THE EFFECTS OF MOUSE MUTANT t^{w1} UPON EMBRYONIC DEVELOPMENT: HISTOLOGICAL AND TISSUE CULTURE STUDIES

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ABSTRACT

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The $\underline{t}$ mutants ($\underline{t}$ haplotypes), a complex group of alleles identified in mice in association with the gene Brachyury ($\underline{T}$), are divided into seven complementation groups. In the homozygous state, alleles from each complementation group affect different stages of early embryonic development. This study examined the effects of one mutant ($\underline{t}^{w1}$) after it was backcrossed into the C3H.B10 strain of mice.

Four different types of experiments were performed in order to study the effect of mouse mutant $\underline{t}^{w1}$ upon the embryonic development of the central nervous system. First, the gene was successfully transferred from a balanced lethal ($\underline{T+}/\underline{+t}^{w1}$) "pen bred" stock to an inbred strain of mice, C3H.B10, in order to reduce the heterogeneous genetic background of the original strain and to permit the study of the action of $\underline{t}^{w1}$ in the absence of mutant genes $\underline{T}$ and $\underline{a}^{t}$. The gene is presently maintained in two strains: a balanced lethal line, $\underline{T+}/\underline{+t}^{w1}$ (generation F_{12}) and a backcross-intercross line (generation N_5F_4).

Second, normal ($\underline{+}/\underline{+}$, $\underline{+}/\underline{t}^{w1}$) and abnormal ($\underline{t}^{w1}/\underline{t}^{w1}$) embryos at days 9 to 15 of gestation were subjected to gross examination after dissection from amniotic sacs and histological examination at the light microscope level. On the basis of gross morphology, mutant embryos could not be easily distinguished from normal littermates

prior to 11 days post-coitus (pc). From 11 to 15 days pc mutant embryos could be identified by their relatively smaller size, retarded growth (younger developmental age than normal littermates), enlarged hearts and degenerating neural tissues.

At the light microscope level, mutant embryos could be identified from serially sectioned material as early as 9 days pc by the presence of pycnotic cells within the mantle layer of the rostral portion of the rhombencephalon. At later stages of development, mutant embryos became more easily recognized in sectioned material by the increased number of pycnotic cells in the ventral rhombencephalon and neural tube. As mutant embryos developed from 9 to 13 days pc, a specific pattern of cellular pycnosis appeared within the neural tube. Pycnotic cells were always confined to the mantle layer and first appeared in the ventral region of the rostral portion of the rhombencephalon. The number of pycnotic cells increased with the age of the embryo along the ventral neural tube in rostral and caudal directions, extended into regions of the dorsal neural tube in the area of the caudal rhombencephalon and continued in the dorsal neural tube towards the tail. Related neural ectoderm structures (otic vesicle, cranial and spinal ganglia) did not appear to be affected. In general, non-neural structures continued to develop even in the presence of an abnormally developing neural tube. By 12 to 13 days pc, mutant embryos were in the process of being resorbed, and non-neural as well as neural structures contained pycnotic cells.

Third, embryonic tailbud, forelimb bud, or heart tissue was cultured *in vitro* to determine whether the mutant was a general cell lethal and to attempt to identify embryonic genotypes prior to the appearance of gross abnormalities. Due to segregation distortion in males (90% $\underline{t}^{w1}$ and 10% $\underline{+}$ bearing sperm fertilize ova), 45% of the embryos within a litter are expected to be $\underline{t}^{w1}/\underline{t}^{w1}$. Successful cellular outgrowth appeared in 94% of all cultures (190 cultures representing embryos from days 9 to 15 *pc*), indicating that the primary action of the gene does not affect all cells of the mutant embryo.

Fourth, determinations of embryonic genotypes by assaying fibroblasts for the presence of two private H-2 antigens (D-2 and 108) were erratic. Results from assays measuring the incorporation of ^{125}I-iododeoxyuridine into the DNA of viable cells following treatment with mouse anti-H-2.2 or mouse anti-H-2^{tw1} and complement suggest that very low concentrations of antigens are present on these embryonic fibroblasts.

Unlike some *t* haplotypes acting earlier in embryonic development which appear to be lethal for all cells of the mutant embryo, the action of gene $\underline{t}^{w1}$ is specific for cells within the neural tube. These studies support the hypothesis that death of homozygous mutant embryos results from death of specific cell types within the neural tube and subsequent aberrant cell-cell interactions and disorganization.

