GENETIC DUPLICATION OF MICE
THROUGH CHEMICAL ENUCLEATION

by

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A Thesis Submitted to the Faculty of the
DEPARTMENT OF GENERAL BIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Amphibian Development</td>
<td>2</td>
</tr>
<tr>
<td>Nuclear Transplantation in Amphibians</td>
<td>8</td>
</tr>
<tr>
<td>Reasons for Failure in Amphibian Transplants</td>
<td>12</td>
</tr>
<tr>
<td>Mammalian Development</td>
<td>20</td>
</tr>
<tr>
<td>Nuclear Transplantation in Mammals</td>
<td>23</td>
</tr>
<tr>
<td>Reasons for Failure of Transplants</td>
<td>30</td>
</tr>
<tr>
<td>in Mammals</td>
<td></td>
</tr>
<tr>
<td>Development of Improved Techniques</td>
<td>33</td>
</tr>
<tr>
<td>METHODS</td>
<td>41</td>
</tr>
<tr>
<td>Preparation of Pasteur Pipettes</td>
<td>41</td>
</tr>
<tr>
<td>Marker</td>
<td>41</td>
</tr>
<tr>
<td>Egg Preparation</td>
<td>41</td>
</tr>
<tr>
<td>Enucleation</td>
<td>42</td>
</tr>
<tr>
<td>Preparation of Donor Nuclei</td>
<td>46</td>
</tr>
<tr>
<td>Cell Fusion</td>
<td>48</td>
</tr>
<tr>
<td>Embryo Culture</td>
<td>49</td>
</tr>
<tr>
<td>Implantation</td>
<td>49</td>
</tr>
<tr>
<td>RESULTS</td>
<td>54</td>
</tr>
<tr>
<td>Preparation of Eggs</td>
<td>54</td>
</tr>
<tr>
<td>Enucleation</td>
<td>54</td>
</tr>
<tr>
<td>Preparation of Donor Nuclei</td>
<td>54</td>
</tr>
<tr>
<td>Cell Fusion</td>
<td>56</td>
</tr>
<tr>
<td>Embryo Culture</td>
<td>56</td>
</tr>
<tr>
<td>Implantation</td>
<td>56</td>
</tr>
<tr>
<td>Combined Treatments</td>
<td>57</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>59</td>
</tr>
<tr>
<td>Accomplishments</td>
<td>59</td>
</tr>
<tr>
<td>Reasons for Failure</td>
<td>59</td>
</tr>
<tr>
<td>Improvements for the Future</td>
<td>60</td>
</tr>
<tr>
<td>Prospect</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Prospects for the Production of</td>
<td>65</td>
</tr>
<tr>
<td>Genetic Duplicates in Mammals</td>
<td></td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>67</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Illustration</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Isolated Balb/c Uterus, Oviduct, and Ovary</td>
<td>43</td>
</tr>
<tr>
<td>2.</td>
<td>Isolated Balb/c Embryo at the 1 Cell Stage</td>
<td>44</td>
</tr>
<tr>
<td>3.</td>
<td>Balb/c Embryo at the 1 Cell Stage After Removal of the Zona with Pronase</td>
<td>45</td>
</tr>
<tr>
<td>4.</td>
<td>Vasectomy of a Balb/c Mouse</td>
<td>50</td>
</tr>
<tr>
<td>5.</td>
<td>Balb/c Oviduct Segments in Culture</td>
<td>51</td>
</tr>
<tr>
<td>6.</td>
<td>Implantation of Embryos into the Uterus of a Pseudopregnant Recipient</td>
<td>53</td>
</tr>
</tbody>
</table>
ABSTRACT

Developmental processes in amphibians and mammals, previous nuclear transplantation experiments, and the reasons for failure in those experiments were analyzed. It was determined that damage to the egg, cell cycle asynchrony, insufficient activation, chromosomal abnormalities, restriction of differentiation due to introduction of foreign controlling substances into the egg with the transplanted nucleus, and the technical difficulty of the procedures combined to cause previous failures. One improvement attempted here was the treatment of isolated nuclei with trypsin to derepress the DNA, followed by spermine to stabilize the condensed chromosome structure and prevent damage during manipulation. Another improvement was the enucleation of the recipient egg by the use of trimethylpsoralen followed by UV-irradiation. When treated nuclei were attached to enucleated eggs by concanavalin A and fused with them by Sendai virus, one egg developed to blastocyst but no further development was observed. Nuclei were also attached to sperm by concanavalin A, suggesting that sterile sperm might be able to carry treated nuclei into eggs at fertilization. Use of small lymphocytes and oleic acid fusion proved unworkable. Suggestions for future work included
serial fusion with derepressed cells, cytochalasin B delay of cleavage, synchronization of donor cells to the recipient's cell cycle, and polyethylene glycol fusion.
INTRODUCTION

There have been many attempts to artificially control the transfer of genetic material into cells of eucaryotes. The successful attempts have included: introduction of exogenous deoxyribonucleic acid (DNA) (Snow and McLaren 1974); transfer of the SV40 genome to rabbit ova by fertilization with sperm onto which the virus was adsorbed (Brackett et al. 1971); viral fusion of isolated mitochondria with mouse cells in tissue culture (Radsak, Sawicki, and Koprowski 1972); incorporation of isolated chloroplasts or isolated chick mitochondria by mouse cells with which they were co-cultivated in tissue culture (Nass 1969); complete reconstitution of normal, reproducing amebae by combination of isolated nuclei, cytoplasms, and cell membranes (Leon, Lorch, and Danielli 1970); fusion of rat karyoplasts with mouse cytoplasts to form indefinitely dividing cell lines (Krondahl et al. 1977).

The most fruitful transfers of genetic information have involved nuclear transplantation. Nuclear transplantation was first successfully carried out in amebae by Commandon and de Fonbrune in 1939, followed by Briggs and King in the frog Rana pipiens in 1952, and subsequently
by other investigators in the ciliate Stentor, Protozoa, the frog *Xenopus laevis*, the salamander *Ambystoma*, the newt *Pleurodeles*, the alga *Acetabularium*, the fungus *Neurospora*, the toad *Bufo*, the fruit fly *Drosophila*, the bee *Apis*, the beetle *Leptinotarsa*, the mouse *Mus*, and the rabbit *Lepus* (Gurdon 1963, 1974b; King 1966; Graham 1969; Bromhall 1975).

Because the most thorough analysis of nuclear transplantation in vertebrates has been done on frogs, the normal development, transplantation experiments, and the reasons for failure of nuclear transplantation in frogs will be presented for comparison with the development, transplant experiments, and reasons for failure in mammals. Of the mammals, attention will be focused on the mouse, which is the subject of choice in the attempt to improve techniques for the production of genetic duplicates outlined below.

**Amphibian Development**

In all discussions about amphibians, where no subject is specified, the subject is the frog *Xenopus laevis*. In cases where a different species is considered, it will be named.

The ovarian oocyte of the normal amphibian synthesizes ribonucleic acid (RNA) but not DNA (Gurdon 1968b). The nucleus of the oocyte is distinguished from that of
other cell types by its enormous volume, diffuse chromatin, and multiple nucleoli (Gurdon 1968a). In Rana pipiens, DNA dependent RNA polymerase accumulates in the germinal vesicle (GV) during oogenesis and is released into the cytoplasm at the breakdown of the GV during ovulation. It is then available to return to the nucleus during embryogenesis in a fashion similar to the o factor discussed below (Hollinger and Smith 1976). At ovulation, the oocyte begins to mature into an egg. It passes through its first meiotic division and synthesizes neither DNA nor RNA (Gurdon 1968b). Development arrests at metaphase of meiosis II.

At fertilization, the egg's cortical granules break down, and the metaphase arrest is ended. In Rana this might be due to neutralization or inactivation of arresting factors by granule constituents (Masui and Markert 1971; Ziegler and Masui 1973). By 15 minutes after fertilization, the egg has completed its second meiotic division and the second polar body is extruded (Graham 1966).

During the next 20 minutes, the sperm and egg nuclei swell and move to the center of the egg (Graham 1966). This nuclear swelling is due to the passage of cytoplasmic protein into the enlarging nucleus and has a close temporal relationship with DNA synthesis and
chromatin dispersion (Gurdon 1968b). Among the cytoplasmic proteins concentrated in the nucleus at this time are non-histone proteins (NHP) (Hoffner and Di Berardino 1977). It is characteristic of activated eggs that DNA synthesis but not RNA synthesis occurs (Gurdon 1968b), and that a factor is found in egg cytoplasm which can suppress RNA synthesis in competent nuclei (Bernstein and Mukherjee 1972).

At about 40 minutes post-fertilization, the sperm nucleus and egg nucleus lie next to each other at the center of the egg. By 50 minutes post-fertilization, DNA synthesis in the nuclei has ceased. At 60 to 70 minutes after fertilization, the nuclei fuse, the nuclear membranes break down, the cell enters mitosis, and the cleavage furrow appears (Graham 1966). In Rana, post-fertilization development takes twice as long, and the first cleavage occurs at about 2 hours after fertilization (King 1966). Mitosis occurs every 20 minutes after the first division, and there is no G1 phase up to 5 hours post-fertilization (Graham and Morgan 1966).

