Order Number 9009751

Studies in transgenic mice: Insertional mutation at the *pcd* locus and absent *de novo* DNA methylation in trophoblast

Krulewski, Thomas Frank, Ph.D.

City University of New York, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

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STUDIES IN TRANSGENIC MICE:

INSERTIONAL MUTATION AT THE *pcd* LOCUS

AND

ABSENT *de novo* DNA METHYLATION IN TROPHOBLAST

by

Thomas F. Krulewski

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1989

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

7/12/89
date

Chairman of the examining committee

7/20/89
date

Executive Officer

Robert J. Desnick

David F. Bishop

Edward H. Schuchman

James R. Roberts

F. Carter Bancroft

George A. Scangos

Supervisory Committee

The City University of New York

ABSTRACT

STUDIES IN TRANSGENIC MICE:

INSERTIONAL MUTATION AT THE *pcd* LOCUS

AND ABSENT *de novo* DNA METHYLATION IN TROPHOBLAST

by

Thomas F. Kruleswki

Adviser: Professor Jon W. Gordon

Two projects involving transgenic mice were undertaken: The characterization of an insertional mutation discovered in one line of animals, and the utilization of the foreign gene insert to report the effects of germline transmission upon DNA methylation.

In the first project 17 lines of transgenic mice produced by pronuclear microinjection with a recombinant plasmid encoding a dihydrofolate reductase cDNA. In pedigree P432 one-quarter of pups derived from crosses of hemizygotes developed severe ataxias at about postnatal day 20. Southern hybridization intensities showed that ataxic animals were homozygous for the foreign gene insert. Extensive histologic investigations demonstrated the degeneration of cerebellar Purkinje cells, retinal photoreceptor cells and olfactory bulb mitral cells, as well as abnormalities of spermatogenesis. Analyses of Giemsa-banded metaphase-arrested chromosomes of transgenic fibroblasts revealed no gross aberrations. When carrier transgenic mice were bred to mice with the spontaneous mutation *Purkinje cell degeneration* (*pcd*) mutant pups were among the litters, thereby establishing

allelism. DNA from transgenic mutants was used to construct a recombinant bacteriophage library which was screened for homology with the foreign gene insert. Two positive recombinant clones were identified as containing flanking mouse genomic DNA. Subfragments of these clones may be used to screen for RNA transcripts of the *pcd* gene. The cloning of *pcd* will allow for molecular analyses of a gene important to the regulation of neuronal development.

In the second project, *de novo* methylation in the absence of parental gene imprinting was studied in five transgenic pedigrees produced by pronuclear microinjection of linearized pBR322 sequences and delivered by Cesaerian section on day 19 of gestation. Placental DNA from founder mice was shown to be strikingly hypomethylated, and perhaps methylation free. Germline transmission was associated with increased placental DNA methylation. These results demonstrate a unique pattern of DNA processing in the placental (trophoblast) lineage, characterized by low or absent *de novo* methylation of unimprinted foreign DNA.

FOREWORD

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Krulewski, T.F., Neumann, P.E. & Gordon, J.W. (1989) Insertional Mutation in a Transgenic Mouse Allelic with *Purkinje Cell Degeneration*, Proc. Natl. Acad. Sci. USA 86:3709-3712

Krulewski, T.F., Rubenstein, W.R. & Gordon, J.W. (1989) Transgenic Mice Reveal Two Distinct Mechanisms of Methylation in the Preimplantation Embryo. submitted

Unrelated publications:

Krulewski, T.F. & Cohen, L.W. (1988) Choriocarcinama of the Stomach: Clinical Characteristics and Pathogenesis, Am. J. Gastroenterology 83:1172-1175

Krulewski, T.F. (1989) Education Enhances Knowledge and Anxiety in Elective Coronary Angiography. Cathet. Cardiovasc. Diagn. in press

Grabowski, G.A., Fabbro, D., Dinur, T., Osecki-Newman, K., Goldblatt, J., Feldman, A., Krulewski, T., Legler, G., Gatt, S. & Desnick, R.J. (1986) Genetic Heterogeneity Of Gaucher Disease:Enzymatic and Immunologic Studies. In: Enzymes of Lipid Metabolism-2. Freysz, L., Dreyfuss, H., Masarel, R. & Gatt, S. eds. (Plenum Press, New York, pgs. 721-734)

ACKNOWLEDGEMENTS

Dedicated most of all to my wife,
who has withstood far more talk
of *bizarre* life forms
than ought to be allowed by law.
You're the only one to explain
so everyone else I know can "get it".
Also dedicated to my parents,
to Regina, Joe, Christopher & Alexandra
and to John, Diane & Heather

Special thanks to Jon Gordon and Bob Desnick
for getting me interested in this field
and to fellow lab members who have helped me along

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INTRODUCTION

Since first described by Jaenisch (1976) and Gordon et al (1980) the applications of transgenic animals has grown. Most uses for transgenic animals exploit the complex cellular interactions that are maintained in these animals, and impossible to reproduce in tissue culture. Many transgenic animals have been produced with the goal of modifying normal genetics by the expression of foreign DNA. Examples of this use include the expression of growth hormone genes to enlarge livestock and the expression of tumor producing genes to induce cellular transformation (For review, see Gordon 1989). Transgenic animals, however, offer significant alternative uses which have received less attention. One such use is the induction of mutations by physical disruption of host chromosomes. The advantage of this system is borne out by the construction of mutant DNA libraries (Figure 1). These libraries may be screened for homology with the foreign insert, which acts as a molecular tag of the mutant gene. Once isolated, the mutant gene may be used to recover the corresponding normal DNA and RNA species. A related strategy was used to clone the dilute (*d*) coat color mutation of mice by screening for homology with an ecotropic murine leukemia virus (Jenkins et al 1981, Hutchinson et al 1984).

Foreign DNA may also be used as inert reporter genes which do not influence the normal machinery but nonetheless provide important information regarding that machinery. This use for transgenic animals may be suited to developmental studies upon constitutional features such as chromosomal methylation, nucleosomal repeat length and interractions with determinator proteins.

This thesis seeks to explore the latter mentioned uses of transgenic animals. In part I, mutation in a line of transgenic animals recreates the spontaneous mouse mutation *Purkinje cell degeneration*, and permits significant progress towards cloning the responsible gene. In part II, pBR322 sequences are used as reporters of DNA methylation in early murine somatic and trophoblastic development. Studies of the effects of parental imprinting upon pBR322 methylation lead to the development of a novel proposal concerning the mechanism of trophoblastic development.

TRANSGENIC MICE

The transgenic mouse results when the chromosome integrates DNA from an exogenous source. Once integrated, the foreign DNA, or transgene, will transmit through the germline as a simple and stable Mendelian trait (Gordon et al 1980, Costantini & Lacy 1981, Wagner et al 1981, Gordon & Ruddle 1981, Brinster et al 1981). Transgenes may be expressed, at times with tissue and temporal specificity (Brinster et al 1981, Swift et al 1984, Storb et al 1984, Palmiter & Brinster 1986, Gordon 1989). Occasionally the transgene will integrate into a restricted number of cells of the founder, causing mosaicism. Mosaics are characterized by a low frequency of transmission to progeny and by greater hybridization with transgene in progeny than in founder (Costantini & Lacy 1981, Palmiter & Brinster 1985, Wilkie et al 1986).

Transgenic mice have been produced by three experimental approaches. Jaenisch (1976) was first to utilize retrovirus to infect the early mouse embryo. With this method, a single copy of the

retroviral genome inserts and induces the duplication of a few base pairs of flanking host DNA (Jahner et al 1985, van der Putten et al 1985, Jaenisch et al 1985). Transgenic mice have also been produced by incorporation into the embryo of DNA-transformed Embryonal Stem or teratocarcinoma cells (Lovell-Badge et al 1985, Stewart et al 1985, Wagner et al 1985, Thompson et al 1989). Since Embryonal Stem cells constitute a small portion of the cells of the blastocyst, the founder animal is invariably a mosaic. If the mosaicism extends to the germline then some descendants will be carriers of the integrated gene. Host genomic effects and foreign gene alterations are expected to be those of DNA-mediated transformation.

The great majority of transgenic mice have been produced by pronuclear microinjection, first accomplished by Gordon et al in 1980. With this method (Figures 2&3) multiple copies of the transgene are usually integrated as a head-to-tail concatamer. It is postulated that concatamers result when the first transgene to integrated serves as a template for the homologous recombination and integration of subsequent transgenes (Palmiter & Brinster 1985). Concatamers might also result from the homologous recombination of independent transgenes in the nucleoplasm prior to integration or from the pre-integration amplification of a smaller number (Gordon 1989). The site of transgene integration is unique and probably random. Integration may be associated with extensive rearrangements of the host genome including deletions (Covarrubias et al 1986, Palmiter & Brinster 1986, Scangos & Bieberich 1987) duplications (Palmiter & Brinster 1986) and translocations (Overbeek et al 1986, Scangos & Bieberich 1987, Mahon et al 1988). In one case the transgene became interspersed with

"islands" of genomic DNA, some of which originated from a distant genomic site (Covarrubias et al 1986). At times cytosine residues within flanking DNA may undergo methylation, potentially inhibiting endogenous gene expression (Jahner & Jaenisch 1985). Transgenes themselves may undergo structural alterations, including reductions and transient increases in copy numbers within the concatamer even long after incorporation (Palmiter et al 1982a, Shani 1986).

Figure 1. Hypothetical Mechanism of Insertional Mutagenesis.

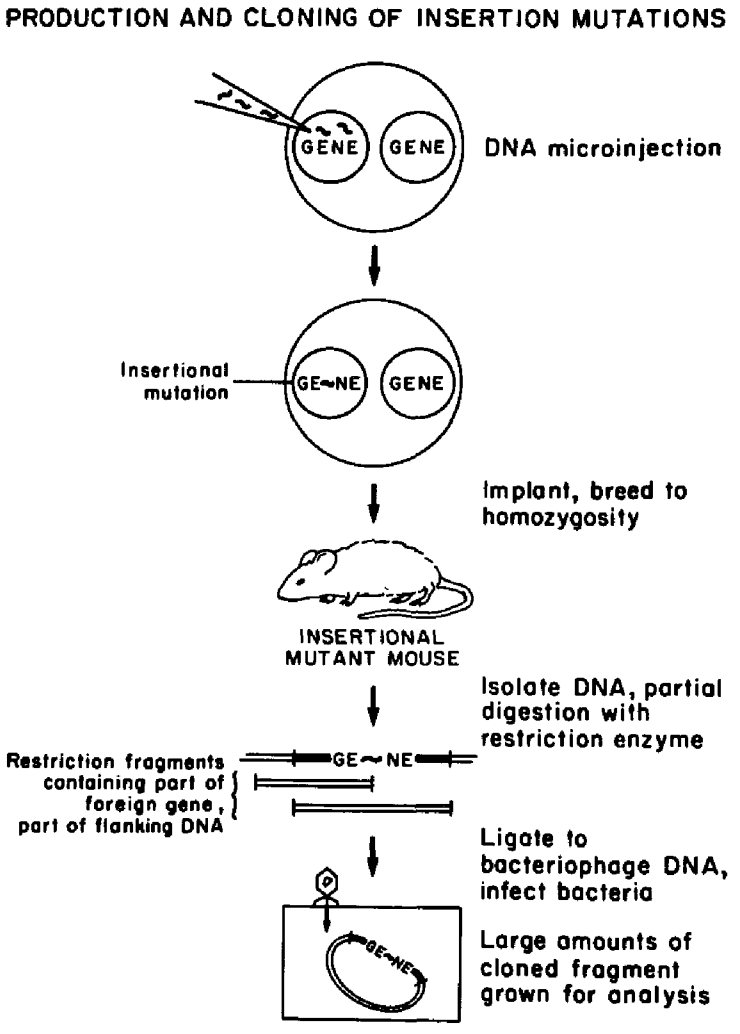


Figure 2. Production of Transgenic Mice.

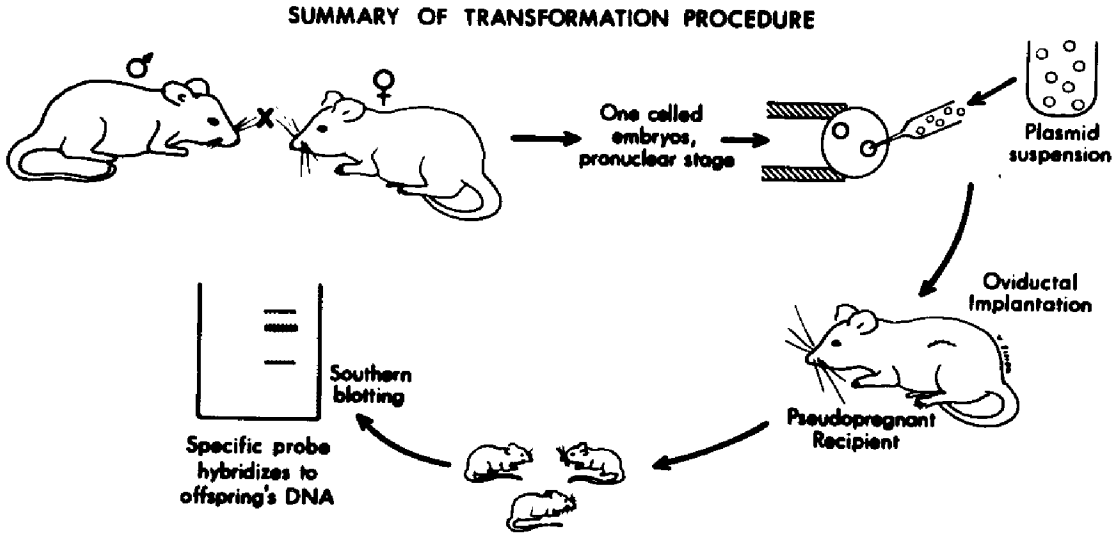
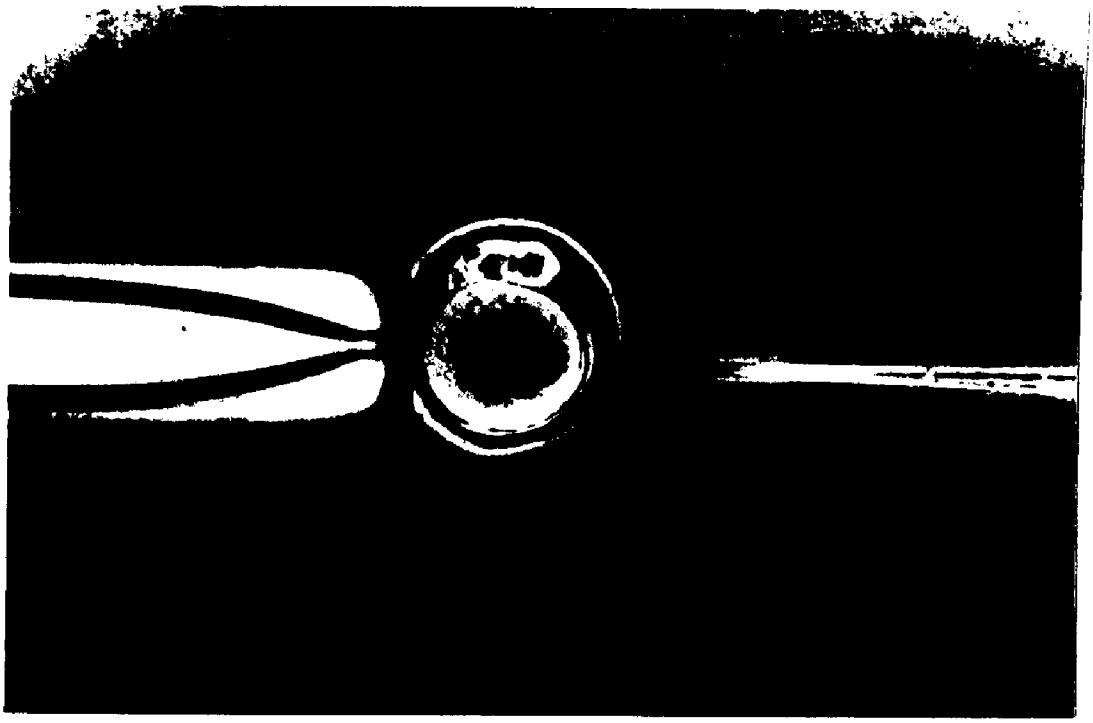


Figure 3. Pronuclear Injection as Seen Through the Microscope.