DEDICATION

To my husband, Bill

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LIST OF ABBREVIATIONS USED IN TEXT AND FIGURES

b	Red blood cell
f	Nerve fiber
L	Marginal layer
M	Mantle layer
m	Mitotically dividing cell
n	Notochord
p	Pycnotic cell
s	Lumen of spinal cord
v	Vertebrae
CR	Crown-rump length
cr	Lumen in region of caudal rhombencephalon
rr	Lumen in region of rostral rhombencephalon
PBS	Calcium-magnesium free phosphate buffered saline
IUdR	Iododeoxyuridine

LITERATURE REVIEW

The intriguing genetic and developmental features of the T-complex in the house mouse (*Mus musculus*) have been recently reviewed (Bennett, 1975; McLaren, 1976; Sherman and Wudl, 1977; Klein and Hammerberg, 1977). The complex, located on chromosome 17 near the major histocompatibility locus (*H-2*), is composed of sets of dominant *T* and recessive *t* mutations. Although the functions of the mutations are unknown, two hypotheses have been formulated to explain the action of the genes: 1) The mutations code for specific cell surface antigens that govern cell-cell interactions during embryonic development (organizational failure hypothesis; Bennett, 1975); or 2) the mutations code for proteins associated with intermediary metabolism of all cells and required for embryonic development (generalized cell lethal hypothesis; Mintz, 1964; Wudl and Sherman, 1976; Wudl, *et al.*, 1977). The following review will summarize the current knowledge about dominant *T* and recessive *t* mutations.

The T Locus

Historical Background: The study of the *T* locus region began in 1924 when Dobrovolskaĭa-Zavadskaĭa identified a male mouse with altered tail morphology (Dobrovolskaĭa-Zavadskaĭa, 1972). The mutation had arisen spontaneously in a laboratory stock of mice, was found to be inherited, and was designated as *Brachyury* (*T*). In crosses

between heterozygous parents (T/+), mice with short tails and normal tails were produced but the total number of progeny was reduced. The lethality of T/T embryos was subsequently confirmed by histological examinations of embryos (Chesley, 1935).

In outcrosses of short tail mice to wild mice, progeny without tails were occasionally observed, instead of progeny with short tails (Dobrovolskaĭa-Zavadskaĭa and Kobozieff, 1932). In crosses between these tailless mice and mice with normal tails, one-half of the progeny had short tails and the other half had normal tails. On the other hand, when tailless mice were intercrossed, only tailless progeny were obtained and the litter size was drastically reduced. The investigators concluded that taillessness resulted from the interaction of Brachyury with another recessive mutation, designated as t. The embryonic lethality of the first t mutation in the homozygous state was later confirmed by Gluecksohn-Schoenheimer (1940).

Dunn and coworkers continued to study the T-complex and found the presence of additional t mutations in laboratory and wild mice (see review by Klein and Hammerberg, 1977). Discovery and identification of t mutations was possible only in the presence of the Brachyury gene.

The T locus is associated with several other gene loci found on chromosome 17 (Figure 1A). The quaking (qk) gene affects the synthesis of myelin in the central nervous system; homozygotes have marked rapid tremors which disappear at rest (Green, 1968). The T complex associated protein (Tcp-1) gene codes for a protein characterized by two dimensional gel electrophoresis and found in high concentrations in testicular cells (Silver, et al., 1979). The low

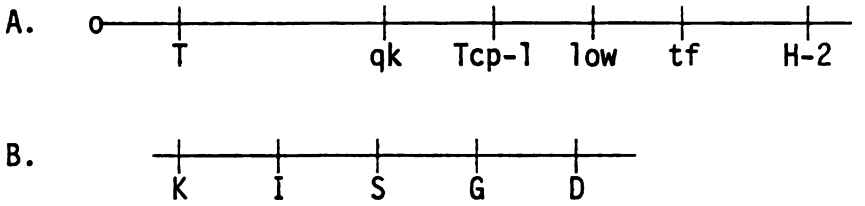


Figure 1. Chromosome 17 of the house mouse.
A. A partial map of chromosome 17 in the house mouse.
B. An expanded view of the H-2 complex.

(low) gene is thought to be a t mutation and causes low segregation distortion (to be discussed later) in males (Lyon and Mason, 1977). The tufted (tf) gene affects hair growth and maintenance (Green, 1968). The tightly-linked group of genes found in the major histocompatibility complex (H-2; Figure 1B) are identified from studies on allograft rejections, are designated as H-2 haplotypes, and code for specific cell surface proteins (Klein, 1975). These loci, located on the proximal portion of chromosome 17, play a role in mapping various factors found in association with t mutations.

The nomenclature used in discussing T and t mutations is complex and often confusing. The superscripts x and y (T^x, T^y, t^x, or t^y) are general symbols used to represent two different mutations. With respect to T mutations, the superscript usually refers to the laboratory in which the mutation was first identified (See Table 1). With respect to t mutations, mutations extracted from wild populations of mice are designated with a superscript w and number (t^{w1}). Mutations discovered in Dunn's laboratory are designated by a serial number in superscript (t^3). Mutations discovered outside of Dunn's

laboratory and derived from laboratory stocks are designated in superscript by initials of the identifying laboratory and a serial number (t^{h2}, MRC Radiobiological Research Unit, Harwell, England; t^{AE2}, Albert Einstein College of Medicine, New York).

