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Mitochondrial DNA in somatic and germinal cells of male rats, and embryo transfer between donors and recipients of known mitochondrial DNA

Acacia A. Alcivar

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MITOCHONDRIAL DNA IN SOMATIC AND GERMINAL CELLS OF MALE RATS, AND EMBRYO TRANSFER BETWEEN DONORS AND RECIPIENTS OF KNOWN MITOCHONDRIAL DNA

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Mitochondrial DNA in somatic and germinal cells of male rats, and embryo transfer between donors and recipients of known mitochondrial DNA

by

Acacia A. Alcivar

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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For the Graduate College

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1987
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INTRODUCTION

Recent experimental evidences indicate that inheritance in the eukaryote is not exclusively controlled by nuclear DNA. On the contrary, an impressive accumulation of experimental data indicate that cytoplasmic inheritance is also an important component of inheritance. The molecular basis for cytoplasmic inheritance was established with the discovery that mitochondria, the organelles responsible for most of the energetic activities of the cell, have circular, double-stranded DNA. Mitochondrial DNA (mtDNA) can be differentiated from nuclear DNA using techniques for DNA analysis and nucleotide differences within the mitochondrial genome can be identified using restriction enzymes. On this basis, mitochondrial populations within and between species can be identified and the transmission of mtDNA can be followed. Alien inflow of mtDNA has occurred in nature, generating interspecies hybrids with regard to their mtDNA. Once incorporated into the cytoplasm, this alien mitochondrial genome is replicated and transmitted to successive generations.

In sexual reproduction, contributions from both parents are transmitted to the nuclear gene pool of the offspring. However, mitochondrial inheritance, in contrast to nuclear inheritance, is only maternal. The maternal inheritance of the mtDNA is explained on the basis of a greater maternal contribution of mitochondria to the embryo. The mtDNA is amplified during oogenesis and the oocyte contains thousands of mitochondria, whereas the spermatozoon contributes very few mitochondria to the oocyte at fertilization. The apparent lack of paternal contri-
bution to the mtDNA constitution of the zygote may also be a protective mechanism which facilitates maternal acceptance of the embryo during attachment and gestation, because the embryo has the same type of mtDNA as the mother.

All of the available evidence indicates that under natural conditions, the embryo will always have the same mtDNA type as the mother. In embryo transfer, however, the embryo may not have the same mtDNA type as the recipient. This difference alone may significantly influence the survivability of the embryo and its development to offspring after transfer.

Biochemical studies, using specific inhibitors of mitochondrial protein synthesis, indicate that mitochondrial transcription is active in pre- and post-implantation embryos. However, the studies reported so far have not clearly defined the role of this mitochondrial activity prior to implantation.

In order to evaluate the role of mitochondria in early embryonic development and the potential impact of cytoplasmic inheritance on genetic improvement of livestock species, I initiated a series of studies using the rat as the experimental model. Among the approaches considered were: the role of the male in the transmission of the mitochondrial genome, the effect of mitochondrial differences on maternal-fetal interaction and embryo survival, and the application of biochemical tools to select and monitor the transmission of mitochondrial populations.

The specific objectives of this thesis were to determine whether:
1) (a) Mitochondria from tunica-free testis and liver, from the same rat, shared the same type of mtDNA.

(b) Mitochondria isolated from somatic and germinal cells of the same rat shared the same morphological characteristics.

2) (a) The survivability of rat embryos after transfer is affected by the mtDNA of the host.

(b) The mtDNA of the embryo influences the postbirth survivability and development of the offspring.

3) (a) Chloramphenicol affects the in vitro development of one- or two-cell rat embryos.

(b) The cleavage response of rat embryos exposed in vitro to different doses of chloramphenicol is affected by the mtDNA type of the embryo.
LITERATURE REVIEW

Mitochondria and Mitochondrial DNA

It is well documented that mitochondria are primarily responsible for cellular respiration and the production of adenosine triphosphate (ATP). The development of techniques for the analysis and manipulation of nuclear DNA, and the application of methods for sequencing of nuclear DNA and RNA to the mitochondrial DNA (mtDNA) have allowed mapping of the mitochondrial genome (see Borst et al., 1977; Rosamond, 1982; Grivell, 1983, for review).

In all metazoan animals studied— from invertebrates to man—the mitochondrion possesses circular, double-stranded DNA, which is distinguishable from the nuclear DNA (Borst et al., 1977; Rosamond, 1982; Grivell, 1983). Most of the structural and functional proteins of the mitochondrion are coded for by the nuclear genome (Borst et al., 1977; Yatscoff et al., 1981; Rosamond, 1982). However, specific proteins are coded for by mtDNA which account for less than 2% of total protein synthesis in the cell (Cascio and Wassarman, 1981). The mitochondrial genome codes for subunits I, II and III of cytochrome oxidase, cytochrome b, subunit 6 of ATPase, two rRNAs, twenty-two tRNAs, and eight proteins which have not yet been identified (Rosamond, 1982).

Different methodologies have been used to characterize the mtDNA of the following species: rat (Francisco and Simpson, 1977; Saccone et al., 1977; Hayashi et al., 1978a,b,; Francisco, Brown, and Simpson, 1979; Brown and Simpson, 1981); bovine, (Laipis et al., 1979, Hauswirth et al., 1980; Anderson et al, 1982); mice (Fischer-Lindahl, 1985), and
human (Hutchinson et al., 1974).

In the rat, two types of mtDNA (A and B) can be identified by the characteristic electrophoretic pattern of mtDNA treated with restriction endonucleases (Francisco and Simpson, 1977; Buzzo, Fouts, and Wolstenholme, 1978; Hayashi et al., 1978a,b; Kroon, de Vos, and Bakker, 1978; Francisco, Brown, and Simpson, 1979). Francisco and Simpson (1977) reported that for 60 rats (Sprague-Dawley, Wistar, and Long Evans Hooded strains) 65% were type A and 35% were type B. MtDNA of either type A or B has been identified in mitochondrial preparations from rat liver (Francisco and Simpson, 1977; Hayashi et al., 1978a,b; Francisco, Brown, and Simpson, 1979), kidney and brain (Francisco, Brown, and Simpson, 1979), or in the pooled organs (kidney, heart, muscle, testes, brain, thymus, and intestine) from a litter of rats (Hayashi et al., 1978b). In these studies in rats, the type of mtDNA extracted from the various tissues was always the same type for a given rat. However, some species exhibit heterogeneity of mtDNA in various tissues from the same animal (Coote, Szabados, and Work, 1979; Bartoov and Fisher, 1980; Hauswirth et al., 1984).

Cytoplasmic Inheritance

There is a general consensus that the mitochondrial genome is inherited maternally in animals (Hutchinson et al., 1974; Francisco and Simpson, 1977; Hayashi et al., 1978a,b; Giles et al., 1980; Hecht et al., 1984). Hutchinson et al. (1974) provided convincing experimental evidence for the maternal inheritance of mitochondrial DNA by determining that the mtDNA of the mother was inherited in the reciprocal
crosses of equine species: the mule and the hinny. The mule is the offspring of a mare (dam) and a donkey (sire); the hinny is the offspring of a donkey (dam) and a horse (sire). The mtDNA pattern of the offspring, mule or hinny, was always the same as the mtDNA pattern of the mother, mare or donkey, respectively. Maternal inheritance of mtDNA has also been demonstrated for the rat (Buzzo, Fouts, and Wolstenholme, 1978; Hayashi et al., 1978a,b; Kroon, de Vos, and Bakker, 1978; Francisco, Brown and Simpson, 1979), human (Giles et al., 1980), and mouse (Ferris et al., 1983; Hecht et al., 1984; Gyllensten, Wharton, and Wilson, 1985).

The maternal inheritance of mtDNA has been explained on the basis that the spermatozoon contributes few or no organelles to the oocyte at fertilization; whereas, the oocyte is rich in mitochondria (Piko, 1975; Piko and Matsumoto, 1976; Bellve and O'Brien, 1983; Wassarman, 1983; Gyllensten, Wharton, and Wilson, 1985). For many animal species, including the rat, the spermatozoal midpiece bearing mitochondria enters the oocyte at the time of fertilization. However, degeneration of these spermatozoal mitochondria has been observed during the pronuclear phase in rat, pig, and rabbit oocytes (Blandau and Odor, 1952; Szollosi, 1965).

Based upon the fact that there is 100-fold more mtDNA in bovine oocytes than in bovine somatic cells, it was suggested that a significant amplification of mitochondrial DNA occurs during oogenesis in the bovine (Michaels, Hauswirth, and Laipis, 1982). In the mouse, mitochondrial DNA is considerably decreased during spermatogenesis (Hecht et
al., 1984), however, the mtDNA did not appear to be altered during spermatogenesis. It has been estimated that each mouse spermatozoon contains about 50 molecules of mtDNA and the number of paternal mitochondrial genomes contributed to the next generation was assumed to be no more than one per thousand of the total amount of mtDNA contributed by the mother (Gyllensten, Wharton, and Wilson, 1985).

It has not been determined whether the mtDNA of the male germline cells is of the same type as that found in the somatic cells from the same individual rat. Morphological changes in the mitochondria of germline cells during spermatogenesis have been reported for the rat (De Martino et al., 1979).

Immunological Aspects of Implantation, Maternally Transmitted Antigen, and Mitochondrial DNA

One of the most intriguing aspects of reproduction is the lack of rejection of the implanting embryo by the mother, despite the antigenic differences between them. The lack of maternal rejection of the embryo has been the subject of intense investigation (see Beer and Sio, 1982; Siiteri and Stites, 1982; Billingham and Beer, 1984, for review). There is considerable evidence for maternal cellular and humoral immune responses to the fetus but, paradoxically, these responses seem to facilitate rather than to impair a gestation to term (Gill and Repetti, 1979; Tachi et al., 1981; Beer and Sio, 1982; Siiteri and Stites, 1982; Billingham and Beer, 1984; Billington and Burrows, 1986). Studies on laboratory rodents have convincingly demonstrated that fetal-maternal incompatibility, especially where major histocompatibility antigens
(MHC) are concerned, often favor blastocyst implantation (Billingham and Beer, 1984). Several hypotheses have been proposed to account for the success of the fetal allograft (see Beer and Sio, 1982; for review). Among the mechanisms or immunoregulatory factors that have been proposed to explain the apparent failure to reject the fetal allograft are: the complete separation of maternal and fetal blood circulations, the uterus as a privileged immunological site, lack of transplantation antigens on the trophoblast and fetus, immunosuppressive hormones, immunosuppressive agents produced by the fetus, and immunoregulatory agents produced by the mother. In addition, blocking antibodies, immune complexes, suppressor cells and macrophages have also been proposed as immunoregulatory factors that prevent rejection of the implanting embryo (see Tachi et al., 1981; and Beer and Sio, 1982 for review).

A maternally transmitted antigen, which seems to be regulated by mtDNA, has been found in some strains of mice (Fischer-Lindahl and Burki, 1982; Fischer-Lindahl and Hausmann, 1983; Fischer-Lindahl, 1985). The identification of this maternally transmitted antigen is the only reported evidence for mitochondrial control of the expression of a cell membrane molecule in eukaryotes (Smith et al., 1983; Huston et al., 1985). This antigen is maternally inherited and two genes contribute to its antigenic expression (Fischer-Lindahl, 1985). One of these genes is a nuclear gene located within the major histocompatibility complex (MHC) of the mouse. The other gene is strictly maternally inherited and several lines of evidence indicate that it is a mitochondrial gene which has been referred to as maternally transmitted factor (Fischer-Lindahl,
1985). The maternally transmitted antigen has only been demonstrated for the mouse. Differences in the expression of maternally transmitted antigen between embryo and recipient did not interfere with the gestation to term of embryos transferred to recipients of different type of maternally transmitted antigen (Fischer-Lindahl and Burki, 1982).