BACKGROUND I/INSERTIONAL MUTAGENESIS

Insertional mutagenesis results when the integration of a foreign gene causes loss of normal host gene function. Estimates of its frequency vary between 7% and 20% of transgenic animals produced (Palmiter & Brinster 1986, Scangos & Bieberich 1987). These values may underestimate the true frequency because dominant mutations that cause embryonic death and mutations with subtle phenotypes can escape detection. On the other hand, unsuccessful attempts to produce mutants may be underreported and associated chromosomal rearrangements that cause developmental defects may mimic insertional mutations. Nonetheless, the estimates agree with notions that roughly 5% of the genome is occupied by genes (Ruddle et al 1981).

In the analysis of mutation produced by gene insertion it is critical to exclude transgene expression as the cause of mutation. One strategy might be to demonstrate that the transgene and the trait cosegregate in a pattern of simple Mendelian inheritance. Southern hybridization anlysis can reinforce the data by detecting a restriction fragment length polymorphism (RFLP) which distinguishes mutant from normal phenotypes. Alternatively, one might show that transgene transcription is identical in mutant and normal mice. Yet none of these analyses definitively prooves insertional mutagenesis. In all cases the transgene might coexist with the mutant phenotype without causing it. Somewhat more compelling evidence for insertional mutation includes the detection of RNA species in normal mice which is absent in mutant mice. Still, transgene expression may cause the degeneration of specific cells, leading to the loss of many RNA species in the mutant

line. Indeed, only by the demonstration of allelism with a previously described spontaneous mutation may one definitively exclude transgene expression as the cause of mutation.

To date, eleven pedigrees have been reported as insertional mutants. Transgene transcription, whether appropriate or aberrant, occurs in the majority. Transcripts of the corresponding genomic loci have been detected in two, and identified as α1(I) collagen RNA in one (Schnieke et al 1983). Seven are recessive traits that cause embryonic death, usually at implantation. Three of the reported mutations cause gross deformities in mice that survive to term. Two of these are allelic with spontaneous mutations called *limb deformity* and *dystonia musculorum* (Woychik et al 1985, Kothary et al 1988). The remaining reported mutation causes distortion of germline transmission (Palmiter et al 1984).

The best characterized mutant transgenic pedigree resulted from the infection of postimplantation embryos with Moloney Murine leukemia virus (Mo-MuLV). Small litter sizes in the Mov13 pedigree suggested an embryonic lethal condition (Jaenisch et al 1983). Demise resulted from progressive necroses of mesenchymal cells leading to hemopericardium or massive hemorrhages of liver or internal carotid artery between embryonic days 11.5 and 12 (Lohler et al 1984). Although transcripts of Mo-MuLV were detected in diverse tissues of hemizygous Mov13 mice, DNA extracted from 35 resorbing embryos was always homozygous for the transgene, indicating that the disorder was recessively transmitted.

Radiolabelled Mo-MuLV was used to recover an *Eco*R1 clone which in Southern blots hybridized with a 14 kilobase pair (kb) band in normal mouse DNA and with 14 kb and 23 kb bands in carrier DNA,

reflecting integration of the 9 kb Maloney provirus. The Movl3 locus clone was shown to share sequence homology with 5200 and 6500 base RNA species (Schnieke et al 1983). Subsequent electron microscopy of human collagen mRNA/Movl3 locus DNA hybrids, sequencing and S1 nuclease mapping demonstrated that the Mo-MuLV provirus had integrated into the first intron of α1(I) collagen gene 19 base pair (bp) from the 5′ boundary in a transcription orientation opposite to that of the collagen gene (Harbors et al 1984). With its defect in collagen, the Movl3 pedigree thus provides a model for the human disorder *osteogenesis imperfecta*.

One might not expect integration into an intron to disrupt transcription. Hartung et al (1986) showed by nuclear run-on assay that transcripts were not initiated at the mutant gene promoter, excluding the rapid degradation of nascent transcripts. Instead it was shown that lack of transcription was associated with altered chromatin structure made apparent by the loss of a DNAse I hypersensitivity site 100-200 bp 5′ to the transcription start site as well as the novel methylation of cytosine residues both 5′ and 3′ to the proviral insert (Breindl et al 1984, Jahner & Jaenisch 1985).

In unrelated studies, Wagner et al (1983) discovered two mutations among six pedigrees of transgenic mice microinjected with a recombinant Human Growth Hormone gene (HUGH). Histologic and molecular studies with both pedigrees indicated recessive conditions causing demise at day 4-5 of gestation, shortly after implantation (Covarrubias et al 1987). Pedigrees HUGH3 and HUGH4 were interbred, producing complementing compound heterozygotes, results that excluded independent mutation at the same locus. Cloned mouse flanking DNA was not reported

to detect an RFLP but was used to screen a hybrid cell panel to map the HUGH4 defect to chromosome 12 and to conclude that the HUGH3 defect was not located on chromosome 12. Expression of growth hormone has not been excluded as the cause of mutation by either allelism or RNA detection. Indeed, complex host and transgene rearrangements have been reported (Covarrubias et al, 1985).

The fourth reported case of insertional mutagenesis resulted from microinjection of a recombinant plasmid, pMK, containing the mouse metallothionein I promoter region and the structural gene of herpes virus thymidine kinase (Palmiter et al 1984). Restriction analyses indicated that one complete and one incomplete copy of the pMK plasmid integrated as an inverted repeat. The founder female transmitted pMK to 7 of its 32 offspring, and hybridization with transgene was 5 to 10 times more intense in offspring. Mosaicism such as this probably results when the transgene is retained in nucleoplasm through a few cycles of cell division before integrating.

PMK carrier males did not transmit the chromosome bearing the transgene to any of three generations of offspring even though litter sizes were normal and equal numbers of female and male pups were sired. Analysis of DNA species isolated from testes and from sperm demonstrated that only about half the expected numbers of sperm carrying pMK reached the cauda epididymis. Since males of other transgenic pedigrees transmit actively transcribing MK genes it follows that pMK disrupted a gene whose post meiotic transcription is important to the development of fertile spermatozoa. Post meiotic transcription in mouse spermatids, although low, has been documented (Monesi et al 1978, Ericson et al 1981).

Sorianno et al (1987) induced a recessive mutation causing arrest shortly after implantation by the infection of embryos with a replication-competant retrovirus containing a bacterial supF gene. Cloning of the mutant locus was simplified by plating recombinant λ Charon4A bacteriophage on a supF- bacterial strain. The unidentified locus, designated as Mov34, codes for an abundantly and ubiquitously transcribed 1700 base polyadenylated RNA species in normal and hemizygous animals. A subclone of this locus was used to screen a mouse brain cDNA library to recover a 1.4 kb cDNA insert which shows evolutionary conservation in several mammals, chicken and Drosophila, but not in yeast.

Mark et al (1985) analyzed a mutation produced by microinjection of a bacteriophage λ recombinant containing the rabbit adult β-globin gene. Homozygous embryos were arrested in development at implantation, whereas hemizygotes inappropriately transcribed the transgene at low levels in skeletal muscle (Lacy et al 1983). *In situ* hybridization to metaphase chromosomes was used to map the recombinant bacteriophage to mouse chromosome 3.

Shani et al (1986) induced a prenatal lethal condition with a recombinant plasmid containing part rat skeletal muscle actin gene and part human embryonic globin gene. Of 45 progeny derived from hemizygous intercrosses 19 were carriers and none were homozygous. Transgene transcription could not be detected in skeletal muscle, heart, kidney, lung, thymus or testes.

Gerlinger et al (1986) reported reduced fertility and homozygous demise by day 9 of gestation resulting from the integration of rabbit β-globin structural sequences with the chicken conalbumin gene

promoter.

Overbeek et al (1986) described the first case of a lethal dominant mutation in transgenic mice with a recombinant Rous Sarcoma virus long terminal repeat/bacterial chloramphenicol acetyltransferase gene (RSV-CAT). Roughly half of embryos resulting from founder matings to wild type or F1 matings to wild type were arrested before day 8 of gestation. But 50% of surviving progeny carried RSV-CAT. The cytogenetic demonstration of a reciprocal translocation and the localization of transgene to the breakpoint explained the survival of carrier progeny (Mahon et al 1988). By strict criterion the lethal dominant pedigree is not due to insertion because the mutation instead was caused by a host genomic rearrangement.

Woychik et al (1985) described an inherited limb deformity characterized by oligodactyly, syndactyly, synostosis of long bones and fusions of bones in transgenic mice. Animals expressing the defect were homozygous for mouse mammary tumor virus long terminal repeat/mouse c-*myc* (MMTV-*myc*). The mutant locus was cloned into a modified cosmid vector which permitted selection for resistance to ampicillin and was, in turn, used to isolate the wild type locus. Restriction fragment analysis and Southern blot hybridization showed that approximately 1 kb had been deleted from the mouse genome as a consequence of integration of MMTV-myc. By screening hybrid somatic cell lines the site was mapped to chromosome 2, distal to band C-1. The inheritance and behavior of the transgenic mutant was similar to that of a spontaneous mutation called *limb deformity* (*ld*) which also maps to chromosome 2. Complementation matings and RFLP analyses showed that the transgenic mutation was allelic to spontaneous *ld*

mutations and that the non-complementing progeny inherited a mutation-linked restriction pattern from both the spontaneous and the transgenic parents (Woychik et al 1985).

Legless, a novel mutation, was found among transgenic mice containing a recombinant Drosophila heat shock promoter (hsp70) and herpesvirus thymidine kinase coding sequences (McNeisch 1988). Homozygotes, as demonstrated by dosage of transgene, expressed great diversity of abnormalities including hindlimbs truncated at the distal femur, forelimbs lacking radius and digits, aberrant or missing olfactory bulbs and facial clefts.

Kothary et al (1988) described mutation of a neural tissue-specific gene, *dystonia musculorum* (*dt*). Homozygotes suffered a severe and specific loss of dorsal spinal root sensory axons at all levels of the spinal cord, as in the spontaneous mutation. The observed expression of mouse hsp68-lac Z transgene in unstressed dorsal midline neural tissue may reflect regulation by the *dt* gene promoter.

In our laboratory a transgenic pedigree with profound gait ataxia was discovered. Analyses have revealed an exciting variety of histologic features, have established allelism to a spontaneous mouse mutation called *Purkinje cell degeneration* and have begun a molecular recovery of the mutant gene. These analyses are described in the present thesis.

PURKINJE CELL DEGENERATION

Purkinje cell degeneration (*pcd*) is an autosomal recessive mutation in the mouse which affects several regions of the central nervous system, the retina and sperm (Mullen & LaVail 1975). *pcd* arose spontaneously in mouse strain C57BR/cdJ and has been mapped to chromosome 13 in the area between A3 and C2 (Southard & Eicher 1977). Homozygotes are identified at about postnatal day 24 (P24) by their slow, hestitating ataxic gait, an obvious behavioral abnormality that accounts for the appelation *pcd* (Mullen et al 1976). Indeed, with the exception of a very few cells in the nodulus all Purkinje cells degenerate between the ages of three and five weeks (see Table I). This degeneration is associated with a predictable secondary atrophy of presynaptic neurons in the inferior olivary complex (Ghetti et al 1987).

The first cytologic abnormality of cerebellum that consistantly distinguishes *pcd* homozygotes is seen by electron microscopy at P15, when affected Purkinje cells aberrantly retain free polysomes between the nucleus and initial segment of the axon, and when an abnormal configuration of rough endoplasmic reticulum is detected (Landis & Mullen 1978). Inclusions in mutant Purkinje cells of immature intracisternal A particles, donut-shaped RNA-like virus particles, and nemotosomes, cytoplasmic organelles normally found in mature sympathetic ganglian neurons, are unique among known mutant murine phenotypes.

Photoreceptor cells begin to degenerate by P25; 25-30% disappear by two months of age, and 100% by twelve months (LaVail et al 1982, Table

II. Degenerating photoreceptors display swollen Golgi apparati and swollen synaptic terminals by P18 (Lavail et al 1982). As the loss of photoreceptor cells progresses, the rod outer segments become shorter and more variable in length. Rod outer segment renewal is normal at P18, but less than half that in littermate controls at two months. The burst of disc shedding, and the number of large phagosomes in retinal pigment epithelium cells soon after light onset are correspondingly reduced by half (LaVail et al 1982). A large number of isolated tubular vesicles 150 to 350 ηm in diameter are seen in the extracellular space adjacent to photoreceptor inner segments. Thought to arise from tubular outpocketings of the inner segments, the vesicles are unique among known forms of retinal degeneration (Blanks et al 1982). A gradient of photoreceptor degeneration is observed between two and ten months of age, being most advanced in the inferior hemisphere, particularly in the far peripheral retina (LaVail et al 1982). Two additional genetic loci, one of them linked to *pcd*, are known to modify the rate of photoreceptor cell loss (O'Gorman & Sidman 1983). The earliest changes of the *pcd* mutation are detected by electron microscopy at P15 and consist of darkly staining amorphous material within and swelling of Muller cell processes (Lavail et al 1982). Muller cell pathology and the limiting of photoreceptor cell pathology to the cytosol suggest that photoreceptor degeneration may be secondary to lack of a diffusable metabolic product.

A slow loss of mitral cells is first observed at age two months, with 80% loss by four months and over 95% loss by eight months (Table III). Degeneration is predictabily accompanied by the loss of axons within the lateral olfactory tract (Greer & Shepherd 1982). The

topography of afferent 2-deoxyglucose input to mutant olfactory bulb is normal and the histologic findings of *pcd* in other olfactory bulb laminae most likely arise as a consequence of mitral cell degeneration (Greer & Shephard 1982, Table III). In view of the continual turnover of olfactory receptor cells and their ability to make synapses onto novel targets, *pcd* appears to be an attractive model for studying the reorganization of synaptic connections (Graziadei & Monti-Graziadei 1980).

A rapid degeneration of discrete populations of thalamic neurons is observed between P50 and P60 (O'Gorman & Sidman 1985, Table III). Severely affected thalamic nuclei, in which a majority of neurons degenerate, include the central division of the mediodorsal nucleus, the ventral medial geniculate, posterior ventromedial, and submedial nuclei and those portions of the ventrolateral and posteriomedial nuclei which immediately surround the medial division of the ventrobasal complex. More subtle cell losses occur during the same period in restricted portions of the lateral ventrobasal, dorsal lateral geniculate, and lateral posterior nuclei, but even at P180, these nuclei are not markedly atrophic. Degeneration within the thalamus does not parallel, and is not attributed to, the distribution of thalamic afferents from cerebellar, retinal or olfactory neuron populations (O'Gorman 1985).

Less is known about the effect of *pcd* upon sperm. Of the three spontaneous alleles of Purkinje cell degeneration described, *pcd* mutant males are infertile while pcd^{2J} mutant males are fertile (Table III).

Cerebellar studies of a *pcd/pcd*<->normal/normal mouse chimera indicate that the defect is intrinsic to the Purkinje cell (Mullen 1977). This finding is consistant with observations that Purkinje and mitral cells, as well as cells of the thalamic ventral medial geniculate, undergo normal neural migration and normal synaptogenesis (Sotello & Alvardo-Mallart 1986). Also, it has been shown that the missing Purkinje cells of adult *pcd/pcd* mice can be cytologically and functionally replaced with embryonic Purkinje cell grafts (Sotelo & Alvardo-Mallart 1986, Sotelo & Alvardo-Mallart 1987). Mitral cell and photoreceptor losses were also expressed by the chimera, but it could not be determined by β-glucuronidase staining whether losses were due to the direct action of the *pcd* locus (Landis & Mullen 1978).