T Mutations: Several spontaneous, and many radiation-induced, mutations have been described (Bennett, 1975; Bennett, et al., 1975). Characteristics common to all T mutations studied to date are: 1) T^x/T^x embryos die in utero at mid-gestation; 2) T^x/T^y heterozygotes also die and thus have not been found to complement each other; 3) recombination has not yet been observed between different T mutations (Klein and Hammerberg, 1977); 4) T^x/T^x embryos may differ phenotypically from T^y/T^y embryos (five phenotypic classes have been recognized; Bennett, et al., 1975); 5) all T mutations interact in trans with recessive t mutations to produce tailless progeny; 6) segregation distortion does not occur in males heterozygous for T; and 7) $T^x/+$ mice, except $T^{hp}/+$, express the wild type protein coded by gene Tcp-1^b (Alton, et al., 1980). The five different phenotypic classes represented by mutations T, T^{hp}, T^h, T^{Orl}, and T^c are described below. A summary of the origin of various T mutations is given in Table 1.

Embryos homozygous for T die at 10-3/4 days of gestation (Bennett, 1975). The placental connections required for nourishment and maintenance of the embryo are not established. The primary defect is thought to involve the inability of the primitive streak to undergo normal differentiation, thereby altering the development of the notochord and mesoderm. In vitro studies have shown that a structurally abnormal T/T neural tube was an effective inducer of cartilage formation in normal somites; however, T/T somites could not produce cartilage in

Table 1

Origin of Various T Mutations

	T Mutation	Origin	Reference
T	Brachyury[1]	Spontaneous in laboratory stock	Dobrovadskaĭa-Zavaskaĭa, 1927
T^{hp}	Hairpin allele[1]	Spontaneous in an AKR mouse	Johnson, 1974
T^{h}	Harwell allele[1]	Spontaneous in laboratory stock	Lyon, 1959
T^{Orl}	Orleans allele[1]	Spontaneous in a Swiss/Orleans mouse	Alton, et al., 1980
T^{c}	Curtailed allele[1]	Radiation-induced	Searle, 1966
T^{Wis}	Wisconsin allele	Spontaneous in laboratory stock	Alton, et al., 1980
T^{J}	Jackson allele	Spontaneous in a BALB/cHu mouse	Bennett, et al., 1975
T^{2J}	Jackson allele	Spontaneous in a C57BL/6 mouse	Bennett, et al., 1975
T^{OR4}	Oak Ridge 4 allele	Radiation-induced	Bennett, et al., 1975
T^{hg}	Hertwig's allele	Spontaneous in laboratory stock	Bennett, et al., 1975

[1]Represents one of the five different phenotypic classes

the presence of a normal neural tube (Bennett, 1958). In aggregation studies involving the association of dissociated normal and mutant embryonic cells, aggregates formed by T/T cells were smaller than those formed by normal cells (Yanagisawa and Fujimoto, 1977b, 1978). Results from these experiments imply that cell surface antigens involved in cell-cell interactions are aberrant. Cell lines established from T/T embryos are also unable to form normal sized aggregates and are being examined in greater detail to identify the cellular aberration caused by the T mutation (Yanagisawa and Fujimoto, 1977a; Yanagisawa, et al., 1980).

Embryos homozygous for T^{hp} die at 7 days of gestation (Bennett, 1975). The viability of the heterozygote is dependent upon the parental origin of the gene (Johnson, 1974). Heterozygotes, whose T^{hp} gene is maternally inherited, die in utero; paternally inherited T^{hp} alleles are not deleterious. In addition to the unusual maternal effects of the gene, the mutation maps as a deletion extending from the T locus distally through the Tcp-1 locus (see Figure 1A; Alton, et al., 1980).

Embryos homozygous for T^{h} die at 8 days of gestation (Lyon, 1959). The embryo sac is half the normal size, and embryonic ectoderm ceases proliferation prematurely.

Embryos homozygous for T^{Orl} die at 6½ to 7 days of gestation when the embryonic ectoderm prematurely ceases to proliferate (Bennett, et al., 1975). The extraembryonic ectoderm and endoderm, ectoplacental cone, and yolk sac are apparently normal. The mutation maps as a deletion extending from the T locus through the qk (quaking) locus (see Figure 1A; Erickson, et al., 1978; Alton, et al., 1980). The

deletion does not include the Tcp-1 locus, as was observed in the deletion for T^{hp}. Hence, the wild type allele ($Tcp\text{-}1^{b}$) is present at the Tcp-1 locus.

Embryos homozygous for T^{c} die at 10½ days of gestation and appear to be similar to embryos homozygous for T (Searle, 1966). However, T^{c}/T^{c} embryos are more severely affected. The posterior body is more abbreviated, the neural folds fail to close, and the anterior limb buds are absent.