It is at the blastula stage that the next dramatic changes occur. In mid- to late-blastula, a spurt of gene activity produces new transcripts before gastrulation. Axolotl blastulae with the o (for ova deficient) mutation never show incorporation of 3H-uridine at this
stage. Their development is arrested at gastrulation. Mutant eggs are thought to lack the o substance, which is synthesized during oogenesis and stored in the cytoplasm. The o substance appears to exert its effect on RNA synthesis when it enters the nucleus at mid- to late-blastula and either binds to cellular structures or is degraded. Up to early blastula stages, the mutant nuclei can be corrected by addition of o substance, but at later stages they have undergone an irreversible loss of this capacity (Brothers 1976). Evidence for synthesis of mRNA during the blastula stage is provided by the fact that in hybrids of R. pipiens, the cleavage rate is determined by the egg cytoplasm, and the sperm nucleus has no effect until the beginning of gastrulation (Briggs, Green, and King 1951). By late blastula, tRNA is synthesized, and by the neurula stage rRNA is made (Gurdon 1968b).

Because it is temporally correlated with synthesis of messenger, it is particularly significant that H1 histone is first synthesized at late blastula (Adamson and Woodland 1974). Histones are synthesized during oogenesis and used until late blastula, but all except H1 are synthesized at all stages (Adamson and Woodland 1974). When H1 histone is removed, RNA polymerase attaching sites increase 10-fold, and specific transcription disappears (Imoh 1978). This control over transcription
specificity has important implications for development, as in *Triturus pyrrhogaster* where the H1 histone/DNA mass ratio increases 3-fold from blastula to neurula at which level it remains constant until hatching. While histone levels are rising, mRNA level is highest at blastula and decreases to a constant level at neurula in *Triturus* (Imoh 1977, 1978), and in *Rana* there is a continuing restriction of the kinds of DNA-like RNA synthesized from neurula to larva (Flickinger 1971).

Before proceeding to a discussion of nuclear transplantation experiments, it is instructive to consider studies in several non-amphibian systems which may be pertinent to amphibian development. Pea cotyledon chromatin, which produces pea seed globulin, is repressed during differentiation to apical bud, but if H1 histone is removed, the chromatin is derepressed, and the globulin/total protein ratio increases. Further removal of other histones reduces this ratio (Bonner 1966). During differentiation, the relative proportions of nuclear non-histone proteins change (Boffa, Vidali, and Allfrey 1976), and the amount of NHP in transcriptionally active cells in culture is greater than in inactive cells (Lau and Ruddon 1977). In steroid-induced differentiation in the chick, the increase and then decrease of NHP to the end of development is paralleled by the capacity of DNA to serve as a
template (O'Malley and Means 1974). Antibodies to nuclear acidic proteins binding to DNA in chromatin are organ specific and change with the stage of development. Furthermore, circular dichroism studies measured a gradual increase in ellipticity, indicating opening of DNA, and removal of proteins from areas of DNA during differentiation (O'Malley and Means 1974). Chick cells stimulated to differentiate by steroids go through the cell cycle from G_1 to M (O'Malley and Means 1974). This is also seen in Rana during differentiation where addition of a specific inhibitor of DNA synthesis (cytosine arabinoside) causes a reversible delay in differentiation, which proceeds essentially normally when the block is removed. Also, in Rana, there is a stimulation of the rate of cell division during induction (Flickinger 1971). A role for histone in cell division is suggested by observations on cells in culture which show that the level of growth-associated H1 histone phosphokinase may control the initiation of mitosis during G_2 (Inglis et al. 1976). The simultaneous occurrence of these events can be seen in the sea urchin, where changes in the relative amounts of NHP types, an increase in NHP content, and an increase in the relative amount of H1 histone occur during development (Gineitis, Stankeviciute, and Vrob'ev 1976).
Nuclear Transplantation in Amphibians

Nuclear transplantation is accomplished in amphibians by activating the egg by pricking with a clean needle followed by mechanical removal (Rana) or removal by ultraviolet (UV) irradiation (Xenopus) of the egg nucleus. A donor cell is then sucked into a micropipette, and passage through the pipette breaks the plasma membrane. The nucleus, surrounded by cytoplasm, is then injected into the recipient egg (Gurdon 1963).

Several experiments have indicated that the results of nuclear transplantation are not artifacts: eggs allowed to retain their maternal nucleus after injection with a donor nucleus develop less well than enucleated eggs injected with a nucleus, demonstrating that retention of the maternal nucleus does not account for development (Briggs and King 1953); passage of Xenopus laevis nuclei through Xenopus victorianus cytoplasm to gastrulation then back to X. laevis eggs results in offspring showing no contribution of the recipient DNA to their development (Gurdon 1961); in R. pipiens, lack of normal development in enucleated eggs injected with embryonic cytoplasm shows that injected cytoplasm is not the cause of development (Briggs and King 1957); in Xenopus, permanent acceptance of skin grafts between members of a clone versus rejection within 1 to 2 weeks.
between siblings of a normal mating supports the idea that the clones are genetically identical (Gurdon 1974b).

Cells from a wide variety of sources have provided nuclei for successful nuclear transplantation, including cells from kidney, heart, skin, lung (Gurdon, Laskey, and Reeves 1975), intestinal epithelium (Di Berardino and Hoffner 1970), single cells from tissue culture (Gurdon and Laskey 1970b) and keratinized skin cells of adult foot webs which are completely differentiated (Gurdon, Laskey, and Reeves 1975). It has also been claimed that transplantation of tumor cell nuclei can give rise to normal development into embryos and tadpoles (McKinnell, Deggins, and Labat 1969), but there has been no proof that the embryos came from cancer cells as opposed to normal cells in the same tissue (Gurdon 1974b). The most normal development from an adult skin cell nucleus was a young frog which died a few weeks after metamorphosis but otherwise appeared normal (Gurdon 1974b). At least 20% and probably 70% of tadpole intestine nuclei contain the genetic information required to form functional muscles and nerve cells (Gurdon 1963). Development of fertile adults has been promoted by 0.3% of nuclei from larval intestinal epithelia (Di Berardino and Hoffner 1970).
The reprogramming of the transplanted nucleus to function in a fertilized egg involves events found in normal development. When mid-blastula nuclei, which synthesize DNA not RNA and divide every 20 minutes, or brain nuclei, which synthesize RNA and almost never synthesize DNA or divide, were injected into growing oocytes, oocytes maturing into eggs, and activated eggs, the transplanted nuclei altered their activities toward that of the recipient cytoplasm within 1 to 2 hours (Gurdon 1968b). Entry of labelled proteins from egg cytoplasm into the brain nuclei correlated with DNA synthesis: nuclei that did not accumulate protein did not synthesize DNA (Gurdon 1974b). HeLa nuclei similarly transplanted into oocytes were shown to take up histones and NHP in amounts that increased with the nuclear volume, indicating that protein binding sites are made available as nuclei enlarge and chromatin undergoes dispersion for RNA synthesis (Gurdon, Partington and De Robertis 1976). It has further been shown that embryonic and larval nuclei concentrate NHP when injected into enucleated fertilized eggs (Hoffner and Di Berardino 1977). These data lend support to a reprogramming concept.

Some restrictions on developmental capacity have been found. There is a wide range of success rates among various species. For example, transplants in Rana
were less successful than in *Xenopus* (Gurdon 1974b). The later the stage of development of the donor nuclei, the fewer complete blastulae and the more severe the abnormalities which were seen. These abnormalities were not corrected by parabiosis with normal embryos and were stable, as indicated by the fact that serial transfers (transplanting a nucleus from a clonal embryo cell into an activated unfertilized egg) of abnormal embryos were abnormal in a similar way. A further indication of restriction due to the stage of the donor is that out of 152 adult frogs obtained in one study, 7 our of 27 advanced-stage donor transplant frogs were sterile, while none of 125 early donor frogs were (Gurdon 1963). A decline in developmental capacity does not necessarily mean a deficiency in genetic content. This is indicated in *R. pipsiens* by the fact that more normal development has been obtained from adult kidney cells than from primary spermatocytes (Gurdon 1974b). At least some of the restrictions are technical in origin, as indicated by the fact that the method of isolating donor cells and the length of time between isolation and transplantation affect the results (Muggleton-Harris 1971). Improved techniques have reduced some of the restrictions. Nuclei from less than normal transplant embryos (partial blastulae) can be retransplanted (serial transfer) and in
some cases give increased success (Gurdon 1963; Di Berardino, and Hoffner 1971). In *R. pipiens*, reduction in temperature during transplantation (from 20°C to 11°C) improves cleavage capacity but not later development, while the addition of the polyamine spermine to the medium significantly improves post-gastrula development. Combining the two effects increases development to the tailbud stage by as much as 118% over controls (Hennen 1970). Such has been the improvement in transplantation techniques that about 70% of *R. pipiens* transfers can develop into normal larvae (about the same as for *Xenopus*) (Hennen 1970), which can be compared with 68% to 75% normal development in naturally fertilized eggs (Briggs and King 1957).