How is it that *pcd* causes the degeneration of apparently unrelated neuronal populations, each beginning at different chronological ages and progressing at different rates? Purkinje and mitral cells are large Golgi type I neurons which project their axons to distant locations. By contrast, photoreceptors are primary afferents with small nuclei of condensed chromatin. One might believe that *pcd* is a disorder of signal transduction because norepinephrine causes an accumulation of cyclic AMP in *pcd* cerebellae that is far greater than in normal mice (Ghetti et al 1981). But this finding is common among the cerebellar neurodegenerative mutants and has been shown in *pcd* to correlate with glial cell activity. Still, the only known deficiency of Guanine nucleotide-binding stimulatory protein, a protein which may profoundly influence cyclic nucleotide concentrations, causes human olfactory dysfunction and therefore may be related to mitral cell losses in

pcd (Weinstock et al 1986). One might also believe the disorder in *pcd* is related to NS-4, a nervous system cell surface antigen detected by polyclonal antibody in high titer in normal mouse cerebellum, retina and sperm (Schachter et al 1975). But the detection has not been shown specific to Purkinje or photoreceptor cells. These unsolved issues underscore the importance of molecularly characterizing *pcd*. Yet, the greater importance of *pcd* may lie in its ability to model a variety of known disorders. In mouse and man there are multiple independent genes which affect both nervous system and male reproductive functions as in *pcd* (Dickie 1966, Sidman et al 1964, Green 1981, Holmes 1907). The slow and progressive degeneration of photoreceptors in *pcd* resembles that seen in human retinitis pigmentosa. Also, in human neurologic syndromes such as Batten-Spielmeyer-Vogt disease, Refsum disease, Laurence-Moon-Biedl syndrome, Bassen-Kornzweig disease, Usher syndrome and some mucopolysaccharidoses, photoreceptor degeneration accompanies cerebellar ataxia, mental retardation, deafness or other abnormality as it does in *pcd* (McKusick et al 1965, Halsey et al 1969, Sorsby 1970). The discovery that a mutant transgenic pedigree is allelic with *pcd*, as described in this thesis, should help to clarify many important issues related to the disorder.

Table I. *pcd/pcd* Purkinje cell findings

degeneration onset/complete	light & EM features
P20/P35	retain polysomes at initial segment, P15
	odd endoplasmic reticulum formation
	nematosome inclusions (unique)
	intracisternal A particle inclusions (unique)
	secondary atrophy of inferior olivary nucleus

Table II. *pcd/pcd* retinal findings

degeneration onset/complete	light & EM features
P25/12months	amorphous densities in Muller processes, P13
	swelling of Muller cells, P13
	150-350 ηm extracellular vesicles IS (unique)
	Golgi body & synapse swelling
	disc synthesis decreased to half
	PE phagosomes decreased to half
	degeneration gradient to peripheral inferior
	phagocytic invasion of retina

Table III. *pcd/pcd* olfactory bulb, thalamus and sperm findings

degeneration onset/complete	light and EM features
thalamic ventral median geniculate (P50/P90)	9 ηm granules at rough ER, P30 polysomes in great abundance centr mediodorsal, post, post ventromed, submed, parts of ventrolat & posteriomed also degenerate
Mitral (P60/8 months)	secondary atrophy of lateral olf tract reduced size of glomeruli thinning of external plexiform layer increased numbers of glial cells normal topology of 2-deoxyglucose input
sperm	*pcd* infertile, pcd^{2J} fertile

MATERIALS AND METHODS I

Plasmids. The plasmid pFR400 (Simonsen & Levinson 1983, Figure 4) is a 4400 base pair (bp) molecule which contains the cDNA of a mutant dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) and the simian virus 40 early promoter (base pairs 1-550).

Production and Identification of the Transgenic Mutant Line. A series of transgenic mice carrying pFR400 were produced by the method of Gordon et al (1980). The number of copies of the foreign gene insert in mutant line P432 was determined by Southern blot hybridization (Southern 1975) using known amounts of pFR400 as a standard. Concatamer analysis was by Southern blot hybridization (Southern 1975) with mutant genomic DNAs digested by restriction enzymes that contain exactly one recognition sequence within pFR400.

Tissue Sections. Mutant mice were deeply anesthetized and perfused with a fixative solution of 4% formaldehyde, 5% acetic acid and 60% ethanol. Paraffin-embedded neural tissues were sectioned at 6-8 μ and stained with hematoxylin and eosin. Testes were fixed in 3% gluataraldehyde in Sodium cacodylate buffer, refixed in 2% OsO_4, dehydrated in ethanol, and embedded in Spurr resin. Sections 1-2 μ in thickness were stained with Toluidine blue. Epididymides were prepared by fixation in Bouin's fixative, embedded in paraffin, sectioned at a thickness of 10 μ, and stained with hematoxylin and eosin.

Library Construction and Screening. DNA from a mutant animal was partially digested with *Mbo*I and fragments 10-20 kb were

electroeluted from agarose. These fragments were used to construct a genomic library in the bacteriophage EMBL3 (Frischauf et al 1983). One million recombinant bacteriophage were screened with pFR400 as probe. Positive recombinants were then rescreened with pBR322, which has homology to pFR400 (Simonsen and Levinson 1983), to exclude cloning of the endogenous dihydrofolate reductase gene. Two positive clones carrying flanking mouse DNA were identified by digestion of bacteriophage minipreparations (Maniatis et al 1982) with *Eco*RI. Since neither EMBL3 cloning arms nor pFR400 has *Eco*RI sites, recombinant clones with such sites were identified as containing mouse genomic DNA. A 2.4 kb *Eco*RI fragment of one clone was shown to lack homology with both pFR400 and mouse repeated elements by failure to hybridize with pFR400 or mouse genomic DNA.

Transgene Dosage Study. A Southern blot of *Sac*I-digested spleen DNA from one normal, four obligate hemizygous transgenic, and five mutant transgenic mice was hybridized simultaneously with a mouse reference probe consisting of the 2.4 kb *Eco*RI fragment cloned from the region flanking the transgene, and with pFR400. The reference probe was added to the hybridization solution at a concentration of 10 ηg/ml, and radiolabelled pFR400 was added at 0.1 ηg/ml. Probes were radiolabelled by random priming using a kit obtained from International Biotechnologies (Prime Time). After exposure of the Southern blot to X-ray film, the relative intensities of plasmid and endogenous genomic bands were determined by scanning densitometry (E-C Apparatus).

Allelic Testing. Hemizygous P432 transgenic males were crossed to pcd^{2J}/pcd^{2J} females. Animals were examined for phenotypic

anomalies characteristic of *pcd*. Normal and affected animals were then studied by Southern hybridization to determine if they carried pFR400.

Production Of Mutant Primary Tissue Cultures. Carrier transgenic males were mated to carrier transgenic females. Embryonic day 18 mutant and carrier pups were identified by Southern blot hybridization (Southern 1975) with the corresponding *Pst*I-digested placentae. Fibroblast cultures were made according to Davisson et al (1981) by skin explant from embryos. Cells were cultured in Dulbecco's Modified Minimal Essential Medium (Maniatis et al 1982) and passed once through trypsinization with addition of Fetal Calf Serum.

Mutant Chromosomal Giemsa Banding And Karyotype Analysis. Transgenic fibroblast cultures were harvested and metaphase-arrested in the presence of velban. Cells were exposed to a hypotonic solution consisting of 0.05% KCL / 0.05% NaCitrate for 8 to 20 minutes, fixed overnight at -4^{0}C in 75% methanol/25% acetic acid, and dropped onto methanol treated slides. Chromosomes were banded by the method of Schnedl (1973) with 1 second exposure to 0.5 mg/ml trypsin solution and 2 minute stain in 4.3% Giemsa, and air blown dry. The karyotype was determined by comparison to the standard idiogram of banding patterns for mouse chromosomes (Nesbitt & Franke 1973).

Transgene Expression Study. Mutant transgenic, obligate hemizygote transgenic and normal non-transgenic age-matched mice were evaluated for expression of pFR400 RNA in brain and cerebellum. RNA extracts were hybridized with radiolabelled pFR400 antisense RNA, digested with RNAse A and T1, separated through polyacrylamide and exposed to film. pFR400 RNA differs from endogenous mouse dihydrofolate reductase RNA at

a single base pair. Thus, a single 275 base band would reflect pFR400 transcription and two bands of 205 and 70 bases would reflect endogenous gene transcription. The antisense construct was produced utilizing the oppositely oriented promoters of pGEM vector sequences.

Chimeric Mouse Production. Aggregation chimeras were made as described by Tarkowski (1961). P432 embryos derived from the matings of albino transgenic carrier males to albino transgenic mutant females, and non-albino normal embryos were collected at the eight cell stage. Zonae were removed by use of acidified Tyrode's solution (Maniatis et al 1982). Embryo pairs were fused in the presence of Phytohemagluttinin and cultured overnight in droplets of Hoppe and Pitts medium (1973) under mineral oil at 37^0C in an atmosphere of 5% CO_2 and 95% air. The next day blastocysts were transferred to the uteri of hosts made pseudopregnant by mating with vasectomised males.

RESULTS I

Line P432 Contains 400 Copies of Transgene as a Head-To-Tail Array. The comparison of hybridization intensities in Figure 5 indicated that the P432 line had integrated approximately 400 copies of pFR400 into the genome. Transgenic genomic DNA was digested with a variety of restriction enzymes each of which contained a single recognition sequence in pFR400. This repeatedly resulted in bands of hybridization with radiolabelled transgene of 4.4 kb unit length (Figure 6). This indicated that the core of the concatemeric transgene insert exists as a head-to-tail array. High molecular weight bands of approximate three-fold unit length and one-tenth hybridization intensity observed with digestion by *Pst*I indicated that this restriction site was destroyed in some copies within the concatamer.

Identification of a Mutant Phenotype in P432 Mice. All 17 transgenic mice lines carrying pFR400 were bred to homozygosity to screen for recessive insertional mutations. In one line, P432, offspring from such crosses displayed abnormal neurologic and reproductive function. Affected P432 animals were normal until approximately 20 days after birth, when they developed a progressively ataxic gait. Eight of 22 affected males were sterile as evidenced by failure to sire progeny after at least three documented matings to normal females. When carrier P432 transgenic mice were mated to normal nontransgenic mice all pups were normal (Table 4). When hemizygous males and females were interbred, 24% of offspring were affected; all of the affected and 60% of the non-affected offspring were later shown to be transgenic ($\chi^2 = 24.11$, $P<<<0.0001$). One fertile mutant

female, when mated to a carrier male, produced 3 transgenic offspring, all of which exhibited the mutant phenotype (Table 4). Thus, genetic analysis indicated that these abnormalities followed a recessive pattern of Mendelian inheritence, with linkage of the phenotype to the transgene. Indeed, no recombination between the transgene and the locus associated with the neurologic trait has been found among 156 animals tested (Table 4).

Histologic Abnormalities in Mutant Mice. Figures 7 through 17 show the associated histopathologic findings in affected animals compared with normal mouse tissues. Purkinje cells are totally absent in affected adult mice (Fig. 8). Degeneration of photoreceptor cells is evident from the progressive thinning of the outer nuclear layer of the retina (Fig. 10). Examination of the olfactory bulb of a 10 month-old affected mouse revealed loss of almost all mitral cells (Fig. 12). The sterile mutant males were found to be azoospermic. Sections of their reproductive organs revealed testes devoid of late spermatids (Fig. 14), and epididymides that contained no mature sperm (Figs. 16 & 17). All mutant female mice tested were fertile, but they produced small litters (Table 4).

Southern Blot Analysis of Mutant Animals Indicated Homozygosity for the Transgene Insertion. A recombinant bacteriophage library was constructed (Figure 18) and screened as described in the methods section. Restriction mapping was performed on recombinant bacteriophage to subclone fragments which could be used to test at the molecular level whether mutant animals were homozygous transgenics. Figure 19 shows the map of one such phage in detail. A 2.4 kb *Eco*RI fragment was subcloned from the mouse genomic DNA of this

bacteriophage and used as a reference probe for the amount of pFR400 in affected and unaffected animals. As demonstrated in Figure 19, digestion with *Sac*I would not be expected to generate a restriction fragment length polymorphism associated with transgene insertion, because a *Sac*I restriction site lies between the 2.4 kb probe and the pFR400 component of the recombinant phage. This probe identified a 15 kb fragment within the mouse genome after *Sac*I digestion (Fig. 20). Radiolabelled pFR400 added simultaneously to the hybridization detected a 4.4 kb band in mutant and hemizygous transgenic mice (Fig. 20, arrow). This band is the size expected for the core of the concatemeric transgene insert, because the 4.4 kb pFR400 molecule contains a single *Sac*I site, and because the low quantity of radiolabelled plasmid included in the hybridization (0.1 ηg/ml) would not be expected to detect minor plasmid-derived bands. When the intensities of the plasmid band relative to that identified by the reference probe were compared by scanning densitometry, it was clear that all mutant animals contained approximately twice as much pFR400 DNA per diploid genome as did hemizygous animals (Fig. 20).

Allelic Testing Indicates Insertion of pFR400 into the *pcd* Locus in P432 Transgenic Mice. Because of the remarkable similarities between P432 homozgous transgenic mice and *pcd* homozygotes, allelism with *pcd* was tested by mating hemizygous p432 males to pcd^{2J}/pcd^{2J} females. In two litters, 7/10 animals were affected with the ataxic disorder. Non-complementation of such closely matched traits suggests allelism. If the trait in the transgenic pedigree is due to insertional mutagenesis, then all mutant animals in these crosses would be predicted to be transgenic mice, while all

phenotypically normal mice would not be expected to carry the transgene. Dot blotting of DNA from these offspring showed that all affected mice were hemizygous for pFR400 and all phenotypically normal animals were not transgenic (Figure 21). That transgne segregation would follow a pattern of allelism with *pcd* as a chance event is highly unlikely ($\chi^2 = 10$, $P << 0.005$). These DNA analyses strengthen the phenotypic analyses which indicated allelism between the pFR400 insertion site and the *pcd* locus. As stated, the observed recombination frequency between the trait and the transgene is 0%. The upper 95% confidence limit is 17% when only the 156 repulsion intercross (Table 4) and 10 backcross offspring are considered. Thus the breeding data confirm that the pFR400 insertion site is most likely between 0 and 17 centimorgans from the *pcd* locus.

Chromosome Analysis Reveals No Evidence Of Rearrangement. The P432 karyotype was found to be normal (Figure 22). This suggests that gross deletions or translocations are not the cause of mutation in the P432 pedigree. The 1.7 megabase pair concatameric insert of pFR400 was not detectible with the resolution afforded by the Giemsa banding method. Future analyses includes the subchromosomal mapping of the *pcd* locus by DNA to DNA *in-situ* hybridization with radiolabelled pFR400 utilizing the banded chromosomes.

Transgene RNA In Mutant Cerebellum. RNAse protection assay was utilized to investigate pFR400 transcription. The 275 base sequence of the pFR400 coding region hybridized intensely with mutant transgenic and was detectable at low intensity with hemizygous transgenic cerebellar extracts (Figure 23). Consistently recurring artifact bands appear in mutant, hemizygote and positive control lanes and do not

correspond to endogenous dihydrofolate reductase transcription. As expected, endogenous gene transcription was not detected in brain and cerebellum.

Eight Chimeric Mice For Future Analyses. On December 31, 1988 eight mice were delivered with obvious coat color chimerism (Figure 24). The photoreceptor cell and Mitral cell defects of *pcd*, however, will not be 90% complete until the mice are 8 months of age. To obtain an unambiguous assessment of whether *pcd* is intrinsic to these affected cell types, DNA-to-DNA *in-situ* hybridization with pFR400 as probe may not be performed until September 1989. Since cells of the pineal gland have been shown to share restricted RNA species with photoreceptor cells (Rodrigues et al 1986), this organ will also be investigated for degeneration. The odds that none of the eight chimeras contains a mutant transgenic component rather than carrier transgenic components is highly unlikely $(1/2)^8$.

Figure 4. Restriction enzyme map of pFR400. The thin line denotes plasmid sequences. Open bars indicate the SV40 promoter (0 to 550) and the HBV polyadenylation sequence (1225 to 1810). The solid bar indicates the dihydrofolate reductase cDNA (580 to 1115). The *Pst*I site was digested prior to microinjection.