Several other spontaneously occurring or radiation-induced mutations have phenotypes similar to one of the mutations (T, T^{hp}, T^{h}, T^{Orl}, or T^{c}) previously described (Table 1). Consequently, new T mutations are classified into one of five different classes on the basis of the homozygous lethal phenotypes and the interactions with different T mutations. The included list of mutations is not meant to be comprehensive but only meant to indicate the different origins of T mutations.

Mutation to dominant T-like alleles occurs spontaneously with high frequency in laboratory stocks and radiation-induced mice (Bennett, 1975; Bennett, et al., 1975). These mutations have not, however, been found in wild populations of mice.

The t Locus

The recessive t mutations are far more heterogeneous and variable than the dominant T mutations. The t mutation in combination with the wild type allele (+/t) has no visible morphological effects; however, the t mutation in combination with the T mutation produces progeny without tails. Embryos homozygous for t can be either viable (t^{v}/t^{v}),

semi-lethal ($\underline{t}^{s}/\underline{t}^{s}$), or lethal ($\underline{t}^{1}/\underline{t}^{1}$) (Bennett, 1975; Klein and Hammerberg, 1977). For purposes of this review, only lethal $\underline{t}$ mutations ($\underline{t}^{1}$) and their effects on embryonic development, sperm maturation and behavior, and genetic recombination will be discussed.

The lethal $\underline{t}$ mutations have several features in common with each other: 1) $\underline{T}/\underline{t}$ mice are viable and usually tailless while $+/\underline{t}$ mice are phenotypically normal; 2) embryos heterozygous for non-complementing lethal alleles ($\underline{t}^{x}/\underline{t}^{y}$) die $\underline{in\ utero}$; 3) male mice heterozygous for lethal alleles in two different complementation groups ($\underline{t}^{x}/\underline{t}^{y}$) are usually viable and sterile whereas female mice are usually viable and fertile; 4) segregation distortion occurs in male mice heterozygous for lethal $\underline{t}$ mutations ($\underline{T}/\underline{t}$ or $+/\underline{t}$) but not in female mice; 5) suppression of recombination occurs between the $\underline{T}$ locus and the $\underline{H\text{-}2}$ locus during meiosis in male and female mice heterozygous for lethal $\underline{t}$ mutations; 6) alleles within the same complementation group generally have the same H-2 haplotype. These characteristics will be discussed in greater detail.

$\underline{Genetic\ Complementation}$: On the basis of genetic complementation, the lethal $\underline{t}$ mutations can be divided into seven complementation groups identified by one typically acting mutation: $\underline{t}^{12}$, $\underline{t}^{w73}$, $\underline{t}^{wPa\text{-}1}$, $\underline{t}^{0}$, $\underline{t}^{w5}$, $\underline{t}^{9}$, and $\underline{t}^{w1}$ (Bennett, 1975; Klein and Hammerberg, 1977; Guénet, $\underline{et\ al.}$, 1980). If two different lethal $\underline{t}$ mutations complement each other, then matings between two different balanced lethal ($\underline{T}/\underline{t}^{x}$ and $\underline{T}/\underline{t}^{y}$) mice will produce offspring with normal tails and no tails (Figure 2A). On the other hand, if two different lethal $\underline{t}$ mutations do not complement each other, then such crosses will only produce offspring without tails (Figure 2B).

Cross:	♂ $\frac{T\text{-}tf}{t^x\text{-}+}$ (tailless)	x	♀ $\frac{T\text{-}tf}{t^y\text{-}+}$ (tailless)	
Frequency of gametes:	$\underline{T\text{-}tf}$ gametes $(1-d_1)$ $\underline{t^x\text{-}+}$ gametes (d_1)		$\underline{T\text{-}tf}$ gametes $(1-d_2)$ $\underline{t^y\text{-}+}$ gametes (d_2)	
Genotypes of expected progeny:	$\frac{T\text{-}tf}{T\text{-}tf}$	$\frac{T\text{-}tf}{t^x\text{-}+}$	$\frac{T\text{-}tf}{t^y\text{-}+}$	$\frac{t^x\text{-}+}{t^y\text{-}+}$
Phenotypes of progeny: A.	dies	tailless	tailless	normal tail
B.	dies	tailless	tailless	dies
Expected frequency of progeny:	$(1-d_1)(1-d_2)$	$(1-d_2)d_1$	$(1-d_1)d_2$	d_1d_2
Expected proportion without SD, $d_1=d_2=0.5$	25%	25%	25%	25%
Expected proportion with SD, $d_1=0.9$ and $d_2=0.5$	5%	45%	5%	45%

Figure 2. Tests for genetic complementation between two different t mutations in the absence or presence of segregation distortion (SD). A. Phenotypes of progeny in the presence of genetic complementation. B. Phenotypes of progeny in the absence of genetic complementation.

