beneficial effect was not present in the within-cycle asynchronous transfers since the reciprocal treatments were not significantly different.

The karyoplasts derived from embryos collected at later stages of the 2nd cell cycle also showed a greater potential to support blastocyst development. This effect was particularly marked when the nuclei were transferred to cytoplasts at the early and mid-stage of the cell cycle. Since the early 2-cell karyoplasts were obtained from embryos collected during or soon after cytokinesis, and therefore before or during the activation of the embryonic genome (Flash *et al.*, 1982), greater development occurred following the transfer of nuclei recovered after the activation of the embryonic genome. These results are contrary to the suggestion that the genomic activation irreversibly restricts nuclei and is responsible for the decreased viability of the reconstituted embryos (Howlett *et al.*, 1987). However, another possible interpretation of these results is that the early karyoplast had still to embark on a prolonged period of nuclear activity while the later karyoplasts had already resumed transcription, at around 41 h after hCG injection (Flash *et al.*, 1982), and may have no longer been releasing substantial amounts of stage-specific messengers into the cytoplasm.

These observations reveal the importance of controlling cell-cycle stage when transferring nuclei between embryos. As the optimum stage of the cycle varies with the stage of the embryo, it will be important to define the ideal combination of cell-cycle stages for each stage of donor and recipient embryo. Transfer at different stages of the cell cycle will also create an opportunity to investigate the influence on subsequent development of factors such as genome differentiation, the influence of stage specific factors and breakdown of the nuclear membrane.

We thank David Parnham and Frances Thomson for help with animal care. L.C.S. is supported by FAPESP-CNPq, Brazil.

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Received 11 April 1988