PCR BASED DETECTION OF THE PREIMPLANTATION EMBRYO DEVELOPMENT (Ped) GENE IN BIOPSIED MURINE PREIMPLANTATION EMBRYOS

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To my parents and Sharon.

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INTRODUCTION

The goal of this project was to utilize the techniques of preimplantation genetic diagnosis (PGD) to develop a polymerase chain reaction (PCR) based method of screening biopsied 8-cell mouse embryos for the presence of the preimplantation embryo development (Ped) gene. All previously described methods of analyzing embryos for the Ped gene and associated mRNA's result in the destruction of the embryo. PGD techniques allow the analysis of one or two cells from the embryo, allowing the embryo to remain viable and to continue to its development to the blastocyst stage. PGD methods can be used to screen embryos of many species for genes related to genetic diseases, disease resistance and susceptibility, production traits, and genetic markers of embryo quality like the Ped gene.

The Ped gene has been mapped to the Q region of the mouse major histocompatibility complex (MHC), also known as the H-2 complex. The effects of this gene include: increased rate of preimplantation embryo cleavage divisions, increased litter sizes, and higher birth weights. Two phenotypes of the Ped gene have been described as Ped fast and Ped slow, corresponding to fast or slow embryonic development respectively. These phenotypic differences can be seen in mouse strains from different H-2 haplotypes. Strains of the H-2b haplotype exhibit the Ped fast phenotype while strains of the H-2f and H-2k haplotypes exhibit the Ped slow phenotype.

Previous research has shown that there are extensive deletions within the Q region of H-2f and H-2k haplotype mice while mice of the H-2b haplotype exhibit all ten Q region genes. This suggests that genes in the Q region of the mouse MHC may be the Ped

gene. The Ped fast phenotype is also known to be associated with embryonic expression of the phosphatidylinositol (PI) linked Qa-2 antigen. It has been shown that removal of the Qa-2 antigen from the cell surface with PI phospholipase C (PI-PLC) or blocking the transcription or expression of the Q7 and Q9 genes, which encode for this PI linked form of Qa-2, reduces the rate of embryonic development. This evidence suggests that the PI linked Qa-2 molecule may be the Ped gene product and that the genes which encode for this form of Qa-2 (Q7 and Q9) may individually or together be the Ped gene.

Many of the Q region genes have been sequenced. It is interesting to note that the sequences of the Q7 and Q9 genes are greater than 99.9% homologous. Transgenic studies have shown that the Q9 gene is definitely involved in modulating preimplantation development. Based on its homology to the Q9 gene, it is probable that the Q7 gene is also involved in modulating early embryonic development.

It is not known if other species have a similar genes controlling preimplantation embryo development, however, it is speculated that at least one domestic species (swine) and humans may have a gene or genes similar to the Ped gene of the mouse. Sequence comparisons suggest that the HLA-6.0 gene in humans and the PD6 gene in swine have characteristics similar to the mouse Q region genes.

The mechanism of action of the Ped gene is not yet understood, however, it may be related to mechanisms shared by other MHC encoded structures. Evidence suggests that Qa-2 antigen expression is increased by γ interferon and that anti-Qa-2 antibodies may cause a mitogenic signal acting through the phosphatidylinositol pathway. Further study is needed to advance our understanding of the Ped gene.

It is well known that the timing of mammalian preimplantation embryo development, in relation to uterine receptivity, is important to the eventual implantation of the embryo. It has been shown that preimplantation embryos not developing in synchrony

with uterine receptivity will not successfully implant in the uterus. No where is this more important than in embryo transfer. The precise timing of embryo transfer, based on the developmental stage of the embryo being transferred and the progression of uterine receptivity, is essential for a successful outcome. The correct timing for embryo transfer is important regardless of the species involved. Transferring a preimplantation embryo too early or too late into a recipient's uterus will normally result in implantation failure, regardless of the quality of the embryo. This phenomenon of transferring slowly developing embryos into an asynchronously developing uterus is extremely prevalent in human *in vitro* fertilization (IVF) and embryo transfer (ET).

The short term goal of this research is to develop a PCR based system of analyzing murine preimplantation embryos for the presence of the Ped gene. The Ped gene has been described as controlling the rate of preimplantation embryo development in certain strains of mice (among other biologic properties). It is postulated, that in most species, the more quickly developing embryo is the embryo which will survive. If this postulate is true, selection of embryos with the genetic potential for fast development could improve the reproductive success of the individual. Selection of fast developing embryos, for this reason, has application, not only in the mouse but in other animal species and even man. The long term goal of the project is to develop techniques of preimplantation genetic analysis that are applicable to other genes and within other species. Definite applications of this research can be seen in analysis of human preimplantation embryos for potential genetic abnormalities and in the analysis of preimplantation embryos of domestic species for sex selection and selection of production traits which currently have genetic markers.

CHAPTER I

REVIEW of LITERATURE

The Major Histocompatibility Complex

All mammalian species have been found to have major histocompatibility complexes. In the mouse the MHC , or H-2 complex, is found on chromosome 17. In man the MHC is located on chromosome 6 and in the bovine it is found on chromosome 23. It is well known that genes within the MHC play important roles in regulation and control of the immune response (Klein, 1986). These roles include, but are not limited to: transplantation (allograft rejection), cell-mediated lympholysis (CML), mixed lymphocyte reaction (MLR), disease resistance and susceptibility, and possibly even reproductive success (Klein et al., 1981).

Genes of the MHC have been placed into three categories, based on the proteins they code for: Class I, Class II, and Class III (Klein, 1979). Because the genes of interest in this study are "Class I-like", this review will limit its discussion to class I genes. Principal class I loci of different species have been given various names or designations. In the mouse, class I loci are referred to as; H-2K, H-2D, and H-2L (Klein, 1986). In the human class I loci are referred to as; HLA-A, HLA-B, and HLA-C (Bodmer et al., 1990). Other species have their own terminology for their respective class I loci.

Classical class I genes code for cell surface proteins which are expressed on almost every cell of the body (Klein, 1982). Classical Class I proteins are expressed on the cell surface as a transmembrane glycoprotein (Strominger, 1980). Products expressed by

Class I and Class II genes regulate immune responses based on the recognition of "self" and "non-self" antigens by cells of the immune system (Klein, 1986). One major role of Class I gene products in regulation of the immune response is the stimulation of T lymphocytes (Stites and Terr, 1991). Other roles for Class I gene products involve lysis of target cells by cytotoxic T cells, disease resistance and susceptibility, and mechanisms of allograft rejection which are thought to in be associated with maternal fetal interactions (Sachs, 1983).

Many species seem to have distinct similarities in the genetic structure of their MHC's. In most species the MHC is found on a relatively small region of a chromosome, usually less than 2.0 centimorgans (cM) in length (Lawlor et al., 1990). It has also been noted that there seems to be a large degree of conservation of genetic information found within the MHC of various species being studied. Recombination or rearrangement of genetic information within the MHC has seemed to cause differences in the order and number of homologous loci; however, the basic function of the MHC in regulation of the immune response has been mostly unchanged. Evidence for this "genetic rearrangement" of the MHC can be seen in the number of duplicated genes and gene pairs, in domestic species, the human, and the mouse (Giphart et al., 1990 and Devlin et al., 1985). Further evidence of this evolutionary conservation of genetic information in the MHC can be observed in the similarities of the linkage maps for this region. Because of the evolutionary conservation of genetic information seen in the MHC it may be possible to study one "model" species and learn much about the functions of various, possibly conserved, MHC genes in many species.

The Murine Major Histocompatibility Complex

The MHC or H-2 complex of the mouse is structured and functions in much the same way as has been previously described. The traditional view of the H-2 complex is as a chromosomal segment bounded by two loci originally chosen because their products were responsible for rapid rejection of foreign tissue grafts (Klein et al., 1981). This chromosomal segment of approximately 1 cM in length is located on chromosome 17 (Warner, 1986) and is broken down into four main regions; K, I, S, and D with the Q and TL regions being outside of the region bounded by K and D. The I region is further broken down into five subregions; I-A, I-B, I-C, I-J, and I-E (Klein, 1981).

It was believed that the genes classically associated with the functions of the MHC, mainly transplantation and immune response, were located between the K and D regions (Flaherty et al., 1990). The traditional view of the genetic organization of the H-2 complex was that each region or subregion controlled several traits and that each trait was controlled by a separate locus (Klein, 1981). These "immune response loci" (Ir) were mapped to the I and K regions of the H-2 complex. Two theories were formed to explain the structure and function of the murine H-2 complex. One theory describes the complex as a maze of regions, subregions, and loci coding for different traits. Each H-2 locus was then described as pleiomorphic in that it controls several functions which could include allograft rejection, CML, MLR, immune response, or immune suppression. The second theory suggests that the physiologic function of the H-2 complex is to guide T lymphocytes in their function of distinguishing "self" from "non-self", and that all other H-2 controlled traits are artificial derivatives of the basic function. These two theories are well reviewed by Klein and colleagues (Klein et al., 1981).

These traditional views of the murine H-2 complex were based on the idea that the H-2 complex was bounded by the K and D regions. Distal to the D region of chromosome 17; however, lie the Q and TL regions which contain genes which structurally and possibly

functionally resemble class I genes. The first TL region gene to be identified was named thymus leukemia antigen (TLa). Its gene product the TL molecule has many similarities to the H-2 molecule produced by classical class I genes (Davies et al., 1967). Ten years later the first two class I genes of the Q region were named; Qa-1 and Qa-2 (Stanton and Boyse, 1976 ; Flaherty, 1976). It is now known that there are at least 20 to 35 class I genes in these two regions (Steinmetz et al., 1982 , Weiss et al., 1984). Current data suggest that these genes may have functional significance (Warner et al., 1987a).

Presently, the most common view of the murine H-2 complex is that the H-2 codes for a large number of proteins which are involved in the immune response. These proteins include class I, class II, and class III molecules. Classical class I genes code for a family of structurally related glycoproteins having a molecular weight of between 39,000 and 45,000 daltons and which are noncovalently or covalently associated with a beta 2 (β-2) microglobulin subcomponent (Hood et al., 1983, Buskin et al., 1986). H-2D, H-2K, and H-2L are considered "classical" class I molecules or transplantation antigens. These molecules are present on a majority of cells in the adult mouse and play important roles in recognition and presentation of peptide antigens to T-cells (Hood et al., 1983). These transplantation antigens show extraordinary polymorphism of the amino acids constituting the antigen binding cleft. This polymorphism contributes to the ability of these molecules to bind and present foreign antigen (Klein, 1986, Hughes and Nei, 1988). Telomeric to the H-2D region are the Q and TLa regions of the H-2 complex. These regions contain between 12 and 20 loci with at least 10 loci lying in the Q region (Weiss, 1987). The functions of the gene products from these loci are not yet fully understood. The biochemical characteristics of many of them; however, are similar to that of the classical transplantation antigens, with a few interesting differences.

Differences of Q and TL Region Gene Products to Classical Class I Molecules

Most Q and TL region gene products share a noncovalent association with a β-2 microglobulin-like classical class I products. Some Q and TL region gene products; however, are of smaller molecular mass than classical molecules and there is little evidence of their participation in classical MHC regulated antigen presentation (Robinson, 1987a). Unlike classical class I products which are transmembrane molecules, some of the Q region gene products have been characterized as being expressed in one of three forms: secreted products, transmembrane proteins, and molecules anchored to the cell surface via phosphatidylinositol (PI) (Cosman et al., 1982, Soloski et al., 1986, Soloski et al., 1988a, Robinson et al., 1987b, Stiernberg et al., 1987). These molecules exhibit limited polymorphism when examined by serologic or sequence analysis (Tewarson et al., 1983, Mellor et al., 1984). Many of these molecules are expressed in hemopoietic cells in a limited number of tissues in adult mice as well as in the developing embryo (Table 1) (Flaherty et al., 1990, Warner et al., 1987b).

Mapping studies have uncovered another important difference between classical class I molecules and Q and TL region gene products. This difference is that the number of class I genes found in the Q and TL regions varies between different H-2 haplotypes. These strain specific differences are seen in the expression, or lack of expression, of Q and TL region gene products and are thought to be results of genetic deletions and fusions of these genes (Table 2) (Mellor et al., 1985, Weiss et al., 1984). Because of these deletions and fusions of genes the Q region of the mouse H-2 complex will differ based on a strain's H-2 haplotype. Because the genes of interest in this research lie in the Q region, further discussion will concentrate on this region of the murine H-2 complex.

Organization of the Q Region of the Murine H-2 Complex

The immunogenetics of the Q and TL regions of the mouse MHC has been well reviewed by Flaherty and colleagues (Flaherty et al., 1990). Four regions of the MHC are believed to be responsible for encoding the majority of the class I genes. These regions are the; K, D, Q, and TL. Most of the genes encoding class I molecules are located in the Q and TL regions, with a maximum of 8 to 10 class I genes being located in the Q region and 10 to 20 class I genes within the TL region (Stanton et al., 1976, Weiss et al., 1984, Winoto et al., 1983). Using genetic analysis of recombinant mouse strains and overlapping cosmid libraries of the various mouse strains and H-2 haplotypes investigators have been able to define the general organization of the H-2 complex. These studies have shown the order and arrangement of class I genes in relation to the centromere as well as variations in the number of class I genes found in the H-2 complexes of the different mouse strains (Weiss et al., 1984, Steinmetz et al., 1982, Muller et al., 1987). The C57BL/6 and C57BL/10 strains of mice, for instance, have ten class I genes in the Q region, the BALB/c strain has only eight Q region genes, and the CBA/Ca strain has only 6 to 7 Q region genes (Weiss et al., 1984). Some strains of mice, such as the A.CA and B10.M strains, are thought to have only one Q region gene.

Ten class I genes have been mapped to the Q region of the H-2 complex of the C57BL/10 mouse strain (Weiss et al., 1984). The standard nomenclature used to name these ten genes is the order of the genes, for the B10 haplotype, within the MHC of mouse (i.e. Q1, Q2, Q3, Q4, Q5, Q6, Q7, Q8, Q9, and Q10). For strains or haplotypes different than the B10 strain the rule is that any gene found to be homologous to a Q region gene in the B10 strain will be given the same name used for the homologous B10 gene followed by a superscript designating its own haplotype (i.e. $Q9^d$) (Flaherty et al., 1990). In cases of gene fusions the names contain the names of the genes that have combined (i.e. $Q8/9^d$).