Reasons for Failure in Amphibian Transplants

Technical difficulties make a contribution to the failure of nuclear transplants. It is known that the poor quality of many *Xenopus* eggs causes developmental abnormalities and that the better development of some serial transfers may be due to the improved chance of getting a good egg (Gurdon 1963). It is essential that the transplanted nucleus contact the egg cytoplasm or the changes necessary for development will not occur, as evidenced by the failure of injected whole cells and injected intact sperm to synthesize DNA (Gurdon 1968a;
Graham 1966). Cells of older stages are smaller and more difficult to dissociate (Briggs and King 1953), and failures have been attributed to this problem, but in a study where the size of cells from different stages was held constant, a differential effect was still observed (McAvoy, Dixon, and Marshall 1975). Direct damage to the egg due to manipulation is also a factor because even saline injection can lead to some abnormal development (Ursprung and Markert 1963). It was thought that exposure of the nucleus to medium caused damage (Briggs and King 1953) similar to that found where damaged nuclei were rejected as transplants by enucleated amebae. Nevertheless, the failure in amebae was due to inability to distinguish a damaged nucleus from a food vacuole (Lorch and Danielli 1953) and failure in amphibia cannot yet be confidently attributed to damage induced by exposure of the nucleus to medium.

The dissimilarity between normal fertilization and artificial activation, where cortical granule breakdown is abnormal, contributes to abnormal development. A situation that could occur during insufficient activation in nuclear transplantation was created in R. pipiens when eggs were inseminated before metaphase II. In those eggs, sperm entry does not activate the egg. The sperm chromatin condenses without DNA synthesis, and the
unreplicated chromosomes are segregated, thrown out into polar bodies or otherwise lost, leading to aneuploidy (Elinson 1977). In *R. pipiens*, cortical granule breakdown and cleavage events are separable, but abnormalities result (Hollinger and Schuetz 1976) and swelling does not occur when granule breakdown is blocked (Masui and Markert 1971). In *X. laevis*, nuclear swelling, and to some extent chromatin dispersion may be linked to granule breakdown (De Roeper and Barry 1976). Injected nuclei seem to compete for swelling agent (Gurdon 1976), and the competition of the irradiated nucleus for this substance (Graham, Arms, and Gurdon 1966) might reduce success. Eggs pricked with a clean needle will be activated but will not undergo parthenogenetic cleavage due to failure of spindle formation unless a "second factor" (a cleavage initiating agent) is introduced (Briggs and King 1953; Maller et al. 1976). This factor is found in all body tissues except unfertilized eggs. The second factor activity on *X. laevis* eggs of extracts of sea urchin sperm is found only in the centriole-containing fraction (Maller et al. 1976). So effective is the activating power of sperm that eggs inseminated with radium-treated sperm develop into partial blastulae without any functional chromosomes (Briggs, Green, and King 1951; Briggs and King 1953). It is now generally
recognized that the amphibian egg usually requires the sperm aster to cleave normally (Briggs, Green, and King 1951) and that the nucleus injected in transplantation is surrounded by perinuclear cytoplasm containing the centrioles (Di Berardino and Hoffner 1970). This might be a better reason for the requirement for cytoplasm to surround the nucleus than the thought that exposure to medium "kills" nuclei (Briggs and King 1953). In fact, when cell homogenates were tried as "protective" media, they gave worse results than the simplest salt solution tried (Briggs and King 1953).

Restriction of differentiation can also be brought about by the introduction of foreign controlling substances into the egg and the inability to respond properly to those substances. When zygotes of R. pipiens are injected with albumin or histone fractions from adult liver cells, cell division ceased and development arrested in late blastula accompanied by chromosomal abnormalities (Ursprung and Markert 1963). Extracts of adult kidney and skeletal or cardiac muscle produced the same effects, but extracts from other animals produced less frequent arrest, indicating species specificity, and extracts of Ranid eggs produced no effects (Kimmel 1963a, -b). When nuclei of arrested eggs were transplanted, arrested development occurred
It has been suggested that the active fraction, which appears to be protein in nature (Melton 1963), may normally regulate gene activity in differentiated chromosomes and that early exposure to it arrests development by blocking gene function (Kimmel 1963a). It has also been suggested that the failure of lens epithelium cells as opposed to adult epithelial cells to support development could be due to an active inhibition of mitosis necessary to maintain a temporary non-dividing state (McAvoy, Dixon, and Marshall 1975). It is known that nuclei from advanced stages do not easily respond to transplantation with a complete reversal of differentiation. When injected into oocytes, blastula nuclei enlarge 250 times and exhibit diffuse chromatin more rapidly than brain nuclei, which only enlarge 40 times, but neither acquires the hundreds or thousands of nucleoli normally found in the oocyte nucleus (Gurdon 1968a). In Rana, the ability to support normal development is at its peak at early gastrula, after which it declines (Briggs and King 1960). The difference in capacity is most profound between early and late stages and is almost non-existent among the early stages or among the late stages. This is indicated by the lack of difference in developmental capacity among various stages of differentiated intestinal epithelial cells (McAvoy, Dixon, and
Marshall 1975; Marshall and Dixon 1977). The distinction between differentiated and undifferentiated cells as opposed to the effect of aging is well illustrated by the fact that adult _R. pipiens_, spermatogonial cells promote more advanced development than juvenile spermatogonial cells (Di Berardino and Hoffner 1971).

Difference in mitotic activity has also been proposed as a biological reason for transplant failure. Somatic cells divide every 1½ days or longer and take 7 hours to replicate their chromosomes, but a transplanted nucleus must finish replication within one hour because the egg divides within 1 to 2 hours after activation (Gurdon 1974a). At blastula, 11% of the cells are in interphase and their transplantability is 34% to 60%, but by early gastrula, 8% of the cells are in mitosis and transplantability has fallen to 15% (Briggs and King 1953). DNA synthesis in gastrula endoderm lasts up to 60 minutes and 45% enter mitosis within 90 minutes post-activation. Only 20% of tadpole endoderm nuclei and no liver, brain, or blood nuclei, which are still synthesizing DNA, enter mitosis by that time (Graham, Arms, and Gurdon 1966). Injected nuclei must enter mitosis with the pronuclei even if they have not synthesized DNA (Graham 1966), which leads to abortive cleavage correlated with clumped chromosomes on the metaphase spindle and non-disjunction
(Gurdon 1963; Gurdon and Laskey 1970a). A caution to this interpretation is raised by the evidence that despite differences in mitotic activity between crest and through gut epithelial cells, they show no difference in success rate (McAvoy, Dixon, and Marshall 1975). In fact, nuclei from cultured epithelial cells promote egg cleavage less successfully than tadpole endoderm nuclei which have a slower division rate (Gurdon and Laskey 1970a).

Mitotic asynchrony is just one aspect of the larger problem of cell cycle asynchrony. Sperm nuclei injected into eggs were only able to synthesize DNA at times when the pronuclei did, not when injected after they had completed their synthesis (Graham 1966). The presence of normal and abnormal nuclei in the same abnormal embryo (such mosaics are inconsistent with damage to the nucleus during transplantation) suggests that the original transplanted nucleus might produce abnormal replicates during early synthesis, but later might become attuned to the division cycle and produce normal replicates, leading to an abnormal and a normal clone of replicates. Late replicating heterochromatin might play a role in preventing complete expression and rapid replication (Di Berardino and Hoffner 1970). All of this is consistent with the evidence that transplanted nuclei which fail to divide until the second division of
the egg often promote more normal development (Gurdon and Laskey 1970a). In a study of Rana lens cells that examined cell cycle effects, the most successful transplantations were with S phase cells, success decreased as mitosis was approached and was least in interphase cells (Muggleton-Harris 1971). Non-chromosomal effects, such as out-of-phase centrioles (Di Berardino and Hoffner 1970) and cell cycle delay due to radiation damage of membranes (Szekely and Copps 1976) during enucleation, could also affect results.

The weight of evidence from work in Rana indicates that developmental arrest is due to chromosomal abnormalities, the severity of which is related to the degree of developmental restriction (Di Berardino and Hoffner 1970, 1971). In most cases, the abnormality is traceable to the initial response of the transplanted nucleus to the cytoplasm of the recipient egg. Many transplants fail to undergo sufficient nuclear enlargement, variable portions of the chromatin remain condensed, and the persistence of condensed chromatin leads to failure of normal replication. This in turn leads to variable deletion in genes required for development, resulting in variable abnormalities (Kimmel 1964; Di Bernardiino and Hoffner 1970). If the chromosomal
abnormality is tetraploidy; some transplants can develop into adults but are sterile (King 1966).

**Mammalian Development**

Because the mouse is the subject of choice in most of the attempts to produce genetic duplicates, where no subject is specified in the discussion that follows, the subject is the mouse. In cases where a different species is considered, it will be named.

Several processes critical for the development of the mammalian embryo occur before fertilization. In the rabbit, concomitant with a nuclear maturation (meiosis I to meiosis II) after ovulation is a cytoplasmic maturation involving significant morphological changes in cell structures (endoplasmic reticulum, Golgi, nucleolus, mitochondria) and qualitative and quantitative changes in protein synthesis (Van Blerkom and McGaughey 1978a). GV breakdown is necessary for meiotic maturation, and after breakdown, there are changes in proteins synthesized, including the appearance of a new DNA polymerase (Schultz and Wassarman 1977). During the time the oocyte is in the ampulla of the oviduct, molecules large enough to carry specific biological activities pass nearly intact from the maternal blood into the oocytes (Glass 1971). In the corresponding process of spermiogenesis, the histones in the sperm are replaced by a basic protein,
called sperm basic nuclear protein (SBNP or masculine), which forms disulfide linkages (Ecklund and Levine 1975; Meistrich, Reid, and Barcellona 1976).