Mitochondrial DNA and Protein Synthesis

Protein synthesis in the developing embryo is influenced by a variety of factors. Among these, mitochondrial transcriptive activity of the mammalian embryo is of special interest because of the relatively large amounts of mtDNA present (Piko and Chase, 1973; Piko, 1975; Michael, Hauswirth, and Laipis; 1982). Biochemical evidence indicates that of the 8 pg of total DNA present in the mouse oocyte, 2 to 3 pg are derived from mitochondria (Piko, 1975).

In the mouse embryo, the mitochondrial genome is transcribed during cleavage and contributes, through the synthesis of rRNA and tRNA, to the establishment of a mitochondrial protein synthesizing system (Piko and Chase, 1973; Cascio and Wassarman, 1981). The role of this transcriptive activity, however, is not clear, because embryonic development and cellular differentiation up to the blastocyst stage were not directly dependent on mitochondrial activity (Piko and Chase, 1973).

Chloramphenicol (CAP) is an antibiotic which is known to inhibit mitochondrial protein synthesis (Piko and Chase, 1973; Koike et al., 1983). This property makes CAP particularly useful for studies related to the expression of mitochondrial DNA and the selection of resistant cell lines (Piko and Chase, 1973; Oerter and Bass, 1975; Koike et al.,
1983; Schieber and O'Brien, 1983). CAP has been used to study mitochondrial protein synthesis in early mouse embryos (Piko and Chase, 1973; Cascio and Wassarman, 1981) or later during organogenesis of rat embryos (Oerter and Bass, 1975; Jager and Bass, 1975). A CAP solution containing 31.2 μg/ml inhibited mitochondrial protein synthesis in two- and four-cell mouse embryos; however, these embryos continued to develop in vitro to the blastocyst stage and developed to term after transfer. Higher concentrations of CAP inhibited the in vitro development of mouse embryos in a dose-dependent fashion (Piko and Chase, 1973).

Somatic cell lines have been derived from rats which are either sensitive or resistant to CAP (Koike et al., 1983). These researchers showed that CAP resistance was associated with a single nucleotide change in two adjacent, highly-conserved, regions near the 3'-end of the large ribosomal RNA gene of mtDNA. Moreover, rat CAP-sensitive somatic cells were made CAP resistant by growing them in the presence of 150 μg CAP/ml (Schieber and O'Brien, 1983). The influence of CAP on protein synthesis has not been determined for rat embryos, nor has it been determined whether or not differences in CAP sensitivity are associated with the two mtDNA types (A or B) reported for the rat (Hayashi et al., 1978a,b).

The rat was used as the experimental animal model for the studies reported in this thesis because only two types of mtDNA have been identified in the rat. In addition, procedures for embryo collection, manipulation, culture, and viability assays for the rat embryo were previously established in the laboratory.
Reproductive Parameters for the Rat

There is little information reported on the reproductive parameters for different inbred strains of rats. There is considerable variation in litter size and reproductive performance within and between inbred strains of rats (Gill, Kunz, and Hansen, 1979). No information has been found detailing reproductive parameters for the Sprague-Dawley strain which was used in my studies. In general, and not including the Sprague-Dawley rats, the duration of the estrous cycle for most inbred rats averages 4 to 5 days (Bennett and Vickery, 1970; Satinder, 1980). The length of pregnancy ranges from 19 to 23 days (Bennett and Vickery 1970; Tofoski and Gill, 1977), and the number of pups at birth varies from 5 to 13 (Bennett and Vickery, 1970; Tofoski and Gill, 1977; Satinder, 1980). The mean number of pups weaned varies from 5 to 8 (Gill, Kunz, and Hansen, 1979; Satinder, 1980, 1984). The sex ratio (male:female) at weaning also varies from 0.80 to 1.42 among inbred strains (Bennett and Vickery, 1970; Gill, Kunz, and Hansen, 1979; Satinder, 1984).

Rat Embryo Transfer

Embryo transfer is a powerful research tool for studies on the reproductive biology of laboratory and farm animals. It has been used to investigate the physiology and endocrinology of pregnancy and to differentiate between maternal effects and those arising from the embryo itself. This technology is also useful in the study of immunological aspects of reproduction, and to test for the developmental potential of
embryos after they have been subjected to experimental treatment or manipulation (Seidel, 1983).

Embryo transfer in rats was first reported by Nicholas in 1933. In a series of surgical transfer experiments using pseudopregnant recipients, Dickmann and Noyes (1960) and Noyes and Dickmann (1960, 1961) established the optimal relationship between embryonic age and the stage of endometrial development for a successful transplantation in the rat. They demonstrated that when day 4 embryos were transferred to diestrous recipients on day 4 or when day 5 embryos were transferred to recipients on day 5 (synchronous transfer), 70% and 58%, respectively, of the transferred embryos survived to term. Others (Mantalenakis and Danezis, 1968; Butcher, Blue, and Fugo, 1969) reported survival rates of embryos of 61% and 73% after synchronous transfer of day 5 embryos to day 5 recipients. In asynchronous transfer experiments, Noyes and Dickmann (1960, 1961) demonstrated that when the embryo donor was on day 5 of diestrus and the recipient was on day 4, more (68%) embryos survived to term than when the donor was on day 4 and the recipient on day 5 (5%). Mantalenakis and Danezis (1968) reported that 67% of the embryos from donors on day 5 developed to term when transferred to recipients on day 4. In these studies, physiological saline, phosphate-buffered Ringer solutions (pH 7.3), or physiological saline mixed with rat plasma were used as the transfer medium.

Lower embryonic survival rates, ranging from 16% (Noyes, Doyle, and Bentley, 1961) to 26% (Yoshida, 1957) have also been reported. Noyes, Doyle, and Bentley (1961) reported that the percentage of embryos that
survived after transfer to day 18 of pregnancy was of 17% when embryos from day 5 albino donors were transferred to day 4 albino recipients and 18% when embryos from day 5 albino donors were transferred to day 4 black recipients. These same authors reported that when day 5 embryos obtained from black donors were transferred to day 4 black recipients, 16% of the embryos survived to day 18 of pregnancy and when day 5 embryos from black donors were transferred to day 4 albino recipients, 11% of the embryos survived to day 18 of pregnancy.

Culture of Rat Embryos

Many studies have been performed to obtain information on the nutrient and specific environmental factors needed to support the cleavage of mammalian embryos in vitro (Brinster, R. L., 1971; Spielmann, 1976; Wright and Bondioli, 1981). Unfortunately, except for embryos from certain strains of laboratory rodents, there are not systems available for the in vitro culture of embryos from the one-cell to the blastocyst stage (Spielmann, 1976). Peluso, Karey and Gruenberg (1983) reported the culture of one-cell rat embryos to the morula or blastocyst stages after 96 h in culture. However, other researchers have not been able to obtain in vitro development of one-cell rat embryos beyond the two-cell stage (Folstad, Bennett, and Dorfman, 1969; Spielmann, 1976). Rat embryos at the eight-cell or morula stages have been successfully cultured to blastocyst stage (Folstad, Bennett, and Dorfman, 1969; Yamamura and Markert, 1981; Dooley, Pineda, and Martin, 1984).
MATERIALS AND METHODS

To perform the studies reported in this thesis, it was first necessary to establish techniques for mitochondrial isolation, purification, and DNA extraction, as well as techniques related to restriction endonuclease digestion and gel electrophoresis. These techniques are described in detail in Experiment 1. Concurrently, I attempted to locate commercial sources of rats with a known mtDNA type. Since a commercial source for mtDNA-typed rats was not found, I started a search to identify the type of mtDNA of littermates, in order to establish a rat colony containing rats of known mtDNA.

Establishment of the Rat Colony

Efforts to obtain mtDNA-typed animals from laboratories that have studied the mtDNA and its inheritance in the laboratory rat (Francisco and Simpson, 1977; Buzzo, Fouts, and Wolstenholme, 1978) were unsuccessful. In order to establish a colony containing the two known types of mtDNA, Sprague-Dawley derived females (SASCO Inc., Omaha, NE) were obtained and mated to males of unknown mtDNA parentage. One littermate resulting from each of these crosses was then sacrificed in order to determine the type of mtDNA for that litter. Mitochondria were isolated from the liver, and the mtDNA was extracted, digested using restriction endonuclease, and electrophoresed as described later in Experiment 1. The first six litters were of mtDNA type A. The seventh, a litter of six rats was of mtDNA type B. The mtDNA patterns obtained were as those reported for the laboratory rat (Francisco and Simpson, 1977; Hayashi et al., 1978a,b; Buzzo, Fouts, and Wolstenholme, 1978).
Littermates of these typed animals were used to develop the rat colony. Animals of known mtDNA type were ear-marked at the time of weaning. Subsequently, females were bred to males of the same mtDNA type (with the exception of the heterologous crosses used for Experiment 1). Females that were found positive to sperm by vaginal smears were considered to have mated on that day, which was designated as day 1. Mated rats were caged individually and observed daily until birth. The offspring were weaned when they were 21 days old.

Animals raised in this colony were the source of experimental animals for all the studies reported in this thesis. While in the colony and for the duration of these studies, rats were caged by sex and type of mtDNA, fed commercial rat food, and maintained in a room with controlled temperature (20 to 22°C) and light (14 hours of light/10 hours of dark).

The following reproductive parameters were recorded for each female that had a litter: length of pregnancy in days, number of pups born, number of pups weaned, sex ratio at weaning, and weight (g) of females and males at weaning day. This information is summarized in Appendix A.

Experiment 1. Mitochondrial DNA from Somatic and Germinal Cells of Male Rats

Objectives

The objectives of this study were to determine whether (a) tunica-free testis and liver cells from the same rat shared the same type of mtDNA, and (b) there were morphological differences in mitochondria
isolated from somatic and germinal cells of the rat.

**Experimental Animals**

Twelve, 30 to 617 day old, Sprague-Dawley derived male rats were used in this study. Of these, seven were of unknown parentage and five were offspring of heterologous crosses performed in our rat colony between rats of known mtDNA type, as follows: 3 rats were from crosses between a female A with a male B and 2 rats were from crosses between a female B with a male A.

**Experimental Procedures**

**Isolation of mitochondria**

Rats were euthanized by cervical dislocation and mitochondria were isolated from the liver and tunica-free testis using a modification of procedures described by Guerra (1974). Immediately after euthanasia, liver and the tunica-free testis was weighed, minced, and washed in mitochondrial wash buffer (0.25 M sucrose, 20 mM HEPES, 1 mM EGTA, [(ethyleneglycol Bis-(B-aminoethylether)N1,N1,N1,N tetracetic acid)], 0.5% fatty acid free bovine serum albumin, BSA; pH 7.5). The chemicals were obtained from Sigma, St. Louis, MO (HEPES, EGTA, and BSA) and Bethesda Research Laboratories, Bethesda, MD (sucrose). Tissue preparation, mincing, weighing, and mitochondrial isolation and purification, were performed at 4 °C. The minced liver and tunica-free testicular tissue from each rat was isolated and each sample was homogenized using a teflon glass-homogenizer (Potter Elvehjem, Fisher Scientific Products, Itasca, IL), in a volume determined by the wet weight of the tissue (1 gm of tissue/10 ml of mitochondrial wash buffer). This homogenate was
filtered through Miracloth (Calbiochem, Behring Corporation, San Diego, CA) and the filtrate was centrifuged at 1000 x g for 10 min. The pellet was discarded and the supernatant was then centrifuged at 9000 x g for 15 min. The supernatant was decanted and the pellet containing mitochondria was resuspended in one-half of the initial volume of mitochondrial wash buffer and recentrifuged at 9000 x g for 15 minutes. The supernatant was again decanted and the pellet resuspended in a mixture of 5 ml of mitochondrial wash buffer and 25 ml of 0.8M sucrose, which was then centrifuged at 9000 x g for 15 minutes to obtain an enriched mitochondrial pellet. Samples of this enriched mitochondrial pellet from the rats known to be mtDNA type A or B were used for electron microscopy (De Martino et al., 1979).