Evolution of the Q Region of the Murine H-2 Complex

Evidence from restriction mapping, probe hybridization studies, and sequencing suggests that the ten Q region genes evolved from the duplication of an ancestral gene and the duplication of a gene pair (Devlin et al., 1985). The Q region genes show very little polymorphism. The genes Q4-Q10 are more closely related to each other than to any other class I gene, even the Q1-Q3 genes. Several theories attempt to explain the conservation of the Q region genes. The first theory suggests that the Q region genes may play an important role and that their conservation is due to selective pressure. The second theory attempts to explain the near identity of the Q7 and Q9 gene as a function of a lower than normal frequency of mutation within this particular chromosomal region. The third theory suggests that gene conversion in this region may act to homogenize the Q region genes (Devlin et al., 1985).

Work by O'Neill and colleagues questions the importance of the Q region genes. This research shows that while mice of the H-2^b haplotype have all 10 of the Q region genes, mice of the H-2^f and H-2^k haplotypes have extensive deletions within the Q region (O'Neill et al., 1986). In the case of the H-2^k haplotype mice, as exemplified by the CBA/Ca strain, there is a deletion within the Q region distal to the Q5 gene and proximal to the Q10 gene. This deletion was shown to remove Q region genes Q6-Q9; however a Q5/9 hybrid may be present (Watts et al., 1989). H-2^f haplotype mice have an even more extensive deletion that seems to remove all but the Q10 gene (O'Neill et al., 1986). Further investigation by Lew and colleagues have shown that in the case of H-2^f haplotype mice the Q10 gene is untranscribed and untranslated. This evidence suggests that these mice have no known functional class I Q region genes (Lew et al., 1986). Work by Goldbard and colleagues; however, suggests that the genetic differences between different H-2 haplotypes have an effect on preimplantation embryo development (Goldbard et al., 1982).

Structure and Homology of the Q Region Genes

The structure of the Q region genes has been found to be similar to other classical class I genes. The first exon codes for the signal peptide of the molecule. Exons 2, 3, and 4 code for the three extra cellular domains; alpha 1, 2, and 3 of the protein. The alpha 3 domain is the most conserved domain of the class I molecule. It is the alpha 3 domain which lies closest to the cell membrane and associates with the β-2 microglobulin. In the Q region genes; Q4, Q6, Q7, Q8, Q9, and Q10 exon five encodes a hydrophobic or transmembrane segment of variable length. In the Q region genes this segment is truncated, as compared to classical class I genes, due the presence of a stop codon in the fifth exon (Flaherty et al., 1990).

Classical class I genes show a high degree of polymorphism. This polymorphism is most prevalent in exons 2 and 3 where most classical class I genes are only 80% homologous. The other six exons of classical class I genes are >95% homologous (Devlin et al., 1985). Q region genes differ from classical class I genes because they show a low degree of polymorphism, even in their noncoding regions (Devlin et al., 1985, Elliott et al., 1989, Tine et al., 1991, Tewarson et al., 1983). Alleles at the H-2K or H-2D loci differ from one an other as much as 10 to 15%. Conversely, alleles of the Q region genes seldom differ by even as much as 1% (Flaherty et al., 1990). Sequence analysis of two Q7 gene alleles, $Q7^b$ and $Q7^d$, show that these two alleles are greater than 99% homologous (Devlin et al., 1985, Steinmetz et al., 1981).

Further sequence analysis has shown that many of the Q region genes share extreme homology. Homology is the strongest between the Q6-Q7 gene pair and the Q8-Q9 gene pair. It is believed that the Q8-Q9 gene pair arose by duplication of the Q6-Q7 gene pair, with Q6 being duplicated to form Q8 and Q7 being duplicated to form Q9. Further evidence for this duplication is shown by sequence analysis of the Q7 and Q9

genes which only differ by a single base substitution out of 959 bases in the first three exons (Devlin et al., 1985, Flaherty et al., 1990). This single base difference between Q7 and Q9 is found in exon 3 and causes a Gln residue in Q7 to be changed to a Glu residue in Q9. This base substitution also causes a Pst I restriction site to be present in Q7 that is not present in Q9 (Devlin et al., 1985, Jaulin et al., 1985, Soloski et al., 1988a). Extreme homology can also be seen between the Q7 and Q8 genes which are greater than 93% homologous with the Q6 gene being almost identical to the Q8 gene (Devlin et al., 1985, Elliott et al., 1989). Because of this extreme lack of polymorphism in both Q region gene alleles and the Q region genes themselves the only real variation in Q region genetics is the presence or absence of the genes themselves and their products (Flaherty et al., 1990). These variations seem to be due to the previously discussed genetic deletions, which seem to be based on the H-2 haplotypes of the various mouse strains (O'Neill et al., 1986).

Transcription and Expression of the Q Region Genes

Six of the ten Q region genes are thought to be transcriptionally active. Transfection experiments with Q1 and Q2 did not show the production of any intra or extra cellular proteins distinguishable with antibodies to either β-2 microglobulin or the Qa-2 molecule (Mellor et al., 1985). Sequencing of these genes by Watts and co-workers determined that these genes are in fact intact; however, no product has been defined (Watts et al., 1989). The Q3 gene has been shown by Weiss and colleagues to be structurally nonfunctional (Weiss et al., 1984). The Q4 gene encodes a secreted product termed Qb-1 which is present in a large number of tissues (Robinson, 1985, Robinson et al., 1988). Q5 is not thought to be transcribed, based on a series of deletions described by Robinson and colleagues which would stop translation at amino acid position 164 (Robinson et al., 1988). The Q10 gene has been shown to be actively transcribed and

translated to Q10 protein in the liver tissue of adult mice (Cosman et al., 1982, Kress et al., 1983).

The remaining four Q region genes; Q6, Q7, Q8, and Q9 have all been shown to be actively transcribed into products which react with anti-Qa-2 antibodies. These genes produce Qa-2 molecules which may range in size from 35 kilodaltons (kDa) to 40-kDa (Mellor et al., 1985, Robinson et al., 1987a). Further studies suggest that all four genes; Q6-Q9, encode for a form of the Qa-2 molecule that is expressed on the cell surface (Flaherty et al., 1985, Stroynowski et al., 1987, Mann and Forman, 1988, Widacki and Cook 1989). Research by Waneck and colleagues suggests that the Q8 gene product has a valine at position 295 instead of the aspartate residue seen in the Q7 and Q9 gene product (Waneck et al., 1988). This aspartate residue is necessary for linkage to a phosphatidylinositol (PI). It was concluded by Waneck and colleagues that the form of Qa-2 encoded by the Q7 and Q9 genes was the PI-linked form (Waneck et al., 1988). This conclusion was further supported by Mann and Forman's work describing the insensitivity of a Q6/Q8 encoded protein to PI phospholipase C (PI-PLC) (Mann and Forman, 1988).

It is the Qa-2 molecule, encoded by the Q7 and Q9 genes, that Warner and co-workers showed to be expressed on the preimplantation mouse embryo from the two-cell through the blastocyst stage of development (Warner et al., 1987b). The presence of the molecule has been shown to be associated with improved preimplantation embryo development (Tian et al., 1992).

Regulation of Q Region Gene Expression

There have been four regulatory sequences identified in the promoter regions of H-2 class I genes which are also shared by the Q region genes. These sequences include two enhancer motifs, designated A and B (Kimura et al., 1986, Vogel et al., 1986). The other

two regulatory sequences include the; IRS or interferon response sequence, which allows class I genes to react to type I and II interferons and the CRE or class I regulatory element which acts to enhance promoter activity in certain types of cells (Vogel et al., 1986, Korber et al., 1987, Sugita et al., 1987, Korber et al., 1988). Work by Robinson and colleagues showed that the expression of the Q4 gene product, Qb-1, increased two fold when cells were stimulated with gamma interferon (γ-IFN) (Robinson et al., 1988). Work by Warner and colleagues suggests that expression of Qa-2 antigen by mouse embryos may be increased by treatment with γ-IFN (Warner et al., 1993).

Cellular function and Qa-2 expression may also be regulated in other ways. Different alleles of Q region genes coding for the Qa-2 molecule may express the molecule at different quantitative levels. Work by Soloski and co-workers suggests that the quantitative variation in Qa-2 antigen expression may be due to either quantitative variation of expression of individual alleles at the transcriptional or post transcriptional level or a gene dosage effect where more than one gene (i.e. $Q7^b$ and $Q9^b$) are encoding for the same Qa-2 molecule (Soloski et al., 1988a). These varying genotypes based on the H-2 haplotype can produce varying phenotypes of Qa-2^{hi} (H-2^b) and Qa-2^{low} (H-2^d) for high or low Qa-2 antigen expression respectively. Studies by Kimura and colleagues suggest that this allelic variation may actually be due to differences in promoter regions of the various Qa-2 encoding alleles (Kimuar et al., 1986). PI-linked Qa-2 may also contribute to cellular proliferation. Work by Gunter and colleagues shows that antibodies to PI-linked antigen sometimes have mitogenic properties (Gunter et al., 1984). This phenomenon of cell proliferation has been shown with Qa-2^+ cell types (Hahn and Soloski, 1989). It is theorized that this mitogenic phenomenon may have something to do with the increased rate of embryonic development seen with Qa-2^+ embryos; however, the mechanism remains unknown.

Biochemistry of the Qa-2 Molecule

The Qa-2 molecule was first described serologically using antiserum developed in Qa-2$^-$ mice against spleen and lymph node cells of Qa-2$^+$ mice (Flaherty, 1976). Serologically the Qa-2 molecule is defined as a specificity mapping to the Q region, of the H-2 complex. This specificity has a tissue distribution characterized by Qa-2 antigen expression by the C57BL/6 (Qa-2$^+$) mouse strain. This specificity can be determined using anti Qa-2 antibodies developed in the B6.K1 (Qa-2$^-$) strain (Flaherty et al., 1990). Linkage studies and molecular genetic techniques have been used to further define the Qa-2 locus as being within the Q region, encompassing the Q6-Q9 genes (Flaherty et al., 1985, Mellor et al., 1985). Expression of the Qa-2 molecule is based on the presence of these genes which in turn is related to the H-2 haplotype of a specific mouse strain.

The Qa-2 molecule has been described as a 34 to 40-kDa molecule which is found to be expressed in a limited number of tissues (Table 1) (Soloski et al., 1986, Soloski et al., 1988a, Flaherty et al., 1990). This molecule is different from classical class I molecules in that it is smaller, has a truncated cytoplasmic domain, and has a different glycosylation pattern (Flaherty et al., 199). Research by Mellor and colleagues has also shown that the Qa-2 molecule is different in that three forms of the molecule have been identified; membrane bound, secreted, and PI-linked (Mellor et al., 1985). Soloski and colleagues found that the PI-PLC sensitive Qa-2 has a molecular weight of 40-kDa while the secreted or soluble form of the molecule has a molecular weight of 39-kDa. This work also showed that the PI-linked form of Qa-2 can be removed with exogenous PI-PLC (Soloski et al., 1988b).

Another theory of how Qa-2 molecules are secreted is supported by Robinson. This theory premises that all Qa-2 molecules are first expressed on the cell surface, some of these molecules are then processed by endogenous lipases which reduce the molecular weight of the molecule by 1 to 2-kDa and cleave the molecule from the cell surface

(Robinson, 1987b). This theory is somewhat refuted; however, by the findings of Waneck and colleagues who showed that sequence differences between the Qa-2 encoding genes of the Q region, especially the Q6-Q8 and Q7-Q9 gene pairs, encode amino acid differences in the respective proteins which control surface attachment of the Qa-2 molecule (Waneck et al., 1988).

Transfection experiments by Soloski and co-workers further supported the findings of Waneck and colleagues. In these experiments the transfected Q7 and Q9 genes were shown to encode an almost identical PI-linked protein which could be removed from the cell surface with PI-PLC. These experiments continued to show these Qa-2 molecules as serologically indistinguishable. Isoelectric focusing; however, showed the Q7 gene product to have a unique basic species and the Q9 gene product to have a unique acidic species (Soloski et al., 1988a). It is the $Q7^b$ and $Q9^b$ genes of the $H\text{-}2^b$ haplotype C57BL/6 mouse that have been shown to encode for the PI-linked form of the Qa-2 molecule. The simultaneous expression of these two genes is what gives this strain of mouse its $Qa\text{-}2^{hi}$ phenotype (Soloski et al., 1988a).

Role of the H-2 Complex in Murine Preimplantation Embryo Development

Research by McLaren and Bowman described "fast" and "slow" developing strains of mice, in terms of their embryonic development, which seemed to be related to their H-2 haplotype (McLaren and Bowman, 1973). These studies lead Verbanac and Warner to hypothesize that genes of the H-2 complex may play a role in early embryonic development. Preimplantation mouse embryos of the $H\text{-}2^k$ haplotype were shown to develop more slowly, from fertilization to blastocyst, and result in fewer cells within the blastocyst than embryos of the $H\text{-}2^b$ haplotype (Verbanac and Warner, 1981). Embryos of the slower developing strains tend to reach the morula stage of development in the same amount of time it takes the quickly developing embryos to become blastocysts. The

cosegregation of the different developmental phenotypes (fast and slow) with the expression of H-2^b and H-2^k antigens lead Verbanac and Warner to describe the H-2 linked preimplantation embryo development (Ped) gene. This H-2 linked gene(s) was thought to influence the timing of early mouse embryo development (Verbanac and Warner, 1981).