The effects of mutations within the seven groups of lethal *t* mutations appear at different stages of embryonic development and affect different cellular structures. Although mutations within the same complementation group are defined as alleles on the basis of genetic non-complementation, studies on different *t* mutations within the same complementation group have shown detectable differences on the type and time of alteration of cellular morphology (Hillman and Hillman, 1975; Spiegelman, 1978). These differences may, however, result from the interactions of the *t* mutation with other genes found in different genetic backgrounds. The alleles at all non-*t* loci found in one population of mice may differ drastically from those found in another population of mice. These allelic variations constitute differences in the genetic background of *t* mutations and may alter the expression of a single mutation such that it has two very different expressions in the two different populations of mice. A brief synopsis of the developmental defect observed in each complementation group is given below. A summary of *t* mutations is given in Table 2.

Embryonic Development: Embryos homozygous for alleles within the first, t^{12}, complementation group represent the earliest acting *t* mutations. The effects of the gene are first visible at day 1 and the embryos die by 2 to 3 days of gestation (Bennett, 1975; Spiegelman, 1975; McLaren, 1976; Klein and Hammerberg, 1977). The embryos reach the morula stage but fail to form the blastocyst. The arrested morulae have large, rounded cells containing fewer polysomes and more lipids than normal morulae (Spiegelman, 1978). The majority of t^{12}/t^{12} embryos are arrested at the late morula stage; embryos homozygous for t^{w32},

Table 2

Summary of *t* Mutations

Complementation Group	Member Alleles	Predominant H-2 Haplotype	T/t Phenotype
t^{12}	t^{12}, t^{w32}	H-2^{t12}	Tailless
t^{w73}	t^{w73}	H-2^{tw1}[1]	Tailless
t^{wPa-1}	t^{wPa-1}	ND[2]	Tailless
t^{0}	t^{0}, t^{6}, t^{30}, t^{h16}	H-2^{w5}	Tailless
	t^{h7}, t^{h13}		Normal[3]
t^{w5}	t^{w5}, t^{w6}, t^{w10}, t^{w11}, t^{w13}, t^{w14}, t^{w15}, t^{w16}, t^{w17}, t^{w37}, t^{w38}, t^{w39}, t^{w41}, t^{w46}, t^{w47}, t^{w74}, t^{w75}, t^{w80}, t^{w81}	H-2^{tw5}	Tailless
t^{9}	t^{4}, t^{9}, t^{w18}, t^{w30}, t^{w52}	ND	Tailless
t^{w1}	t^{w1}, t^{w3}, t^{w12}, t^{w20}, t^{w21} t^{w71}, t^{w72}	H-2^{tw1}	Tailless

[1]Exception to classification of *t* mutations on the basis of H-2 typing and genetic complementation.

[2]ND - not yet determined.

[3]These *t* mutations retain the *L*-factor but have lost the *T-int* factor.

a second member of the $\underline{t}^{12}$ complementation group, are arrested at the early morula stage (Hillman and Hillman, 1975). Studies involving $\underline{t}^{12}/\underline{t}^{12} \leftrightarrow \underline{+}/\underline{+}$ and $\underline{t}^{w32}/\underline{t}^{w32} \leftrightarrow \underline{+}/\underline{+}$ chimeras show that the mutation $\underline{t}^{w32}$ appears to alter the cell surface and prevents the integration of $\underline{t}^{w32}/\underline{t}^{w32}$ cells with normal cells whereas the $\underline{t}^{12}$ mutation permits the interaction of $\underline{t}^{12}/\underline{t}^{12}$ cells with normal cells (Mintz, 1964; Spiegelman, 1978). The cell surface differences produced by $\underline{t}^{12}$ or $\underline{t}^{w32}$ mutations apparently interfere with the establishment of tight junctions between cells (Spiegelman, 1978).

Embryos homozygous for the allele within the second, $\underline{t}^{w73}$, complementation group are recognized at day 3 and die at days 4 to 5 of gestation (Bennett, 1975; McLaren, 1976; Hammerberg and Klein, 1977). Embryos reach a blastocyst stage, induce a decidual reaction in the uterus, and die shortly after implantation (Spiegelman, et al., 1976). The trophectoderm and ectoplacental cone fail to form a close association with the uterine decidua. The mutation appears to alter the function of the trophectoderm.

Embryos homozygous for the allele within the third, $\underline{t}^{wPa-1}$, complementation group die after implantation (Guénet, et al., 1980). The specific tissue affected by $\underline{t}^{wPa-1}$ is unknown. The mutation is the most recently discovered $\underline{t}$ mutation in wild mice and appears to complement all other $\underline{t}$ mutations.

Embryos homozygous for alleles within the fourth, $\underline{t}^{0}$, complementation group are first detected at 5½ days and die at 6 to 7 days of gestation (Bennett, 1975; McLaren, 1976; Klein and Hammerberg, 1977). The egg cylinder fails to lengthen and the ectoderm does not differentiate into the embryonic and extraembryonic components.