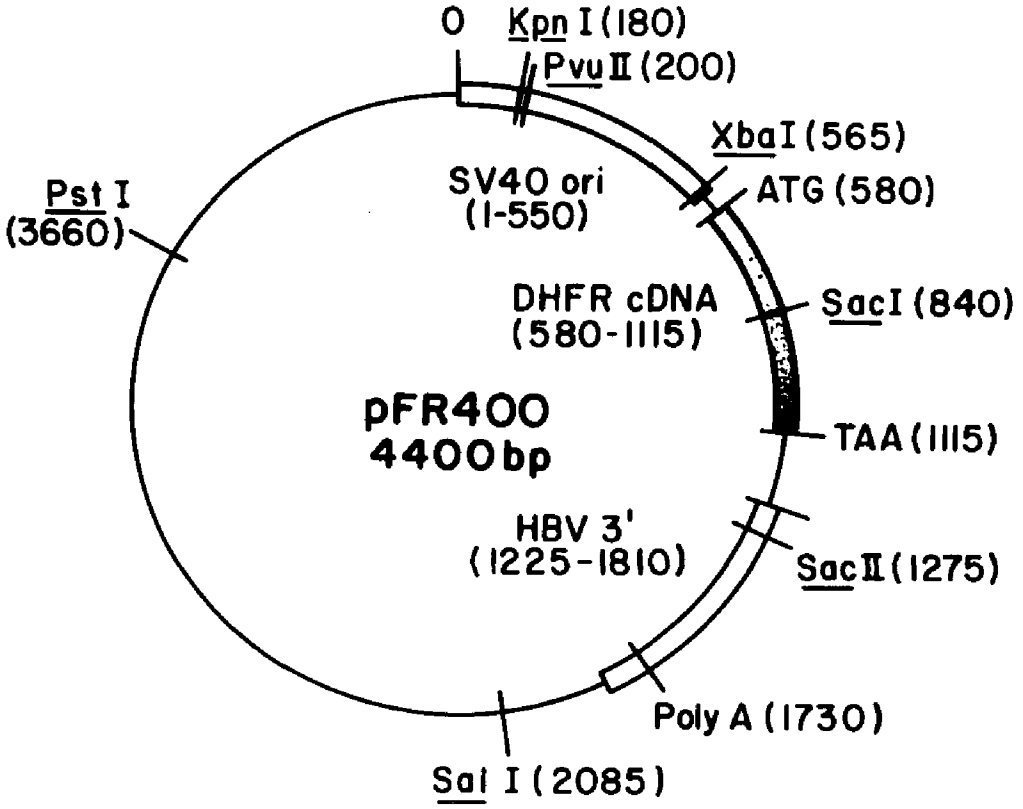


Figure 5. Hemizygous transgenic cells contain 400 copies of pFR400. Hybridizations of radiolabelled pFR400 with 1 πg purified plasmid and 1.4 μg obligate hemizygote transgenic DNA (lower row, in duplicate) would be equal if one copy of transgene had integrated per cell. The upper row contains 1 πg purified plasmid DNA (leftmost) and factor 2 multiples of this amount. The lower dots hybridization intensities lie between the upper row dots corresponding to 256 and 512 copies of transgene per cell.

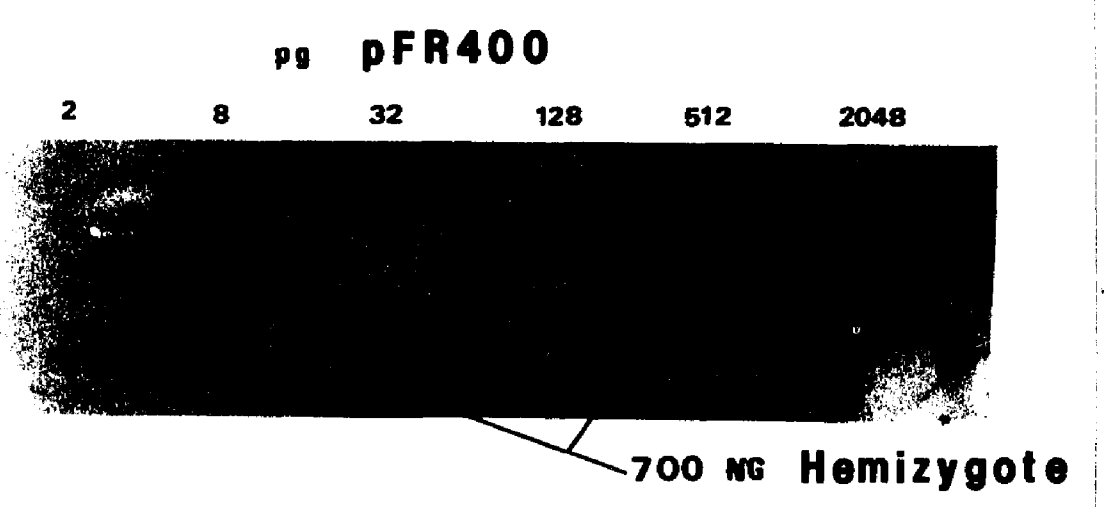
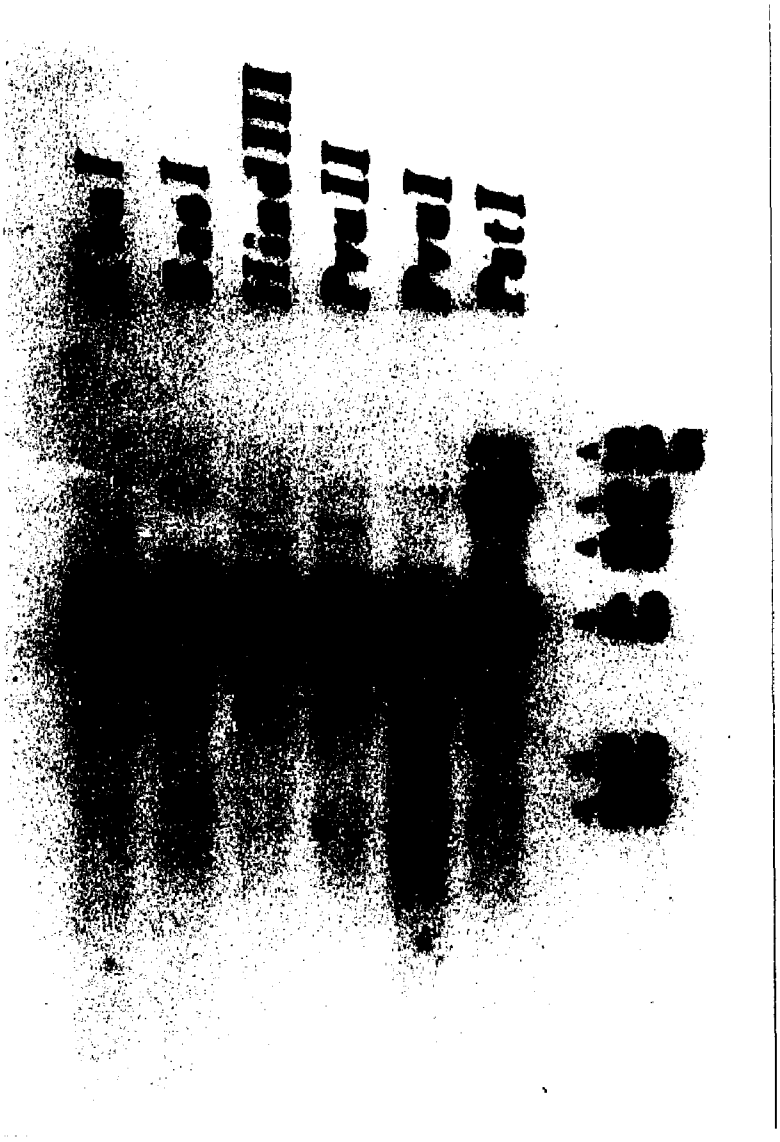
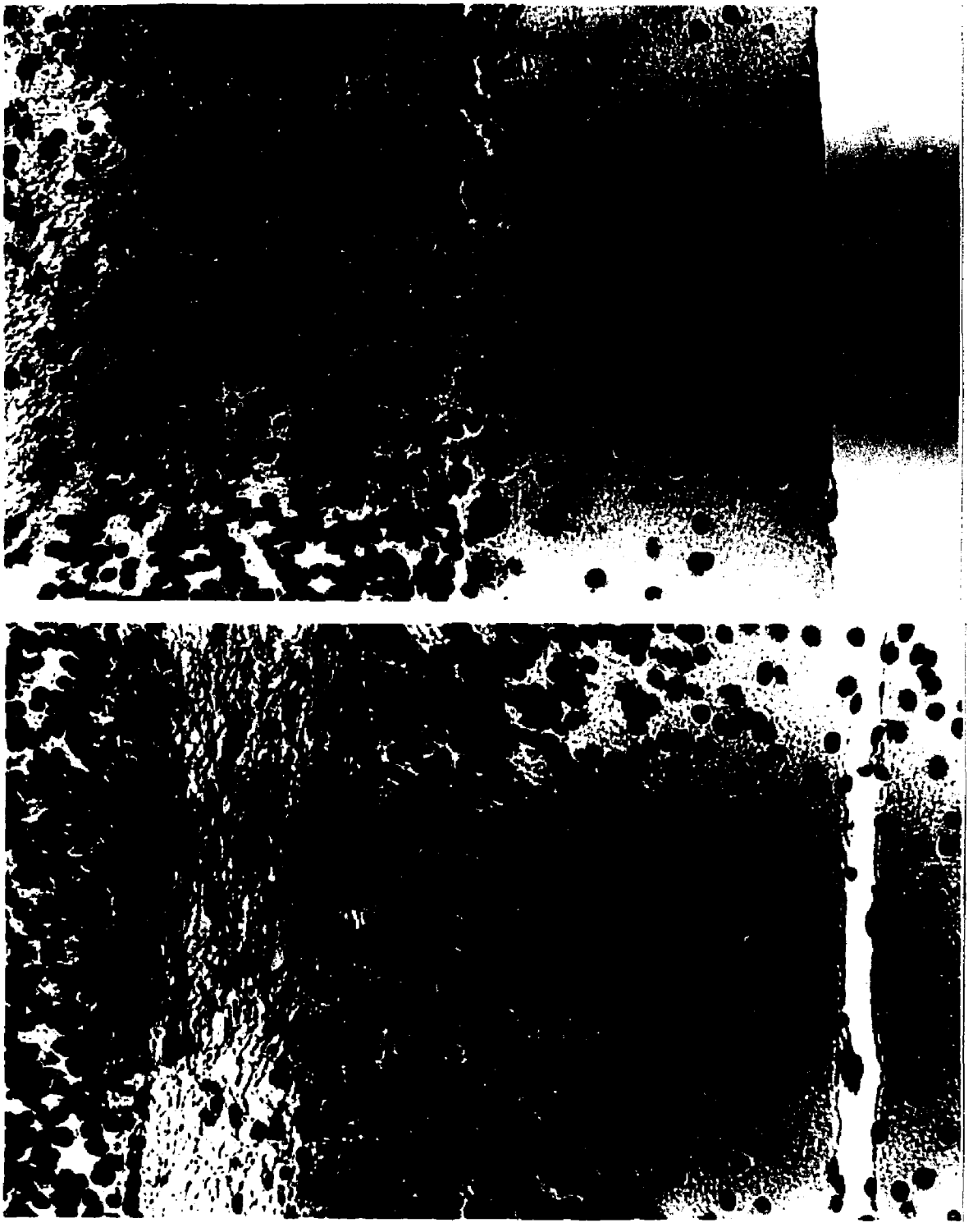


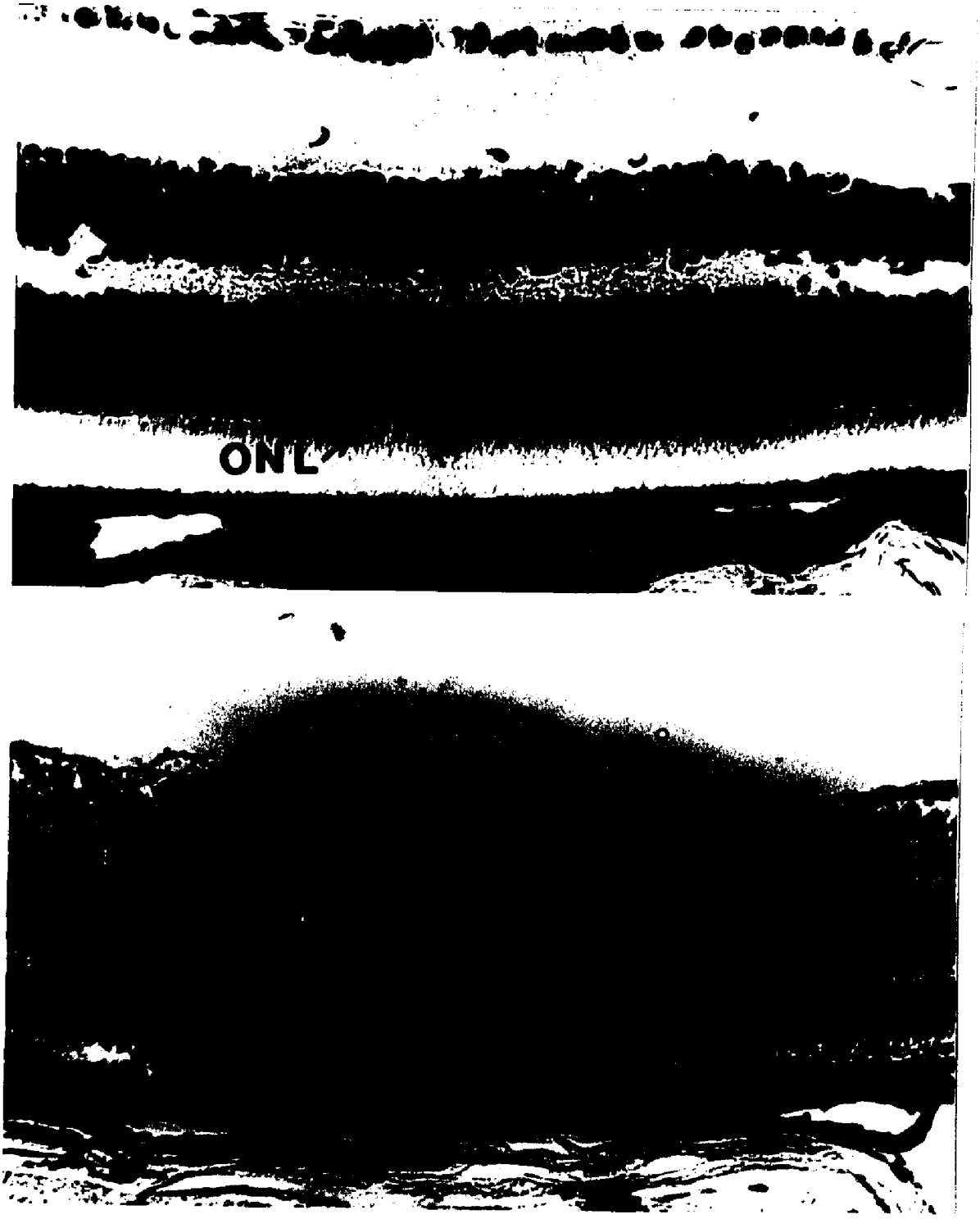
Figure 6. Head-to-tail array of transgene insert. DNA samples were digested with a number of restriction enzymes that recognize a single site within pFR400. Integration of a single copy of the transgene would be likely to produce bands different in size from the injected unit, as digestion would generate fragments composed partially of flanking mouse genomic DNA. In contrast, digestion of concatamers yields bands of high intensity with the same mobility as the 4.4 kb injected sequence.