Congenic strains of mice that differed only in the genes of the Q region were then used to investigate the importance the Q region genes in regulating preimplantation embryo development and to investigate the existence of the Ped gene. These findings again suggested that mouse embryos of the H-2^k haplotype develop more slowly and have fewer cells in their blastocyts, possibly due to a later first cleavage division and slower subsequent cleavage divisions (Table 3) (Goldbard et al., 1982a). To investigate the genetic transmission of the gene(s) Goldbard and colleagues did a series of F1, F2, and back crosses. The findings from these studies suggest that the H-2 gene(s) involved were, in fact, dominantly expressed (Table 4) (Goldbard et al., 1982b). Other findings by Goldbard and Warner suggest that H-2 haplotypes of congenic mouse strains, other than the b and k haplotypes, also show variations in their rate of embryonic development (Table 3). These variations can be best seen at certain optimal times during preimplantation development (Goldbard and Warner, 1982). The variations associated with the Ped gene are due to the timing of the first cleavage division and subsequent divisions rather than being due to the timing of ovulation and fertilization, which do not seem to be affected by the Ped gene (Goldbard and Warner, 1982). By linkage analysis and comparative genetic mapping of the H-2 complexes of the congenic mouse strains being studied Warner and co-workers were able to map the Ped gene to the Qa-2 locus of the mouse MHC (Warner, 1986).

Biological Properties of the Ped Gene

Two functional alleles of the Ped gene are thought to exist: Ped gene fast and Ped gene slow based on fast or slow embryonic development respectively. The phenotypes, associated with these alleles, have been shown to be expressed both *in vivo* and *in vitro*. Because these phenotypes are expressed *in vitro* as well as *in vivo* it is thought that Ped gene expression is an intrinsic property of the embryo and not dependent on the uterine environment (Brownell and Warner, 1988). Biological properties, other than the rate of cleavage divisions, are also thought to be under the Ped gene's control. These properties are all probably related to the rate of development. They include; embryonic survival, litter size, birth weights, and weaning weights. Other properties, which are not thought to be under the control of the Ped gene, have also been described. These properties include; time of ovulation, time of mating, and time of fertilization (Table 5) (Warner, 1986, Jin et al., 1992). The mechanism of action of the Ped gene is not yet understood; however, the identity of the Ped gene and its product are beginning to become clear.

Identification of the Ped Gene

Further identification of the Ped gene necessitated using congenic strains of mice that differed only within the Q region of the H-2 complex. The Q region of the mouse MHC is a likely location of the Ped gene. This is due to the fact that mouse embryos of the H-2^k and H-2^f haplotypes exhibit the Ped "slow" phenotype (Goldbard and Warner, 1982, Goldbard et al., 1982a, Goldbard et al., 1982b, and Verbanac and Warner, 1981). The Q region of these two haplotypes (H-2^k and H-2^f) has been shown to have extensive deletions of many of the Q region genes (O'Neill et al., 1986). Mouse embryos of H-2 haplotypes with all ten Q region genes intact (i.e. H-2^b) exhibit the Ped "fast" phenotype (Goldbard and Warner, 1982, Goldbard et al., 1982a, Goldbard et al., 1982b, and Verbanac and Warner, 1981).

The sequences of many of the Q region genes have been elucidated using molecular genetic techniques (Devlin et al., 1985, Soloski et al., 1988a, Elliott et al., 1989, and Watts et al., 1989). Comparative sequence analysis shows these genes to be extremely homologous, especially the Q6, Q7, Q8, and Q9 genes. Numerous studies have shown that these genes all encode for proteins which react with anti-Qa-2 antiserum (Waneck et al., 1987, Waneck et al., 1988, Soloski et al., 1988a, Stroynowski et al., 1987, and Elliott et al., 1989). Warner and colleagues utilized the B6.K1 and B6.K2 strains of mice, which differ only in the Q region of the H-2 complex, to find that the fast Ped gene phenotype was seen in concordance with Qa-2 antigen expression by the embryo. Embryos not expressing the Qa-2 molecule exhibit the slow Ped gene phenotype (Warner et al., 1991). These findings lead to speculation that the Qa-2 molecule may be the Ped gene product and that the Ped gene may be one, some, or all of the Q6-Q9 genes which encode the Qa-2 antigen (Warner et al., 1991).

Ped Gene Product the Qa-2 Antigen

Studies by Tian and colleagues further supported the findings that Ped fast embryos express the Qa-2 antigen while Ped slow embryos do not. These studies also showed that Qa-2 antigen being expressed by these embryos was sensitive to cleavage with PI-PLC. The most suprising finding of the studies by Tian and co-workers was that when the Qa-2 molecule being expressed by Ped fast embryos was removed with PI-PLC the embryonic development rate slowed down in comparison to non-treated controls. Ped slow embryos similarly treated with PI-PLC did not show any significant reduction in their rate of development (Table 6) (Tian et al., 1992). These findings again suggested that the Qa-2 antigen was in fact the Ped gene product.

Jin and co-workers investigated the expression of class I genes in the embryo. Slot blot hybridization analysis of total class I RNA showed that class I genes are expressed in

increasing levels from the oocyte to the blastocyst stage of preimplantation development. Jin and colleagues also used reverse transcription polymerase chain reaction (RT-PCR) to investigate the transcription of the Q7 and Q9 genes. The Q7 and Q9 genes were selected as likely candidates because they are known to encode for the PI-linked form of the Qa-2 molecule (Waneck et al., 1988). Results of the RT-PCR studies showed that the Q7 and Q9 genes are expressed at the two cell stage through the blasocyst stage of preimplantation development. By crossing Ped fast phenotype (C57BL/6) males with Ped slow phenotype (CBA) females Jin and co-workers were able to show that the Q7 and Q9 genes are not expressed in zygotes but start at the two cell stage when the embryonic genome is activated (Jin et al., 1992).

To investigate the effect of blocking the expression of the Q7 and Q9 genes on Qa-2 antigen expression and subsequent preimplantation embryo development Xu and colleagues designed antisense oligonucleotides specific to the Q7 and Q9 genes. These antisense oligonucleotides were hypothesized to block transcription and or translation of the Q7 and Q9 genes; however, the mechanism by which this is accomplished is not clear. Results of these studies showed that treatment of Ped fast embryos with the Q7/9 antisense oligonucleotides did reduce Qa-2 expression by preimplantation mouse embryos. The rate of embryonic development was also slowed (Table 7) (Xu and Warner, 1992). Expression of other class I genes was shown to be unaffected by the Q7/9 antisense treatment. Embryos treated with Q7/9 sense oligonucleotides showed Qa-2 expression levels and developmental rates consistent with non-treated controls. Ped slow embryos treated with sense or antisense oligonucleotides showed no effects (Xu and Warner, 1992, Xu et al., 1993). The results from these studies support the theory that the Q7 and/or Q9 gene(s), which encode the PI-linked Qa-2 molecule, may be the Ped gene. By blocking transcription of the Q7 and Q9 genes or the subsequent translation of Q7/Q9 RNA into

Qa-2 antigen, the Ped fast phenotypic traits can be changed to those more closely resembling the Ped slow phenotype.

Transgenic Mouse Embryos With the Ped Fast Phenotype

Recent experiments by Xu and co-workers have continued to work toward identification of the Ped gene at the molecular level. In one study transgenic mice were produced by Xu and colleagues by injecting the $Q9^b$ transgene from the C57BL/6 (Ped fast) strain of mouse directly into fertilized oocytes (one cell zygote) of the CBA/Ca (Ped slow) strain which lack Q region genes Q6-Q9 (Robinson et al., 1989 and Mellor et al., 1991). The resulting transgenic embryos were evaluated for rate of preimplantation embryo development and expression of the PI-linked Qa-2 antigen encoded by the Q9 gene. The results of this study showed that the transgenic embryos expressed the Qa-2 antigen and exhibited the Ped fast phenotype of embryonic development (Xu et al., 1994). Breeding studies involving the transgenic mice showed that the Q9 transgene was transmitted to progeny as would be normally expected in F1, F2, and back crosses, with both heterozygous $Q9^+/Q9^-$ and homozygous $Q9^+/Q9^+$ progeny exhibiting the Ped fast phenotype (Xu et al., 1994).

Comparing the preimplantation embryo development rates of the Q9 transgenic embryos to those produced by B6.K1 (Ped slow) x B6.K2 (Ped fast) crosses shows that the transgenic embryos do not develop quite as fast as their counterparts from the B6.K1 x B6.K2 crosses. This is most probably due to the lack, in the transgenic embryos, of other Qa-2 encoding genes (Q7 especially) which are present in the embryos from the B6.K1 x B6.K2 crosses (Xu et al., 1994). Although the results of this study suggest that the Q9 gene and its product are definitely involved in modulating the rate of preimplantation embryo development it is still unclear if the Q9 gene is acting alone or in concert with other Qa-2 encoding genes.

Ped Gene Mechanism of Action and Maternal Selection

The mechanism of action of the Ped gene is currently unknown. It is possible that the mitogenic properties of antibodies to PI-linked molecules such as the Qa-2 molecule, as reported by Gunter and colleagues, may play a role in stimulating an increased rate of cleavage divisions in the embryo (Gunter et al., 1984). This mechanism may act through the phosphatidylinositol pathway much like as has been shown in other cell types which express the Qa-2 antigen (Hahn and Soloski, 1989).

There may also be an environmental or maternal component to the increased embryonic survival rate shown with Ped positive embryos. Gamma interferon (γ-IFN) has been shown to modulate Qa-2 antigen expression by preimplantation embryos. Treatment with γ-IFN increases Qa-2 expression and increases the rate of development of C57BL/6 mouse embryos (Warner et al., 1993). This effect could be caused by the IRS or interferon response sequence found in the 5' flanking region of the genes encoding Qa-2 (Vogel et al., 1986, Korber et al., 1987, Sugita et al., 1987, Korber et al., 1988).

Maternal selection for embryos expressing the Ped gene product (Qa-2 antigen) has also been shown. Studies by Exley and colleagues crossed Ped fast heterozygotes with Ped slow homozygotes. The embryos resulting from the matings were collected 89-96 hours after the matings. These embryos were expected to be 50% Ped positive and 50% Ped negative. The embryos were analyzed via PCR for the presence of the Q7 and Q9 genes. Surprisingly 72% of the embryos were found to have the Q7 and Q9 genes (Ped^+) and only 28% were Ped^- (Exley and Warner, 1995). These findings suggest a maternal selection based on Qa-2 antigen expression during early embryonic development.

Ped Gene in Other Species

There are no known genes in any species other than the mouse that have been identified as modulating the rate of embryonic development. However, varying rates of

preimplantation embryo development in many species, including swine and humans, suggest that such genes may exist (Buster et al., 1985, Bolton et al., 1989, Pope et al., 1986, Ford et al., 1988, and Pusateri et al., 1990). In swine, embryonic development and litter sizes have been shown to be related to SLA haplotypes (Bazer et al., 1988, Conley et al., 1988, and Ford et al., 1988). In the human, different rates of preimplantation embryo development have been reported by *in vitro* fertilization (IVF) programs (Cummins et al., 1986 and Bolton et al., 1989). Human embryos developing at the highest rates have been reported to have the best chance for pregnancy in IVF (Claman et al., 1987, Puissant et al., 1987, and Levy et al., 1991).

Molecular genetic studies suggest that genes similar to the Q region genes of the mouse also exist in the human and the pig. The HLA-6.0 gene of the human is a non-classical class I gene which may encode for the human equivalent of the Qa molecule. The HLA-6.0 gene has been classified by Geraghty and co-workers. The promoter region of HLA-6.0 more closely resembles the mouse Q region gene promoter regions than those of any classical human class I gene. HLA-6.0 also has premature termination codon in exon 6 which truncates the cytoplasmic domain of its product much like the Q region genes of the mouse. In structure the HLA-6.0 gene most closely resembles the Q7 gene; however, it is not known if the HLA-6.0 gene product is attached to the cell surface via PI (Geraghty et al., 1987). Expression of the HLA-6.0 transgene results in the expression of a surface class I molecule that is smaller than classical class I molecules, much like the Qa-2 antigen (Shimizu et al., 1988).

The PD6 gene in swine also shares some of the same characteristics as the mouse Q region genes. The PD6 gene is only 55% homologous to the other classical class I genes of the swine. The gene also has a premature termination codon (exon 7) like the mouse Q region genes and the human HLA-6.0 gene; however, the size and structure of the gene is similar to other classical class I of the pig. PD6 seems to share the IRS found

in its 5' flanking region with the mouse Q region genes. Transcription of PD6 increases with α and β interferon treatment. The PD6 gene product is also seen in a limited distribution of tissues, like the Q region gene products (Ehrlich et al., 1987 and Singer et al., 1987).

Preimplantation Genetic Diagnosis

Preimplantation embryo manipulation has become common place for many domestic and laboratory species. With the advent of human IVF, human embryos are also now manipulated. Preimplantation genetic diagnosis (PGD) is a branch of the field of assisted reproductive technology (ART). PGD is being used to diagnose genetic abnormalities in the preimplantation embryos of many species, including the human. The same techniques utilized to diagnose or recognize genetic disorders carried by an embryo can also be used to classify the presence or absence of other genes of interest. The basis for PGD involves removing a small number of cells from a preimplantation embryo for analysis while the rest of the embryo remains viable for a future transfer. As more is learned about the genomes of the various species PGD will become a more and more useful technique.

Preimplantation Embryo Biopsy Techniques

Preimplantation biopsy is the removal of a small number of cells for analysis, from a preimplantation embryo. To be successful the cells must be removed in such a way as to leave the embryo viable. The first successful PGD technique was accomplished by Gardner and Edwards in 1968. Rabbit blastocysts were sexed by removal of a piece of the trophoblast, the sexed embryos were then transferred to recipient does and the diagnosis of sex confirmed in the offspring (Gardner and Edwards, 1968). These same techniques are still in use today in attempts to sex blastocysts from many domestic species.

Currently, the embryo biopsy techniques being developed involve the removal of one or two blastomeres from a four to eight cell embryo. Mouse embryo model systems have been used to develop the techniques for these procedures. These microsurgical procedures involve making a hole in the zona pellucida of the embryo either by mechanical (microneedle) or chemical (acid Tyrode's solution) means and then removing one or more blastomeres. The blastomeres can either be removed by extrusion, or placing pressure on the zona to squeeze the blastomere out or the blastomere can be aspirated out of the zona with a micropipette with approximately the same diameter as the blastomere (Roudebush et al., 1990, Tarin and Handyside, 1993). These techniques allow one or two blastomeres to be removed from four or eight cell embryos respectively with greater than 90% of the biopsied embryos developing to the blastocyst stage (Roudebush et al., 1990 and Takeuchi et al., 1992). The most commonly used procedure for biopsying murine eight cell embryos has been describe by Godron and Gang. The embryo is held in place by a holding pipette while a hole is "drilled" in the zona pellucida with acid Tyrode's solution. A micropipette with the approximate diameter of the blastomeres is pushed through the hole and one or two blastomeres are aspirated into the pipette. These cells are then used for genetic analysis. It is reported that greater than 94% of murine 8-cell embryos biopsied in this manner will survive to reach the blastocyst stage (Gordon and Gang, 1990). The 8-cell stage seems to be the optimal time to biopsy both murine and human embryos (Tarin and Handyside, 1993). Embryo biopsy can also be performed on preimplantation embryos of other species. Later embryonic stages require different biopsy techniques.