Attempts to culture trophectoderm and/or inner cell mass of $\underline{t}^0/\underline{t}^0$ and $\underline{t}^6/\underline{t}^6$ embryos *in vitro* have failed (Wudl and Sherman, 1978; Hogan, *et al.*, 1980). Examination of β-glucuronidase activity in $\underline{t}^6/\underline{t}^6$ cells cultured *in vitro* shows that macromolecular synthesis stops at the egg cylinder stage (Wudl and Sherman, 1978). The primary effect of the $\underline{t}^6$ mutation has been hypothesized to be a metabolic lesion which kills inner cell mass cells quickly but has a more gradual effect upon trophoblast cells.

Embryos homozygous for alleles within the fifth, $\underline{t}^{w5}$, complementation group are recognized at 6½ days and die by 8 to 10 days of gestation (Bennett, 1975; McLaren, 1976; Klein and Hammerberg, 1977). Embryonic and extraembryonic ectoderm forms, but the growth and maintenance of the egg cylinder stops. Attempts to culture inner cell masses of $\underline{t}^{w5}/\underline{t}^{w5}$ embryos *in vitro* have failed, and the lethal period *in vitro* corresponds to that observed *in utero* (Wudl and Sherman, 1976; Hogan, *et al.*, 1980). However, *in vitro* culture of $\underline{t}^{w5}/\underline{t}^{w5}$ trophectoderm in the absence of the inner cell mass has been successful (Sherman, 1975). The differential results in the ability to culture inner cell mass (embryonic derivative) or trophectoderm (extraembryonic derivative) imply that various cell types are not equally sensitive to the effects of the *t* mutation or that death of trophectodermal cells is due to secondary degenerative effects resulting from the primary lesion in the inner cell mass (Wudl and Sherman, 1976).

Embryos homozygous for alleles within the sixth, $\underline{t}^9$, complementation group are first visible at 6 days and die at 8 to 10 days of gestation (Bennett, 1975; McLaren, 1976; Klein and Hammerberg, 1977). Formation and differentiation of the primitive streak begins, but

mesodermal differentiation is aberrant. Mesodermal cells are abnormal in shape, fail to establish intercellular junctions, and are deficient in microfilaments (Spiegelman, 1975; Bennett, 1978; Snow and Bennett, 1978). *In vitro* cultures of t^{w18}/t^{w18} embryos, ectopic implants and mutant ↔ normal chimera studies show that some cells are capable of surviving beyond the usual period of lethality exhibited by mutant embryos *in utero* (Wudl, *et al.*, 1977). An explanation for the ability of some embryonic cells to survive in an environment outside of the uterus is not readily apparent.

Embryos homozygous for alleles within the seventh, t^{w1}, complementation group have been reported to be visible as early as day 9 and die between 11 to 21 days of gestation. The neural tube forms but cells in the ventral half of the neural tube become pycnotic and die (Bennett, *et al.*, 1959a, b).

Sperm Maturation and Behavior: The effects of lethal *t* mutations in the heterozygous state on sperm maturation and behavior fall into two categories: 1) the effects causing segregation distortion in *T/t* or *+/t* males mated with females; and 2) the effects causing sterility in t^x/t^y males (Bennett, 1975; Klein and Hammerberg, 1977). With respect to the cause of segregation distortion in males, in which fertilization of ova by sperm from *+/t* or *T/t* males does not usually result in typical Mendelian ratios of expected phenotypic offspring, recent studies have shown that sperm bearing lethal *t* mutations are not morphologically defective and have the same life span as sperm bearing the wild type allele (McGrath and Hillman, 1980a). The distortion does not occur in metaphase I of meiosis since equal numbers of *t*- and *+*-bearing chromosomes are present (Hammerberg and Klein,

1975a). Transmission frequency of $\underline{t}^{12}$-bearing sperm *in vitro*, for example, is Mendelian (McGrath and Hillman, 1980b) and is identical to the transmission frequency *in vivo* when matings are delayed until the time of ovulation (Braden, 1958). Braden (1958) has hypothesized that sperm bearing lethal *t* mutations are physiologically superior to sperm bearing the wild type allele and that this physiological advantage is time dependent. The superiority of *t*-bearing sperm is expressed in normal matings but not in delayed matings, implying that the sperm are responding to extrinsic factors present in the uterine and oviducal environments.

The sterility of $\underline{t}^{x}/\underline{t}^{y}$ male mice may be due to lack of spermatozoan maturation (McGrath and Hillman, 1980b). Histological and ultrastructural studies indicate that no unique spermatid or spermatozoan defects are found in sperm produced by $\underline{t}^{x}/\underline{t}^{y}$ males (Hillman and Nadijcka, 1978a,b, 1980; Nadijcka and Hillman, 1980). However, fewer spermatozoa are found in the female reproductive tract (Tucker, 1980). Results of *in vitro* fertilization of ova with sperm from $\underline{t}^{x}/\underline{t}^{y}$ males in which all female-derived physical and physiological barriers are removed show that fertilization is still not possible (McGrath and Hillman, 1980b). The sperm from $\underline{t}^{x}/\underline{t}^{y}$ males resemble immature normal sperm in their ability to fertilize ova. Thus, the decreased number of sperm is not the primary cause of sterility. The lack of spermatozoan maturation may result from their inability to mature en route through the epididymis, to undergo capacitation, or to arrive at the site of fertilization.