After fertilization, during anaphase II of the second meiotic division, the sperm loses its SBNP, becomes less condensed and then becomes compact again to form the pronucleus (Ecklund and Levine 1975). *In vitro* experiments on sperm decondensation suggest that this might occur due to the combined action of a reductase (to reduce the disulfide bonds) and a protease, such as trypsin (Gall and Ohsumi 1976). Such dispersion of chromatin in the fertilized sea urchin egg is believed to allow acquisition of egg proteins (present prior to fertilization) which enable the sperm to participate in replication and transcription (Kunkle and Longo 1975). After formation of the pronuclei, DNA synthesis begins (Graham 1973).

The fertilized egg contains a store of mRNA that is utilized during early development (Van Blerkom and McGaughey 1978b), and protein synthesis occurs in the one-celled egg and continues throughout development (Graham 1973). Nevertheless, fertilization to the 8-cell stage in the rabbit is characterized by the gradual disappearance of specific polypeptides synthesized during oocyte maturation (Van Blerkom and McGaughey 1978b), and
in the mouse, an RNA synthesis suppressor is found in the egg cytoplasm and no nucleolar synthesis occurs (Bernstein and Mukherjee 1972). DNA synthesis begins 4 to 11 hours after fertilization and continues for 4 hours (Sirlin and Edwards 1959; Abramczuk and Sawicki 1975). By the 4-cell stage, nucleoli are synthesized (Bernstein and Mukherjee 1972) and both tRNA and rRNA are found (Graham 1973). Unlike the amphibian, this early genetic activity may be important as early as the 2-cell stage as indicated by blockage of development by actinomycin D (Baker 1971; Graham 1973) but may not be important until after the 8-cell stage as indicated by alpha amanitin blockage at that point (Van Blerkom and McGaughey 1978b). Still, there is no evidence of morphogenetic factors at the 4- and 8-cell stages and any cell of those stages could determine a blastomere (Hillman, Sherman, and Graham 1972). At these early stages, the presence of the zona pellucida is necessary to prevent adhesion of the embryo to the walls of the oviduct and it is only at and after the 8-cell stage that it is not required (Modlinski 1970).

The genetic contribution of the sperm is not required until the morula stage, as indicated by the fact that gross chromosomal abnormalities introduced by the sperm do not show developmental effects until then
(Graham 1974). When the mouse blastocyst is about to implant, it contains about 64 cells (Graham 1971b) which have differentiated so that the cells on the outside are unable to form the embryo and the cells on the inside are unable to develop on their own or induce a decidua (Graham 1973). The presence at late blastocyst of enzymes produced by the sperm DNA indicates that mRNA is being made (Graham 1973). It is also at this point that the $G_1$ phase of the cell cycle and the late duplication pattern of one X chromosome appear (Mukherjee 1976). By 6.5 days, the cell cycle is 5 hours in some areas of the embryo and as little as 2.5 hours in others (Papaioannou et al. 1978).

**Nuclear Transplantation in Mammals**

There have been 13 reports of nuclear transplantation in mammalian eggs (Graham 1969, 1971a, -c, -d; Baranska and Koprowski 1970; Lin and Oh 1970; Tarkowski 1971; Lin, Florence, and Oh 1972, 1973; Bernstein and Mukherjee 1972; Bromhall 1975; Uehara and Yanagimachi 1976, 1977). In two cases the rationale was purely technical: 2- and 4-cell embryos were placed on a monolayer and fused with the cultured cells to examine patterns of RNA synthesis (Bernstein and Mukherjee 1972); whole sperm or sperm nuclei were injected into hamster eggs to observe their maturation into pronuclei.
(Uehara and Yanagimachi 1976, 1977). In each of the other cases, there was a serious attempt to further the production of genetic duplicates.

Graham (1969) was the first to attempt production of genetic duplicates in mammals. Cells of mouse embryos were dissociated in 0.25% trypsin after mechanical removal of the zona pellucida. Cells were teased away from splenic tissue and bone marrow cells were flushed out to serve as nuclear donors. Donor cells were from mice which had a T6 translocation marker. UV-irradiated Sendai virus (also called hemagglutinating virus of Japan [HVJ]) was used as the agent to introduce nuclei into the recipient cells by cell fusion. To protect the fragile 1- and 2-celled mouse embryos with which the donors were fused, only the donor cells were exposed to high HVJ concentrations, and once fusion had begun, the cells were diluted in 50% serum to sequester the fusion activity. The fusion of embryonic cells (from 2- and 4-cell stage embryos) with unfertilized eggs or 2-celled embryos resulted in no cleavage. Spleen cells fused with fertilized or unfertilized eggs cleaved (some abortively) but this result is difficult to interpret because unfertilized eggs will cleave in culture after zona and cumulus removal. Exposure of 2-cell embryos to fusion with bone marrow cells slowed entry of recipients into mitosis by
about 2 hours. Such embryos had an abnormal cell number 24 to 48 hours later and at 72 hours, the cell number was one-third normal, although the embryos appeared to be morphologically normal. Bone marrow nuclei were found in one or rarely two cells of 4-celled embryos. No marker was seen at the 12-cell stage 2 days after fusion. The 2-celled embryos were in $G_2$ when fused with the bone marrow nuclei, which swelled slightly then decondensed without DNA synthesis as the recipient cell entered mitosis.

Graham (1971a, -c, -d) has reported other attempts to induce development of genetic duplicates. There was a reduction in ovum lysis when beta-propiolactone (BPL) was used to inactivate the HVJ before use, and when the ovum was exposed to virus for only one to two minutes before sequestration (the donor cell was exposed for a longer period). Fusion occurred during the first six hours. Mouse strains not susceptible to 2-cell block (inability of eggs of most mouse strains to develop through the 2-cell stage in culture) allowed development in culture. Embryos were transferred to pseudopregnant recipients. By fusing 1-celled eggs and, with more success, blastomeres of 2-celled eggs, morphologically normal tetraploid and diploid/tetraploid mosaic blastocysts were obtained and transferred to uteri of recipients. On the 10th day, pseudopregnant females were
killed, and where development had occurred at all, large
trophoblastic outgrowths without fetal or embryonic deriv­
avitives were found. In one experiment, of 36 blastocysts
implanted, 2 such outgrowths were found. In all cases
where 2-celled recipients were used, the fused blasto­
meres developed more successfully than the unfused.
Because it was known that the largest blastomeres tend
to form outside of the morula and give rise to the tropho­
blast, the 4-celled embryo was surrounded by several
others in an attempt to force the larger tetraploid cells
to develop into inner cell mass. Using just such a tech­
nique, it had previously been shown that 40% of the divi­
sion products of the central embryo would be found in
the inner cell mass, and such chimaeras formed of an
aggregate of 14 morulae around a central morula had
developed into apparently normal 13 day embryos (but
these were resorbed on day 16) (Hillman, Sherman, and
Graham 1972). Mice were born from 6-embryo chimaeras,
but they showed the coat color of the recipient (surround­
ing) embryos. From the previously mentioned 2N/4N
mosaics, 3 diploid young were born. A further attempt to
induce some participation of the donor nuclei in develop­
ment was made by fusing embryonic fibroblasts and adult
bone marrow cells to 8- to 16-cell embryos of different
genotype. Donor cell nuclei were found mainly in the
peripheral cytoplasm of the outside cells. From 152 implanted blastocysts 25 mice were born and none showed the donor coat color. In another experiment where embryos were surrounded by 5 unfused morulae, 5 mice were born from 225 implants but none showed the marker. In these experiments, Graham (1971a) was able to greatly increase the cell number of developing embryos by using hybrid females and by culturing fused blastomeres in pairs. Graham (1971a) also attempted fusion of haploid parthenogenones, but reported no positive results.

In other experiments in the mouse, no investigator has had as much success as Graham. Baranska and Koprowski (1970), using BPL-inactivated HVJ, fused unfertilized eggs with a variety of cells in culture: primary or secondary mouse fibroblasts; mouse fibroblasts infected with Moloney sarcoma virus; mouse cell lines JCS-V9 and MC57G; Chinese hamster tumor cells, HT-1; African green monkey kidney cells, CV-1; African green monkey lymphocytes; W18V_a2 human fibroblasts transformed by SV40 virus. Mitotic cells were labelled with ^3H-thymidine. A high proportion of cells degenerated, but such fusion had an enhancing effect on the survival of eggs. Balb/c fibroblasts infected with Moloney sarcoma virus showed the most cases of cleavage (70%, 23% non-dividing, 6 degenerating). It was also noted that the exposure to cold
required for HVJ fusion did not adversely affect development. Although heat inactivated Newcastle disease virus (NDV) did not fuse, Lin and his associates were able to use live Newcastle disease virus to induce fusion and found that it caused less lysis than HVJ (Lin and Oh 1970; Lin, Florence, and Oh 1972). When about 15 isolated blastomeres from 8- to 16-cell embryos were fused, multinucleated giant cells were formed, and when cervical lymph node cells plus NDV were injected into the perivitelline cavity of late 2-celled mouse eggs (in which one blastomere had been damaged or destroyed) fusion was observed. In a later experiment (Lin, Florence, and Oh 1973), when UV-inactivated HVJ was used to fuse cervical lymph node or femur bone marrow cells with a blastomere inside the zona pellucida as before, development to the 4-cell stage was observed. When 8-celled embryos, in which 1 or 2 cells were damaged, were injected with virus, some of the cells fused and development proceeded to the morula stage. Tarkowski (1971) has found that where two eggs or two blastomeres were fused to produce tetraploid embryos, they contained less than one-half the normal cell number due to the treatment or the longer tetraploid cell cycle. Tarkowski (1977) has attempted to produce haploid eggs for fusion after fertilization by irradiation with a UV microbeam or by submitting the egg to
colchicine at the second meiotic division (in some eggs the entire nuclear apparatus is expelled) but these techniques did not produce consistent results. Recently bisection of 1-celled fertilized eggs has resulted in viable haploids that would be ideal for such fusion (Tarkowski 1977).