Genetic Analysis of Single Cells

Once cells have been removed from an embryo they can be analyzed for the genes of interest. There are several ways to accomplish this genetic analysis. All but DNA (deoxyribonucleic acid) amplification using PCR are outside the scope of this project and

will not be discussed. PCR has opened a new fronteir in genetic analysis. Nowhere is this technique more important than in the genetic analysis of single cells, where the amount of DNA available for starting the analysis is severely limited (Arnheim et al., 1990). The PCR technique allows specific DNA sequences from a single cell to be amplified by as many as a billion times (Saiki et al., 1988, Navidi and Arnheim, 1991).

PCR is facilitated by the use of DNA polymerase from Thermis aquaticus (Taq) a thermophilic bacterium which lives at temperature exceeding 95^{o}C. PCR requires thermal cycling through three basic temperature steps. The first step is a heating step (94^{o}C) to cause denaturation of the double stranded DNA into two single strands. The second step allows annealing (37-65^{o}C) of primers, 15 to 30 base pair (bp) single strands of DNA, specific for segments at the ends of the sequence which is to be amplified. The third step is the primer extension step (72^{o}C) where the Taq polymerase attaches to the complex of the primer and single stranded template. The polymerase enzyme then extends or "builds" new DNA complimentary to the DNA template in the 5' to 3' direction. As these steps are repeated in subsequent cycles there is a logarithmic increase in the number of amplified products bounded by the primers. At the end of 30 to 40 cycles the product of interest should be present in almost a billion copies. PCR products can then be analyzed using standard gel electrophoresis techniques.

Table 1. Tissue distribution of serologically defined Q and TL region antigens. (Flaherty et al., 1990).

Antigen	Positive Tissues	Positive Subpopulations
Qa-2	S, THY, LN, M,B, PEM, L, LEU	HSC, NK, T, B, E, M, G
Qa-3	S, LN	T, B, NK
Qat-4	S, LN	T, B, NK
Qat-5	S, LN	T, B, NK
Qa-6	S, LN	T
Qa-m7	S, THY, LN, B,M	HSC, T, B
Qa-m8	S, THY, LN, B,M	HSC, T, B
Qa-m9	S, THY, LN, B,M	HSC, T, B
TL	THY, LEU	ITHY
Qa-1	S, THY, LN, B,M	HSC, T, B, NK

Note: Abbreviations: S- spleen, THY- thymus, ITHY- immature thymocytes, LN- lymph node, PEM- preimplantation stage embryo, L- liver, LEU- leukemia, HSC- hematopoietic stem cell, T-T cell, B-Bcell, NK-natural killer cell, ER-erythrocyte, M- macrophage, G-granulocyte.

Table 2. Expression of Q and TL Region Antigens in Commonly Used Mouse Strains. (Flaherty et al., 1990).

Strain	H-2	Qa-2	Qa-3	Qat-4	Qat-5	Qa-6	Qa-m7	Qa-m8	Qa-m9	Qa-11	Tla	Qa-1
C57BL/6	b	+	+	+	+	+	+	+	+	+	-	b
BALB/cBy	d	-	-	-	-	-	-	-	-	-	c	b
BALB/cJ	d	+	+	+	-	+	*	*	*	*	c	b
B10.A	a	+	+	+	-	+	-	-	+	+	a	a
A	a	+	+	+	-	+	-	-	-	-	a	a
B6.K1	b	-	-	-	-	-	-	-	-	*	-	b
B6.K2	b	+	+	+	+	-	+	+	+	-	-	b
DBA/2	d	+	+	+	-	+	+	-	-	*	c	b
DBA/1	q	+	-	+	-	+	+	-	-	*	-	b
RIIIS/J[a]	r	-	-	-	-	-	-	-	-	-	-	c
A.CA[b]	f	-	-	-	-	-	-	-	-	-	c	c
AKR/J	k	-	-	-	-	-	-	-	-	-	-	b
A.SW	s	+	+	+	+	-	*	*	*	-	-	b

Note: A plus (+) indicates the presence of the antigenic specificity; a minus (-) indicates its absence; a asterisk (*) indicates that the strain is not typed.

[a] In certain cases, haplotype expression is inferred from the typing of B10.RIII.

[b] Strain type is inferred from the typing of A strain.

Table 3. **Association of Slow Development with the H-2^k Haplotype. (Goldbard et al., 1982a, Warner, 1986)**

Mouse Strain	H-2 Haplotype	No. Embryos Scored	Mean Cell No. / Embryo (SE) 89 h post hCG[a]	Rate of Development
A	a	54	32.9 (1.5)	Fast
C57BL/10Sn	b	48	33.1 (2.1)	Fast
DBA/2	d	53	30.5 (1.4)	Fast
CBA	k	45	18.9 (1.0)[b]	Slow
C3H/He	k	40	24.3 (0.4)[b]	Slow
C57BR/cdj	k	55	18.7 (1.0)[b]	Slow
DBA/1	q	47	30.2 (1.5)	Fast
SJL	s	48	31.9 (2.0)	Fast

[a] Embryos were collected 89 h post-hCG injection and the mean cell no. / embryo determined by the method of Tarkowski (1966).

[b] Significantly lower than the other inbred strains at $P<0.01$ level.

Table 4. **Analysis of F1 Crosses.**
(Goldbard et al., 1982b)

Source of Embryos	Mean Cell no. / Embryo (SE)	P*
C57BL/10Sn	33.1 (2.1)	-
B10.BR	22.8 (1.3)	<0.001
F1 Cross 1	29.7 (1.6)	NS
F1 Cross 2	28.6 (1.2)	NS

* Comparison to C57BL/10Sn by Student's t-test.

NS = Not Significant

Table 5. Biological Properties of the Ped Gene[a]. (Jin et al., 1992)

Parameters Measured	Effect of the Ped Gene
Time of ovulation	No
Time of mating	No
Time of fertilization	No
Time of first cleavage division	Yes
Rate of *in vivo* cleavage	Yes
Rate of *in vitro* cleavage	Yes
Time of implantation	Unknown
Gestation time	Unknown
Embryo survival	Yes
Size of litters	Yes
Weight at birth	Yes
Weight at weaning	Yes

[a] Based on Verbanac and Warner, 1981; Goldbard et al., 1982a; 1982b; Goldbard and Warner, 1982; Warner, 1986; Warner et al., 1987a; 1987b; 1988; 1991; Warner, 1987; Brownell and Warner, 1988.

Table 6. **Removal of PI-PLC-Sensitive Antigens Slows the Rate of Development of Preimplantation Mouse Embryos. (Tian et al., 1992)**

	Control		PI-PLC Treated		
Mouse Strain	**n**	**No. Cells/ embryo +/- SEM**	**n**	**No. Cells/ embryo +/- SEM**	**Significance**
C57BL/6	21	31.2 +/- 1.9	22	24.5 +/- 2.3	$p < 0.001$
CBA	14	20.0 +/- 2.7	12	21.6 +/- 3.4	$p > 0.10$
B6.K2	23	28.4 +/- 4.1	31	15.9 +/- 0.4	$p < 0.001$
B6.K1	20	19.6 +/- 3.1	14	19.6 +/- 1.5	$p > 0.10$

Table 7. Cleavage rate of preimplantation mouse embryos cultured with antisense and sense oligonucleotides to Q7/9 mRNA.[a] (Xu et al., 1993)

Mouse Strain	Oligos	n	Cells/Embryo +/- SEM	P Value
CBA/Ca	s-LP and s-α1	27	9.29 +/- 0.78	
CBA/Ca	a-LP and a-α1	27	9.88 +/- 0.78	NS
C57BL/6	s-Globin	41	15.66 +/- 0.60	
C57BL/6	a-Globin	36	16.07 +/- 0.38	NS
C57BL/6	s-LP	139	16.12 +/- 0.48	
C57BL/6	a-LP	148	14.42 +/- 0.45	< 0.05
C57BL/6	s-α1	96	16.21 +/- 0.66	
C57BL/6	a-α1	134	13.44 +/- 0.40	<0.001

[a] Two-cell embryos were cultured for 48 h in the presence of 7μM oligonucleotides, the embryos fixed, and the nuclei counted (Goldbard et al., 1982a).

Oligonucleotides: s-sense, a-antisense, Globin-mouse hemoglobin β, LP-leader peptide of Qa-2, α1-α1 domain of Qa-2, n-number of embryos assayed, P Values from Student's t-test, NS-not significant.

CHAPTER II

MATERIALS and METHODS

Study Design

This project was designed to investigate two problems. First the effect on continued embryonic development of biopsying preimplantation embryos from two different genetic strains (CBA/Caj and C57BL/6) known to have different Ped gene genotypes. Second the ability of a PCR based analysis system to determine the correct genotype off an embryo from only two biopsied cells. The first problem was investigated by randomly separating embryos from each strain into either the control (non-biopsy) group or into the "treatment" group to be biopsied. The number and percentage of embryos from each group reaching the blastocyst stage was then observed. A blind study was designed to investigate the second question. Two cells removed via biopsy from embryos of each strain were placed in coded sample tubes. The genotypes of the cells in the sample tubes were unknown to the investigator until the samples had been processed and analyzed with the PCR based system. The number and percentage of correctly identified genotypes for all of the embryos analyzed and for biopsied embryos from each strain were determined and compared to evaluate the effectiveness of the PCR based system.

Mouse Embryos

Frozen eight cell mouse embryos from two different strains were purchased from Embryotech Laboratories, Wilmington, MA (Plate I). Embryos of the C57BL/6 strain

were selected due to their fast embryonic development. Embryos of the CBA/Caj strain were selected for use based on their slow embryonic development. These strains have been previously shown to be of the Ped fast and Ped slow phenotype respectively (Xu and Warner, 1992, Xu et al., 1993). Previous research has also shown that the C57BL/6 strain, having the H-2^b haplotype, has all ten Q region genes and expresses the Q7 and Q9 genes as a PI-linked form of the Qa-2 molecule (O'Neill et al., 1986, Jin et al., 1992, Tian et al., 1992). Conversely the CBA/Caj strain, having the H-2^k haplotype, has a deletion for the Q region genes Q6-Q9 and does not exhibit Qa-2 antigen expression (O'Neill et al., 1986, Jin et al., 1992, Tian et al., 1992). All mouse embryos were shipped to our laboratory on liquid nitrogen vapor and kept in liquid nitrogen at -196^o C until ready for use.

Embryo Thawing

The embryos were thawed following the procedures suggested by Embryotech Laboratories (Appendix A). The straws containing the embryos were first exposed to room temperature (20 to 24^{o}C) air for 2 minutes. The straws were then placed in a 37^{o}C water bath for 1 minute, removed from the water bath and dried. The straws were carefully cut at both ends and the contents of the straw pushed out into a Falcon tissue culture dish. The embryos were located under a steriomicroscope and transferred, using a finely drawn Pasteur pipette with mouth suction, into another Falcon tissue culture dish containing three 50 microliter (μl) drops of a hepes buffered synthetic human tubal fluid (modified HTF) medium (Irvine Scientific, Santa Ana, CA) at room temperature. The modified HTF medium was supplemented with 3% human serum albumin (HSA) (Irvine Scientific). The embryos were consecutively tranferred through the three modified HTF drops to dilute any residual freezing medium. After "washing" the embryos were transferred into a 250 μl drop of modified HTF medium, also at room temperature, where

they remained for a minimum of 10 minutes to rehydrate. After the 10 minute rehydration period the embryos were washed through three consecutive 50 µl drops of bicarbonate buffered HTF with 3% HSA (Irvine Scientific) under light mineral oil at 37°C, to remove any residual hepes buffer. The embryos were next placed in a Falcon tissue culture dish containing a 250 µl drop of HTF with 3% HSA under oil. These dishes were placed in a 5% CO_2 in air incubator at 37°C for 1 hour to allow the embryos to equilibrate and stabilize.

Embryo Biopsy and Culture

After one hour the 8-cell embryos of each individual strain were randomly sorted into either the biopsy or control group. The control embryos of each strain were cultured together in 250 µl drops of HTF with 3% HSA under oil in the 5% CO_2 in air incubator at 37°C. Embryos in the biopsy group were washed through three consecutive 250 µl drops of modified HTF with 3% HSA under oil at 37°C. The embryos were then each placed in individual 25 µl drops of modified HTF with 3% HSA under oil in a Falcon tissue culture dish, 5 drops per dish. The dishes containing the embryos to be biopsied were kept on a warming tray at 37°C until the biopsies were attempted.

Embryo biopsies were accomplished using the facilities of the Division of Reproductive Endocrinology and Infertility in the Department of Obstetrics and Gynecology at The Ohio State University. Embryo biopsy methods have been previously described by Tarin and Handyside (Tarin and Handyside, 1993) (Appendix B). The embryos to be biopsied were visualized using a Nikon Diaphot (Nikon, Melville, NY) inverted microscope. Embryo micromanipulation was accomplished with Narishige micromanipulators (Narishige USA, Greenvalie, NY). Micropipettes were made with borosilicate glass, on a Sutter pipette puller, (Sutter Instrument, Novato CA). They were of varying diameters, dependent on the tool being made. The embryo was held in place

via a holding pipette while a small hole was made in its zona pellucida with acid Tyrode's solution (Appendix C) (Plates II, III, and IV). Once the hole was made the embryo was moved within the medium droplet, away from any residual acid solution. Two blastomeres were removed from the embryo by inserting an aspiration micropipette, with approximately the same inner diameter as the blastomere, through the hole in the zona and pulling the blastomere out with gentle suction (Plates V, VI, and VII).