Genetic Recombination: The distance between the *T* locus and *H-2* locus is approximately 15 cM (Bennett, 1975; Klein and Hammerberg, 1977).

In the presence of lethal t mutations, however, only 1%-2% recombination occurs during meiosis between the T locus and the H-2 locus. The recombination suppression effect observed in the presence of lethal t mutations is not absolute since recombinants can be identified. Various hypotheses proposed to explain the suppression effect include the presence of deletions or structural rearrangements in moderately repetitive DNA (iDNA) intercalated between structural genes (Geyer-Duszynska, 1964; Lyon and Mason, 1977).

Lyon and coworkers have investigated the recombinant t mutants in order to determine the cause of recombination suppression and to attempt to map the lethal t mutations (Lyon and Meredith, 1964a,b,c; Lyon and Bechtol, 1977; Lyon and Mason, 1977; Lyon, et al., 1979a). Their results suggest that suppression is due to either an intrinsic failure of the chromatin material to participate in crossing-over during meiotic pairing or a mismatching of normal or abnormal heterochromatin, thus preventing chiasma formation (Lyon, et al., 1979a). Recently, experiments involving recombination between two chromosomes carrying extensive overlapping segments of t chromatin suggest that recombination suppression is not due to an intrinsic inability of t chromatin to undergo crossing-over but due to mismatching of normal and variant chromatins (Silver and Artzt, 1981). Regions of DNA in two over-lapping t mutations, t^{w12}, and t^{h17}, are homologous and will permit normal crossing-over whereas regions of + DNA and t DNA are non-homologous and will inhibit crossing-over.

The evidence described above does not eliminate the possibility of deletions occurring within similar regions and causing the mismatching of chromatin. However, the looped out chromosomal material

found on cytological examinations of chromosomes in some t mutant mice (Geyer-Duszynska, 1964) and thought to represent deleted regions of DNA could also result from regions of non-homologous pairing in an otherwise normal chromosome. More studies are needed to distinguish between the two hypotheses.

Three properties usually associated with t mutations include: 1) the interaction with T mutations to produce tailless progeny; 2) the presence of a segregation distortion factor in males; and 3) the lethality of developing embryos homozygous for t. The various phenotypes of mice produced following exceptional recombination events indicate that t mutations may have at least three altered regions of the chromosome, each coding for one of the three properties usually found in t mutations (Lyon and Mason, 1977). Since the mutation covered an extended region of the chromosome and includes more than one locus, the t mutations with all three properties are now referred to as complete t haplotypes. The region interacting with Brachyury (T) to produce taillessness is referred to as T-int (Figure 3) and maps close to T (Lyon and Mason, 1977). The A region causes low segregation distortion in the presence of the wild type allele and interacts with T-int and L to give a high segregation distortion ratio (Lyon and Mason, 1977; Hammerberg, 1981). The L region is responsible for lethality observed in homozygous t/t embryos and maps in the tf region (Lyon and Mason, 1977).

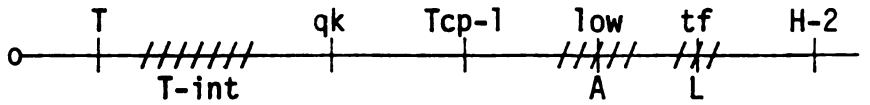


Figure 3. Proposed regions of the tail-interacting factor (T-int), segregation distortion factor (A), and lethality factor (L) in complete t haplotypes.

Another gene locus recently identified and mapped within the T - H-2 interval is Tcp-1 (Silver, et al., 1979; Silver, et al., 1980; Danska and Silver, 1980). The Tcp-1 gene codes for a protein with a molecular weight of 63,000 daltons that is found on all cellular surfaces, but appears in greatest quantities on testicular cells (0.4% of total protein; Silver, et al., 1979). Three alleles have been identified: $Tcp\text{-}1^{b}$ is found in wild type and T mutant mice; $Tcp\text{-}1^{a}$ is associated with complete t haplotypes; and $Tcp\text{-}1^{n}$ (null allele) is associated with the T^{hp} mutation.

Specific H-2 haplotypes are also associated with specific t mutations (Hammerberg and Klein, 1975b,c; Hammerberg, et al., 1976; Hauptfield, et al., 1976). Since recombination suppression exists and includes the H-2 locus, t and H-2 appear to be inherited as a unit, a "super gene" (Snell, 1968). A strong correlation exists between the t mutation and H-2 haplotype. Alleles within a complementation group carry the same H-2 haplotype; alleles within different complementation groups are usually associated with different H-2 haplotypes (see Table 2).