The most recent attempt to produce genetic duplicates is that of Bromhall (1975) in the rabbit. Donor cells were from $^3$H-thymidine-labelled morulae, one group of which was reversibly arrested in mitosis using nitrous oxide. Nuclei were introduced by microinjection (as with amphibian eggs) or by fusion of cells using UV-irradiated HVJ (which did far more damage than microinjection). Synchronized nuclei tended to fuse with recipient nuclei, but non-synchronized nuclei did not, became pycnotic, and were later found in one of the blastomeres. About 7% of injected eggs extruded the egg nucleus spontaneously during metaphase II as a pseudo-polar body. Some fused enucleated embryos developed to the 18-to 26-cell stage at a normal rate and labelling was present in about half the nuclei. Nuclear volumes were consistent with diploidy. Nevertheless, conclusive proof of nuclear transplantation into an enucleated egg was not shown.
Reasons for Failure of Transplants in Mammals

Technical problems can be a cause of failure. This includes inability to successfully perform difficult procedures such as culture of embryos in explanted oviducts in order to circumvent the 2-cell block (Graham 1969). Manipulation of the egg, including treatment with pronase to remove the zona, reduces development to blastocyst by 10% (Graham 1971c). The mouse egg is so small (about 30 microns in diameter) that addition of the volume of cytoplasm present in another embryonic cell can distort cell organization (Graham 1969). Where unfertilized eggs were used, the subnormality of artificial activation plays a role. Artificially activated eggs do not extrude cortical granules. In addition, artificially activated eggs show cytokinesis abnormalities and subnormal changes in the zona pellucida, even when the cell looks normal and forms a pronucleus (Mintz and Gearhart 1973; Graham 1971a). In the hamster, sperm nuclei injected into unfertilized eggs became partially swollen and irregular in shape (Uehara and Yanagimachi 1977). Furthermore, activating stimuli seem to damage eggs (Graham 1971a). Another complication is that hyaluronidase treatment (used to remove cumulus cells) artificially activates eggs which can then develop to the blastocyst stage before disintegrating (Graham 1971a). This makes interpretation difficult without karyotyping.
Cell cycle effects are found in mammalian cells just as they are in amphibian cells. In fusion of a mitotic cell with an interphase cell (including spermatozoa [Johnson, Rao, and Hughes 1970]), the nuclear membrane can break down and premature chromosome condensation (PCC) can occur in the interphase cell without development of a mitotic spindle. In such condensation, in G1/M hybrids, the G1 nucleus condenses into single chromatids (Rao and Johnson 1972), but these condensed chromatids are longer than normal and show uncondensed areas (Rao and Johnson 1974). These uncondensed areas may lead to the sort of problems seen in S phase cells fused with M cells where the S nuclei lose chromosomes (Gordon 1975). These results do not support Graham's (1969) suggestion that the recipient should be in G1 or S, and, in fact, later results indicate that a G2 nucleus introduced into an S cell divides in synchrony but a G1 nucleus introduced into a G2 cell becomes pycnotic (Graham 1971c). Synchronization of donor and recipient cycles may be difficult. It has been suggested (Graham 1969) that adult bone marrow nuclei might require several days to swell and participate in division.

When eggs were not enucleated, the consequences of tetraploidy have been blamed for poor results. Heteroploidy is usually fatal, possibly due to regulatory imbalances. For example, X-inactivation has not been
observed by XXY individuals (Bomsel-Helmreich 1971). Development to blastocyst, such as in haploid (bisected) eggs with half the normal number of cells (Tarkowski 1977) may be misleading because, as noted previously with reference to X heterochromatin, genetic interactions and regulation become critical at blastocyst, not before. This was well demonstrated when blastomeres from 4-celled mouse eggs were inserted into empty zonae and transferred to pseudopregnant oviducts. Development was observed to blastocyst but few blastocysts implanted. Because blastocyst formation occurred at about the same time as in intact eggs, an insufficient number of cells at cavitation may have been the cause of failure (Rossant 1976). Tetraploid cells, with their relatively slow rate of cleavage and low viability were virtually eliminated by the 10th to 13th day from 2N/4N hybrid mosaic embryos (Tarkowski, Witowska, and Opas 1977). That survival of groups of cells depends upon cleavage rate is indicated by experiments involving the production of chimaeras. A chimaera was shown to form when cells from the inner cell mass (ICM) of one blastocyst were injected into the blastocyst cavity of a recipient differing by as much as 24 hours in age (Graham 1971b; Gardner 1971). Injection of diploid parthenogenic ICM's into the cavity of normal blastocysts resulted in a low proportion of cells from
the parthenogenone in the chimaera (Surani, Barton, and Kaufman 1977), and where embryonal carcinoma cells, with an even greater difference in cleavage rate, were used, a very low level of donor cells was found in the chimaera (Papaioannou et al. 1978). That Graham (1971c) had observed no difference in the early cleavage rate of tetraploids and diploids may be due to the presence in the tetraploids of immediate cleavage, leading to an unequal distribution of chromosomes. This would obscure the cleavage rate just as it did in parthenogenones, whose ultrastructure revealed irregularities in nuclear and cellular division, fragmentation of nuclei, and persistence of nucleoli during mitosis (Graham and Deussen 1974; Solter et al. 1974).

Development of Improved Techniques

An analysis of the above evidence suggested several areas in which technical or biological improvements could be made. Lin (1975) felt that the major obstacle to success in the production of genetic duplicates was enucleation. Lin and his associates (1973) also felt that in fusion with unfertilized mouse eggs, the somatic cell nucleus did not always reach the center of the egg as the pronuclei do in normal embryos. He suggested the use of small cells to correct this. Another problem was the repression of differentiated nuclei that not only
caused retardation of development but also resulted in chromosomal abnormality, which was the greatest source of developmental arrest.

Graham (1969) suggested that enucleation could be accomplished by the injection of colchicine into the egg donor at a definite time after coitus. Such an injection, as noted above, can result in the expulsion of the female pronucleus (Tarkowski 1971). Graham (1971c) also reported enucleation of eggs in tissue culture by activation with hyaluronidase followed by exposure to colchicine (40% were enucleated). On closer examination, however, it becomes evident that separating enucleated eggs from the many forms of heteroploidy produced by colchicine in eggs is impractical (McGaughey, 1975). Such treatments with colchicine also interfere with fertilization and cleavage in the mouse (McGaughey and Chang 1969).

Fortunately, a better method does exist. The chemical trimethylpsoralen reacts with pyrimidine bases to form monoadducts which, in the presence of UV-irradiation, react photochemically with a pyrimidine base in the complementary strand to produce an interstrand crosslink. Intrastrand crosslinks are not produced so that RNA is not affected. Irradiation at 10 erg/m$^{-2}$/sec. for 60 seconds was found to be sufficient to kill virtually all Chinese hamster cells in one experiment (Ben-Hur
and Elkind 1973). This method has a strong advantage in that crosslinks are formed only during irradiation (Cole 1970). Thus, psoralen can be introduced, DNA crosslinked by irradiation, and psoralen washed out before fusion with the donor nucleus, which is, therefore, unaffected. Trimethylpsoralen has been used in this way to effect partial genetic transfer between two Chinese hamster ovary cell lines (Chasin and Urlaub 1975). Because psoralen crosslinking requires irradiation at 365 nm., the DNA is safe from UV-induced crosslinking. Likewise, the energy level of irradiation is below that required to do gross damage to cellular structures like the centrioles (Edwards 1957b). Even if some UV damage were done, UV-induced delay of cleavage and mitosis might have a beneficial effect on the mouse egg as it has been credited with having on the Xenopus egg (McAvoy, Dixon, and Marshall 1975).