Biopsied blastomeres were transferred, with a finely drawn Pasteur pipette and mouth suction, into a 0.5 milliliter (ml) Eppendorf PCR tube containing 5 µl of a previously described potassium hydroxide lysis buffer (Appendix D) (Wu et al., 1993). The tubes containing the lysis buffer and biopsied cells were immediately centrifuged at 20 X G for 5 minutes to place the buffer and cells in the bottom of the tube. The tubes were then frozen at -20°C until genetic analysis could be performed.

The biopsied embryos of each individual strain were washed through three drops of HTF with 3% HSA and placed in a 250 µl drop of HTF with 3% HSA in a Falcon tissue culture dish. This dish was then placed in the 5% CO_2 in air incubator at 37°C to allow continued development of the embryos. Embryonic development was determined for both the control and biopsy group, as well as for each individual strain after 36 hours in culture. The percentage of embryos in each group reaching the blastocyst stage was recorded and compared for each group and mouse strain.

Blastomere Preparation for PCR

The method of preparing blastomeres for PCR has been previously described by Wu and colleagues (Wu et al., 1993). Tubes containing the lysis buffer and biopsied blastomeres were thawed and placed in a 65°C water bath for 10 minutes. After 10 minutes the tubes were removed and 5 µl of a neutralization solution was added to each tube (Appendix D). The contents of the tube were overlayed with one drop of sterile

molecular biology grade mineral oil. The tubes were then placed in a 97°C heat block for 15 minutes to denature the DNA strands. After 15 minutes the tubes were removed directly to ice to keep the DNA strands in their denatured state. The tubes were kept on ice while they were prepared for PCR.

Hemi-Nested Primers for the Q7 and Q9 Genes

Due to the homology of the Q region genes it was necessary to develop hemi-nested primers to amplify specific sequences of the Q7 and Q9 genes (Devlin et al., 1985). Previous research has shown that the C57BL/6 strain exhibits all of the 10 known Q region genes while the CBA/Caj strain of mouse has deletions for Q region genes Q6-Q9 (O'Neill et al., 1986). The deletion of the Q6-Q9 genes in the CBA/Caj mice made it possible to design primers which should amplify regions specific to the Q7 and Q9 genes in the C57BL/6 mice which are not present in the CBA/Caj mice. These primers were designed to be specific enough to amplify just DNA from the Q7 and Q9 genes and not from the other related class I genes which have known sequences and are known to be present in both the C57BL/6 and CBA/Caj mouse strains.

Primer design was facilitated by the Oligo primer selection program (National Biosciences, Inc., Plymouth, MN) (Rychlik and Rhoads, 1989). All primers were synthesized by Operon (Operon Technology, Inc., Alemeda, CA). Three primers were designed to allow maximum specificity for Q7 and Q9 amplification (Table 8). Two different forward primers and a common reverse primer where used to facilitate "hemi-nested" amplification. All three primers were designed to anneal specifically at locations where the Q7 and Q9 genes showed the highest degree of polymorphism to other known class I genes. Both hemi-nested amplifications amplify DNA surrounding the single sequence difference found between the Q7 and Q9 genes. This difference creates a Pst I restriction site in the Q7 gene that is not present in the Q9 gene (Devlin et al., 1985). This

restriction site variation was specifically incorporated into our hemi-nested PCR products. Additional mismatches near the 3' ends of the primers were incorporated to increase the specificity of amplification toward the Q7 and Q9 genes and away from other class I genes (Kwok et al., 1990, Ferrie et al., 1992).

The first set of hemi-nested primers amplifies a 491 base pair (bp) sequence fairly homologous within all of the Q region genes. This sequence begins toward the end of the third exon and continues into the third intron. The second set of primers amplifies a 91 bp sequence, specific to the Q7 and Q9 genes. This PCR product ends at the same location in the third intron as the first amplified product, due to the common reverse primer. It is the presence of this 91 bp product that denotes the presence of the Q7 and Q9 genes and thus the Ped positive genotype.

PCR Conditions for Hemi-Nested PCR

PCR and electrophoretic analysis of amplified products was carried out in the Animal Genetics Laboratory in the Department of Animal Sciences at The Ohio State University.

Both embryonic samples and control DNA from C57BL/6 and CBA/Caj mice were amplified via the hemi-nested method. To facilitate a blind analysis of the embryonic samples the tubes were coded so that the genotypes of the embryonic samples were unknown until the analysis was complete. Genotypes of the control DNA from each strain were known and were used as comparisons to the results of the embryonic amplifications

The first of the hemi-nested PCR reactions was carried out in a 30 µl reaction volume. The PCR "cocktail" mixture containing water, primers, 10X PCR buffer (USB, Cleveland, OH), and dNTP's (Perkin Elmer Cetus, Norwalk, CT) was added directly, through the overlaying mineral oil, to the tubes containing the denatured blastomeres. The concentrations for the various reactants were as follows: primer (set #1) 10 picomolar

(pM), 1.5 millimolar (mM) MgCl, 50 mM KCl, 10 mM Tris HCl (pH 8.3), and 20 micromolar (μM) of each dNTP. All tube were placed in a DNA thermal cycler for temperature cycling (Perkin Elmer Cetus). After a "hot start" of 94°C for 10 minutes 1 unit of *Taq* DNA polymerase (Perkin Elmer Cetus) was added to the reaction tubes. The samples were then subjected to 31 cycles with the following cycle parameters: denaturation 94°C for 45 sec., annealing 60°C for 1 min., and extension 72°C for 1 min.. The final cycle had an extra extension step of 72°C for 5 min.. Amplified samples were held at 4°C until further processing could be accomplished.

The second PCR step was accomplished with reaction conditions identical to the first, except for the use of the second primer set. The sample preparation prior to PCR was slightly different, however. Samples from the first PCR reaction were processed to concentrate the 491 bp PCR product and purify it by removing molecules less than 100 bp in length, including any extraneous products, excess primers, and dNTP's left over from the first PCR reaction. This was accomplished by subjecting 28 μl of the first PCR samples to chromatographic purification on a Chroma Spin 100 chromatography column (Clonetech Laboratories, Inc., Palo Alto, CA). Twenty one microliters of the purified products were then used for the second PCR reaction. The second PCR sample concentrations and volumes as well as cycle parameters and durations were identical to that of the first PCR except that the second primer set, which amplifies a 91 bp sequence of the Q7 and Q9 genes, was used.

Gel Electrophoresis

Hemi-nested PCR samples were prepared by mixing 16 μl of the sample with 2 μl of a formamide loading buffer (95% formamide, 0.5% bromophenol, and 0.5% xylene cyanol). A 6% acrylamide gel was prepared (Appendix E). The gel was placed on a vertical electrophoresis apparatus (Bio-Rad Life Science Group, Hercules, CA) with 1X

TBE electrophoresis buffer. The apparatus was attached to a power supply and the gel allowed to warm for approximately 60 minutes until it reached 50°C. Once the gel had reached the desired temperature, the sample well was flushed several times with 1X TBE buffer and a shark's tooth sample comb put in place. The previously prepared 18 µl samples were pipetted into individual sample wells. Four micorliters of a 100 bp DNA ladder (Gibco BRL, Gaithersburg, MD) were also pipetted into sample wells at each side of the gel to use as size references. Electrophoretic separation of the PCR products was continued for 2 hours (Appendix E). After 2 hours the gel was removed and placed in 40% methanol for at least 30 min. to prepare the gel for staining.

Detection of DNA Bands

DNA bands were detected on the gel by silver staining with the Bio-Rad silver staining kit (Bio-Rad Life Science Group) (Appendix F). Bands from the embryonic samples were compared to the 100 bp DNA ladder for size determination and compared to the bands seen in the lanes containing control DNA to determine their genotype.

Table 8. Hemi-nested primers for amplification of Q7 and Q9 specific sequences, primer sequences and product sizes.

Primer[a]	Sequence	Product (bp)
1st Set		
Q7Q9NEW.FP	5' GGAGCCTGAGGACCGCAAAG 3'	
Q7Q9.RP	5' GCAGCACCAGCTCCTGTGTG 3'	491
2nd Set		
Q7Q9.FP	5' AGGCATACCTGGAGGGCACG 3'	
Q7Q9.RP	5' GCAGCACCAGCTCCTGTGTG 3'	91

[a] Forward Primers (FP) and common Reverse Primer (RP).

Plate I. **Photograph of Murine 8 cell embryo taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. The indivdual cells and the zona pellucida of the embryo can be clearly identified.**

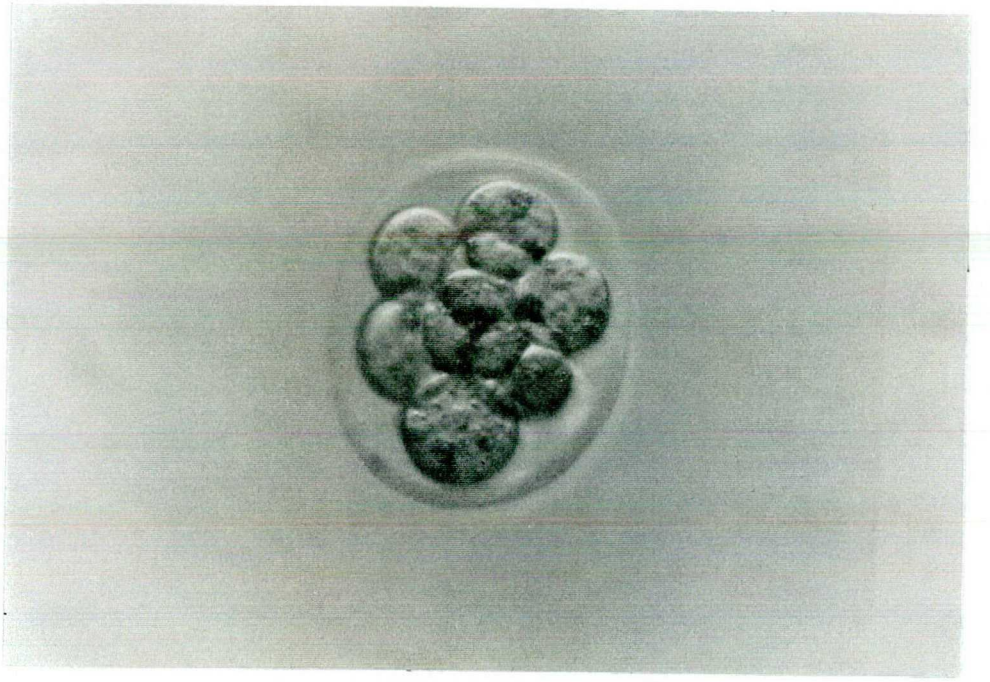


Plate II. **Photograph of a Murine 8 cell embryo taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. The embryo is being held in place with gentle suction through a holding pipette attached to the Narishige micromanipulators.**

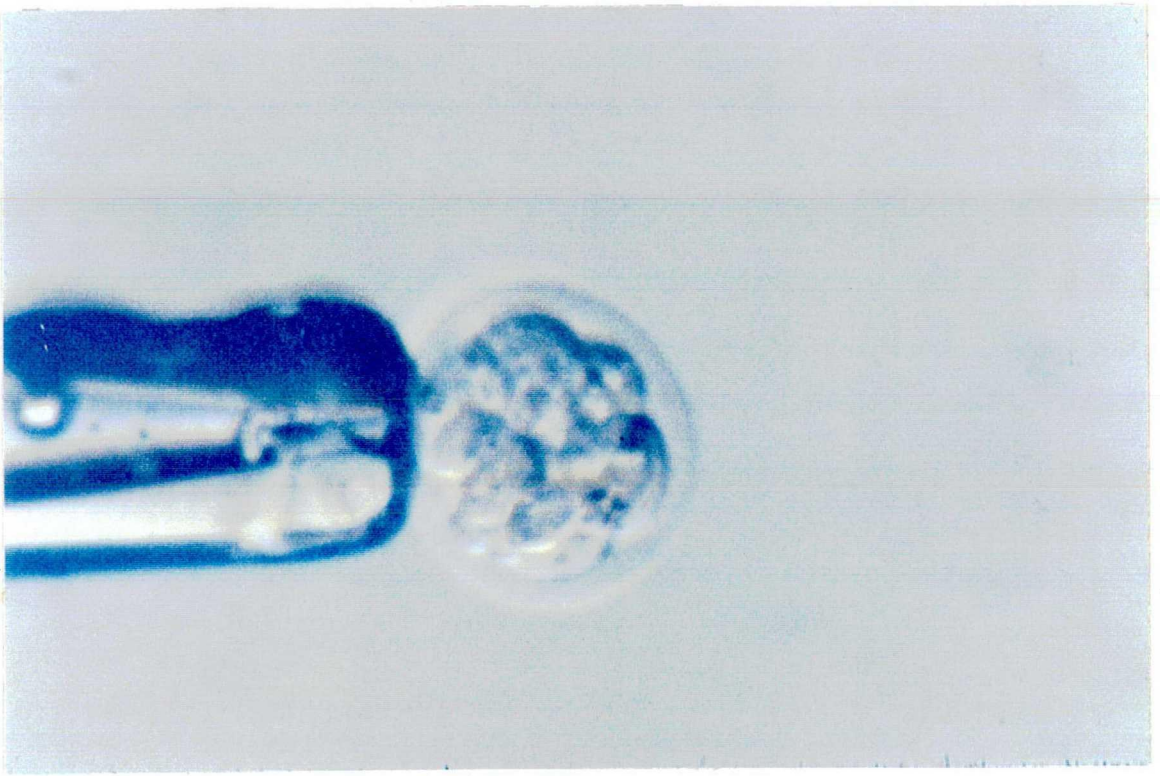


Plate III. **Photograph of a Murine 8 cell embryo being "zona drilled", taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. While the embryo is being held by the holding pipette (on left) a smaller microtool (Assisted hatching needle) (on right), attached to the right side of the micormanipulators and containing acid Tyrode's solution, is used to "drill" a small hole through the zona pellucida.**