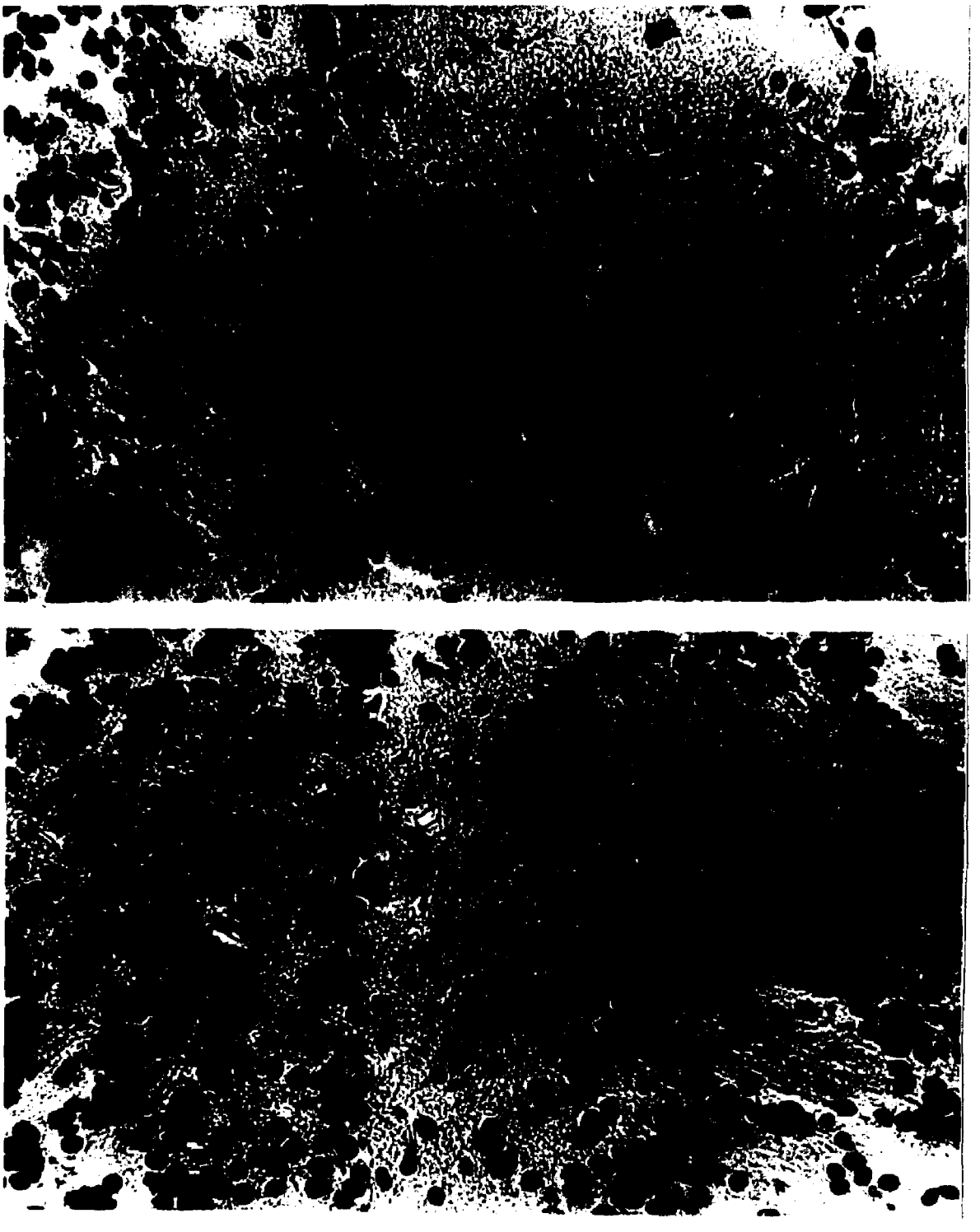
Figures 7 & 8. Photomicrographs of cerebellar cortex. Purkinje cells are indicated by arrows in age-matched control (upper panel) but absent within adult mutant transgenic mice (lower panel).



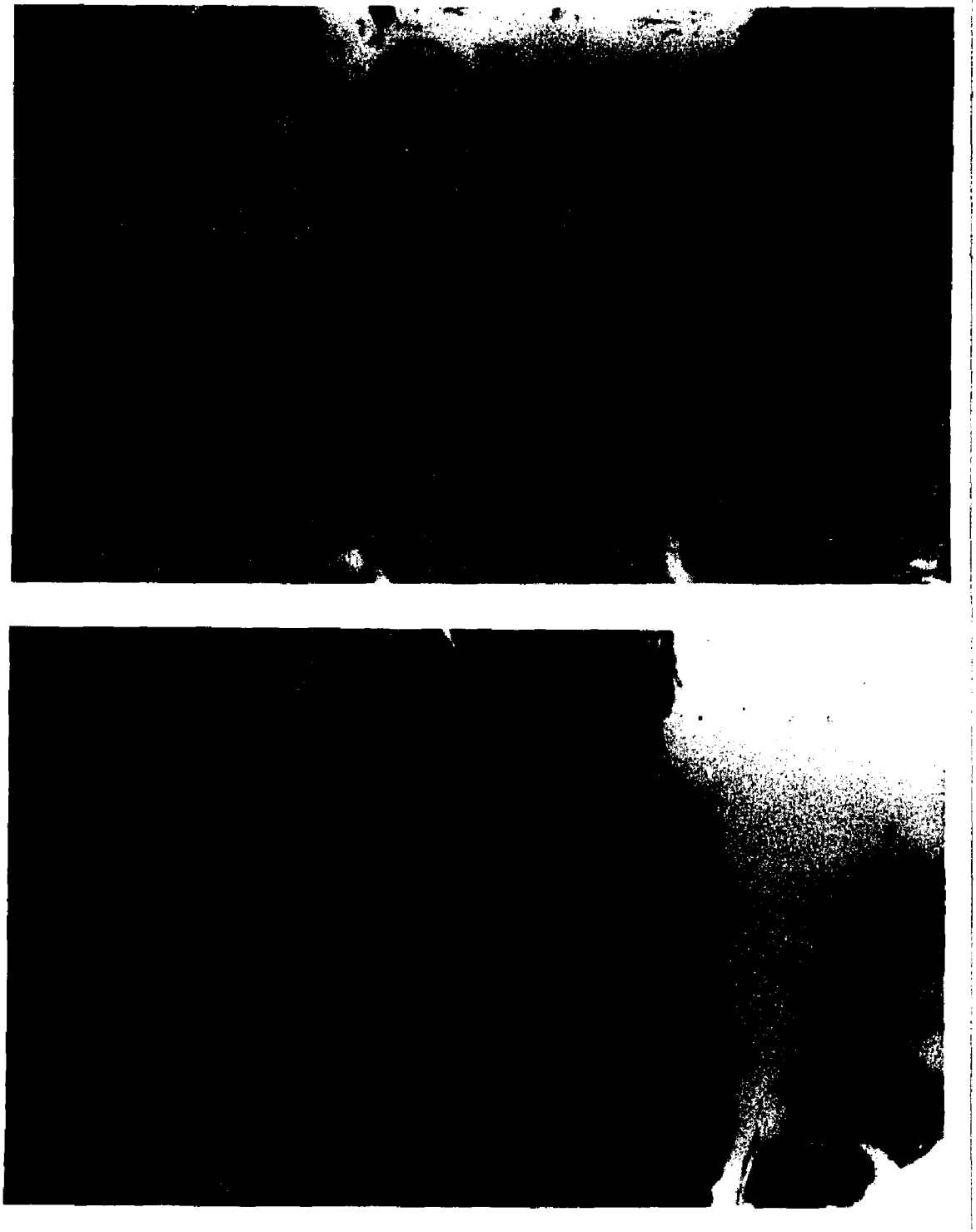
Figures 9 & 10. In wild-type retina the outer nuclear layer (ONL) contains the perikarya of photoreceptors (upper panel). In mutant retina, the ONL is thinned due to loss of photoreceptors (lower).



Figures 11 & 12. In wild-type olfactory bulb several mitral cells are indicated by arrows (upper panel). In mutant olfactory bulb (lower) a rare surviving mitral cell is indicated.



Figures 13 & 14. Photomicrograph of wild-type testis reveals the condensed heads of numerous late spermatids (upper panel), while that of the transgenic mutant has no late spermatids (lower panel).



Figures 15 & 16. Wild-type epididymis (upper) is filled with mature sperm while mutant epididymis (lower) contains no mature sperm.

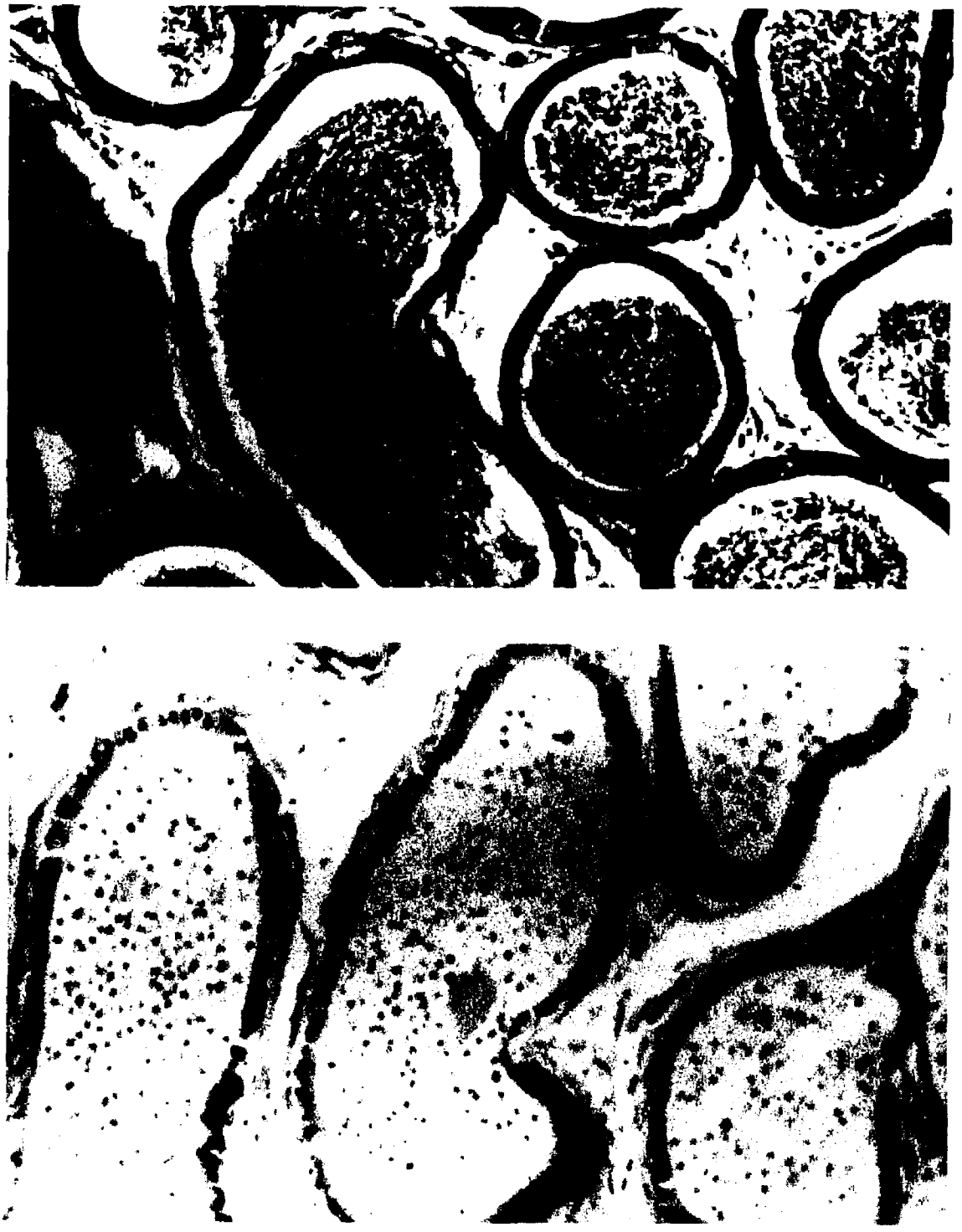


Figure 17. Higher power magnification of mutant epididymis reveals binucleate and round spermatids which have been sloughed off due to developmental arrest.

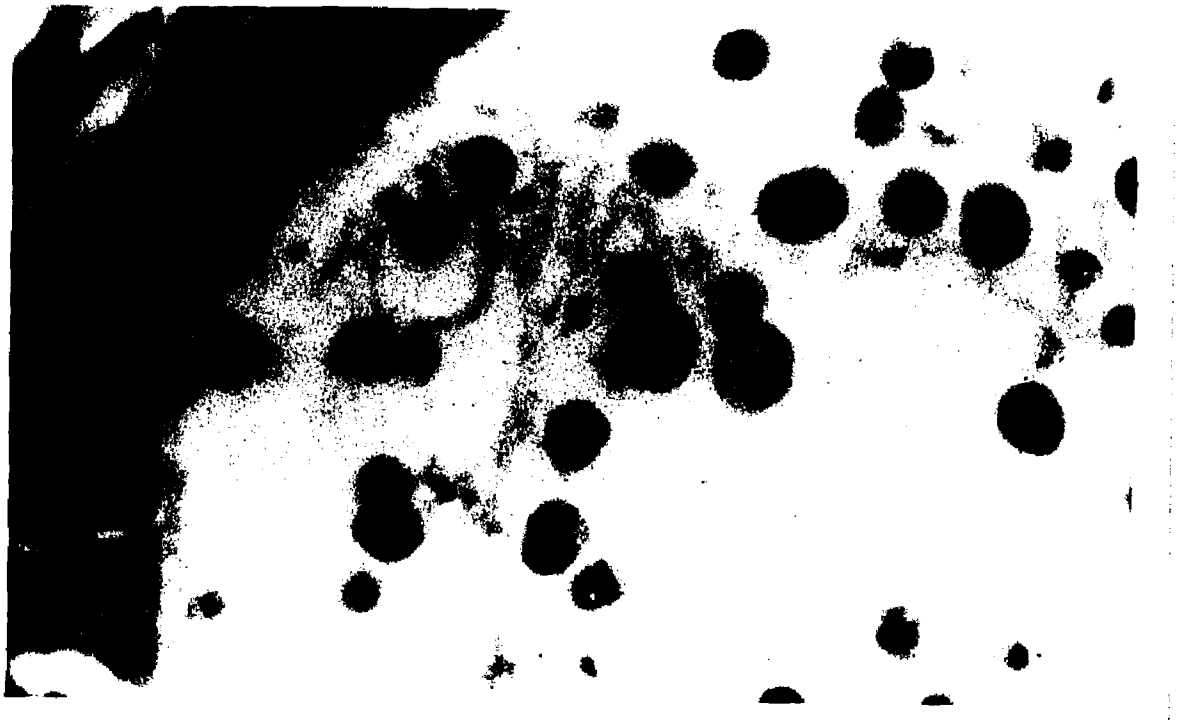
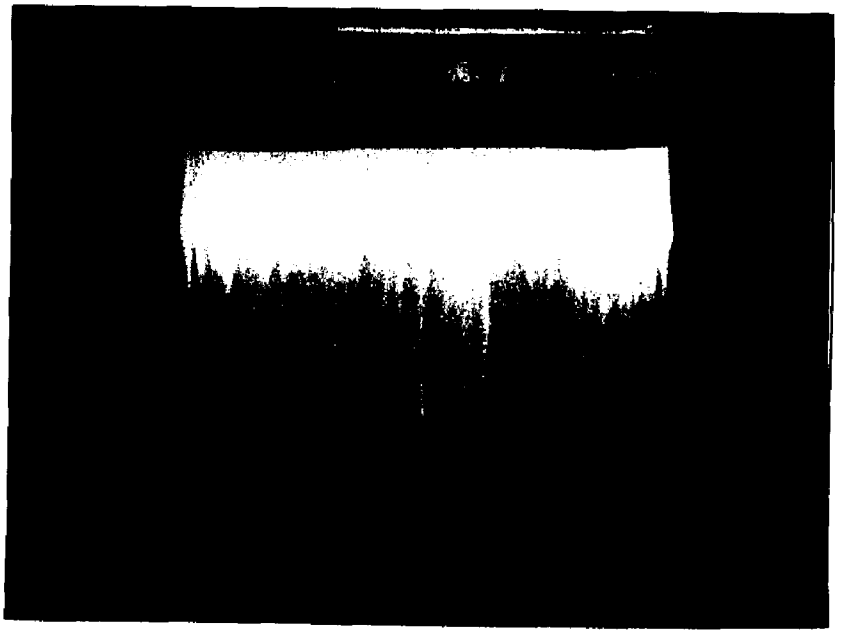


Figure 18. Size-selection of transgenic mutant DNA for production of the bacteriophage library. DNA was exposed to *Mbo*I restriction enzyme for increasing durations to determine the optimal degree of partial digestion (A). Mass preparation fragments between 10 and 20 kb were excised from the gel (B), re-extracted and electrophoresed through agarose to demonstrate purity (C).