The requirement for smaller cells as nuclear donors suggested several possibilities. Small lymphocytes have relatively little cytoplasm and large nuclei, and they can be isolated from spleen or bone marrow (Miller and Phillips 1969). The use of minicells would have less restrictive donor tissue requirements. Production of minicells is accomplished by centrifugation of inverted cell monolayers in the presence of cytochalasin B.
The karyoplast or "mini-cell" pulled out of the cytoplasm has only 10 to 20% of the original amount of cytoplasm and is surrounded by an intact plasma membrane (Ege et al. 1974). To carry the process one step further, isolated nuclei could be used. Nuclei of adult cells have been isolated in a procedure similar to that of Chauveau, Moule, and Rouiller (1956) for microinjection into Xenopus eggs (Graham, Arms, and Gurdon 1966). Furthermore, fusion of isolated mitochondria with evidence of retained function (Radsak, Sawicki, and Koprowski 1972) implies the possibility that isolated nuclei could be introduced into cells by fusion. Even if a nucleus introduced by fusion should lack a functional membrane, problems might not arise because current evidence suggests that the membrane of the male pronucleus arises predominately from endoplasmic reticulum within the egg and only to a minor extent from the sperm nuclear membrane (Longo 1976). Such isolated nuclei have receptors for concanavalin A (Con A) (Monneron and Segretain 1974) as do mouse eggs (Johnson et al. 1975), and eggs exposed to Con A treated nuclei could be monitored to determine the number of nuclei attached. Eggs with the appropriate number of nuclei attached by Con A could be fused with those nuclei by HVJ, which has been shown to be bound by Con A to cells which can then induce hemolysis.
This implies that fusion could be induced, although this has not been shown (Gordon 1975). If HVJ fusion should be inhibited by Con A, as are other types of viral fusion (Ludwig, Becht, and Rott 1974), other fusion agents, such as oleic acid (Ahkong et al. 1973), are available. If nuclei were attached to sperm, which have Con A receptors (Nicolson et al. 1977), it is possible that the process by which microvilli reach out from the ovum during fertilization to engulf the sperm head (Thibault 1971; Yanagimachi and Noda 1970), might draw in an attached nucleus as well. Perhaps the most advantageous aspect of the use of isolated nuclei is the ability to treat them directly to modify their structure.

The outstanding problem in nuclear transplantation has been the presence in the donor nucleus of repressor proteins (histones and NHP) which interfere with the synthetic processes and mitosis and prevent the egg's NHP and histones from mediating the appropriate gene activities. It is no coincidence that lysine-rich histones, such as H1, are often deficient in early embryos (Gurdon and Woodland 1970) because lysine-rich histone blocks DNA availability for template function more than other types and one-third of the initiation sites for DNA polymerase are in lysine-accessible zones (Janakidevi 1978). Lysine-rich histones are also known to inhibit
DNA synthesis in cell-free systems (Wang 1969). Trypsin, which preferentially hydrolyzes arginine and lysine bonds, has been used to digest about 70% of the total histones in isolated nuclei, whose structural integrity was preserved by the addition of soybean trypsin inhibitor, and in this way to produce enhanced RNA synthesis (Allfrey, Littau, and Mirsky 1963). In addition, nuclei isolated from G₁ cells have been stimulated to synthesize DNA at S rates by treatment with trypsin (Brown and Stubblefield 1975b). However, it must be noted that this DNA synthesis level could have been due in part to DNA from fragmented cells adsorbed to the nucleus (Brown and Stubblefield 1975a). In any event, removal of the histones only removes part of gene masking (Paul 1971), probably because among the NHP are fibrillar, contractile-like proteins which are involved in condensation of the chromatin in differentiated versus undifferentiated cells (Paulin et al. 1976) and in the gastrula, which has 6 times the NHP of sperm (Gineitis, Nivinskas, and Vrob'ev 1976). Scanning electron microscopy of metaphase chromosomes reveals a surface composed of a skein of fibers (Korf and Diacumakos, 1978). It is possible that trypsin might aid chromatin decondensation by digesting fibrillar proteins, but Allfrey, Littau, and Mirsky (1963) have reported that trypsin had no effect on NHP.
Once repressive and contractile proteins have been removed, it may be that the extended chromatin, especially the denatured regions, might be susceptible to damage. It would be advisable to promote condensation during transfer. The polyamine spermine has been shown to have tight, highly stereospecific bonding to DNA by a covalent linkage between the basic groups of the spermine and the highly acidic DNA phosphates (Tabor and Tabor 1964; Rao and Johnson 1972). The resulting neutralization leads to a reduction in repulsive forces and a net increase in the strength of cohesive forces such as Van der Waal's and hydrogen bonding. In this way structures such as the DNA double helix are strengthened, as confirmed by the ability of polyamines to facilitate the formation of double-stranded polymers from single-stranded nucleotides (Tabor and Tabor 1964). The condensation of chromatin into discernible chromosomes is known to be initiated and maintained by Mg$^{2+}$, spermine, and some chromosome-specific proteins (Rao and Johnson 1972). Such a natural role for spermine is suggested by the fact that the response of cells in culture to growth stimulus is an increase in spermine production, while a signal for cessation of growth causes cells to stop production and to secrete polyamines (Melvin and Kier 1978). High concentrations of polyamines are found in hormone-stimulated
tissues, tumor cells, regenerating liver, and embryonic systems, including the sea urchin where an increase in polyamine levels is seen after fertilization and before each cleavage division (Russell 1973; Kusunoki and Yasumasu 1976). Other beneficial effects have been observed, including stimulation of in vitro activity of RNA polymerase I and the synthesis of 18S and 28S RNA after microinjection of putrescine (a polyamine) into mature X. laevis oocytes (Russell 1973). It has also been suggested that spermine might displace histones and thus speed up DNA replication (Hennen 1970), and that spermine might delay cleavage by sequestering microtubular protein (McAvoy, Dixon, and Marshall 1975). Whatever its effects, spermine is naturally present during insemination, mostly in the seminal plasma (Tabor and Tabor 1964) but also in the spermatozoa (Bamberg, Weiser, and Desser 1975).
METHODS

Preparation of Pasteur Pipettes

Pasteur pipettes were drawn to between 0.13 and 0.35 mm. for use in oviduct flushing and egg manipulation. For puncture of the zona pellucida, micropipettes of 10 microliter capacity (Corning) were drawn to the smallest possible diameter possible using manual techniques. The micropipettes were then fitted by melting to the end of a Pasteur pipette and were used with a pipette bulb as were the larger pipettes. Pipettes were autoclaved inside modified test tubes in a procedure devised by Mintz (1971b).

Marker

As a marker, coat or eye color was used. Eggs, sperm, vasectomized males, and embryo transplant recipients were albino (Balb/c) mice. Nuclear donors were male, brown (C3H) mice. The eye color is determinable before birth, and the coat color is determinable some time after birth.

Egg Preparation

Mature female albino mice were injected with 10 to 15 IU of pregnant mare serum (PMS) (Calbiochem) and
48 hours later with 10 to 15 IU of human chorionic gonadotropin (HCG) (Calbiochem). The females were then placed with 1 to 2 mature male albino mice. The next morning, the females were examined for evidence of mating (mating plugs) and successfully mated females were sacrificed. Up to 30 eggs/mouse were flushed, using medium, from the excised oviducts. Figure 1 shows the isolated reproductive tract of a Balb/c mouse. In Figure 1, the tract is intact on the left, and, on the right, the ovary (above) is separated from the oviduct and uterus (below). When found, egg masses were broken up by placing them in 300 IU/ml. of hyaluronidase (Calbiochem) in phosphate-buffered saline (PBS) (Rafferty 1970). The sort of 1-celled, Balb/c embryo obtained in this way can be seen in Figure 2. The zona pellucida was removed by exposing the eggs to 0.5% pronase (Calbiochem) in Hank’s salt solution (Mintz 1962). A Balb/c egg, after removal of the zona pellucida, can be seen in Figure 3. The demembranated eggs were manipulated in glassware treated with Siliclad. In some cases, eggs were mechanically demembranated by using a micropipette to puncture the zona.

**Enucleation**

Demembranated eggs were introduced into a solution of $1 \times 10^{-6}$ M trimethylpsoralen (Chasin and Urlaub 1975; Ben-Hur and Elkind 1973; Cech and Pardue 1976) in embryo.
Figure 1. Isolated Balb/c Uterus, Oviduct, and Ovary
Figure 2. Isolated Balb/c Embryo at the 1-Cell Stage
Figure 3. Balb/c Embryo at the 1-Cell Stage after Removal of the Zona with Pronase
culture medium. The trimethylpsoralen was kindly supplied by the Paul B. Elder Company of Bryan, Ohio. The eggs were then cultured at 3°C. for 30 minutes. Next, the eggs were irradiated at a distance of 1 meter from a UV source with peak emission at 365 nm., filtered to allow no emission below 300 nm. (Watson, Bauer, and Vinograd 1971), and an output of 1.25 ergs/cm.²/sec. measured at 30 cm. using a photodiode (Pin Photo Receptor, Santa Monica). Exposure to UV lasted 60 seconds. The eggs were then washed twice, once in PBS and once in medium.

**Preparation of Donor Nuclei**

When small lymphocytes were used, they were isolated according to the procedure of Miller and Phillips (1969). Pieces of excised spleen in PBS were aspirated through a 1 ml. syringe, pelleted in a centrifuge tube, and resuspended in 2 ml. of 0.3% bovine serum albumin (BSA) in PBS. This suspension was layered on top of a 10 ml. linear gradient of 0.5% to 2% BSA in PBS, which was in turn layered on top of 5 ml. of 2% BSA in PBS. The gradient was then refrigerated at 4°C. for 3 hours. 1 to 2 ml. fractions were then collected.