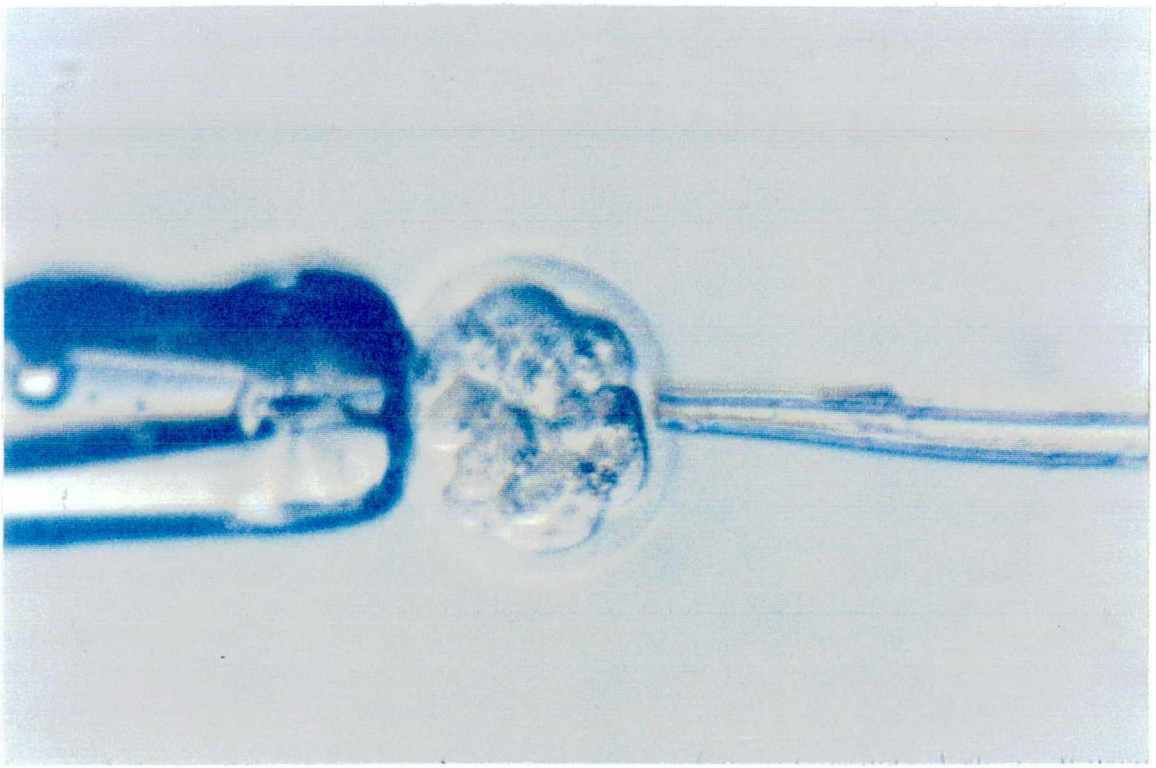


Plate IV. **Photograph of a Murine 8 cell embryo, after "zona drilling", taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. Once the hole has been made, one of the embryo's 8 cells or blastomeres can be seen protruding out through the gap in the zona pellucida.**

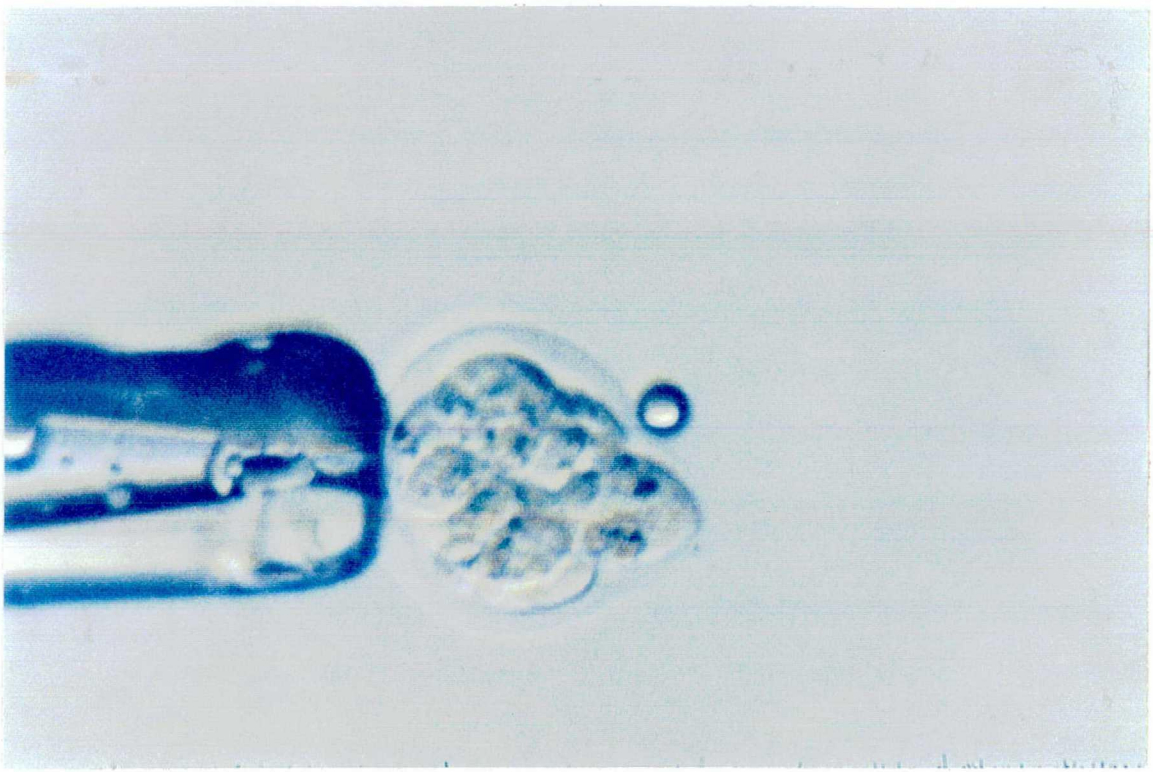


Plate V. **Photograph of a Murine 8 cell embryo, being biopsied, taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. Using the controls of the right side micromanipulator, to which it is attached, the micro aspiration pipette is carefully positioned against the cell to be removed.**

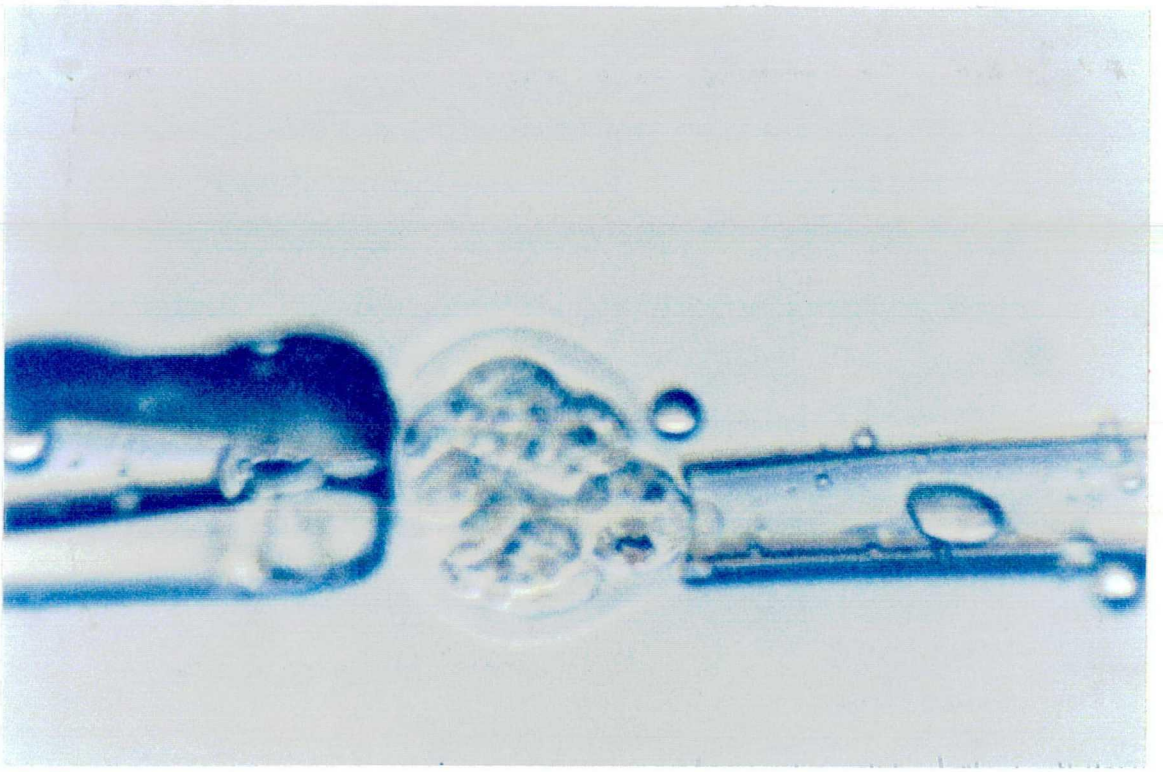


Plate VI. **Photograph of a Murine 8 cell embryo, being biopsied, taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. Gentle suction applied to the micro aspiration pipette can be seen to draw the blastomere into the pipette. A micro aspiration pipette that is too large will not allow suction on the desired blastomere. A small micro pipette will possibly rupture the cell.**

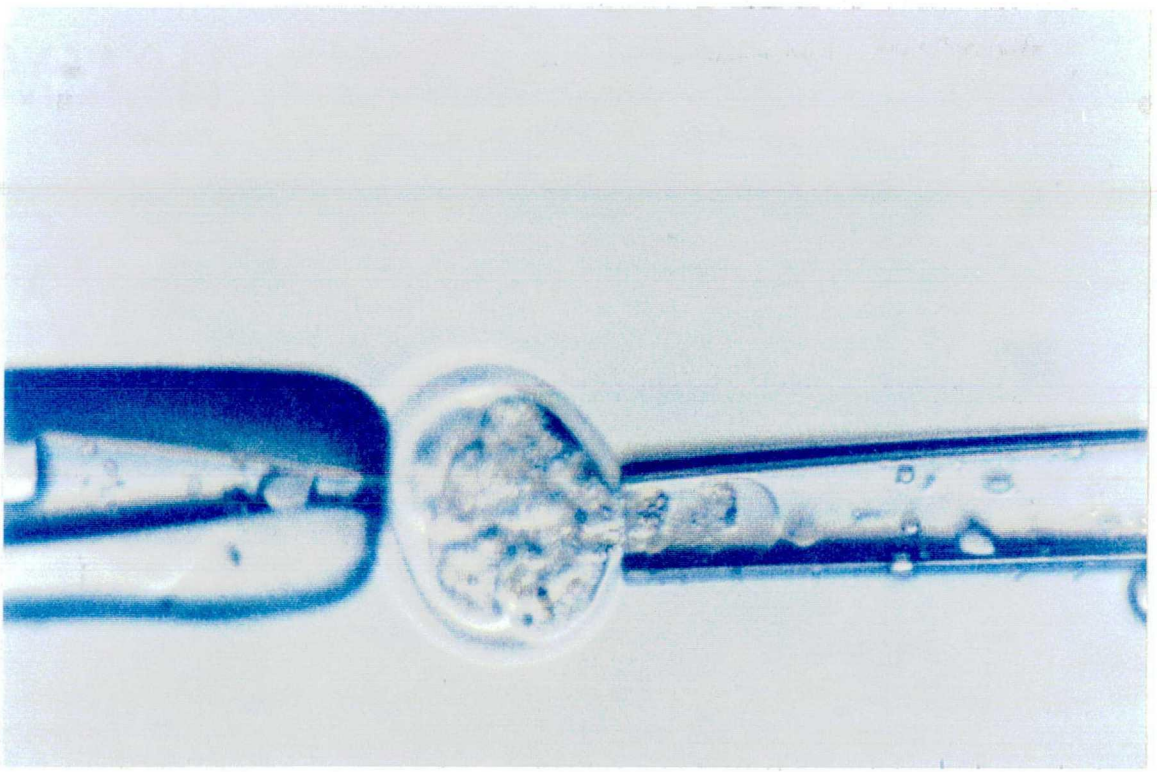
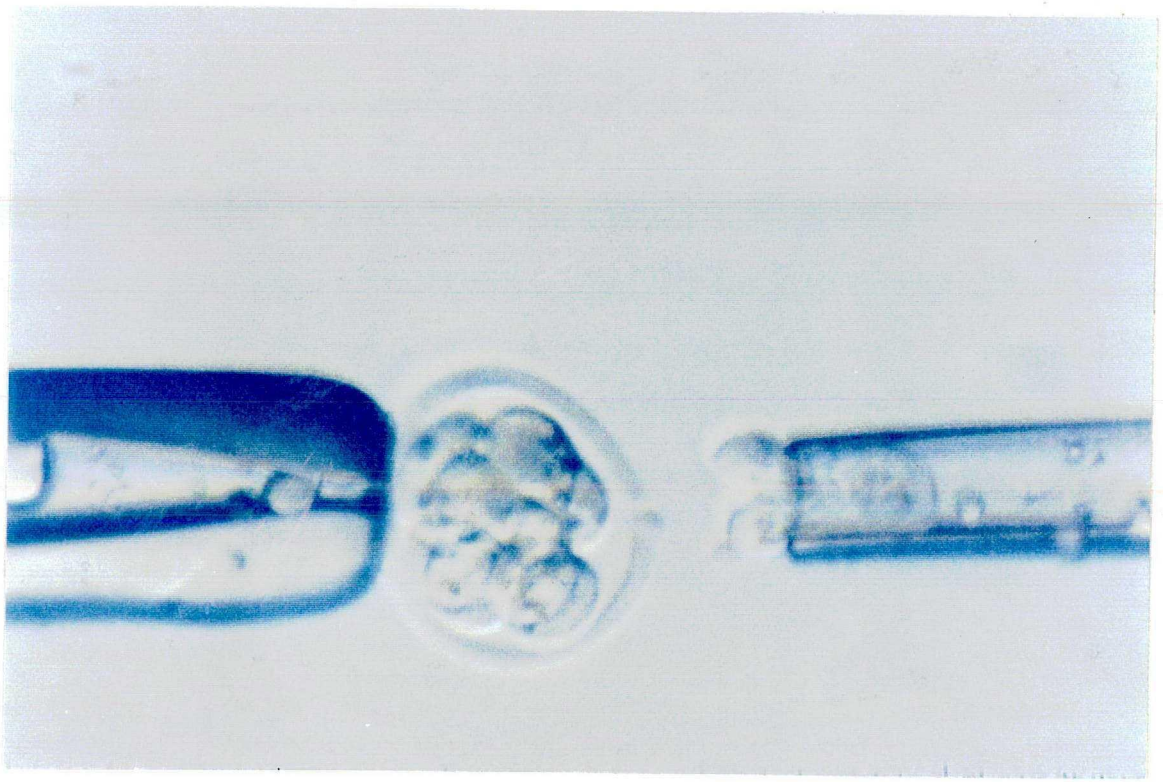


Plate VII. **Photograph of a Murine 8 cell embryo, being biopsied, taken on a Nikon Diaphot inverted microscope using the 40 X objective and Hoffman Modulation optics. Once the blastomere has been partially drawn into the aspiration pipette the pipette can be slowly withdrawn using the micromanipulator controls. By carefully withdrawing the aspiration pipette the blastomere will be removed from the embryo, as seen below.**



CHAPTER III

RESULTS

Embryonic Development to the Blastocyst Stage

Embryos of the C57BL/6 strain from both the control and biopsy groups developed to the blastocyst stage within 24 hours of thawing (Plate VIII). In the control group 50 out of 53 embryos or 94% developed to the blastocyst stage. In the biopsy group 29 biopsied embryos out of 36 or 81% reached the blastocyst stage within 24 hours of thawing. Biopsy of the C57BL/6 embryos was shown to significantly effect the percentage of embryos developing to the blastocyst stage at the $P<0.05$ level, however, significance was not seen at the $P<0.01$ level.