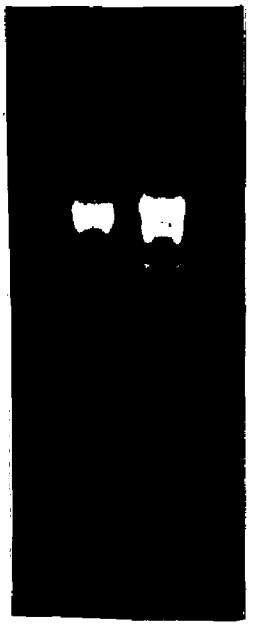


Figure 19. Restriction enzyme map of recombinant bacteriophage cloned from the transgenic mutant genomic DNA library. Wavy lines represent sequences derived from pFR400. Hatched boxes represent repeated sequences in mouse DNA. The underlined 2.4 kb *Eco*RI fragment was employed as a probe in Figure 20. Restriction enzyme sites are abbreviated as follows. B:*Bam*HI, Bg:*Bgl*I, E:*Eco*RI, H:*Hin*dIII, P:*Pst*I, Pv:*Pvu*II, S:*Sac*I, Sa:*Sal*I.

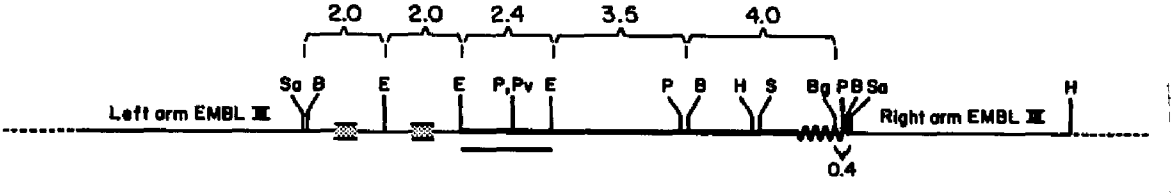


TABLE IV. Breeding data of the transgenic pedigree.

	HF X NM	NF X HM	HF X HM	AF X NM	AF X HM
No. of litters	2	7	20	1	1
No. of pups born	18	84	156	4	3
No. of pups affected	0	0	38	0	3
Per cent affected	0	0	24	0	100
Per cent carriers among non-affected	NT	68	60	NT	NT

M indicates a male parent, F a female parent. N: normal mouse, H: hemizygous transgenic mouse, A: affected transgenic mouse, NT: not tested.

Figure 20. Cosegregation of double doses of transgene per diploid genome with the mutant phenotype. Numbers below the bands indicate the ratios of upper to lower band intensities as recorded by scanning densitometry. The positions of *Hin*dIII fragments of λ DNA, used as size markers, are shown at right.

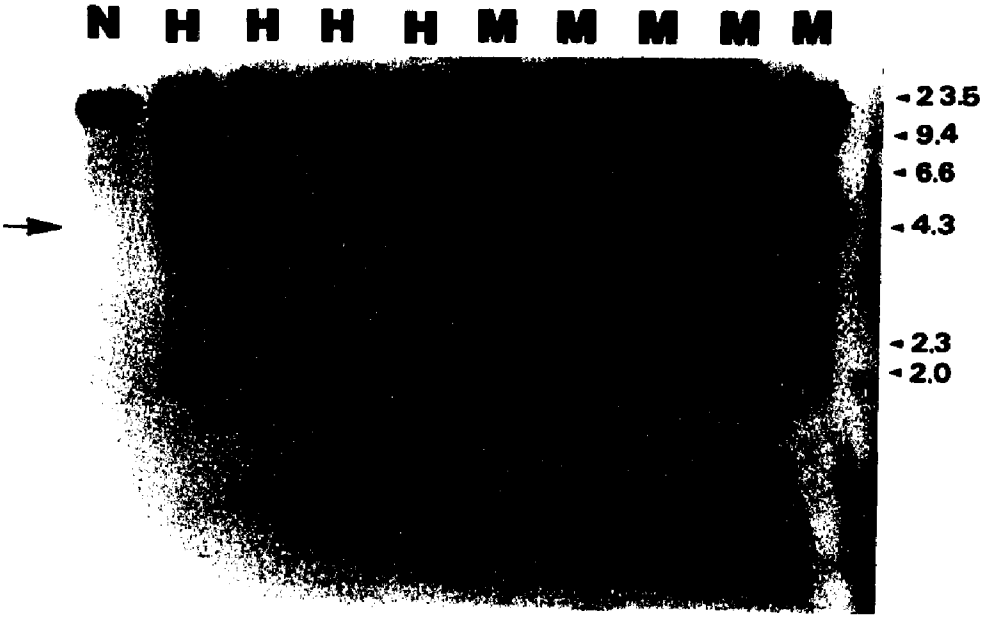


Figure 21. Dot blot hybridization of 2 μg of spleen DNA obtained from progeny of crosses between hemizygous transgenic mice mated with *pcd*^{2J} homozygotes. The probe used was pFR400. M indicates mutant animals; N, normal animals.

Figure 22. Metaphase spread of Giemsa-banded fibroblasts derived from primary tissue culture of carrier transgenic embryo skin.

Figure 23. RNAse protection assay of samples collected from brain and cerebellum of mutant transgenic (M), obligate hemizygote transgenic (H) and normal non-transgenic (N) animals. The 275 base pFR400 RNA species is indicated by the arrow. Positive sense pFR400 RNA (p) and yeast T RNA (n) were included in the hybridization and RNAse digestion as positive and negative controls respectively.

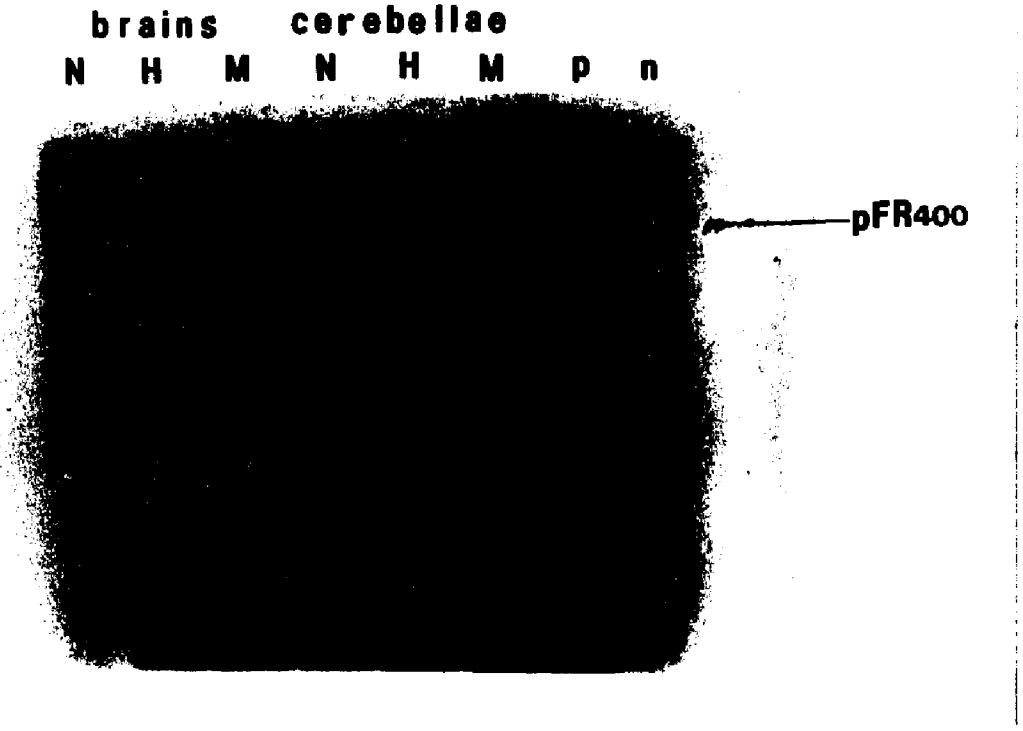
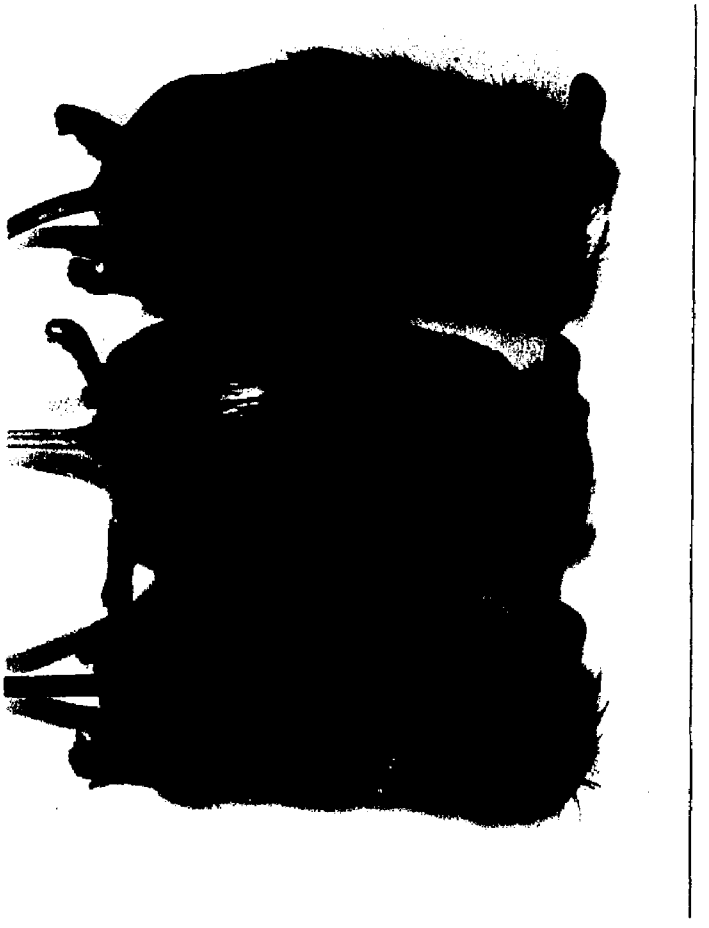


Figure 24. Coat color chimerism of three of eight animals produced from the aggregation of albino mutant transgenic and pigmented normal mouse embryos.



DISCUSSION I

In our mouse colony a transgenic line established by microinjection of the recombinant plasmid pFR400 exhibits a severe motor disorder and male infertility when male and female hemizygotes are crossed. Genetic analysis indicates that the mutant phenotype results from recessive insertional mutagenesis. Southern analyses further support this conclusion, showing that affected animals carry twice the amount of pFR400 per diploid genome as unaffected members of the line do.

Although persuasive, the above data do not formally differentiate insertional mutants from those which might arise from transgene expression. Hemizygous transgenic mice might express pFR400 at a level not sufficient to cause abnormalities, while additive expression of 2 transgenes in homozygous mice could exceed a threshold of expression beyond which developmental abnormalities are induced. This possibility was addressed by testing the P432 mutation for allelism with spontaneous mouse mutations known to affect Purkinje cell development. The behavioral and histologic features of P432 mutants bore striking similarities to *pcd/pcd* mice, with the same cell types of the central nervous system affected, and with severe impairment of spermatogenesis. These findings encouraged us to test this transgene insertion site for allelism with *pcd*. The finding that double heterozygotes for the transgene and the *pcd* allele manifest the mutant phenotype constitutes a positive allelism test. This demonstration, coupled with the finding of a normal karyotype in transgenic animals, provides compelling evidence that the disorder in the transgenic pedigree resulted from insertion into the *pcd* locus.

It is not surprising that some transgenic mutant males were sterile while others were fertile. Whereas *pcd* mutant males are infertile, pcd^{2J} mutant males are not. Since the transgenic pedigree was established on a hybrid background (Gordon 1986) breeding may result in cosegregation of the transgene with modifying alleles which alter the effect of the insertional mutation upon male fertility.

It is unclear why a high intensity of hybridization with pFR400 RNA was found in mutant cerebellum. Transcription most likely results from activity of one of two promoters-that within the SV40 early region of the foreign gene insert and the other, a hypothetical promoter, within flanking mouse DNA just upstream of the integration site. Since these promoters would have different transcription start sites, a second RNAse protection assay could distinguish their activities. A new radiolabelled antisense RNA probe should extend upstream into the SV40 region, from pFR400 bases 180 to 1115 for example (Figure 4). Protection of a 275 base species would represent activity of the SV40 promoter while protection of a larger species would suggest activity of an upstream mouse DNA promoter. If an upstream promoter were found to be responsible for pFR400 transcription in mutants, the the *pcd* gene would be the most likely source of upstream promoter. One could then hypothesize that the *pcd* promoter was stimulated by a negative feedback mechanism to produce high levels of pFR400 RNA rather than *pcd* RNA because the transgene had integrated just downstream of the *pcd* promoter.

As noted previously, we have cloned material flanking pFR400 in p432 mice. Initial tests for RNA homologous to the cloned material have been negative. However, as with several other insertional

mutations, continued chromosome walking and the screening of libraries enriched for Purkinje cell or photoreceptor cell cDNAs should lead to isolation of coding sequences of the *pcd* gene. *pcd* may thereafter come to provide an important model of photoreceptor cell loss. Also, because known mutations of many independent genes affect both neurologic and reproductive tissues, it is reasonable to hypothesize that these genes are members of a family, and as such, share sequence homology. If this is the case, cloning of the *pcd* locus may lead to the isolation of a number of related genes with similar functions. Even if this proves not to be the case, continued analysis of the transgenic pedigree should lead to isolation of the *pcd* gene, and thereby contribute significantly to our understanding of mammalian development.

BACKGROUND II/PARENTAL IMPRINTING

Parental imprinting is the differential modification of the maternal and paternal genomic contributions to the zygote. The concept of imprinting was formulated to explain observations that maternally and paternally derived genes may carry equivalent genetic codes but nonetheless function unequivalently. Such observations partially contradict Mendelian laws. Classical genetics, by extrapolation, would instead predict equal functions of sister chromatid genes with identical base sequences. But unequal functions of some analogous genes within autosomal sister chromatids may be a requirement for normal development. Studies have shown this true in mealy bug (Sapienza et al 1987), sunfish (Whitt et al 1972), kangaroo (Sharman 1971) and mouse. For example, mouse embryos containing either two female pronuclei or two male pronuclei have been created by exchange of pronuclei between zygotes (Barton et al 1984, McGrath & Solter 1984, Surani et al 1984, Anderegg & Markert 1986). In embryos with two female genetic components, fetal development is good but extraembryonic membrane and placental development is poor, resulting in embryonic demise. In mouse embryos with two male genetic components the reverse is true-with good extraembryonic membrane development and poor development of the embryo proper. Other evidence derives from matings between mice with different chromosomal translocations that duplicate specific maternal and paternal chromosomal regions (Searle & Beechey 1978). The combinations of maternal or paternal duplications may be lethal or result in different phenotypes than those ordinarily seen. Specifically, the *hairpin tail* mutation of mouse is known to be

lethal only when maternally transmitted (McGrath & Solter 1984). Evidence of imprinting also exists in man. For example, the juvenile form of Huntington's disease appears to be inherited preferentially from the male parent and the infantile form of myotonic muscular dystrophy is inherited preferentially from the female parent (Merritt et al 1969, Bartlett et al 1987).

The reason for the uneven parental contributions to the conceptus is not yet understood, and most hypotheses have been directed instead at elucidating the mechanisms of parental imprinting. This search has sought factors which can be established during or before gametogenesis, persist stably through DNA replication and cell division in the conceptus, and be erased in the germ line to be differentially established once more in sperm and egg genomes. The methylation of DNA has received much attention as a possible mechanism of parental imprinting.

DNA METHYLATION

Differential patterns of DNA methylation have been shown capable of regulating the expression of specific genes (Doerfler 1983), of chromosomal domains (Naveh-Many & Cedar 1981) and of whole chromosomes (Monk 1986). Growing evidence suggests that methylation may also be a mechanism of parental imprinting. Differential methylation dependent upon the parental gender of origin has been shown in 4 out of 5 transgene loci studied by Sapienza et al (1987), 1 out of 7 studied by Reik et al (1987) and 1 out of 10 studied by Swain et al (1987). Although counterexamples exist, the majority of studies that have

reported differential modification find that transgenes are less methylated when paternally derived than when maternally derived. The evidence does not necessarily indicate that DNA methylation is the primary mechanism of imprinting, however. Another form of imprinting could occur as a result of the binding of specific proteins to certain chromosomal regions, which would then be protected from undergoing *de novo* methylation. In this way, undermethylated foreign gene inserts could be expressed subsequently in specific tissues at specific developmental stages. The eventual expression of these genes would require in addition an appropriate combination of enhancer sequences and *trans*-acting factors. This explanation is compatable with observations that the influence of germline origin results in the same degree of methylation of transgenes in all somatic tissues while somatic expression is often confined to a tissue in which the enhancer is expected to be functional (Reik et al 1987, Sapienza et al 1987, Swain et al 1987).