Mitochondrial DNA extraction

Mitochondrial DNA was extracted from the enriched mitochondrial pellet. The pellet was resuspended in 2 ml of TRIS [Tris (hydroxy-methyl) aminomethane hydrochloride]-EDTA (ethylenediamine-tetracetic acid) buffer (10 mM TRIS; pH 8.0, 1 mM EDTA), 200 ul of a 20% solution of sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO) in TRIS-EDTA buffer and 3 ml of TRIS-EDTA-saturated phenol (Bethesda Research Laboratories, Bethesda, MD). The tube containing the mixture was then gently hand-shaken for 5 minutes, then centrifuged at 5000 x g for 5 minutes. The aqueous supernatant was removed and reextracted with 3 ml of TRIS-EDTA saturated phenol, mixed, and centrifuged, as above. This procedure was repeated twice. The aqueous phase was then removed, precipitated with 0.3 ml of 2.5 M sodium acetate, pH 5.2 (SIGMA, St. Louis, MO), and
two volumes of 100% ethanol, and then centrifuged at 5000 x g for five minutes. The supernatant was discarded and the pellet was mixed with 5 ml of 70% ethanol to obtain a uniform suspension. The suspension was centrifuged at 5000 x g for five minutes. The supernatant was decanted and the final pellet containing mtDNA was dissolved in TRIS-EDTA buffer in the amount of 1 ml of buffer for the liver samples and 0.1 ml of buffer for the tunica-free testicular preparations (testis mtDNA). Alliquots of the liver or testis mtDNA preparations were used for restriction endonuclease digestion and gel electrophoresis.

Restriction endonuclease digestion and agarose gel electrophoresis

The standard reaction mixture for digestion contained: 10 µl of liver or testis mtDNA, 1.5 µl high salt buffer (100 mM NaCl, 50 mM TRIS, pH 7.4; 10 mM MgSO4), 1.0 µl of EcoRI endonuclease (10 units/µl; Bethesda Research Laboratories, Gaithersburg, MD), and 2.5 µl distilled water. The ingredients were mixed in a 1.5 ml Eppendorf tube and digested for two hours at 37 C. Two µl of a tracking dye (15% Ficoll and 0.25% bromophenol blue) was added to each reaction mixture and each sample was loaded into a single well of an agarose gel. Electrophoresis using 1% agarose was performed at 75 V for 1 h using TRIS-Borate-EDTA (89 mM TRIS, pH 8.3; 89 mM Borate, 3 mM EDTA) buffer in a horizontal gel electrophoresis chamber. For liver samples, 10 µl of the mtDNA digest were loaded into each gel. For testicular samples, preliminary electrophoretic analyses indicated that the volume of 10 µl of mtDNA digest produced banding patterns which were marginally detectable, therefore a 30 µl of the mtDNA digest was selected and used for each gel. After
electrophoresis, gels were stained with ethidium bromide (1 μg/ml) to locate the mtDNA bands. Mitochondrial DNA bands were visualized with medium wavelength ultraviolet light and photographed through an orange filter using Royal Pan Kodak film (Eastman Kodak, Rochester, NY).

To roughly estimate, after the fact, the relative frequency of germinal versus nongerminal cells in the tunica-free testis preparations, samples of minced, tunica-free testis were obtained from a 364 day old rat of known parentage and mtDNA type. The samples were homogenized as described for the mitochondrial isolation and purification and the homogenates were then prepared for light microscopy. Attempts to identify mtDNA patterns using agarose gel electrophoresis in mtDNA obtained from rat epididymal spermatozoa were unsuccessful (Appendix B).

Experiment 2. Transfer of Rat Embryos to Recipients with the Same or Different Type of Mitochondrial DNA

Objectives

The objectives of this experiment were to determine whether (a) the survivability of rat embryos after transfer is affected by the type of mtDNA of the recipient, and (b) the type of mtDNA of the embryo influences its survival and development after transfer.

Experimental Design

A 2 x 2 factorial design (Table 1) was used to examine the effects of transfer of rat embryos of mtDNA type A or B into recipient females type A or B. A total of 48 recipients were used.
Table 1. Experimental design for experiment 2

<table>
<thead>
<tr>
<th>mtDNA of embryo</th>
<th>mtDNA of recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>A</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
</tr>
</tbody>
</table>

Experimental Animals

Proestrous or estrous rats, of mtDNA type A or B, 61- to 369-days old, were used in this experiment.

Experimental Procedures

Preparation of donor and recipient rats

The procedures used to prepare embryo donors or recipients, as well as to collect and transfer embryos, were modifications from published studies (Dickmann and Noyes, 1960; Noyes and Dickmann, 1960, 1961; Vickery, Erickson, and Bennet, 1969; Nagai et al., 1985).

All rats used as recipients had at least one previous pregnancy and had at least one postpartum estrous cycle. To determine the stage of the cycle, vaginal smears were performed daily between 1000 and 1400 h. Rats used as donors were placed with a male of the same type of mtDNA for mating. Mating was confirmed by the presence of spermatozoa in the vaginal smear on the following morning. Rats that were sperm positive were considered to be in Day 1. For the purpose of analysis of data,
rats that did not produce embryos were not included in the study. In order to transfer embryos obtained from day 5 donors into day 4 recipients, as will be described later, once donors were found to be sperm positive, suitable recipients were then caged with two vasectomized males and left overnight in order to induce pseudopregnancy. Preliminary studies using only one vasectomized male per recipient suggested a male effect on the number of females that became pregnant after embryo transfer. Thereafter, two vasectomized males were placed with each recipient to ensure a higher pseudopregnancy rate and minimize variability between recipients. A total of four pairs of vasectomized males were used in this experiment. In order to ensure sufficient number of embryos for transfer two to three donor rats were provided for each recipient. Food and water were withheld from donors and recipients the day before embryo transfer was performed.

**Embryo collection**

Embryos were collected between 0800 and 1200 h from donors that were euthanized on the morning of day 5, 4 days after found to be sperm positive. The uterine horns were removed, placed in tissue culture dishes (75 x 25 mm; Falcon Division, Becton, Dickinson & Co., Oxnard, CA) containing 2 ml of modified BMOC-3 media (Brinster, 1971; Appendix C), and flushed three times with 0.5 ml of the same media, using a 0.50 ml glass syringe attached to a blunted 1/2-inch long, 24-gauge needle. The embryos were removed from the uterine horns by flushing and they were located in the culture dish using a dissecting microscope. Embryos were recovered and placed in 20 x 15 mm (Falcon Division, Becton,
Dickinson & Co., Oxnard, CA) tissue culture dishes which contained 2 ml of BMOC-3 medium. The stage of embryonic development was determined at a magnification of 100x, using an inverted microscope with phase contrast illumination. Embryos were either classified as morula, early blastocyst, blastocyst, expanded blastocyst, or as degenerated and/or fragmented embryos. An embryo containing more than 16 cells that were coalesced to form a compact mass, but had not yet developed a blastocoele, was considered a morula. An embryo that had formed a small blastocoele was considered to be an early blastocyst. A blastocyst was an embryo which had an expanded blastocoele but did not completely fill the zona pellucida. An expanded blastocyst was an embryo that had expanded to fill the zona pellucida. Embryos were considered fragmented and/or degenerated if they had condensed cytoplasm or if the blastomeres were irregular and of unequal size. The procedure to flush the uterine horns, and to recover and classify the embryos required 40 to 60 minutes for every 2 donors used.

Embryos were washed twice in BMOC-3 media and only embryos classified as morulae or blastocysts were transferred. At least 20 embryos (10 per uterine horn) were transferred to each recipient. The decision to use this number of embryos in this experiment was based on preliminary findings which indicated that pregnancy rates were improved when more than 16 embryos were transferred into the uterine horns of each rat. Embryos and medium were loaded into a 5 μl micropipette (Wiretrol, Drummond Scientific Co., Broomall, PA) that had been beveled and fire-polished. This loaded pipette was then left in an incubator at 37°C.
with 5% CO2 in air for 10 to 20 minutes, before the transfer was completed.

**Surgical embryo transfer**

Embryos collected on day 5 were transferred surgically between 1000 and 1300 h into recipients anesthetized with ether that were in day 4 of pseudopregnancy (day 1 = first day of mating with vasectomized males) on the morning of the day of transfer. The uterine horns were exposed via a 1 cm abdominal incision using a clean but nonsterile technique. A small, cutting edge surgical needle was passed through the wall of the uterus near the uterine bifurcation. The micropipette containing the embryos was inserted through this puncture and the media containing the embryos was gently released into the uterine cavity. Care was taken to ensure that air was not injected along with the embryos. Furacin powder (Life Science Products, St. Joseph, MI) was sprinkled into the abdominal cavity and the abdomen was, then, closed with cotton suture. The transfer procedure lasted 10-15 minutes and most recipients recovered from the ether anesthesia within 30 minutes. Recipients were caged individually after surgery and observed daily until delivery of pups.

**Statistical analysis**

Analysis of variance (Snedecor and Cochran, 1980) was used to determine whether or not the mtDNA of the donor affected a) the number of embryos and b) the developmental stage of embryos collected. Rao's test (Rao, 1952) was used to test for differences and interaction among treatment groups in the ratio of females that became pregnant (females
Chi-square test, with one degree of freedom (Snedecor and Cochran, 1980) was used to test for differences in the embryo survival rate (number of pups born/number of embryos transferred to recipients that became pregnant). Analysis of variance was also used to test for differences and interaction among groups in the length of gestation, number of pups born, number of pups weaned, and weight at weaning of male and female pups. As additional, unplanned observation, the survival rate of pups to weaning day (number of pups weaned/number of pups born) was obtained and analyzed by the Rao test.

For all of the aforementioned comparisons, statistical significance was established at P<0.05. Tukey's w-procedure was used to test for differences between means for which the analysis of variance indicated a significant F-ratio.

Experiment 3. Chloramphenicol Effect on the In Vitro Development of the One-cell Rat Embryo

Objectives

The objectives of this experiment were to determine (a) the effect of different doses of Chloramphenicol (CAP) on the in vitro development of one-cell rat embryos, and (b) whether the type of mtDNA of the embryo influences the cleavage response of the embryos.
Experimental Design

One-cell embryos were randomly assigned to treatment groups corresponding to 5 different doses of CAP (Table 2). A minimum of 20 embryos were included in each group.

Table 2. Experimental design for experiment 3

<table>
<thead>
<tr>
<th>Embryo mtDNA</th>
<th>Chloramphenicol, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
</tr>
</tbody>
</table>

Experimental Procedures

Embryo collection

To collect embryos, proestrous or estrous, 71 to 368 day old rats were caged for mating with males of the same type of mtDNA. Sperm positive females (day 1) were euthanized by cervical dislocation between 1000 and 1600 h and the embryos were recovered by flushing the oviducts, as described for experiment 2. The embryos were washed twice in the same media used to flush oviducts. At least one embryo from each mtDNA type A or B rat donor was allotted to each of the five treatment groups (Table 2).