None of the eighty CBA/Caj embryos thawed developed to the blastocyst stage (Table 9). These results differ significantly from the development of the C57BL/6 embryos in both the control and biopsy groups.

Correct Identification of Embryonic Genotypes from Biopsied Cells

Out of a total of 80 biopsy samples 38 (48%) were correctly identified as either Ped gene positive (Ped +) or Ped gene negative (Ped -) corresponding to the C57BL/6 and CBA/Caj mouse strains respectively. No significant difference was seen in the ability to determine either the Ped + or Ped - genotypes correctly. These genotypes were determined correctly 53% and 43% of the time respectively (Table 10).

Errors in correctly identifying the embryonic genotype were related to the unexpected amplification of the 91 bp PCR product in the tubes containing cells biopsied from the CBA/Caj embryos. This product was only expected to be seen in the PCR tubes containing C57BL/6 cells. Also responsible for errors in genotype identification were amplification failures in the tubes containing C57BL/6 cells. Both of these types of errors can be seen on the silver stained acrylamide gels run for this project (Plate IX).

Plate VIII. **After biopsy, 80% of the C57BL/6 embryos reached the blastocyst stage of development. A photograph of two Murine blastocysts, an early blastocyst and an expanded blastocyst, can be seen below. The stage of the blastocyst can be determined by the size of the blastocoel cavity.**

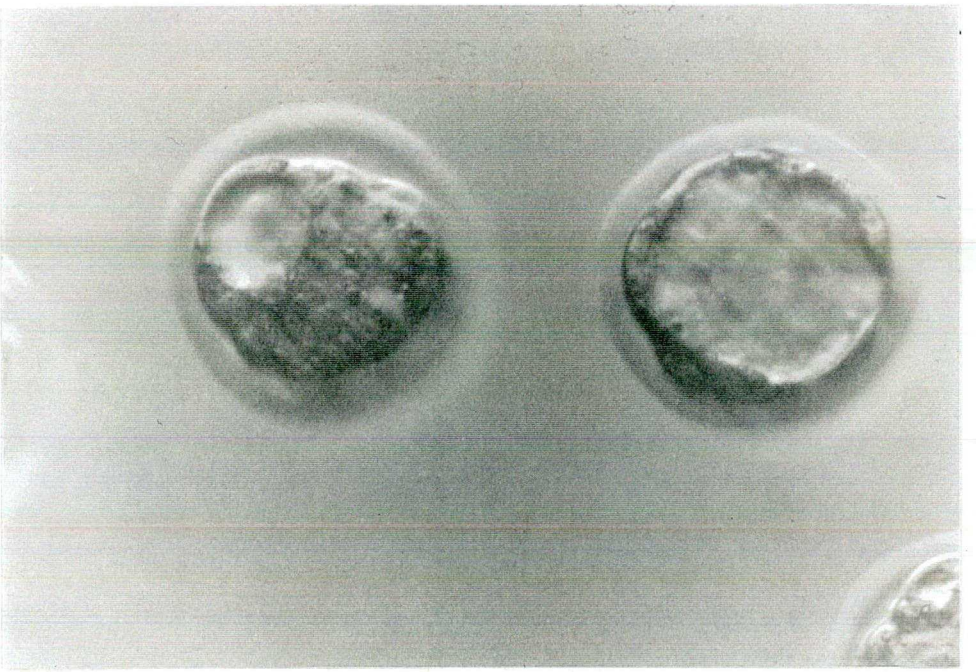


Table 9. Development, to the blastocyst stage, of control and biopsied CBA/Caj and C57BL/6 embryos .

Strain	Group	N	No. Blastocysts (%)	P Value[a]
C57BL/6	Control	53	50(94)	-[a]
	Biopsy	36	29(81)	< 0.05[b]
CBA/Caj	Control	40	0(0)	< 0.000004[c]
	Biospy	40	0(0)	< 0.000004[c]

[a] Percentages of embryos developing to the blastocyst stage were compared to the blastocyst percentage of the C57BL/6 control embryos using the Z test for sample proportions.

[b] Biopsy of C57BL/6 embryos was shown to significantly effect the percentage of embryos developing to the blastocyst stage at the $P < 0.05$ level but not at the $P < 0.01$ level .

[c] After thawing none of the CBA/Caj embryos continued to develop. This differed significantly from both a and b.

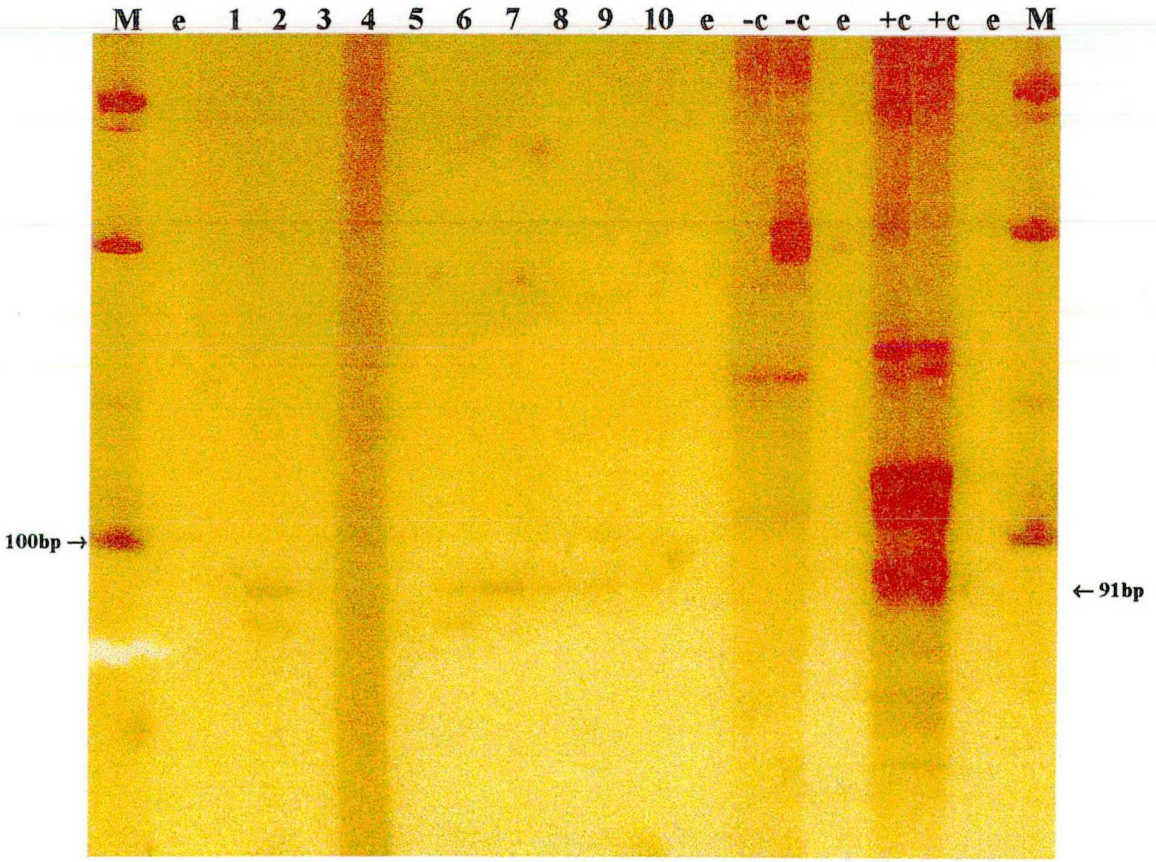
Table 10. Percentage of embryonic genotypes correctly identified from two biopsied cells.

Biopsy Source	N	No. Correctly Identified (%)	P Value[a]
All Biopsies	80	38(48)	NS
C57BL/6	40	21(53)	NS
CBA/Caj	40	17(43)	NS

[a] Percentages of correctly identified genotypes were compared for all biopsies and for those from each mouse strain using the Z test for sample proportions. Differences were not significant (NS).

Plate IX. **A computer generated scan of a silver stained acrylamide gel can be seen below. The 91bp band is the product generated by the Q7/Q9 heminest PCR primers. The contents of the sample lanes are as follows:**

M - 100bp marker
e - empty lanes
-c - PCR amplified control DNA from CBA/Caj mouse.
+c - PCR amplified control DNA from C57BL/6 mouse.
1-10 - biopsy samples (Actual genotypes: 1-Ped$^-$, 2-Ped$^-$, 3-Ped$^+$, 4-Ped$^+$, 5-Ped$^-$, 6-Ped$^+$, 7-Ped$^-$, 8-Ped$^+$, 9-Ped$^+$, 10-Ped$^-$)



CHAPTER IV

DISCUSSION

It is well known that the timing of mammalian preimplantation embryo development, in relation to uterine receptivity, is important to the eventual implantation of the embryo. In embryo transfer this timing and the quality of the embryo being transferred are of extreme importance to the establishment of a successful pregnancy.

In the mouse fast and slow developing strains, in respect to preimplantation embryo development, have been identified (McLaren and Bowman, 1973). These differences in preimplantation development rates have been linked to the H-2 haplotype of the mouse strain (Verbanac and Warner, 1981). Study of various genes within the mouse major histocompatibility complex has lead to the belief that certain H-2 genes may be responsible for controlling the rate of early embryonic development (Verbanac and Warner, 1981). The gene or genes responsible for the increased developmental rate has been termed the preimplantation embryo development (Ped) gene (Goldbard and Warner, 1982). The biological properties of the Ped gene are believed to be increases in the time of the first cleavage division, rate of *in vitro* and *in vivo* cleavage, litter size, birth weights, weaning weights, and improved embryonic survival.

Study of congenic strains of mice has shown that the slowly developing strains have a deletion in the Q region of the H-2 complex. This deletion removes some or all of the ten Q region genes (O'Neill et al., 1985). In all strains of mice lacking the Q region genes Q6-Q9 the slow development phenotype is observed. This evidence and the results

of other genetic mapping studies have allowed the Ped gene to be mapped to the Q region of the mouse H-2 complex.

It is now believed that the identity of the Ped gene is that of the Q7 and/or Q9 genes. These genes are believed to have evolved from an identical ancestral gene through gene duplication (Devlin et al., 1985). These genes differ by only a single base pair in the first three exons. The Q7 and Q9 genes have been shown to be expressed as the PI-linked Qa-2 molecule on preimplantation embryos (Warner et al., 1987b). The expression of these genes and the presence of this molecule have been shown to be associated with improved preimplantation embryo development (Tian et al., 1992).

It was the purpose of this study to develop a PCR based method of analyzing two cells, removed from an 8 cell mouse embryo, to determine the presence or absence of the Q7 and Q9 genes and thus determine the Ped gene genotype of the embryo. This type of preimplantation genetic diagnosis is already being used to determine the sex of embryos from domestic species as well as to screen human and domestic species embryos for some types of genetic disease. In this project it was hoped that this type of analysis would allow detection of the Q7 and Q9 genes, which have been shown to be related to the quality of the embryo. Embryo quality is presently still subjectively evaluated based on morphologic parameters. This success of this project would have given a more objective alternative to determining embryonic quality.

The results of this research show that only 48% of the genotypes of the embryos biopsied could be correctly determined. The failure of this PCR based system can probably be traced to the extreme homology of the Q7 and Q9 genes to the other Q region genes. The CBA/Caj strain of mouse, used as the Ped^- strain, has a deletion for the Q region genes Q6-Q9. The remaining six Q region genes Q1-Q5 and Q10 remain intact (O'Neill et al., 1985). These genes, along with other class I genes, share extreme homology to the Q7 and Q9 genes. It is possible, due to the limited availability of original

template DNA found in the PCR tubes containing only two cells, that during the initial PCR cycles the primers allowed amplification of DNA from genes other than Q7 and Q9. Subsiquent PCR cycles would then have amplified these initial incorrectly produced PCR products. Also to facilitate successful amplification from the low DNA concentration found in 2 cells the stringency of the PCR conditions were reduced. This may have played a role in the misamplification seen in the samples containing cells from the Ped^{-} CBA/Caj embryos.

Also investigated in this research was the effect of biopsy on preimplantation embryos of two strains of different Ped gene genotype. The results of this line of investigation give further strength to the idea that the Q7 and Q9 genes are related to improved embryo quality. In the embryonic development experiments 81-94% of the C57BL/6 embryos developed to the blastocyst stage. Conversely none of the embryos of the CBA/Caj strain developed to this stage. This difference was probably due to the reduced quality of the CBA/Caj embryos, which were unable to survive the freezing and thawing processes successfully. This line of investigation was successful. The results further support the current view that the Ped gene is related to improved quality in the preimplantation embryo.