When isolated nuclei were used, pieces of excised liver or spleen were rinsed and homogenized in a solution of 0.05 sodium glycerol phosphate (Sigma) in 2.2 M sucrose. The homogenate was then filtered through
sterile gauze, and then centrifuged, decanted, and resus­pend ed twice (Chauveau, Moule, and Rouiller 1956; Georgiev, Ermolaeva, and Zbarskii 1960; Zbarskii and Georgiev 1959). The nuclei isolated in this way were then treated with 0.5 mg./ml. trypsin (Sigma) plus 0.4 mg./ml. soybean trypsin inhibitor (Sigma) in PBS. After 30 minutes of incubation, 0.1 mg./ml. of inhibitor (Allfrey, Littau, and Mirsky 1963), 50 micrograms/ml. of Con A (Sigma) (Monneron and Segretain 1974), and 1.99 mg./ml. of sperm­ine (Sigma) (Hennen 1970) were added. In some cases, nuclei were exposed to ova in a 100:1 ratio. In other cases, nuclei in a 1:1 ratio were exposed to spermatozoa isolated from the cauda epididymis (Nicolson et al. 1977), and the resulting mixture was then cultured with disem­bered ova (Whittingham 1968a) for the purpose of in vitro fertilization.

When minicells were to be used, a piece of connec­tive tissue (deep fascia) was cut from beneath the skin of a mouse and exposed to 3 ml. of 0.75% trypsin in calcium­magnesium free PBS in culture at 37°C. until cells detached. The supernatant was centrifuged and the pelleted cells were resuspended in Ham's F-10 medium (GIBCO) plus 10% fetal calf serum (GIBCO) in a petri dish containing discs cut from the bottom of Falcon tissue culture dishes. If sufficient cells had been grown to
form a confluent monolayer on the discs, the discs would have been placed, cells down, in 5 ml. of medium (Ham's F-10 plus 10% fetal calf serum) in a centrifuge tube containing 10 micrograms/ml. of cytochalasin B (Aldrich) dissolved in 10% dimethylsulfoxide and centrifuged. The pelleted material (minicells) would have been resuspended in PBS (Prescott and Kirkpatrick 1973).

**Cell Fusion**

For oleic acid fusion, nuclei that had been treated with concanavalin A (as above) were added to eggs in a dilution approximating 100:1, nuclei : eggs. Eggs with attached nuclei were placed in 0.1 ml. of oleic acid (Sigma) that had been sonicated in 6 ml. of PBS containing 0.264 g./l. of CaCl$_2$ and 80 mg./ml. of dextran sulfate (Dextran 60 C, Sigma) until the lipid had dispersed. After 5 minutes, the cells were washed in PBS (Ahkong et al. 1973).

For fusion by means of HVJ, HVJ was cultured in fertile hen's eggs, isolated, and inactivated using a 2% beta-propiolactone solution (Steplewski and Koprowski 1970; Rao and Johnson 1972; Giles and Ruddle 1973). Eggs with attached nuclei (as above) were exposed to a solution of HVJ (128 HAU/ml.) at 4°C. for 15 minutes. The cells were then washed in medium containing serum to stop the fusion reaction (Graham 1969).
**Embryo Culture**

Oviduct donors were cycled using PMS and HCG in the same way that egg donors were. The cycled females were then mated with vasectomized males. The operation for vasectomy is shown in Figure 4. On day 4, oviducts were excised and the ampullary portions isolated. Embryos at the 1-cell stage were pipetted into the isolated oviduct segments, which were then placed on a raft of sterile teabag paper floating in 0.5 ml. of Ham's F-10 medium (Whittingham 1968b) and cultured under 5% CO$_2$, 5% O$_2$, and 90% N$_2$ in a vacuum desiccator at 37°C. (McGaughey and Van Blerkom 1977) for 48 hours. Figure 5 shows isolated oviduct segments in culture. The oviduct segments were then flushed, and the embryos were cultured in embryo culture medium until they reached the blastocyst stage. The embryo culture medium was Brinster's medium (Brinster 1965) that had been modified according to Pinsker and Mintz (1973). All media were Millipore filter-sterilized, collected directly into Vacutainers (evacuated tubes), and gassed with 5% CO$_2$, 5% O$_2$, and 90% N$_2$ through a Millipore filter. Media were prepared in advance and stored at -70°C. until used.

**Implantation**

Recipient females were cycled with PMS and HCG one day behind the egg donors and were exposed to
Figure 4. Vasectomy of a Balb/c Mouse
Figure 5. Balb/c Oviduct Segments in Culture
vasectomized males. The uteri were exposed through incisions above the kidneys on the dorsum of the animal. A bent, 30 gauge syringe needle was used to puncture the uterus. Blastocyst stage embryos were introduced into the puncture using a pipette (Rafferty 1970). The arrangement of instruments in this operation can be seen in Figure 6.

Alternatively, in the cases where the zona surrounded the embryo or was only punctured, the uteri were exposed (as above) and the eggs were introduced by means of a pipette into the ovarian bursa at the mouth of the oviduct (Runner 1951).
Figure 6. Implantation of Embryos into the Uterus of a Pseudopregnant Recipient
RESULTS

Preparation of Eggs

Upon hormone-induced superovulation C3H females regularly produced 20-30 eggs/mouse but Balb/c females usually produced 10-15 eggs/mouse. Balb/c eggs were far more fragile than C3H eggs, and losses during manipulation were high. These factors reduced the number of eggs after manipulation to about 5-6/mouse. Because 5 to 6 females / experiment were used as egg donors, this meant that 25-36 eggs were used in each experiment. Balb/c eggs appeared to survive mechanical demembranation better than enzymatic demembranation.

Enucleation

Psoralen treatment did no gross damage to denuded eggs. In a study on the effect of psoralen treatment, of 10 control eggs and 8 eggs treated with psoralen plus UV, no psoralen-treated eggs survived after 3 days in oviduct culture while 6 control eggs survived.

Preparation of Donor Nuclei

Small cells, presumably small lymphocytes, were concentrated from spleen tissue with about 10%
contamination by larger cells. This technique was aban­
donned due to the technical difficulties involved with improving the results.

Nuclei were regularly and reproducibly isolated from spleen or liver tissue with little or no contamina­
tion by cell fragments. The trypsin, spermine, and Con A treatments produced no visible damage to the nuclei. As long as care was taken to maintain the nuclei in suspen­
sion by periodic shaking, very little clumping occurred in the presence of Con A. Con A-treated nuclei were observed to be attached to mouse ova within one minute of addition of nuclear suspension to the ova in culture. Nuclei were also observed to attach to sperm in 1:1 suspensions. Of 50 sperm observed in one case, 10 had attached nuclei and of these 1 sperm had more than one nucleus attached and 2 sperm had become attached to a small clump of about 8 nuclei. Where a single nucleus was attached to a sperm, a reduction in freedom of movement was observed, but the sperm was still able to move. In vitro fertilization attempts did not result in the attachment of any sperm (with or without attached nuclei) to any eggs.

Mouse cells of line L929 and mouse primary fibro­
blasts were cultured on round plastic cover slips. The L929 cells approached confluency, but the fibroblasts remained sparse after 4 days in culture.
**Cell Fusion**

In oleic acid fusion, the oleic acid coated the eggs and oleic acid droplets swirling around the egg made it difficult to monitor the fusion and difficult to find the eggs. From gross observation, the nuclei appeared to fuse with the eggs to which they had previously attached after Con A treatment, but the layer of oleic acid coating could have obscured distinct nuclei.

The Sendai virus had a titer of 128 HAU/ml. in the concentrated solution after isolation and inactivation with BPL. Therefore, the concentrated solution was used for fusion purposes. It was not clear from gross observation whether Con A-attached nuclei fused with the egg or not. Of 10 treated eggs, 1 survived oviduct culture to be implanted in a pseudopregnant female. No evidence of development was found 2 weeks later when the recipient female was sacrificed.

**Embryo Culture**

Untreated embryos were regularly cultured for 3 days in explanted oviducts with 60-75% success. Such embryos appeared grossly normal.

**Implantation**

No evidence of development was ever found 2 weeks after implantation of either treated or control embryos.
in a pseudopregnant female. When 19 fertilized eggs (5 with ruptured zonae, 14 control) were placed in the ovarian bursa, no evidence of development was found after 2 weeks.