Chloramphenicol concentrations

Stock solution of CAP (Sigma Co., St. Louis, MO) containing 1000 μg/ml was made up daily in BMOC-3 media. From this stock solution, dilutions of CAP were made immediately before embryo culture to prepare the five different concentrations of CAP (Table 2).
Embryo culture

Embryos for each treatment group were cultured under paraffin oil at 37°C in 5% CO₂ in air in 100 μl droplets of medium containing the allocated CAP concentration. A phase contrast microscope was used to evaluate embryonic morphology and cleavage after 24 and 48 h of culture, and the developmental stage was recorded. Embryos that cleaved at least once during the 48 h culture period were considered viable. Because of the fact that rat embryos do not develop past the two-cell stage when cultured in vitro, the viability of the embryos at the end of the culture period (48 h) was further evaluated by incubating them for 15 minutes in 30 μl of a micromolar (240 μM) solution of eosin B (Dooley, Pineda, and Martin, 1984). Embryos that were partially or totally stained with eosin B were considered dead.

Statistical analysis

Split-plot analysis of variance were used to test for the effect of mtDNA, CAP, and time, on the ratio of embryos that cleaved and viability of embryos (Snedecor and Cochran, 1980). The error terms for main effects and interactions are presented on Appendix D.

Experiment 4. Staining Response to Eosin B of Two-cell Rat Embryos Cultured in Medium Containing Chloramphenicol

Objectives

The objectives of this experiment were to determine whether (a) the viability of two-cell rat embryos, as evidenced by the staining with eosin B, was affected by different CAP concentrations, and (b) the type
of mtDNA of the embryo influences the staining response of embryos after treated with CAP.

**Experimental Design**

Two-cell rat embryos were randomly assigned to the different treatment groups, as described for Experiment 3. A minimum of 20 embryos per group were used (Table 3).

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Chloramphenicol, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
</tr>
</tbody>
</table>

**Experimental Procedures**

**Embryo collection**

Embryos were collected from rats of known type of mtDNA, as described for experiment 3, except that the embryos were obtained from donors euthanized between 1000 and 1600 h of day 2.

**Chloramphenicol concentrations**

Chloramphenicol concentrations were prepared as described for experiment 3.

**Embryo culture**

Culture and evaluation of embryo was the same as described for experiment 3.
Statistical analysis

Because of the known fact that two-cell rat embryos do not undergo further cleavage in vitro, the cleavage response was not analyzed. Chi-square analysis (Snedecor and Cochran, 1980) with 1 degree of freedom was used to test for differences in the staining response of embryos exposed to eosin B after treatment with CAP.
RESULTS

Experiment 1. Mitochondrial DNA from Somatic and Germinal Cells of Male Rats

Two types of mtDNA, A or B, were identified in the rats used in this study (Figs. 1-3) by the banding pattern obtained after electrophoresis of liver or testicular (Figs. 2 and 3) mtDNA preparations digested with EcoRI restriction endonuclease. For the seven rats of unknown parentage, the mtDNA types for both liver and testis were as follows: 3 rats (1, 2, and 3, Fig. 2) exhibited mtDNA type A and 4 rats (4, 5, 6, and 7, Fig. 2) exhibited mtDNA type B. In all cases, the two tissues from each animal had the same type of mtDNA. The electrophoretic patterns for the mtDNA from the testis were consistently fainter than the electrophoretic patterns for the liver mtDNA preparations. No other differences were detected between the patterns for mtDNA from the liver and testes from the same animal.

For the 5 rats, which were offspring of parents whose mtDNA type was known, the type and relative distribution of the mtDNA type was as follows: 3 offsprings from crosses between a female type A with a male type B were of mtDNA type A (rat 8, 9, and 10, Fig. 3) and 2 offsprings from crosses between a female type B with a male type A were of mtDNA type B (rat 11 and 12, Fig. 3). Again, the electrophoretic patterns for the mtDNA isolated from the liver and testicular cell preparations from the same rat were identical.
As estimated by light microscopy, homogenates from tunica-free testes from a reference rat were examined at 100X and 400X. About 80% of the cells freed by the homogenization procedure were germinal cells at various stages of differentiation. Sertoli cells were rarely observed. These homogenates also contained a considerable amount of cellular debris whose origin could not be ascertained.

The mitochondria isolated from homogenates of tunica-free testes were smaller (Fig. 4BT) and contained vacuolated intracristal spaces which were considerably more swollen than those in the mitochondria isolated from liver cells (Fig. 4BL). The morphological characteristics of mitochondria isolated from liver or tunica-free testes from mtDNA type A rats did not appear to differ from those of mtDNA type B rats.

Experiment 2. Transfer of Rat Embryos to Recipients with the Same or Different Type of Mitochondrial DNA

The total number of embryos collected from donors of mtDNA type A and B and their distribution by developmental stage, are shown in Table 4. A total of 1,220 embryos were collected from 101 donor rats. The mean (+ SD) number of embryos recovered per rat was 12 ± 2. The mean number of embryos collected from rats of mtDNA type A was not significantly different (P>0.05) from the number of embryos collected from rats of mtDNA type B. However, there was a trend (P<0.09) for rats with mtDNA type B to produce more recoverable embryos than for rats of mtDNA type A. A total of 595 embryos were collected from 51 females type A (mean ± SD: 12 ± 2) and 625 embryos were collected from 50 females type B (mean ± SD: 13 ± 3).
Fig. 1 Electrophoretic pattern of mitochondrial DNA type A (AL) extracted from the liver of a 30-day old male rat and mitochondrial DNA type B (BL) extracted from the liver of a 59-day old male rat.
Fig. 2 Electrophoretic pattern of mitochondrial DNA (A or B) extracted from the liver (AL, BL) and testicular (AT, BT) mitochondrial preparations from rats 1 through 7, for which the type of mitochondrial DNA was not previously known.
Fig. 3  Electrophoretic pattern of mitochondrial DNA type A (rats 8 through 10) or B (rats 11 and 12) from liver (AL, BL) and testicular (AT, BT) mitochondrial preparations of offsprings from parents of known type of mitochondrial DNA
Fig. 4 Composite pictures made from electronmicrographs of mitochondria isolated from the testes (T; x 27,000) and liver (L; x 13,000) of a 241-day old rat of mitochondrial DNA type B
The stage of embryonic development at the time of collection was not significantly affected (P>0.05) by the type of mtDNA of the donor. Of the 595 embryos of mtDNA type A, 14% (85/595) were morula, 26% (153/595) were early blastocysts, 35% (206/595) were blastocysts, 20% (120/595) were expanded blastocysts, and 5% (31/595) were fragmented and/or degenerated. Of the 625 embryos of mtDNA type B, 11% (67/625) were morula, 25% (155/625) were early blastocysts, 42% (263/625) were blastocysts, 18% (114/625) were expanded blastocysts, and 4% (26/625) were fragmented and/or degenerated. A trend (P<0.08, Table 4) was observed for more embryos at the blastocyst stage to be from donors of mtDNA type B than from type A (42% vs 35%, respectively).

The pregnancy rate after transfer of embryos obtained from donors of known mtDNA (types A and B) into recipients of known mtDNA (types A and B) are shown in Table 5. Pregnancy rate was not significantly affected (P>0.05) by the type of mtDNA of the recipient or embryo. Pregnancy rates for type A embryos transferred into recipients of type A and type B were 42% (5/12) and 67% (8/12), respectively. Pregnancy rates for type B embryos transferred into recipients of type A and type B were 50% (6/12) and 58% (7/12), respectively. During the daily observations, several rats appeared to be pregnant, yet these rats did not deliver offspring. Hence, they were not considered to be pregnant, by the criteria used in this study.
Table 4. Total number and developmental stages of day 5\textsuperscript{a} rat embryos collected from donors of mtDNA types A or B

| mtDNA donors | No. of embryos | Embryo developmental stage | Morula | Early Blastocyst | Expan Blast | Degen/ Frag.
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>51</td>
<td>595 (12±2)</td>
<td>85</td>
<td>153</td>
<td>206</td>
<td>120</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>625 (13±3)</td>
<td>67</td>
<td>155</td>
<td>263</td>
<td>114</td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>1220 (12±2)</td>
<td>152</td>
<td>308</td>
<td>469</td>
<td>234</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Day 1 of pregnancy = day that a vaginal smear was positive to sperm.

\textsuperscript{b} The number of embryos and the distribution of the embryos by developmental stages were not affected (P>0.05) by the type of mtDNA of the embryo. A trend (P<0.08) was observed for more embryos at the blastocyst stage to be from donors of mtDNA type B than from type A.
Table 5. Pregnancy rates\(^a\) after transfer of embryos to recipients (mtDNA type A or B)

<table>
<thead>
<tr>
<th>mtDNA of recipient</th>
<th>mtDNA of embryo</th>
<th>ratio A</th>
<th>ratio B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA of embryo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>5/12</td>
<td>8/12</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>6/12</td>
<td>7/12</td>
</tr>
</tbody>
</table>

\(^a\)Pregnancy rate: ratio formed by the number of rats that gave birth to at least 1 pup (numerator), and the total number of females to which embryos were transferred (denominator). Ratios were not different (\(P>0.05\)) for the recipient or embryo, and interaction recipient x embryo was also not significant (\(P>0.05\)).

Embryonic survival to birth (number of pups born/number of embryos transferred to recipients that remained pregnant) was not significantly different (\(P>0.05\); Table 6) for any transfer group. The survival rate for type A embryos transferred into recipients of type A and type B were 12% (13/110) and 14% (28/196), respectively. Survival rates to birth for type B embryos transferred to recipients of type A and type B were 11% (15/140) and 13% (22/176), respectively. In one case, a female type B which had given birth to 5 pups became sick and had to be euthanized 24 h later. This rat had 5 fetuses in her uterus which had not been delivered probably because of extensive uterine adhesions.
Table 6. Embryonic survival to birth\textsuperscript{a} after transfer of embryos into recipients (mtDNA type A or B)

<table>
<thead>
<tr>
<th>mtDNA of Recipient</th>
<th>mtDNA of embryo</th>
<th>No. of rats pregnant</th>
<th>ratio</th>
<th>No. of rats pregnant</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>5</td>
<td>13/110</td>
<td>8</td>
<td>28/196</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6</td>
<td>15/140</td>
<td>7</td>
<td>22/176</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Embryo survival to birth: ratio formed by the number of pups born (numerator) and the number of embryos transferred to recipients that remained pregnant (denominator). Ratios were not affected (P>0.05) by the type of mtDNA of the recipient or embryo.

The length of pregnancy was not different (P>0.05; Table 7) among treatment groups. When embryos type A were transferred to recipients type A or B, the mean ± SD length of pregnancy was 24 ± 1 days for both groups. When embryos type B were transferred to recipients type A or B, the length of pregnancy was 24 ± 1 and 23 ± 1 days, respectively.

The mean number of pups born per rat was not affected (P>0.05; Table 8) by the type of mtDNA of the recipient or embryo. The mean number of pups born to recipients type A or B that were transferred with embryos type A were 3 ± 1 and 4 ± 2, respectively. The mean number of pups born to females type A or B that were transferred with embryos type B were 3 ± 1 and 3 ± 3, respectively.
Table 7. Length of pregnancy (days) after transfer of embryos

<table>
<thead>
<tr>
<th>mtDNA of recipient</th>
<th>No. of pregnant rats</th>
<th>Pregnancy length</th>
<th>No. of pregnant rats</th>
<th>Pregnancy length</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>24 ± 1</td>
<td>8</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>24 ± 1</td>
<td>7</td>
<td>23 ± 1</td>
</tr>
</tbody>
</table>

The length of pregnancy was not affected (P>0.05) by recipient or embryo and the interaction recipient x embryo was also not significant (P>0.05).