CHAPTER V

CONCLUSION

The goal of using the techniques of preimplantation genetic diagnosis (PGD) to develop a PCR based method of screening mouse embryos for the presence of the Ped gene was not successfully accomplished in this project. Only 48% of the embryos analyzed were correctly "diagnosed". This project was successful; however, in developing more information which further supports the importance of the Ped gene to embryonic quality. The techniques of preimplantation embryo biopsy and single cell PCR, necessary for PGD, were also established in our laboratory. These techniques will most certainly be used in future projects.

It is clear that the selection of higher quality embryos utilizing the techniques of PGD has important implications for the future. Definite applications for this type of research can be seen in the analysis of human preimplantation embryos for potential genetic abnormalities and in the analysis of preimplantation embryos of domestic species for sex selection and selection of production traits which currently have genetic markers.

Ethical considerations also must be evaluated, especially if this type of research is to be utilized on human embryos. While screening of human embryos for genes which cause disease is clearly advantageous to the human species, selection of human embryos for other genes related to aesthetic traits may be ethically questionable. These moral and ethical questions must be asked. The technology necessary for this type of selection will soon be availible.

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APPENDIX A

Frozen Mouse Embryo Thawing Technique
(Revised from Embryotech Laboratories Thawing Instructions)

1. Expose the straw to room temperature air for 2 minutes.

2. Place the straw in a 37°C water bath. The straw must remain in the water bath for at least 1 minute.

3. Remove the straw from the water bath and wipe off any excess water.

4. Expel the contents of the straw as a single drop into a sterile Petri dish in the following manner:

   a. Using a pair of scissors, cut the straw between the lower heat seal and the column of medium (FIGURE 1).

   b. Make a second cut to bisect the cotton plug at the PVA section (FIGURE 2).

   c. Using the stylet push down on the remaining cotton plug, expelling the contents of the straw into the sterile Petri dish. **(No diluent present in straw.)**

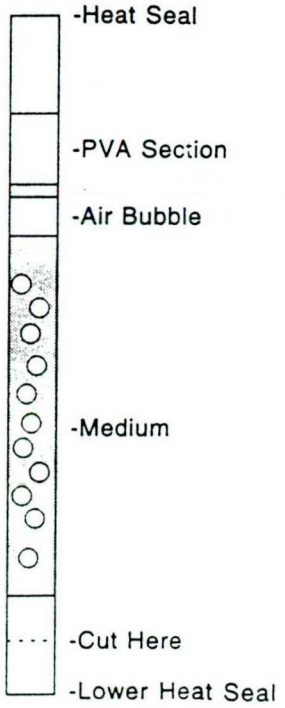

FIGURE 1

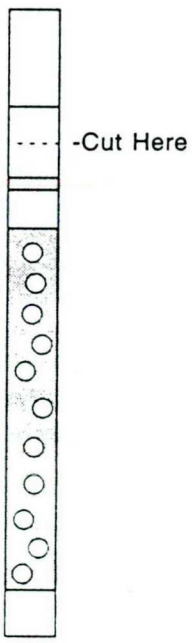

FIGURE 2

5. Immediately transfer the embryos into hepes buffered (modified) HTF medium at room temperature at a pH of 7.0 to 7.4.

6. Allow the embryos to rehydrate for at least 10 minutes and wash in bicarbonate buffered HTF medium.

7. Place dishes containing HTF medium and embryos into a 5% CO_2 in air incubator, at 37°C for 30 to 60 minutes.

8. The embryos are now ready for use.

APPENDIX B

Embryo Biopsy Technique
(Revised from Cornell University Micromanipulation Manual)

1. Ensure that the controls of the two micromanipulator arms are in vertical position, and that free movement is possible in all directions.

2. Place the holding pipette on the left microtool-holder of the micromanipulator, and tighten it.

3. Fill the well of an empty slide with biopsy medium or place a drop of medium in a culture dish.

4. Carefully move the embryo-holding pipette into the biopsy medium.

5. Draw biopsy medium into the holding pipette until it is filled 2cm. This step has two purposes: (1) To prevent suction of biopsy medium containing the embryo, and (2) To prevent the production of air bubbles in the droplet of medium containing the embryo.

6. Place assisted hatching (AHA) needle on the right arm of the micromanipulator, and tighten it.

7. Fill the well of an empty slide with acid Tyrode's solution or place a drop in the culture dish containing the biopsy medium, overlay the drops with oil.

8. Carefully move the AHA needle into the embryo biopsy medium.

9. Aspirate acid Tyrode's solution until the AHA needle is filled to 2cm.

10. Place an embryo in the drop of biopsy medium and visualize the embryo with 10x objective.

11. Manually place the embryo-holding pipette near the embryo without touching the overlaying oil.

12. Using the coarse controls of the left micromanipulator arm move the holding pipette in the medium and approach the embryo.

13. Once the holding pipette is left of the embryo, apply gentle suction until the embryo is attached to the needle. Position the embryo. Use the 20 or 40x objectives.

14. Apply more suction in order to have a good grip on the embryo.

15. With the coarse controls of the left arm of the micromanipulator lift the holding pipette until the embryo is detached from the bottom of the slide, but not so much that it is touching the oil overlaying the biopsy medium. This step allows easier maneuvering.

16. Center the AHA needle.

17. Lower the needle using the coarse controls of the right micromanipulator arm.

18. Focus the needle and position it adjacent to the targeted blastomere/s to be biopsied.

19. Ensure that both pipette tips and the targeted blastomere/s are in focus.

20. Use only the fine controls of both micromanipulator arms.

21. Touch the zona pellucida adjacent to the targeted blastomere to be biopsied with the AHA needle containing the acid Tyrode's solution.

22. Expel acid Tyrode's solution moving the needle tip up and down alongside the zona pellucida next to the targeted blastomere. Keep expelling the acid solution until a hole has been made in the zona.

23. Evaluate the size of the hole. Continue dissolving the zona if the hole is smaller than the blastomere to be biopsied.

24. Elevate the AHA needle out of the biopsy medium by using the coarse control of the right micromanipulator arm.

25. Move the embryo to another area of the biopsy medium to reduce its exposure to the acid solution.

26. Replace the AHA needle with a biopsy pipette. This needle should have an inner diameter approximately equal to the diameter of the blastomere to be biopsied. It may be necessary to try different size biopsy pipettes.

27. Lower the biopsy pipette near the embryo.

28. Use the coarse control of the right micromanipulator arm to immerse the biopsy pipette in the medium and place it adjacent to the blastomere to be biopsied.

29. From now on use only the fine controls of both micromanipulator arms.

30. Focus the blastomere to be biopsied, the blastomere biopsy pipette, and the hole in the zona.

31. Enter the hole in the zona pellucida with the biopsy pipette and touch the targeted blastomere.

32. Draw the blastomere carefully into the pipette using suction until at least half of the blastomere is inside the pipette.

33. Pull the blastomere out by pulling the embryo in one direction and the blastomere in the other.

34. Release the blastomere into the medium by expelling the biopsy pipette.

35. Check that the blastomere biopsied has a nucleus. If it does not have a nucleus, repeat steps 31-35 to obtain another nucleated blastomere.

36. Elevate the blastomere biopsy pipette out of the biopsy medium by using the fast control of the right micromanipulator arm. Be careful not to suck up the biopsied blastomere/s in this process.

37. Gently release the embryo from the holding pipette.

38. Move the holding pipette out of the visual area avoiding air bubbles and suction near isolated blastomeres.

39. Take the slide or dish containing the biopsied embryo and blastomere from the micromanipulator stage.

40. The embryo can be washed 3-4 times in culture media and returned to its culture dish.

41. The blastomere/s can now be pipetted into PCR tubes or fixed for FISH.

APPENDIX C

Preparation of 1 Liter of Acid Tyrode's Solution (AT)
(pH = 2.3 to 2.4, preferably 2.35)

KCL	0.20g
$CaCl_2 \bullet 2H_2O$	0.20g
$MgCl_2 \bullet 6H_2O$	0.10g
*$NaH_2PO_4 \bullet H_2O$	0.05g
Glucose	1.00g
PVP (Polyvinyl-pyrrolidone)	4.00g
H_2O	1 liter

Titrate to pH = 2.3-2.4 with HCl

*If anhydrous NaH_2PO_4 is used, use 0.04g

APPENDIX D

Blastomere Lysis Method with Lysis and Neutralization Buffers (from Wu et al., 1993)

Lysis Buffer			**Neutralization Buffer**		
KOH	200mM		KCL	300mM	
DTT	50mM	(dithiothreitol)	Tris-HCl	900mM	(pH 8.3)
			HCl	200mM	

Procedure:

1. Pipette 5 µl of lysis buffer into 0.5 ml Eppendorf PCR tubes.
2. Pipette biopsied blastomeres, in 1-2 µl of biopsy medium, into PCR tubes containing lysis buffer. (The tubes can be frozen at -20°C and stored at this time).
3. Place tubes containing blastomeres in lysis buffer into a 65°C water bath for 10 minutes.
4. Remove tubes from the water bath and add 5 µl of the neutralization buffer.
5. Centrifuge and overlay with one drop of sterile mineral oil.

APPENDIX E

6% Acrylamide Gel Preparation and Electrophoresis Conditions

Necessary Reagents for a 6% Acrylamide Gel

Urea	33.6g
Acrylamide/Bis	12.0ml
TBE 10X	8.0ml
H_2O	30.0ml
Temed	175µl
Ammonium Persulfate	175µl (must be made up weekly)

Gel Prep. Procedure

1. Weigh out urea into a 250 ml beaker.
2. Pipette correct volumes of acrylamide/bis, TBE 10X, and H_20 into beaker.
3. Place stir bar into beaker and allow contents to mix until urea is completely in solution.
4. While the contents of the beaker are mixing prepare the gel plates.
    A. Wash plates with 70% ethanol.
    B. Allow plates to air dry.

C. Place spacers between plates and clamp the plates together with spring clamps.

D. Tape sides and bottom of plates. Double taping of bottom of plates to prevent leakage.

E. Remove spring clamps and clamp sides of plates together with screw clamps (4 screw clamps will cover the entire lenghts of both sides of the plates).

5. Once urea has dissolved and the plates have been prepared, the temed and ammonium persulfate can be added to the gel mixture.

6. Immediately remove the gel mixture from the stir plate and draw it into a 30cc syringe.

7. Pour the gel by carefully expelling the contents of the syringe to avoid air bubbles forming in the gel.

8. Place a doubled shark's tooth comb, up side down, into the top of the liquid gel making sure that it is evenly placed.

9. Clamp the comb in place with the spring clamps.

10. Allow the gel to solidify for at least 45 minutes.

Electrophoresis Procedure

1. Once the gel has solidified remove the bottom sets of tape.

2. Carefully remove the up side down comb and excess gel material above the plates.

3. Wet the front panel and gaskets of the Bio-Rad vertical gel electrophoresis apparatus with 1X TBE buffer to insure a good seal.

4. Place the gel into the gel apparatus.

5. Fill the upper and lower chambers with 1X TBE buffer.

6. Attached the power supply lines to the correct poles of the apparatus.

7. Check to see that the setting on the power supply is set for constant power.

8. Turn on the power supply and slowly bring the power to 25-30 watts.

9. Allow the gel to warm at this level until its temperature reaches 50°C (Approximately 1 hour).

10. Once the gel has warmed, turn off the power supply and remove the lines.

11. Flush the sample well several times with 1X TBE buffer.

12. Carefully place a doubled shark's tooth comb into the sample well.

13. Place samples, controls, and size markers in individual sample wells created by the teeth of the shark's tooth comb.

14. Reattach the power supply lines to the apparatus.

15. Check to see that the settings remain on constant power.

16. Run the gel at 30 to 40 watts until the desired separation has been accomplished.

APPENDIX F

Bio-Rad Silver Staining Kit for the Detection of DNA Bands

Necessary Silver Stain Kit Reagents

40% METOH	400ml METOH + 600ml H2O
10% ETOH	100ml ETOH + 900ml H2O
5% Acetic Acid	30ml Glacial Acetic Acid + 570ml H2O
Developer	32g stock developer + 1 liter H2O
Oxidizer	30ml oxidizer concentrate + 270ml H2O
Silver Stain	30ml silver stain concentrate + 270ml H2O
Nitric Acid	

Procedure

1. Prepare a glass dish by washing it with nitric acid. Allow the nitric acid to sit in the plate for approximately 15 minutes. Then discard the acid (back into its container) and thoroughly rinse the dish with distilled water. This washing should remove any contaminating DNA from the dish.

2. Once electrophoresis is complete carefully remove the gel from the apparatus (you must first remove the buffer from the upper chamber).

3. Carefully remove the tape from the sides of the plates.

4. Separate the plates carefully so that the gel remains on the smaller of the two plates.

5. Place the plate containing the gel in the acid-washed dish and carefully pour the 40% METOH over the gel to loosen it from the glass plate.

6. Once the gel is lose from the plate, carefully remove the plate from the dish.

7. The gel should remain in the 40% METOH for at least 30 min.; however, the gel can remain at this step for a longer period of time (overnight) if necessary.

8. After at least 30 min., the 40% METOH can be removed and the gel soaked in 10% ETOH for 15 min. This step should be repeated 2 times.

9. After the second 15 min.has been completed the ETOH can again be removed and the gel incubated in the oxidizer solution for 8 min.

10. The oxidizer should be removed after 8 min. and the gel washed 4 consecutive times with distilled water (8 min. per wash). This step should be repeated until all of the yellow color has been removed from the gel.

11. The water should then be removed and replaced with the silver stain solution for 20 min.

12. The silver stain solution can be removed after 20 min. and excess solution and diluted and removed by washing with distilled water for 2 min.

13. A small amount of developer can then be added to the dish. Once a smokey precipitate appears, this developer should be removed and the gel immediately washed with distilled water.

14. The water should then be removed and half of the remaining developer added to the dish for 3 min.

15. After 3 min. the developer should be removed and replaced with the remaining unused developer.

16. The gel should now be watched carefully. Once it reaches the desired contrast the developer should be removed and the reaction stopped by adding the 5% acetic acid. (*Note: The longer the developer is in contact with the gel the darker the gel will become.)

17. The gel can remain in the acetic acid for an indefinite period of time.