DNA methylation may be assessed with the isochizomeric pair of restriction enzymes *Msp*I/*Hpa*II. Whereas both enzymes recognize the four basepair motif 5'CCGG3', *Hpa*II cannot cleave when the internal cytosine residue is methylated (Waalwijk & Flavell 1978). Comparison of the distributions of DNA digestion with these enzymes therefore reflects methylation at the aforementioned internal cytosine residues.

In eukaryotic somatic tissues approximately 2-5% of cytosines are methylated. These occur as CpG dinucleotides and hence are symmetrically situated on both DNA strands. Hemimethylated DNA produced by DNA replication becomed fully methylated by the action of a

maintenance methylase. *de novo* methylation in the absence of a hemimethylated template also is well-established. Evidence of *de novo* methylation derives from the finding of foreign gene methylation in transgenic mice produced by manipulation with non-methylated DNA (Jahner & Jaenisch 1985, Hadchouel et al 1987, Palmiter et al 1987).

TROPHOBLASTIC HYPOMETHYLATION

Extraembryonic tissue DNA is generally less methylated than somatic tissue DNA. The finding has been reported in the comparative analyses of total DNA (Razin et al 1984), of repetitive sequences (Chapman et al 1984, Monk et al 1987) as well as a number of unique gene sequences (Stanford et al 1985). This finding may be difficult to interpret in light of the substantial methylation seen in the very early embryo and the limited number of cell divisions which preceed placental determination. One would expect the cells of the early placenta to feature at least moderate DNA methylation. But Monk et al (1987) have found that preimplantation development is associated with loss of methylation as compared to that seen in the very early embryo. This loss of methylation may reflect either a passive dilutional effect in which DNA methylation is not replicated along with cell division or it may reflect an active *demethylase* activity. Nonetheless, as preimplantation development progresses, the embryonic and extraembryonic lineages are progressively and independently remethylated to different final extents (Monk et al 1987). Thus, differential methylation may be a mechanism initiating or confirming the differential programming of the germ layers as well as a mechanism

of parental imprinting.

INHERITANCE OF FOREIGN GENE INSERTS

Previous studies that demonstrate *de novo* methylation of donor genetic material were conducted before it was appreciated that parental imprinting may dictate patterns of methylation of genes transferred through the germline. But transgenes are capable of reporting *de novo* methylation exclusive from imprinting methylation. In the tissues of founder animals produced by microinjection the foreign gene insert is "inherited from the microneedle" rather than through the germline. If the DNA selected for microinjection was unmethylated then any methylation of the insert in founder must reflect *de novo* activity. Also the foreign gene insert in founder DNA is free from the potential methylating effects of germline transmission. In this thesis we have therefore focused upon the methylation of placental DNA in founder to determine the roles of *de novo* activity and parental imprinting in shaping the pattern of DNA methylation seen in that tissue.

MATERIALS AND METHODS

Plasmids. The plasmid pBR322 is a 4363 bp molecule with 26 5'CCGG3' recognition sites for the methylation-inhibited restriction enzyme *Hpa*II and its methylation insensitive isoschizomer *Msp*I. A portion of the pBR322 source was treated with *Hpa*II methylase prior to microinjection. Complete methylation of the *Hpa*II sites was confirmed by demonstrating resistance to digestion with a 100-fold excess of enzyme.

Production and Identification of Transgenic Mice. Transgenic mice carrying both un-methylated and premethylated sources of pBR322 were produced by the method of Gordon et al (1980) after linearization with *Pst*I. Transgenic animals were identified by dot blot hybridization of 3 μg of placental DNA. Animals were confirmed by progeny testing to transmit the foreign gene insert to approximately half of all pups derived from matings with normal mice. This indicated that the transgenic mice were not genetic mosaics for the foreign gene insert, a condition which could be associated with integration of pBR322 molecules at different sites in the placental and somatic genomes, thereby giving rise to different methylation patterns dependent upon site of integration (Wagner et al 1981, Burki & Ullrich 1982).

Recovery of Placentae. Founder mice and subsequent generations were delivered by Cesaerian section on day 19 of gestation. Placentae were harvested for DNA analysis and the corresponding pups were marked by tail or toe clipping.

Placental and Somatic Methylation Studies. Pups were reared to

adulthood, bred, and studied for the degree of methylation in placenta and in select somatic tissues. To directly compare the methylation status of genes before and after parental imprinting, DNA methylation in placentae and somatic tissues of founder animals was compared to that seen in the same pedigree after transmission of pBR322 through both the male and female germlines. Methylation was assessed by the digestion of 15 μg purified tissue DNA to completion with *Hpa*II, separation through agarose and transfer to nylon membrane (Southern 1975). DNA fragments greater than 10 kb in size were transilluminated for 40 seconds with short wave ultraviolet light to aid in their transfer. Blots were then hybridized to pBR322 labelled by random priming. The degree of transgene digestion, which correlated inversely with methylation of *Hpa*II sites, was determined both by direct examination of blots and by scanning densitometry with an E-C apparatus.

As control for the completeness of each *Hpa*II or *Msp*I digest, 0.1 μg intact λ DNA was included in all reaction mixtures reported. Following hybridization to radiolabelled pBR322, nylon filters were stripped and rehybridized to radiolabelled λ DNA. Since λ DNA is not methylated at cytosine residues, complete *Msp*I and *Hpa*II reactions should digest λ into the same patterns of low molecular weight fragments.

RESULTS II

Somatic DNA Is Consistently Heavily Methylated. DNA methylation was compared in kidney, liver, lung, spleen and testes of a single F1 transgenic animal obtained by mating with a founder produced by microinjection of unmethylated pBR322. The consistently heavy methylation of somatic tissues in the F1 animal is evidenced by hybridization with large molecular weight fragments despite complete digestion with *Hpa*II (Figure 25, lanes d-h). A similar result is observed with kidney DNA in the corresponding founder animal (Figure 25, lane a). Indeed, the finding of significant methylation in the embryo proper is generalizable to all somatic tissues and to all generations studied (see Figures 30 & 31 lanes a, d & g). Figure 26 shows that the differences between placental and somatic *Hpa*II digestions are apparent with ethidium bromide staining and therefore are not unique to pBR322. This figure also serves as a control to indicate that the differential methylation patterns of pBR322 (Figure 25) are not due to differential DNA loading onto the agarose gels.

Founder Placenta Is Hypomethylated. Tissues from three pairs of transgenic founder animals were investigated for DNA methylation. Placental DNA was compared to spleen DNA, a representative somatic tissue. The heavy methylation of spleen pBR322 is demonstrated by an intense hybridization at high molecular weights (Figure 27). The remarkable hypomethylation of pBR322 in placental DNA is evidenced by a low intensity of hybridization at high molecular weights and their partial reciprocation at low molecular weights (Figure 27). The lower overall signal in placental DNA suggests that some *Hpa*II fragments

are not of sufficient size for detection with Southern hybridization at 65^{0}C. In fact, when pBR322 is digested to completion with *Hpa*II, 19 of the 26 fragments generated are less than 200 bp in length. The visual interpretation of these results is confirmed by scanning densitometry scanning shown in Figure 28.

Figure 29 shows hybridization of the identical blot to radiolabelled λ DNA after the filter had been stripped to remove pBR322 hybridization. Distinctive, fully-digested λ bands are evident. This control was performed with confirmation of complete digestion for all blots reported but only shown here.

Unimprinted Placenta Does Not Maintain Transgene Premethylation. Two lines of transgenic mice were produced by the pronuclear microinjection of pre-methylated pBR322. Figure 30 lane b shows that even after premethylation, the placenta of the founder animal exhibits markedly low methylation of the foreign gene insert. Methylation is more significant in subsequent generations (Figure 30 lanes c & d). To demonstrate that differences in placental DNA methylation between founder and progeny transgenics are unique to pBR322, the filter was stripped of label and rehybridized using total mouse genomic DNA as probe. This probe gives a view of the methylation status of repeated elements in the genome. As shown in Figure 31, the pattern of methylation of endogenous repeat elements in transgenic founder placental DNA is quite similar to that of progeny.

Germline Transmission Increases Methylation. After passage through either the male or female germlines, placental DNA methylation remained low relative to DNA extracted from somatic tissues. Unexpectedly, placental methylation was more extensive in transgenic progeny than in

the founder animal (contrast lanes b & c of Figure 30).

Differential Methylation Associated With Germline Transmission. Since *de novo* methylation is either absent or at low levels, placental tissue is a logical choice to investigate the effects of parental imprinting upon DNA methylation. Figures 32 & 33 demonstrate the effects of passage through the male and female germlines upon transgene methylation in three generations of transgenic mice. With both the unmethylated pedigree (Figure 32) and the premethylated pedigree (Figure 33) transmission through the male germline resulted in greater placental methylation than passage through the female germline (contrast lanes e & f).

Figure 25. Placental DNA hypomethylation is revealed by tissue DNAs of a transgenic pedigree produced by pronuclear microinjection of unmethylated pBR322. (a&b) founder or G0 kidney and placenta. (c-h) G1 placenta, kidney, liver, lung, spleen and testis.

Figure 26. Differential DNA methylation of soma and placenta is seen with ethidium bromide staining of agarose gels. (a&b) G0 kidney and placenta. (c-h) G1 placenta, kidney, liver, lung, spleen and testis.

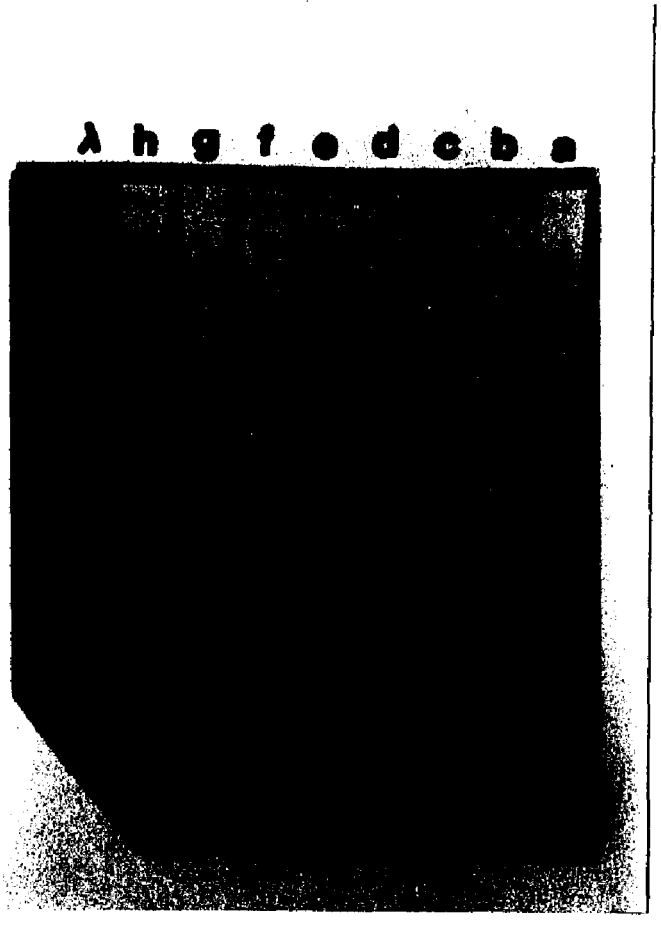


Figure 27. *Hpa*II-digested placental (p) or splenic (s) DNA samples of three founder animals. Most apparent in the rightmost pair, somatic DNA hybridization with pBR322 is greater at high molecular weights while placental DNA hybridization is greater at low molecular weights. M indicates molecular weight markers produced by digestion of 0.5 μg bacteriophage λ DNA with *Hin*dIII. These markers are visualized in Figure 29.

Figure 28. Scanning densitometry of X-ray film from hybridization with radiolabelled pBR322 of *Hpa*II-digests of DNA from the placentae (P) and corresponding spleens (S) of three founder animals.

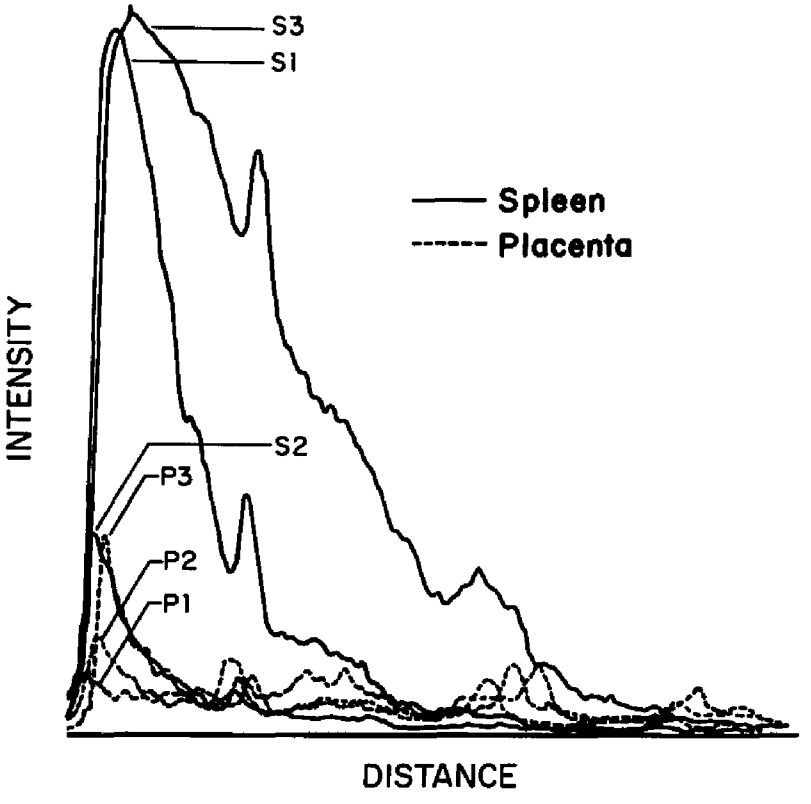


Figure 29. 0.1 μg λ DNA was included in all digests to confirm the completion of each enzyme reaction. Filters were reprobed with radiolabelled λ DNA to yield distinctive, fully-digested λ bands for each experiment reported. The reprobing shown in this figure corresponds to the filter shown in Figures 27 & 28.

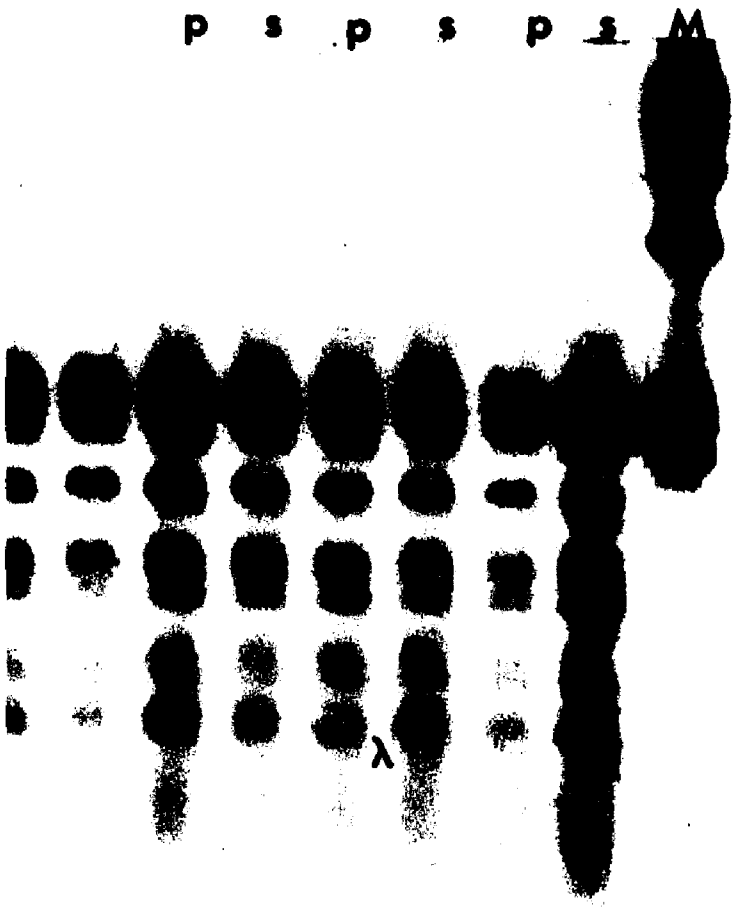


Figure 30. Germline transmission increases placental DNA methylation. (a&b) premethylated G0 kidney and placenta. (c) female transmitted G1 placenta. (d) male transmitted G2 placenta. Radiolabelled pBR322 was used as probe.