**Combined Treatments**

As noted above, 1 egg survived isolation, zona removal, attachment of nuclei, exposure to HVJ, and oviduct culture. It was implanted in a pseudopregnant female, but no sign of development could be found after 2 weeks. Table 1 indicates the various combinations of procedures that were tried. As is evident from the data presented there, the combinations of successful procedures were exhausted. This being the case, of the combinations of procedures attempted in this study, NVS fusion of isolated nuclei with mouse ova, enucleated by psoralen treatment, gave the most promising results.
Table 1. Combination of Procedures Tested for Effect on Success

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>Preparation of donor eggs</th>
<th>Enucleation</th>
<th>Preparation of donor nuclei</th>
<th>Fusion</th>
<th>Oleic acid</th>
<th>Sendai virus</th>
<th>Fertilization by sperm with attached nuclei</th>
<th>Embryo culture</th>
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<tr>
<td>Oleic acid</td>
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<tr>
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<tr>
<td>Fertilization by sperm with attached nuclei</td>
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<td></td>
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<tr>
<td>Embryo culture</td>
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<td>+</td>
<td>+</td>
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<td></td>
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<tr>
<td>Implantation</td>
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<td>+</td>
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<td>+</td>
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</table>

* = both procedures performed in the same experiment

NA = not attempted
DISCUSSION

Accomplishments

The experiments discussed above represent the first report of attachment of isolated nuclei to sperm and attachment to and fusion with ova. More importantly, suggestive evidence is provided that treatment with trimethylpsoralen plus UV irradiation is effective in enucleating mammalian eggs for nuclear transplantation. The above experiments also represent the first attempt to treat nuclei to remove histones in order to allow reprogramming during nuclear transplantation.

Reasons for Failure

No firm conclusions may be drawn from the data presented due to the small number of eggs involved. The small numbers and the fragility of Balb/c ova no doubt contributed to the lack of success in transplantation. It is also known that Balb/c mice show a relatively exceptional degree of malsynchrony between embryo stage and the timing of implantation initiating factor in the uterus (Mintz 1971a), which could have further confounded results.
Improvements for the Future

Many improvements could be made in the egg-handling techniques employed in this series of experiments. Beyond using a strain of mice with hardier eggs or a better chance of implantation, a strain could be used that does not show the 2-cell block (Whitten and Biggers 1968), or another species of animal could be used. The rabbit, for example, has many advantages, including the size of its egg, which is three times the size of a mouse egg (Bromhall 1975). If a situation required microsurgical enucleation, the procedure would be far easier in the large rabbit egg than it has been in the mouse (Modlinski 1975). Mechanically demembranated eggs have been shown to behave more normally at fertilization than eggs exposed to enzymes (Wolf, Inoue, and Stark 1976), but both microsurgical enucleation and mechanical demembranation increase the difficulty of working with large numbers of eggs. For example, the use of medium containing 30 IU/ml. of hyaluronidase increases the ease of the time-consuming step of oviduct flushing (Whitten 1971), which may outweigh the disadvantages. A better approach than avoidance of enzymes might be the use of medium containing 50% serum, which stops pronase action and increases pH stability of the medium during handling (Mintz 1971b). Better equipment,
such as a stage with a heated culture well, could increase survival. Although no adverse effects were noted in a study where mouse embryos were recovered and kept at room temperature for periods up to 3 hours (Whittingham 1971), another study found that 16% more experimentally manipulated eggs survived at 37°C and that the likelihood of development of such eggs was increased (Modlinski 1975). It might be suspected that cooling might be beneficial because cooling retards cleavage more than DNA synthesis in the frog, as shown by a cooling-induced reduction in the number of mitotic cells which did not exhibit karyokinesis (Hennen 1970). Nevertheless, cooling has been shown to cause a slowing down of development in the mouse (Mintz 1971b) which could cause problems at the blastocyst stage similar to those encountered with tetraploids. Gurdon (1976) has found improved response of nuclei after transplantation when the enucleation procedure is geared to the cell type, and many of the procedures he tested on mammalian cell types could be substituted for the procedure originated by Chauveau, Moule, and Rouiller (1956) used here. Finally, an important improvement to be made over other nuclear transplantation attempts in mammals is the use of a better marker for following development than \(^{3}H\)-thymidine labelling. \(^{3}H\)-thymidine affects pre- and post-implantation vigor of
labelled embryonic cells, and with extensive labelling, blastocysts show little or no inner cell mass formation (Kelly and Rossant 1976). If an appropriate trisomic or an appropriate strain producing an early-expressed enzyme variant could be found, it might provide a better label.

If the problem of enucleation has been solved by the use of psoralen, the only formidable obstacle which remains in the path of the production of genetic duplicates in mammals is the derepression of the transplanted nucleus. Histones, which inhibit DNA and RNA synthesis, seem to compete for binding sites with NHP, which can reverse histone inhibition (Wang 1969), but the specificity of NHP control of expression requires that the NHP have access to the DNA before the addition of histones (Gurdon and Woodland 1970). The problem would seem to be to provide some mechanism whereby the egg NHP could gain access to the transplant DNA. If it is shown that the manipulations of isolated nuclei employed in this study do not result in damage that interferes with development, the removal of histones by trypsinization could well solve this problem. The introduction of such nuclei at fertilization by Con A attachment to sperm should give the nuclei the maximum amount of exposure to a normally-fertilized egg's cytoplasm. The sperm DNA could be inactivated by exposure to X-rays (Edwards
1957a) or the alkylating agent TEPA (tris-(1-aziridinyl)-phospine oxide) (Beil, Bauman, and Graves 1976), which appears to do less damage to the spermatozoa. Spermatozoa with one attached nucleus could be isolated by centrifugation, which has already been used to separate haploid from diploid rabbit spermatozoa (Beatty and Fechheimer 1972), or by use of a gradient (Evans, Douglas, and Renton 1975). That the centrifugation method succeeded in the rabbit holds particular promise because rabbit spermatozoa have been shown to possess Con A binding sites (Nicolson et al. 1977). If such measures are not adequate to provide the time required for depression, additional time might be provided by delaying cleavage. In the rabbit, in vitro fertilization using spermatozoa from the distal corpus of the epididymis has been shown to result in delayed cleavage (Orgebin-Crist and Jahad 1977). A more controllable delay might be achieved by the use of cytochalasin B. Cytochalasin B acts on actin filaments to cause a disappearance of contractile microfilaments found in the contractile ring that produces the cleavage furrow in mouse eggs (Beams and Kessel 1976, Opas and Soltynska 1978) without affecting bundles of microfilaments and microtubules deeper in the cell (Beams and Kessel 1976) and without interfering with nuclear division (Carter 1967). The timing of application is critical
because when newly fertilized eggs are exposed to cytochala
sin B for 5 to 8 hours, formation of the second polar body is
suppressed, survival is reduced and the rate of development is
delayed (Balakier and Tarkowski 1976). However, cleavage can occur
without damage if cytochalasin B is applied before cleavage begins
(Balakier and Tarkowski 1976).

Nevertheless, if enzymatic removal of histones or manipulation of
cleavage does not prove to be workable, control of the cell cycle is a likely alternative.

Hormone-induced differentiation (casein synthesis) has been shown to require cell division, and it was suggested that this made the cell especially susceptible to enviromental factors capable of eliciting changes in cell function (Stockdale and Topper 1966). Further, Gurdon and Woodland (1970) have suggested that mitosis in embryonic systems is a time of gene reprogramming when cytoplasmic proteins have access to the nucleus. Specifically, the swelling of the nucleus and chromatin dispersion after fertilization are similar to events during reconstitution of the nucleus at telophase. Donor cells could be synchronized using a thymidine block or nitrous oxide (Rao and Johnson 1972), and synchronized cells in G_2 could be enucleated using the technique of Prescott and Kirkpatrick (1973) to produce a minicell in G_2 that could be fused
with an egg. The $G_2$ nucleus would wait until the egg ended $S$ and $G_2$ before proceeding into mitosis (Rao and Johnson 1972). Graham (1969) recognized that the introduction of a $G_2$ nucleus might be fruitful. If continued presence of quantities of H1 histone interfered with success, it is possible that $G_2$ minicells could be fused with cytoplasts of embryonic or other rapidly dividing cells which lack or have reduced levels of H1, grown to confluence, synchronized, and enucleated to produce a second generation of cells for fusion with the egg. Such cellular reconstruction and growth to confluency has been demonstrated using mouse L and A9 cells (Lucas and Kates 1976). If cells (such as lymphocytes) which do not attach to surfaces need to be employed, a technique now exists for cytochalasin B enucleation of such cells following attachment of the cells to a surface to which Con A has been linked (Gopalakrishnan and Thompson 1975). A final improvement might be made by the use of polyethylene glycol as the fusion agent, especially during fusion with the egg, because of the greater control over fusion conditions (Pontecorvo 1975).

Prospects for the Production of Genetic Duplicates in Mammals

It is clear that the ability to produce genetic duplicates of mammals would be of enormous value to
science, industry and medicine. A hybrid frog (X. laevis \( X \times X. gilli \)), which yields female offspring genetically identical to the mother due to a defect in the meiotic mechanism, has already come into use in immunogenetic experiments (Kobel and Du Pasquier 1975). A mammalian clone would be of much greater value to mammalian genetics. The argument that there will be nontrivial maternal (Gross 1978) effects in the attempts to clone does not diminish the value of the technique for most genetic purposes. Possible medical uses of genetic duplication and a general discussion of the controversy over alleged human cloning attempts can be found elsewhere (Gruber 1978). Such a discussion is certainly not premature when human fertilized eggs can be grown to implantation in culture (Steptoe, Edwards, and Purdy 1971), which is a critical point of similarity with the mouse.

The demonstration in this study of the applicability of several new techniques, particularly chemical enucleation employing trimethylpsoralen plus UV, to the production of genetic duplicates in the mouse has advanced the day when such production and its attendant benefits will be achieved.
LIST OF REFERENCES


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