The mean number of pups weaned per rat was not different between treatment groups (P>0.05; Table 9). The mean number (± SD) of pups weaned at day 21 per rat after transfer of type A embryos into recipients of type A or B was 2±1 and 3±2, respectively. When type B embryos were transferred into recipients of type A or B, the mean number (± SD) of pups that were weaned on day 21 was 3±1 and 4±2, respectively.

The survival rate of pups to weaning on day 21 was affected by the type of mtDNA of the embryo (P<0.01; Table 10) but not by the mtDNA of the recipient or the interaction between them (P>0.05). Pups born from type A embryos transferred to recipients of type A or type B survived to weaning on day 21 at 92% (12/13) and 79% (22/28), respectively. Pups born from type B embryos transferred to recipients of type A and B, survived to weaning on day 21 at 49% (6/15) and 55% (12/22), respectively.
Table 8. Number of pups born per rat after transfer of embryos to recipients (mtDNA type A or B)\(^a\)

<table>
<thead>
<tr>
<th>mtDNA of recipient</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA of embryo</td>
<td>No. of rats</td>
<td>No. of pups</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>

\(^a\) The mean number of pups born per rat was not significantly affected (P>0.05) by the recipient or embryo, and the interaction recipient x embryo was also not significant (P>0.05).

Table 9. Number of pups weaned per rat after transfer of embryos into recipients (mtDNA type A or B)\(^a\)

<table>
<thead>
<tr>
<th>mtDNA of recipient</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA of embryo</td>
<td>No. of rats</td>
<td>No. of pups weaned</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) The mean number of pups weaned per rat was not affected (P>0.05) by the recipient or embryo and the interaction recipient x embryo was also not significant (P>0.05).
Table 10. Survival rate to weaning\textsuperscript{a} of pups that were born after transfer of embryos into recipients (mtDNA type A or B)

<table>
<thead>
<tr>
<th>mtDNA of embryo</th>
<th>mtDNA of recipient</th>
<th>ratio (%)</th>
<th>ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>12/13 (92)</td>
<td>22/28 (79)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6/15 (49)</td>
<td>12/22 (55)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Survival rate to weaning: ratio formed by the number of pups surviving to weaning on day 21 (numerator) and the number of pups born (denominator). Survival rate to weaning was affected (P<0.01) by the type of mtDNA of the embryo but not (P>0.05) by the type of mtDNA of the recipient. The interaction recipient x embryo was also not significant (P>0.05).

The weaning weights by sex of pups are shown in Tables 11 and 12. The weight of the female pups was affected (P<0.05; Table 11) by both the type of mtDNA of the recipient and embryo and there was a trend (P<0.10) for an embryo x recipient interaction. Female pups born to type B recipients that received embryos of mtDNA type A weighed significantly more (P<0.05) than female pups born to type B recipients that received embryos of mtDNA type B (mean ± SD: 64 ± 9 and 47 ± 11, respectively). The weights at weaning of female pups mtDNA type A or B born to type A recipients were not different (P>0.05, mean ± SD: 49 ± 12 and 45 ± 3, respectively, Table 11).

The weaning weight of male pups (Table 12) was not affected (P>0.05) by the type of mtDNA of the embryo or recipient. The interaction embryo x recipient was also not significant (P>0.05).
Table 11. Weights (g) of female pups weaned 21 days post parturition

<table>
<thead>
<tr>
<th>mtDNA of embryos</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pups</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>mean SD</td>
<td>49</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
</tr>
</tbody>
</table>

The weaning weight of the female pups was significantly affected (P<0.05) by the type of mtDNA of the recipient or the embryo, but the interaction recipient x embryo was not significant (P>0.05).

Means in the same row or column which do not have a common superscript letter are different (P<0.05).

Table 12. Weights (g) of male pups weaned 21 days post parturition

<table>
<thead>
<tr>
<th>mtDNA of embryos</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pups</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>mean SD</td>
<td>51</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
</tr>
</tbody>
</table>

The weaning weights of the male pups was not affected (P>0.05) by the type of mtDNA of the recipient or embryo, and the interaction recipient x embryo was also not significant (P>0.05).
Experiment 3. Chloramphenicol Effect on the In Vitro Development of the One-cell Rat Embryo

The cleavage of rat embryos from the one- to the two-cell stage was affected (P<0.05; Table 13) by CAP and by time. After 24 h of culture, the percentage of embryos (types A and B) that cleaved at CAP concentrations of 1000, 500, 250, 125, and 0 ug/ml (treatments 1 through 5, respectively) were 0%, 8%, 47%, 86% and 83%. At 48 h of culture, the percentage of embryos (types A and B) that cleaved were 0%, 16%, 57%, 94% and 82%, for treatments 1 through 5, respectively. Analysis of means for the effect of dose of CAP indicated that the cleavage ratio of embryos at CAP concentrations of 1000 or 500 ug/ml were not different (P>0.05) but the cleavage ratio of embryos for these two concentrations was significantly less (P<0.05) than any of the other three concentrations. Only embryos cultured in 500 ug CAP/ml were affected (P<0.05) by culture time. More embryos cleaved in the group treated with 500 ug CAP/ml by 48 h (16%) than by 24 h (8%).

The type of mtDNA of the embryo did not affect significantly (P>0.05) the cleavage ratio of the one-cell rat embryo after 24 h and 48 h of in vitro culture for any of the five different concentrations of CAP. At 24 h of culture (Table 13) the percentage of embryos type A cultured in CAP (treatments 1 through 5) that cleaved from the one- to two-cell stage were 0%, 10%, 44%, 81%, and 82%, respectively, and the percentage of embryos type B, for treatments 1 through 5 that cleaved from the one- to two-cell stage were 0%, 6%, 51%, 92%, and 85%, respectively. After 48 h of culture, the percentage of embryos for treatments
1 through 5 that cleaved were 0%, 15%, 50%, 91%, and 78% for mtDNA type A embryos, and 0%, 17%, 65%, 97%, 87% for embryos mtDNA B, respectively. Two embryos (type A) from treatment 5 were lost (Table 13). The interactions mtDNA x CAP, and mtDNA x time x CAP were not significant (P>0.05). None of the one-cell rat embryos developed beyond the two-cell stage during the 48 h culture period.

Table 13. Ratio of one-cell rat embryos that cleaved to two-cell stage in medium containing Chloramphenicol within 24 and 48 h of culture.

<table>
<thead>
<tr>
<th>mtDNA of</th>
<th>No. embryos</th>
<th>No. rats</th>
<th>Chloramphenicol, µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>A</td>
<td>22</td>
<td>229</td>
<td>0/35</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>207</td>
<td>0/33</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>436</td>
<td>0/68</td>
</tr>
</tbody>
</table>

Observations at 48 h:

| A        | 22           | 229     | 0/35 | 7/48 | 25/50 | 43/47 | 38/49 |
| B        | 20           | 207     | 0/33 | 8/47 | 28/43 | 37/38 | 40/46 |
| Total    | 42           | 436     | 0/68 | 15/95 | 53/93 | 80/85 | 78/95 |

a Ratio formed by the number of embryos that cleaved (numerator) and the total number of embryos cultured.

b Two embryos were lost. The ratio of cleavage was not affected (P>0.05) by the type of mtDNA of the embryo donor. Cleavage in CAP at 1000 and 500 µg/ml was significantly different (P<0.05) from the other CAP concentrations. Cleavage of embryos at 500 µg CAP/ml was affected by time (P<0.05).
The percentage of embryos which stained with eosin B after 48 h of \textit{in vitro} culture is shown in Table 14. The staining response was affected (P<0.05) by CAP but not by the type of mtDNA of the embryo.

**Table 14. Viability\textsuperscript{a} of embryos after 48 h of culture in media containing different CAP concentrations**

<table>
<thead>
<tr>
<th>mtDNA</th>
<th>Chloramphenicol, (\mu g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>A</td>
<td>35/35</td>
</tr>
<tr>
<td>B</td>
<td>33/33</td>
</tr>
<tr>
<td>Total</td>
<td>68/68</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Ratio formed by the number of embryos that stained with eosin B (numerator) and the total number of embryos exposed to the dye (denominator). Embryo viability at 1000 and 500 \(\mu g/ml\) were significantly different to other CAP concentrations.

\textsuperscript{b} One embryo in media droplet that was contaminated stained.

Analysis of means for the effect of dose of CAP indicated that the staining response of embryos at CAP concentrations of 1000 and 500 \(\mu g/ml\) were not different (P>0.05); however, the staining response for these two concentrations was significantly different (P<0.05) from the staining response for any of the other concentrations. Embryos exposed to 250, 125, and 0 \(\mu g\) CAP/ml did not stain when exposed to eosin B, even when including those embryos which had condensed cytoplasm or irregular blastomeres. One embryo type B which was not exposed to CAP (0 treatment group) stained with eosin B after 48 h. This embryo was in a
culture dish containing medium that was turbid and appeared to be contaminated. All of the embryos exposed with 1000 μg CAP/ml stained when exposed to eosin B after 48 h of culture and were therefore considered to be dead (Table 14). At 500 μg CAP/ml, 56% (27/48) of embryos mtDNA type A and 49% (23/47) of embryos mtDNA type B stained by the eosin B.

Experiment 4. Staining Response to Eosin B of Two-cell Rat Embryos Cultured in Medium Containing Chloramphenicol

As anticipated, none of the two-cell rat embryos cleaved after 48 h of culture. Only CAP at 1000 μg/ml caused appreciable morphological degeneration and death of the embryos. Rat embryos of mtDNA types A and B stained at 100 and 91%, respectively, in the group treated with 1000 μg CAP/ml. The staining response was not affected (P<0.05) by the type of mtDNA of the embryos. CAP doses of 500, 250, 125, and 0 μg CAP/ml did not cause morphological degeneration of embryos. Embryos appeared normal and excluded the eosin B since they did not stain (Table 15). One blastomere of a two-cell rat embryo type B was stained at 48 h in the group treated with 500 μg CAP/ml.
Table 15. Viability\textsuperscript{a} of two-cell rat embryos after 48 h of culture in media containing different CAP concentrations

<table>
<thead>
<tr>
<th>type of mtDNA</th>
<th>Chloramphenicol, ( \mu \text{g/ml} )</th>
<th>1000</th>
<th>500</th>
<th>250</th>
<th>125</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>17/17</td>
<td>0/19</td>
<td>0/18</td>
<td>0/19</td>
<td>0/22</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>20/22</td>
<td>1/20\textsuperscript{b}</td>
<td>0/20</td>
<td>0/21</td>
<td>0/24</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Viability was estimated by the ratio formed by the number of embryos that stained with eosin B (numerator) and the total number of embryos exposed to dye (denominator). Embryo viability at 1000 \( \mu \text{g} \) CAP/ml was less (\( P<0.05 \)) than the other concentrations of CAP.