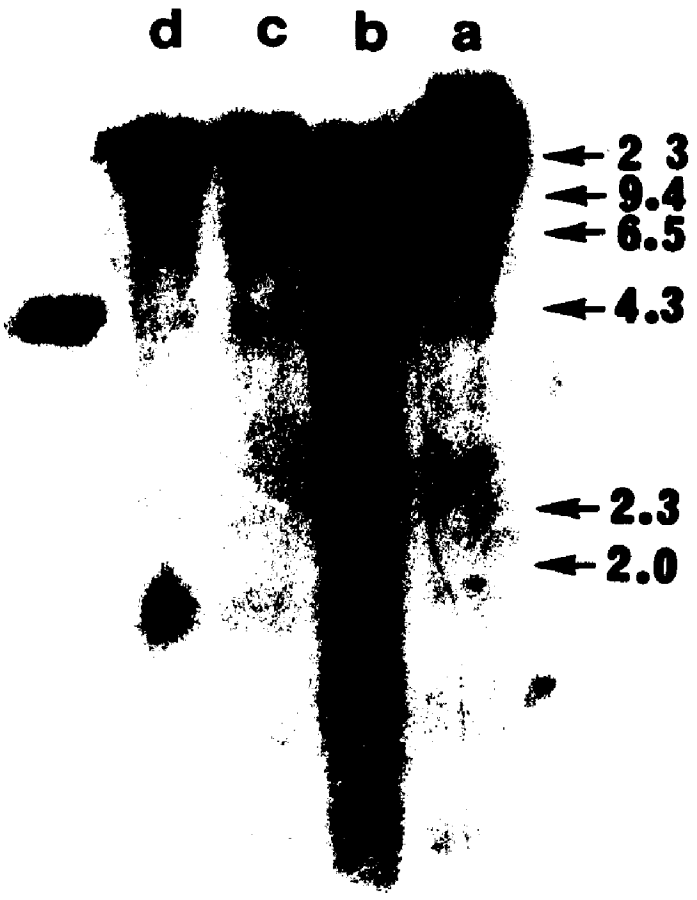


Figure 31. The filter in Figure 30 was stripped and reprobed with radiolabelled mouse total cell DNA to illustrate the distribution of mouse repeat elements shown here. (a&b) premethylated G0 kidney and placenta. (c) female transmitted G1 placenta. (d) male transmitted G2 placenta. The distributions of hybridizations in lanes b, c & d are similar.

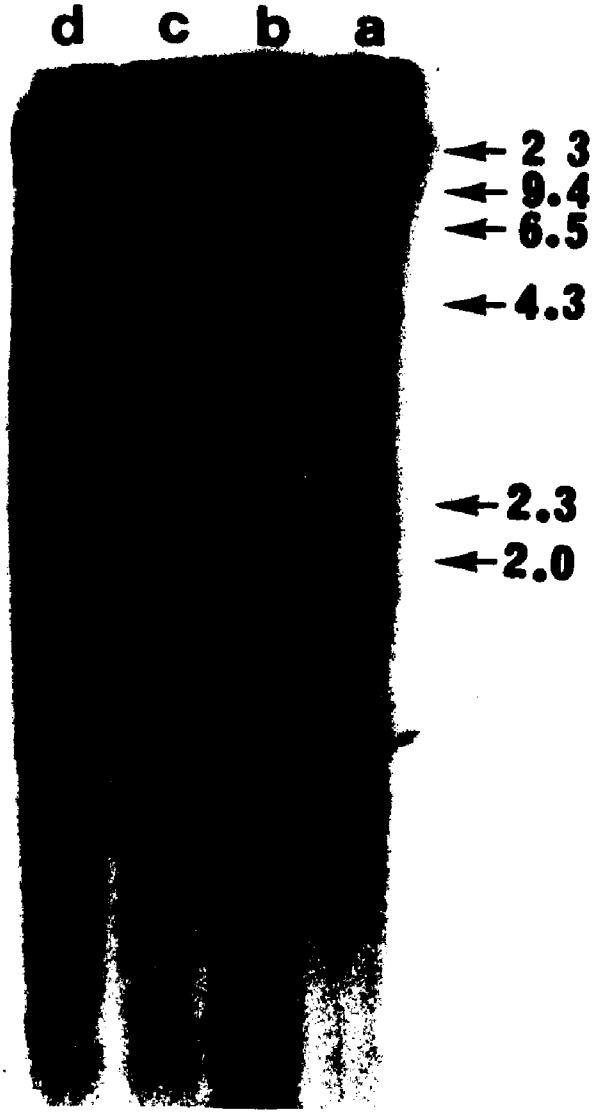


Figure 32. *Hpa*II-digests of tissue DNA from an unmethylated pBR322 pedigree. (a) G0 kidney. (b&c) male transmitted G1 placenta and soma. (d&e) male transmitted G2 soma and placenta. (f&g) female transmitted G2 placenta and soma.

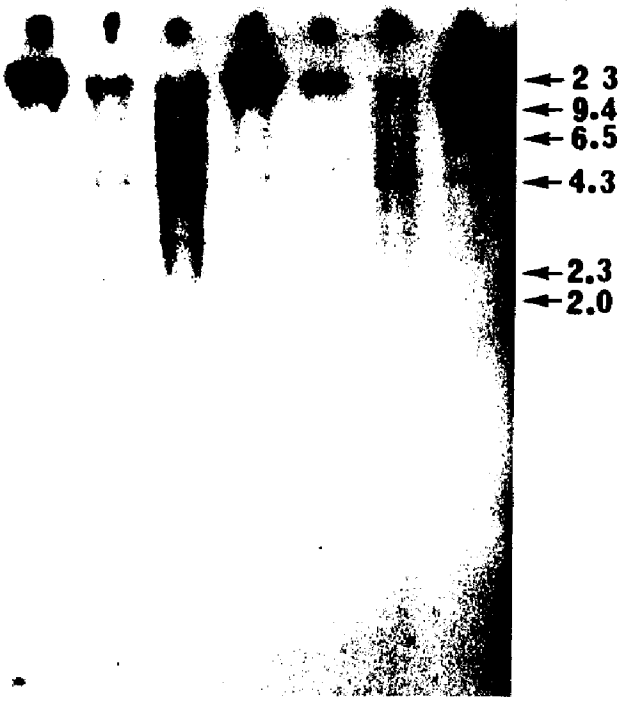
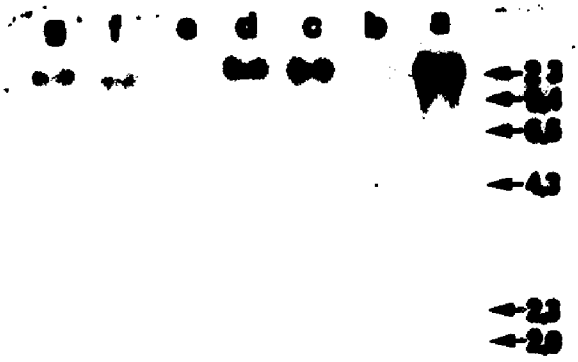


Figure 33. *Msp*I and *Hpa*II-digested premethylated pBR322 pedigree tissue DNA. The arrow shows that hybridization at low molecular weight bands is evident with *Msp*I (M) digestion of founder kidney DNA. (a) G0 kidney. (b&c) female transmitted G1 placenta and soma. (d&e) male transmitted G2 soma and placenta. (e&f) female transmitted G2 placenta and soma.



DISCUSSION II

To study *de novo* methylation of DNA in the absence of parental gene imprinting, five lines of transgenic mice were produced. Placental DNA from founder animals which had inherited pBR322 "from the microneedle" and which were therefore free from parental imprinting, displayed striking hypomethylation. Subsequent transmission through the male or female germlines to progeny was associated with increases of placental DNA methylation. When transgene was premethylated with *Hpa*II methylase prior to microinjection, unimprinted placenta was unable to maintain DNA methylation through the action of maintenance methylase.

The DNA of founder placenta was shown to be markedly hypomethylated (Figure 27), yet a comparison to *Msp*I-digested material shows that a small amount of hybridization to high molecular weight *Hpa*II fragments was observed in founder placenta. The most immediate explanation for this low signal of high molecular weight hybridization is that a correspondingly low level of methylation exists in founder placenta. But conventional methods for the recovery of placenta permits the contamination by small amounts of white blood cells present within the vasculature. Thus, the low intensity of high molecular weight hybridization seen in founder might also reflect contamination by non-placental tissues. An examination of placental DNA made pure from contaminating tissues might instead reveal no hybridization at high molecular weights, reflecting a complete absence of DNA methylation. Such an experiment would conclusively determine if the DNA methylation of founder placenta is at low levels or entirely

absent. Another approach to this problem might be to use the polymerase chain reaction to amplify pBR322 sequences in the DNA of individual cells of the placenta. Methylation can thereafter be determined in the amplified segments.

The polymerase chain reaction may also be useful in determining if the aforementioned loss of methylation in early murine development is passive or active. A passive lack of maintenance methylase activity associated with cell division predicts that the loss of DNA methylation could not proceed faster than by 50% per cell devision. Active demethylation might proceed at any rate. In this study DNA would be extracted from single cells at proceeding developmental stages and digested with *Hpa*II. A pBR322 fragment bounding a *Hpa*II site would be selected as target for amplification. Only in methylated fragments would the target be undigested and therefore permit amplification. If the results indicate that a specific stage of cell division yields a near complete loss of DNA methylation of the amplified pBR322 fragment then demethylase activity would be the likely explanation.

The observed differences between placental and somatic *de novo* methylation might have resulted exclusively from a faithful maintenance of conditions established in early cleavage. A transgenic pedigree was therefore produced with the premethylated pBR322 source to assess that possibility. In the experiment, the trophoblast cells were presented with heavily methylated material which had not been subject to parental imprinting in founder tissues. The observation that placental DNA of this founder was nonetheless hypomethylated indicates that hemimethylase activity is incapable of maintaining methylation in an

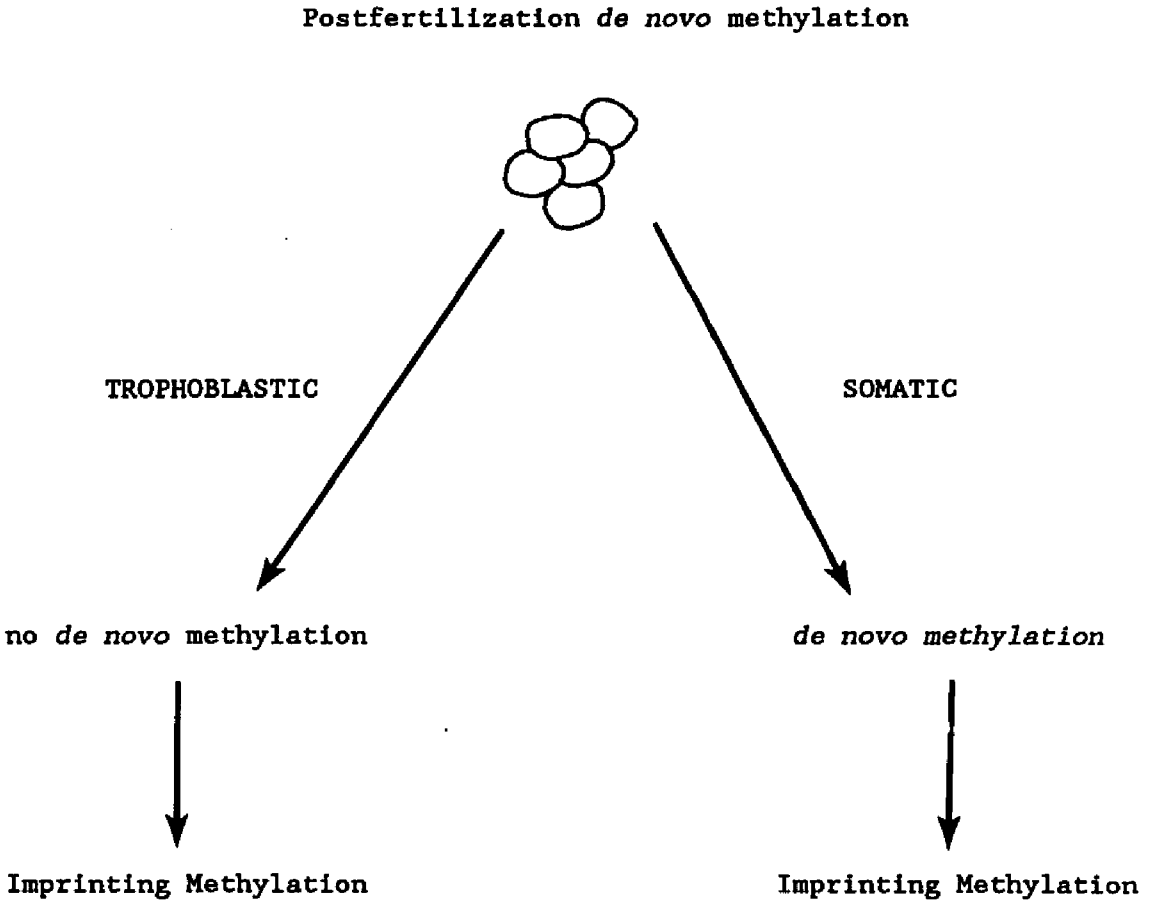
unimprinted substrate.

The patterns of methylation seen with the foreign gene inserts suggest that cells of the cleaving embryo, once determined to become trophoblast, are unable to methylate DNA *de novo*. It has previously been established that DNA methylation is very low in fetal germ cells, high in early cleaving embryos, and again low in blastocysts (Manes & Menzel 1981). Methylation at preimplantation stages prior to the 8-cell stage is also substantial, though a small portion of fragments are of low molecular weight (Manes & Menzel 1981). We believe these results, in conjunction with the present findings, are best explained by proposing a two tiered system of DNA methylation (Figure 34): After fertilization, DNA is rapidly and extensively methylated by a *de novo* mechanism. This initial methylation cannot be maintained by cells determined to become the trophoblast. In both the trophoblast and the embryo proper, a second methylation activity determined by parental imprinting exists. This latter mechanism accounts for most or all methylation seen in placental DNA, but for a relatively small amount of the methylation seen in DNA of the embryo proper. Our findings further suggest that methylation dictated by imprinting does not exist as a residuum of the initial *de novo* methylation process. Were this the case, placental pBR322 methylation would be expected to be the same in founder animals as that seen after germline transmission. Thus our data indicate that methylation dictated by imprinting exists as a distinct *de novo* mechanism. When imprinting is invoked cannot be determined from the present data.

The finding that blastocysts, which are largely composed of cells destined to become extraembryonic tissues, have markedly low methylation (Monk et al 1987), indicates that the inability to maintain the methylation applied during early cleavage is a very early marker of trophoblast determination. Since only a few cell divisions occur between trophoblast determination and blastocyst formation, the loss of methylation in trophoblast must occur very shortly, perhaps immediately, after segregation from the embryo proper. Thus the differential ability to methylate DNA may be one of the earliest distinguishing features of trophoblast formation.

Only conjecture can explain why somatic tissues may possess a *de novo* methylation mechanism which trophoblastic tissues do not. Perhaps placental DNA methylation is less critical to the survival of the organism. In the relatively undermethylated placenta, for example, leaky gene expression and aneuploidy are common even among healthy conceptuses. The greater levels of methylation seen in somatic tissues, therefore, may reflect stricter demands for the regulation of genomic activity imposed upon the embryo in order to assure survival. It is similarly unknown whether the *de novo* activity of soma evolved independently of a placental evolution or whether the activity first evolved in both tissues, and was subsequently lost in placenta. This latter issue can be addressed, however, by a thorough species-to-species examination of founder placental DNA methylation.

Figure 34. Proposed scheme for the mechanism of methylation shows that placenta has no *de novo* DNA methylation capacity.



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