\textsuperscript{b} Only one of the two blastomeres stained with eosin B.
DISCUSSION

Experiment 1. Mitochondrial DNA from Somatic and Germinal Cells of Male Rats

The two types of mtDNA (A and B) previously described for the rat were identified in the rats used in this study (Francisco and Simpson, 1977; Hayashi et al., 1978a,b; Francisco et al., 1979). For each of the 12 rats, the liver and testicular mitochondrial preparations shared the same type of mtDNA. This suggests that the type of mtDNA, as identified by the electrophoretic banding pattern of EcoR1 digested mtDNA, is not altered during differentiation, development, and spermatogenic activity of the male gonad. To confirm these results for the rat, hybridization studies with radiolabelled probes (Hecht et al., 1984) need to be performed. However, my findings are consistent with findings reported for the mtDNA isolated from the testis of the mouse (Hecht et al., 1984). These researchers determined that the nucleotide sequence of mtDNA from testicular germinal cells undergoing meiosis was not different from that of spermatozoa obtained from the cauda epididymidis (Hecht et al., 1984). In their studies, the testicular mtDNA decreased 8- to 10-fold between meiosis and the end of spermatogenesis.

No attempt was made in this study to quantitate the mtDNA extracted from the testicular mitochondrial preparations. The difference in dilution required to obtain visible bands after electrophoresis of the EcoR1-digested mtDNA from the tunica-free testis suggest that the content of mtDNA per gram of testes is about 10-fold less than in liver. The bands observed in the electrophoretic banding pattern for the mtDNA
isolated from the testicular mitochondrial preparations were consistently fainter than those for the mtDNA extracted from the liver. This may reflect differences in the mtDNA content between mitochondria or differences in the number of mitochondria in the germinal cells from the testis and somatic cells from the liver. Differences in mitochondrial morphology were found in rat somatic and germinal cells. These differences are similar to those previously reported for the rat (De Martino et al., 1979) and may reflect differences in the metabolic activities of these tissues. The differences in the intracrystal spaces of mitochondria isolated from the testicular preparations as compared to mitochondria isolated from liver preparations are similar to those reported for mitochondria within cells, as observed in tissue sections (De Martino et al., 1979).

The maternal inheritance of the mtDNA in the rat was anticipated in view of previous reports (Hutchinson et al., 1974; Hayashi et al., 1978b; Francisco, Brown, and Simpson, 1979; Hecht et al., 1984). Indeed, the results of this study confirmed the maternal inheritance of mtDNA, as demonstrated by the fact that 5 offsprings from parents of known mtDNA type inherited the mtDNA type A or B from the mother. These results further suggest that either paternal mitochondria are not transmitted to the offspring or that the mtDNA from the spermatozoal mitochondria are diluted to levels below detection. Furthermore, morphological studies have indicated that spermatozoal mitochondria degenerates during the first cleavage divisions of the embryo (Szollosi, 1965).
Experiment 2. Transfer of Rat Embryos to Recipients with the Same or Different Type of Mitochondrial DNA

The total number of embryos recovered from the donors and the stage of embryonic development were not affected by the type of mtDNA. The trend (P<0.09) observed for greater total number of embryos recovered at the blastocyst stage from females of type B should be regarded with caution because the statistical analysis that resulted in this trend was based on the data from a selected sample since it was obtained only from those donors from which embryos were recovered.

The specific purpose of this experiment was to determine whether or not the survivability of rat embryos was affected by the type of mtDNA of the recipient. The mtDNA of the recipient did not affect the number of females that remained pregnant to term (pregnancy rate) or the number of offspring born indicating that differences in the type of mtDNA between embryos and recipients is not a factor for a successful gestation to term during the first pregnancy after transfer. However, a one-time transfer may not have allowed the recipient's uterine cells to react to the embryo with foreign mtDNA at the implantation site. It may be that additional transfers to the same recipient would influence embryo survival. Further studies are needed to confirm or deny this assumption.

The overall 54% pregnancy rate after surgical transfer of embryos obtained in this experiment was similar to the 50% pregnancy rate reported by Noyes, Doyle and Bentley (1961) and lower than the 68% pregnancy rate reported by Noyes and Dickmann (1960) or the 67% pregnancy rate
reported by Mantalenakis and Danezis (1968). These differences could be ascribed to a number of factors such as differences in techniques, animal and strain variation, and other unforeseen variables, including the time when pregnancy was evaluated. For instance, the highest (68%) of these pregnancy rates (Noyes and Dickmann, 1960) was obtained by counting fetuses on day 18 of pregnancy and not pups born. On these basis, the overall 54% pregnancy rate obtained in this experiment is believed to be within the range of pregnancy rates for embryo transfer reported by others.

The most widely accepted measure of embryo viability is their survival to term. In this study, although there was a low survival rate to birth (11-14%) in those animals that remained pregnant, embryonic survival was not affected (P>0.05) by the type of mtDNA of the recipient or the mtDNA of the embryos transferred. The low survival rate obtained in this experiment could have been the effect of surgical trauma to the recipient animals, as evidenced by the observation that 75% (12/16) of the type A recipients and 69% (9/13) of the type B recipients had adhesions after the experiment was completed. Furthermore, survival rate could have been affected by the degree of estrous cycle synchrony between the embryo donor and the recipient females. Since recipients were mated with vasectomized males and for donors, mating was determined only by the presence of spermatozoa in vaginal smears, the actual time of mating and fertilization was unknown for either the donor or recipient. It is possible that the actual stage of development of the embryo, which was determined, and the actual endocrine stage of the
recipient uterus, which was assumed, might have been asynchronous for 6 to 12 h before or after the assigned stage of embryonic development. However, similar survival rates after transfer of rat embryos have been reported by others. Noyes, Doyle and Bentley (1961) reported an overall 13% of embryos surviving to term when reciprocal transfers between two rat strains (albino and black pigmented) were performed. Nagai et al. (1985) also reported a low 11% survival rate for Wistar embryos after transfer and a 25% survival rate for F1 embryos (Wistar x Brown Norway).

If there was an interactive, deleterious effect on embryo survival during gestation caused by differences between the mtDNA of the embryo and that of the mother, the low survival rate of transferred embryos and the small number of animals used may have precluded to detect these differences among treatment groups. However, the experimental design used ensured that the conditions for the embryo collection and transfer to the recipients, which may have subsequently affected embryonic development, were the same for both mtDNA type A and B embryos. Thus, the conclusion that mtDNA type did not affect pregnancy rates and embryo survival is believed to be valid despite the low survival rates to birth obtained.

Similarly, the type of mtDNA did not affect the length of pregnancy, the number of pups born, the number of pups weaned, and the weight of male pups at weaning day, providing additional evidence to conclude that a difference of mtDNA between embryo and recipient is not a factor to be considered when embryo transfers are to be performed. On this basis, and considering all the results of this experiment, the signifi-
cantly greater weight at weaning for female pups may not reflect a true biological effect caused by differences on the mtDNA type because data from our established rat colony (Appendix A) from 929 female pups weaned indicated no difference on body weight for females mtDNA type A or B.

Experiment 3. Chloramphenicol Effect on the In Vitro Development of the One-cell Rat Embryo

As anticipated in view of the previous results in the literature, none of the one-cell rat embryos developed beyond the two-cell stage, corroborating the in vitro block for the two-cell stage of the rat embryo. CAP at 1000 and 500 μg/ml significantly inhibited the in vitro development of one-cell rat embryo. The inhibition of development at high doses of CAP could be due to the inhibitory effect of CAP on mitochondrial respiration (Piko and Chase, 1973). The cleavage of embryos to the two-cell stage during the 48 h of culture in 250, 125, and 0 μg CAP/ml indicated that CAP at doses lower than 500 μg/ml did not interfere with mitochondrial activity.

The type of mtDNA of the embryo was not a factor affecting the in vitro cleavage of embryos. Koike et al. (1983) have shown that the resistance and/or sensitivity to CAP of rat somatic cells was linked to a single nucleotide change in the mtDNA. Again, these results suggest that mtDNA activity in undifferentiated cells, like those of the one-cell embryos, is different to the mtDNA activity in somatic cells. Koike et al. (1983) showed that 150 μg CAP/ml changed a rat cell line that was CAP-sensitive into a resistant one.
Experiment 4. Staining Response to Eosin B of Two-cell Rat Embryos Cultured in Medium Containing Chloramphenicol

Only embryos exposed to 1000 ug CAP/ml evidenced degeneration and stained with eosin B. Embryos exposed to 500, 250, 125, and 0 µg CAP/ml did not degenerate nor did they stain when exposed to eosin B. The mtDNA of the rat embryos did not affect the staining response of the embryos after exposure to CAP. Rat embryos at the two-cell stage did not stain at 500 µg CAP/ml, as observed for the one-cell rat embryo. This suggests that the difference in response between the one- and the two-cell embryos may be related to either metabolic or genetic regulation differences between these stages. Norris, Barton, and Surani (1985) have shown distinctly different protein profiles for the one-cell rat embryo, when compared to embryos at the four-cell or blastocyst stage. The results of experiments 3 and 4, with regard to the effects of CAP on the mtDNA of early embryos, are consistent with observations of other researchers, who reported that oxidative phosphorylation of the mitochondria becomes important only at the time of implantation, when the rat embryo starts to grow in mass (Jager and Bass, 1975; Oerter and Bass, 1975).
SUGGESTED FUTURE STUDIES

Although the rat is a widely used laboratory animal, only few studies have been reported on embryo transfer for this species. The low embryo survival reported in this thesis, which is not different from the results of others, clearly are not satisfactory, and demand more studies to understand the requirements and improve the technique in order to obtain higher success rate after transfer.

From the experiments of this thesis, there appears to be a need for studies testing the possibility that hybrid F1 embryos (A x B vs B x A) will survive transfer better than inbred embryos (A x A vs B x B). Moreover, experiments should be performed to test for the effect on embryo survival of the concurrent transfer to the same recipient of embryos type A and B.

Studies to test the possibility that repeated transfer to the same recipient of embryos with different mtDNA type affects embryo survival, should be performed to conclusively disprove an interactive effect of mtDNA on embryonic survival. I believe this to be important because of the wide application of embryo transfer to livestock species.

All available evidence using EcoR1 endonuclease, including those of this study, indicate that the laboratory rat has only two mtDNA types. This, coupled to the fact that the mtDNA is inherited maternally, provides a suitable species and genetic markers for other studies related to cytoplasmic hybridization, nuclear transplantation, generation of chimaeric embryos, as well as for studies correlating specific traits to a given mtDNA make-up.
REFERENCES


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To Abelino, Ivan, Bruce and the Mongolitas, Patricia, Beatriz, Marilyn, Flavia, Lynn, and Gilda, I give my thanks for their friendship. Our many late nights at Dugan's helped me keep my sanity. Finally, I would like to thank Bill for showing me the subways of Boston, especially Park Station.
APPENDIX A:

REPRODUCTIVE PARAMETERS OBTAINED FROM THE RAT COLONY

To fulfill the objectives reported in this thesis it was first necessary to establish a rat colony containing the two mtDNA types (A and B) known to exist in the rat. After determination of the mtDNA type of an animal, littermates of this typed animal were then used to develop the colony. Subsequently, females were bred to males of the same mtDNA type. Pregnant rats were caged individually and observed daily until parturition. To establish base lines of reproductive parameters for internal control, the following reproductive parameters were recorded for each female bred: number of pups born, gestation length in days, number of females and males weaned, sex ratio at weaning, and weight (grams) of females and males at weaning day. The information obtained was analyzed by analysis of variance (Snedecor and Cochran, 1980) and significance established at P<0.05. The percentage of females that carried all pups to weaning day, and further analyses on the length of gestation obtained was analyzed by chi-square with 1 degree of freedom.

Reproductive Parameters

Number of pups born

A total of 1,011 pups were born to 92 females of mtDNA type A and 1,008 pups were born to 85 females mtDNA type B (Table A1). The mean number (± SD) of pups born to females mtDNA type B (12 ± 2) was higher (P<0.05) than those born to females type A (11 ± 2).
Number of pups weaned

The number of pups weaned were 945 and 892 for mtDNA types A and B, respectively (Table A1). The mean number (± SD) of pups weaned was not different (P>0.05) for females mtDNA type A (10 ± 2) or B (11 ± 3). The weaning rate (number of pups born/number of pups weaned) was 93% for females type A and 88% for females type B and the ratios from which these percentages were derived were not different (P>0.05). The cause for the postnatal losses of 7% and 12% for types A and B, respectively, could not be ascertained.

Table A1. Litter size at birth and at weaning, and weaning rates for offsprings of mtDNA type A and B rats

<table>
<thead>
<tr>
<th>mtDNA type</th>
<th>No. rats</th>
<th>Number of Pups born (Mean + SD)</th>
<th>Number of Pups weaned (Mean + SD)</th>
<th>Survival to weaning (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>92</td>
<td>1,011 a (11 ± 2)</td>
<td>945 a (10 ± 2)</td>
<td>93 a</td>
</tr>
<tr>
<td>B</td>
<td>85</td>
<td>1,008 b (12 ± 2)</td>
<td>892 a (11 ± 3)</td>
<td>88 a</td>
</tr>
</tbody>
</table>

Means in the same column which have a different superscript letter are significantly different (P<0.05).

The percentage (not shown in tables) of the females that carried all pups to weaning day was higher (P<0.05) for females of mtDNA type A (65%; 60/92) than for females of type B (46%, 39/85).

Length of gestation

The average length of gestation (Table A2) was not different (P>0.05) for females mtDNA type A and B (22 ± 1 days for both groups).
The range, however, varied from 19 to 25 days for females type A and from 17 to 25 for females type B. Further analysis of the frequency of distribution of pregnancy length indicated that the number of females with pregnancy length of 21 and 22 days was greater (P<0.05; Table A2) for females type A (15 and 45, respectively), than for females type B (3 and 28%, respectively). The number of females with pregnancy length of 23 days was greater (P<0.05) for females type B (42) than for females type A (20).

Table A2. Length of pregnancy for females mtDNA type A and B bred to males of the same mtDNA type

<table>
<thead>
<tr>
<th>mt DNA rats Type</th>
<th>Length of pregnancy in days</th>
<th>Mean ± SD, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 92</td>
<td>0 1 2 15 45 20 7 2 22 ± 1</td>
<td>a a a</td>
</tr>
<tr>
<td>B 85</td>
<td>1 1 3 3 28 42 6 1 22 ± 1</td>
<td>b b b</td>
</tr>
<tr>
<td>Overall</td>
<td>1 2 5 18 73 62 13 3 22 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Means within columns which have a different superscript letter are different (P<0.05).

Weight of the offspring at weaning

The sex ratio (male:female) of pups indicated that the sex ratio was 1.01 and 1.12 for females type A and B, respectively.

The percentage of females and males pups weaned at 21 days was not different (P>0.05; Table A3) for mtDNA type A (50% females vs 50% males)
and B (51% females vs 49% males) animals.

Mean body weight (g) at weaning for females and males was not different (P>0.05) for either mtDNA type A (46 ± 9 for females vs 48 ± 10 for males), or mtDNA type B (46 ± 8 for females vs 49 ± 8 for males) rats.

Table A3. Number and weaning weight (g) of females and males offsprings of mtDNA type A or B rats

<table>
<thead>
<tr>
<th>mtDNA</th>
<th>No. females weaned (%)</th>
<th>No. males weaned (%)</th>
<th>a Sex ratio</th>
<th>b weaning weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Females</td>
</tr>
<tr>
<td>A</td>
<td>471 (50)</td>
<td>474 (50)</td>
<td>1.01</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>B</td>
<td>458 (51)</td>
<td>434 (49)</td>
<td>1.12</td>
<td>46 ± 8</td>
</tr>
</tbody>
</table>

a Sex ratio: number of males (numerator) and number of females (denominator).

b Mean ± SD. F-ratios for weaning weights of either males or females were not significantly affected (P>.05) by the type of mtDNA.

This is believed to be the first attempt to relate the mtDNA type to the reproductive parameters in the rat. Litter size and frequency of distribution of length of gestation are two parameters that appear to be influenced by the mitochondrial genome. Even though this assumption is made from data collected in a rat colony and not from experiments designed for this specific purpose, the data are suggestive enough to warrant further studies.
APPENDIX B: ADDITIONAL STUDIES ON RAT MITOCHONDRIAL DNA

The studies indicated below were performed after experiment 1 of this dissertation was completed. The objective was to complement the results of Experiment 1 by determining whether or not the type of mtDNA could be identified in mitochondria isolated from rat spermatozoa recovered from the cauda epididymidis. Several attempts to identify the mtDNA pattern were not successful. Additionally, this section also includes information regarding mtDNA derived from rat blood platelets (obtained from the same animals used to collect spermatozoa) as well as on the use of other restriction endonucleases on rat mtDNA.

Collection of Mitochondria from Spermatozoa Isolated from Rat Cauda Epididymidis

The procedure used to collect the mitochondria from rat spermatozoa was a modification of the procedure of Hecht and Kennington (1983). The rats were euthanized and the cauda epididymidis were sectioned and expressed in dishes containing phosphate buffer (0.146 M sodium chloride, 0.008 M sodium phosphate, pH 7.2). In the first trial, the spermatozoal concentration from 2 males of mtDNA type A was 70 x 10^6 in 4 ml of medium. No somatic cells were observed at this step. The medium containing the epididymides was gently agitated for 10 min and then was allowed to settle at room temperature. The liquid phase which contained the spermatozoa was decanted (the sperm count was 62.5 x 10^6 in 4 ml). The epididymides were agitated again in 3 ml phosphate buffer and the above procedure was repeated. The liquid phase contained 30 x 10^6 sper-
matozoa per ml. Removal of the fragments of epididymal tissue was first attempted by filtering 1 ml of the combined supernatants through Miracloth (Calbiochem, San Diego, CA). No sperm were obtained in the filtrate. Subsequently, the supernatants were combined and the tissue fragments removed by filtering 6 ml of the solution through a polypropylene mesh monofilament with a mesh opening of 500 μm (CMP-500, Small Parts Inc, Miami, FL). The filter was then washed by passing an additional 2 ml of PBS through it using an experimental filtration kit (Nucleopore Corporation, Pleasanton, CA 94506). After filtration, the sperm count was 59 x 10^6/ml. Spermatozoal suspension was concentrated by centrifugation for 10 minutes at 5000 X g.

Mitochondria isolation from epididymal spermatozoa

The spermatozoal pellet was resuspended, centrifuged and washed three times with 5 ml of 0.45% saline to lyse somatic cells released from the epididymides. The sperm concentration was 1 x 10^7/ml. The washed spermatozoal pellet was resuspended in 0.50 ml of mitochondrial buffer (0.25 M sucrose, 0.02 M TrisHCl, pH 8.0) and sonicated 15 times at 4 °C for 20 sec, interspersed with 40 second cooling intervals at a setting of 4 with a Branson Sonifier (Model W185). The sonicate was centrifuged at 2500 X g for 10 min to pellet spermatozoal heads and this pellet was discarded. The supernatant was then centrifuged at 8000 X g for 20 min to pellet mitochondria. Samples were stored for mtDNA extraction, EcoR1 digestion, and gel electrophoresis, as described in the Materials and Methods of this dissertation. No mtDNA pattern could be detected with the above preparation.
In a second trial, two male rats were used and modifications to the above procedure were introduced in regard to the volumes of buffer used and the filtration procedure. Rat caudal epididymal spermatozoa were isolated as previously described. The supernatant with the spermatozoa was decanted and the epididymis were again resuspended in 10 ml of PBS. Spermatozoal concentration was 95 x 10^6/ml and a total volume of 20 ml. Filtration was performed by using nylon cloth to speed up the collection procedure. The filtrate was centrifuged at 5000 X g for 15 min and washed three times with 0.45% saline to lyse the somatic cells. The final pellet containing 105 x 10^6 spermatozoal per ml was resuspended in 3 ml of mitochondrial buffer.

This suspension was sonicated as previously described. The sonicate was centrifuged at 2500 X g. The pellet containing both head and tails was saved this time (tube 1; 600 x 10^6/ml in a total volume of 3 ml). The supernatant was centrifuged at 10000 rpm for 20 min to pellet mitochondria (tube 2). Mitochondrial pellets were frozen and the mtDNA was then extracted as follows: Tube 1 (heads and tails), 1 ml of TRIS-EDTA buffer, 100 ul of sodium dodecyl sulphate (SDS) and 1.5 ml phenol were added. Tube 2 (mitochondria) 0.5 ml of TE, 50 ul SDS and 0.9 ml phenol were added. The mixtures were then agitated by hand for 5 min and centrifuged at 5000 rpm for 5 min. Phenol extraction was repeated two times and the mtDNA was precipitated by adding 0.08 ml of sodium acetate in two volumes of 100% ethanol and the precipitate was frozen overnight. The precipitate was then washed in 4 ml of 70% ethanol and the mtDNA was suspended in 50 ul of TRIS-EDTA buffer. This mtDNA solu-
tion was used for the digestion procedure (40 ul were vacuum dried first in an Eppendorf tube) and then 10 ul of the mtDNA solution were used to make up the final digestion reaction. Gel electrophoresis indicated no pattern of mtDNA for any of the two tubes.

From these preliminary results, the assumption was made that the relatively low number of spermatozoa recovered was the primary reason for not obtaining an identifiable mtDNA electrophoretic pattern. On a preliminary trial using ejaculated bovine sperm, a faint banding pattern was obtained with this procedure. Therefore, a third rat trial was performed using 22 males of known mtDNA, as follows: 10 males were of mtDNA type A (60-68 day old), 10 males were mtDNA type B (56-61 day old), and 2 males were mtDNA type B (270-283 days). Rats were first anesthetized with ether, bled by heart puncture to collect blood, and euthanized by cervical dislocation. Both testes and epididymides from each animal were dissected free. To maximize the yield of spermatozoa, testicular and epididymal spermatozoal were pooled for each group of animals. The mtDNA extraction and EcoRI gel electrophoresis of mitochondria isolated from tunica-free testis were performed as described in the Materials and Methods section of this dissertation. Testicular weight (g) and blood volumes (ml) obtained from these animals were:

<table>
<thead>
<tr>
<th></th>
<th>total testicular weight (g)</th>
<th>blood volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 males type B</td>
<td>4 testes: 6.06</td>
<td>6</td>
</tr>
<tr>
<td>10 males type B</td>
<td>20 testes: 25.67</td>
<td>40</td>
</tr>
<tr>
<td>10 males type A</td>
<td>20 testes: 22.30</td>
<td>22</td>
</tr>
</tbody>
</table>
Rat cauda epididymides were slit opened in 10 ml of PBS and the solutions containing these epididymides were gently agitated for 10 min at room temperature. After sedimentation, the liquid phase containing sperm was decanted and saved. The epididymides were again resuspended in 10 ml PBS and the agitation procedure repeated. These two volumes were pooled, filtered through a nylon cloth to remove tissue fragments, and refrigerated overnight. The epididymal spermatozoal concentrations at this step were as follows:

- **2 males B** = 450 x 10^6/ml/male
- **10 males B** = 1500 x 10^6/ml/male
- **10 males A** = 250 x 10^6/ml/male.

The samples were centrifuged at 5000 x g for 10 min to pellet the spermatozoa. The pellets were then washed three times with 0.45% saline (10 ml) to lyse somatic cells and centrifuged at 5000 g for 15 min. The final pellets were resuspended in 5 ml of mitochondria buffer and the suspensions were sonicated fifteen times for 20 sec with 40 sec cooling intervals, using a Branson Sonifier (Model W185). The sonicate was centrifuged at 600 x g for 15 min. Light microscopy indicated that the pellets contained mostly heads and fragmented tails. The supernatant contained mitochondria and fragmented tails. Both fractions (pellet and supernatant), were separately used for the mtDNA extraction. The head pellet was diluted in 5 ml of mitochondrial buffer. The buffer and the supernatant (mitochondria) fraction were counted and contained as follows:
The supernatant samples were then centrifuged at 8000 X g for 20 min. The pellets from the supernatant fraction were supposed to contain the mitochondria, whereas the pellet of the head fraction supposedly contained spermatozoal head and tail fragments. To extract mtDNA, all samples were dissolved in 2 ml TRIS-EDTA (TE) buffer, 200 ul SDS, and 3 ml of TE-saturated phenol.

Samples were hand-shaked for 5 min and centrifuged at 2500 X g for 30 min. The aqueous phase was resuspended in 2 ml TE-saturated phenol, shaked for 5 min and centrifuged again at 5000 rpm for 5 min. To the final aqueous phase, 0.3 ml of 2.5 M sodium acetate and 2 volumes of 100% ethanol were added. Samples were left in the freezer overnight. After thawing, the solutions were centrifuged at 5000 rpm for for 10 min. The final pellet was carefully washed with 70% ethanol, vacuum dried, and resuspended in 100 ul of TRIS-EDTA buffer.

Neither the mitochondrial fraction, nor the head fraction gave an electrophoretic pattern that could be readily identified from the rat cauda epididymal spermatozoa, under the conditions described above. However, following the same procedure, samples from ejaculated bull spermatozoal that were processed in the same way showed a distinct pattern (although it had additional bands to those obtained from bovine
liver mtDNA, suggesting some nuclear contamination).

Mitochondria Isolation from Rat Blood Platelets

Pooled blood samples were from the group of animals indicated above, as well as from two more groups of animals (one group contained 4 males type B, 29-days old from which 6 ml of blood were collected, the other group contained 7 males type B, 26-days old from which 7 ml of blood were collected). The procedure used here is identical to the procedure used for bovine blood platelets (Giles et al., 1980).

Platelet isolation and mtDNA extraction

Blood samples were centrifuged at 1000 g for 15 min at 20 C using a Beckman TJR centrifuge. The plasma was decanted and the pellet containing red blood cells was recentrifuged and a second plasma sample obtained. These two plasma samples were pooled. Using light microscopy, a plasma sample obtained at this step appeared to contain 95% platelets and 5% nucleated cells. The pooled plasma was centrifuged at 9000 X g for 10 min at 4 C. The supernatant was discarded and the pellet was dissolved in 5 ml of mitochondria buffer, centrifuged at 9000 g for 15 min at 4 C, and the supernatant was discarded. The pellet was suspended in 2 ml TRIS-EDTA buffer, 200 ul SDS, and 3 ml of TRIS-EDTA saturated phenol. This solution was agitated for 5 min and centrifuged at 5000 x g for 10 min. The aqueous phase was decanted and 2 ml of TRIS-EDTA saturated phenol was added, agitated for 5 min, and centrifuged at 5000 rpm for 10 min. To the final aqueous phase, 0.3 M of 2.5 sodium acetate, pH 5.2 and two volumes of 100% ethanol were added. The suspension was stored overnight at -20 C. After thawing, the samples
were centrifuged at 5000 g for 10 min, washed carefully with 70% ethanol, vacuum dried, and diluted in 100 ul of TRIS-EDTA buffer.

Restriction endonuclease digestion and gel electrophoresis were performed as follows: 30 ul of the mtDNA solution was vacuum dried in 1.5 ml Eppendorf tubes. The same tubes used for the digestion mixture contained 10 ul mtDNA, 4 ul of EcoR1, 1.5 ul of high salt buffer, and 2.5 ul of distilled water. After incubation for 2 h at 37 C, 2 ul of a tracking dye was added and gel electrophoresis was performed at 100 V for 3 h.

A mtDNA fragment pattern was obtained from approximately 40 ml of rat blood, under the conditions described above. The pattern obtained was from the group of the 10 males of mtDNA type B, but no patterns were obtained from other samples. Unexpectedly, the pattern that was observed was not the same as that found in both somatic and germinal cells of these same animals. To further investigate this finding, the mtDNA of the same samples was digested with HindIII, a restriction endonuclease that is known to give similar fragments of mtDNA in samples that have two different EcoR1 fragment patterns. Results indicated that, indeed, the HindIII-treated mtDNA pattern of the testicular samples was the same for all the animals on the three groups, as opposed to those differences observed with EcoR1 mtDNA (types A or B), and that the mtDNA from blood platelets was, again, different to the somatic and germinal cell mtDNA from the same animal suggesting the possibility of nuclear contamination. All of the testicular samples showed the expected fragment patterns when digested with EcoR1.
APPENDIX C:
CULTURE MEDIA FOR RAT EMBRYOS

The media used for *in vitro* culture and short term storage of embryos during this study were the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Brinster's</th>
<th>BMOC-3</th>
<th>Biggers</th>
<th>Peluso</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>94.90 M</td>
<td>94.60 mM</td>
<td>94.70 mM</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>4.78 mM</td>
<td>4.78 mM</td>
<td>4.78 mM</td>
<td></td>
</tr>
<tr>
<td>CaCl2.2H2O</td>
<td>1.29 mM</td>
<td>-</td>
<td>1.71 mM</td>
<td></td>
</tr>
<tr>
<td>KH2PO4</td>
<td>1.19 mM</td>
<td>1.19 mM</td>
<td>1.91 mM</td>
<td></td>
</tr>
<tr>
<td>MgSO4.7H2O</td>
<td>1.19 mM</td>
<td>1.19 mM</td>
<td>1.91 mM</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>5.56 mM</td>
<td>5.56 mM</td>
<td>5.56 mM</td>
<td></td>
</tr>
<tr>
<td>NaHCO3</td>
<td>25.07 mM</td>
<td>25.07 mM</td>
<td>25.07 mM</td>
<td></td>
</tr>
<tr>
<td>Lactic acid (Na)</td>
<td>24.85 mM</td>
<td>21.55 mM</td>
<td>21.55 mM</td>
<td></td>
</tr>
<tr>
<td>Na Pyruvate</td>
<td>0.51 mM</td>
<td>0.33 mM</td>
<td>0.55 mM</td>
<td></td>
</tr>
<tr>
<td>Calcium lactate</td>
<td>-</td>
<td>1.71 mM</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BSA, Frac V</td>
<td>5 g/l</td>
<td>4 g/l</td>
<td>4 g/l</td>
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</tr>
<tr>
<td>K-Penicilline G</td>
<td>100 IU/ml</td>
<td>100 IU/ml</td>
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</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>50 ug/ml</td>
<td>50 ug/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Brinster's medium was adjusted to pH 7.4, kept refrigerated at 4°C, and used within 2 weeks. Peluso's medium was gassed with 5% CO2 in air for 15 minutes, the pH was adjusted to 7.4, and the medium was kept...
refrigerated at 4°C. At the time of usage, 5 ml of Pelusso's medium and 5 ml of autoclaved paraffin were mixed in a tube and left in an incubator at 37°C overnight. Bigger's medium was filtered and gassed with 5% CO2 in air for 15 min before refrigeration at 4°C.

Preliminary Studies on the in vitro Culture of Rat Embryos

I attempted to culture one-cell rat embryos using the procedure published by Peluso, Karey, and Gruenberg in 1983. These researchers reported the successful culture of one-cell rat embryos to the blastocyst stage. Further details on the culturing procedure were obtained through personal communication with Dr. Peluso (see media components above) and numerous attempts were made to repeat their results. After culturing 181 embryos at the one-cell stage for 48 h, following the procedure reported by Peluso, Karey, and Gruenberg (1983) none of these embryos developed to the 4- or 8-cell stage after 48 h of culture. Of these embryos, only 48% (86/181) cleaved to the two-cell stage and 32% were fragmented or degenerated during the 48 h of culture.

In a second trial, the effect of 2 culture temperatures (37 vs 39°C) was examined, using the Peluso medium. (Body temperature in the rat is 38.1°C.) After 48 h in culture, 64% (27/42) of the embryos cultured at 37°C and 39% (17/44) of the embryos cultured at 39°C cleaved to the two-cell stage.

A third and final trial was performed to study the effect of three media shown in the Appendix on the cleavage rate of one-cell rat embryos cultured at 37°C for 48 h. Commercial BMOC-3 medium (Grand Island Biological Company, Grand Island, NE) was used, along with two other media
which were prepared following published procedures (Peluso, Karey, and Gruenberg, 1983; and Biggers 1971). After 48 h in culture, the percentage of one-cell embryos that cleaved to two-cell stage were: 79%, 38%, and 81% for Peluso, Biggers, and BMOC-3 media, respectively. None of these embryos developed beyond the two-cell stage. Based on these results, the BMOC-3 medium was selected for use in the studies related to transfer and culture of early rat embryos that were part of this thesis. The medium was no longer purchased but prepared in our laboratory with slight modifications to the procedure published by Brinster (1971). The medium was adjusted to pH 7.4 and kept refrigerated to use within 2 weeks, without filtration.
APPENDIX D:

STATISTICAL ANALYSIS FOR EXPERIMENT 2

The classes used in the Analysis of Variance procedure were:
Females (FEM), mitochondrial DNA (DNA), culture time (TIME),
and Chloramphenicol (DRUG).
The dependent variables were: cleavage and eosin B

The model used was:
\[
\text{Cleavage Eosin} = \text{DNA FEM(DNA) TIME DNA*TIME}
\]
\[
\text{Time*FEM(DNA) DRUG DNA*DRUG}
\]
\[
\text{DRUG*FEM(DNA) TIME*DRUG}
\]
\[
\text{DNA*TIME*DRUG TIME*DRUG*FEM(DNA)}
\]

Analysis of Variance:

Dependent variable: Cleavage

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>ANOVA SS</th>
<th>F Value</th>
<th>PR &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1</td>
<td>0.17144300</td>
<td>0.05</td>
<td>0.8198</td>
</tr>
<tr>
<td>TIME</td>
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<td>154.44126984</td>
<td>147.47</td>
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<tr>
<td>DNA*TIME</td>
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<td>0.19942228</td>
<td>0.19</td>
<td>0.8266</td>
</tr>
<tr>
<td>DRUG</td>
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<td>202.86349206</td>
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<td>0.0001</td>
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<tr>
<td>DNA*DRUG</td>
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<td>0.86802309</td>
<td>0.25</td>
<td>0.9091</td>
</tr>
<tr>
<td>TIME*DRUG</td>
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<td>68.95555556</td>
<td>45.59</td>
<td>0.0001</td>
</tr>
<tr>
<td>DNA<em>TIME</em>DRUG</td>
<td>8</td>
<td>1.34565657</td>
<td>0.89</td>
<td>0.5253</td>
</tr>
</tbody>
</table>

Dependent variable: Eosin

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>ANOVA SS</th>
<th>F Value</th>
<th>PR &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
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<td>0.01</td>
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<tr>
<td>DRUG</td>
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<td>3.59843639</td>
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<td>0.0024</td>
</tr>
<tr>
<td>DNA*DRUG</td>
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<td>0.00000000</td>
<td>0.00</td>
<td>1.0000</td>
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</table>