In summary the suppression of recombination between T and H-2 are thought to arise from mismatching of chromatin. As a result, genes

found within the interval are inherited as a unit and the term t haplotype is used instead of t mutation or allele to define this set of genes.

Purpose of Thesis

The alleles found in the complementation group of t mutations acting late in embryonic development, t^{w1}, have not been studied extensively. The t^{w1} mutations appear to act after organogenesis has begun whereas all other t mutations act prior to the initiation of organogenesis. In the one study that has reported the effects of t^{w1} mutations on embryonic development, data obtained from embryonic studies involving t^{w1}, t^{w3}, t^{w12}, and t^{w20} were summarized and emphasis was placed on the effects observed after 13 days of gestation (Bennett, et al., 1959a,b). Complementation studies of t^{w1}, t^{w3}, t^{w12}, and t^{w20} with mutations from other complementation groups indicate that the t^{w1} haplotypes differ drastically with respect to their complementation ability (Lyon, et al., 1979b). Since little information was available on the early effects of the t^{w1} mutation and since non-complementing t^{w1} haplotypes probably differ in their effects on embryonic development (as noted for other non-complementary t haplotypes), the t^{w1} mutation was selected for the study of the gene action at early time periods of development.

Research on t^{w1} was divided into four phases. First, the gene was transferred from the original "pen bred" stock to an inbred strain of mice, C3H.B10, in order to establish more vigorous breeding lines, to eliminate the heterogeneous genetic background, and to examine the effects of t^{w1} in the absence of genes T and a^t. Second, normal

and abnormal embryos at days 9 to 15 of gestation were subjected to gross examination after dissection from amniotic sacs and histological examination by light microscopy in order to determine the effects of $\underline{t}^{w1}$ on embryonic development. Third, primary explants from mouse embryos derived from C3H.B10-$\underline{t}^{w1}$ matings were cultured *in vitro* in order to determine if: explants would attach to the substrate and cellular outgrowth would continue longer *in vitro* than *in vivo*; any specific cell types from primary explant outgrowth would become established *in vitro*; and cell lines could be subcultured from primary explants. Fourth, the H-2 types of cells grown *in vitro* were assayed in an attempt to identify mutant from normal embryos without sacrificing tissue for histological examination.

MATERIALS

Paraplast, melting point 56-57°C, microcentrifuge tubes (250µl capacity), 3" x 1" glass slides, and Permount were purchased from Fisher Scientific, Fair Lawn, New Jersey. Glass coverslips and 35 mm sterile tissue culture dishes were purchased from Corning, Corning, New York. Falcon MicroTest II plates and lids were purchased from Becton, Dickinson & Co., Cockeysville, Maryland.

H-2 typing reagents D-2, D-23, D-28, and D-33, prepared by Drs. G. Snell and M. Cherry, were obtained from the Transplantation and Immunology Branch NIAID of the National Institute of Health, Bethesda, Maryland. Fetal calf serum, CMRL 1066 (10x), mycostatin, rabbit complement, and trypan blue were purchased from Grand Island Biological Co., Grand Island, New York. Penicillin G and streptomycin sulfate were purchased from Calbiochem-Behring Corp., La Jolla, California. Hematoxylin and eosin were obtained from MC/B, Norwood, Ohio. Albumin fixative came from Harleco, Gibbstown, New Jersey. Bovine pancreas type III trypsin, fluorodeoxyuridine, glutamine, and asparagine were purchased from Sigma, St. Louis, Missouri. ^{125}I-iododeoxyuridine (NEX 072) was obtained from New England Nuclear, Boston, Massachusetts. MEM/Hanks balanced salt solution, MEM, and non-essential amino acids (100mM) were purchased from International Scientific Institute, Inc., Cary, Illinois. Flow Laboratories, Rockville, Maryland, was the source of 100 mM sodium pyruvate. All other chemicals were analytical reagent grade.

C3H mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. Breeding pairs of C3H.B10 mice were kindly provided by Dr. D. Shreffler, University of Michigan, Ann Arbor, Michigan. Breeding pairs of $\underline{T}$-$\underline{tf}/\underline{t}^{w1}$-$\underline{+}$ mice were kindly provided by Dr. H. O. McDevitt, Stanford University, Stanford, California. The C57BL/10ScSn strain was already established in the laboratory by Dr. James H. Asher, Jr.

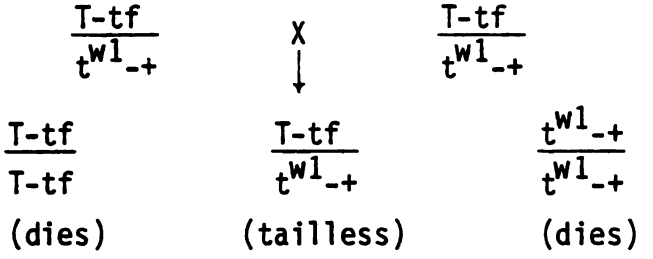
METHODS

Animal Breeding

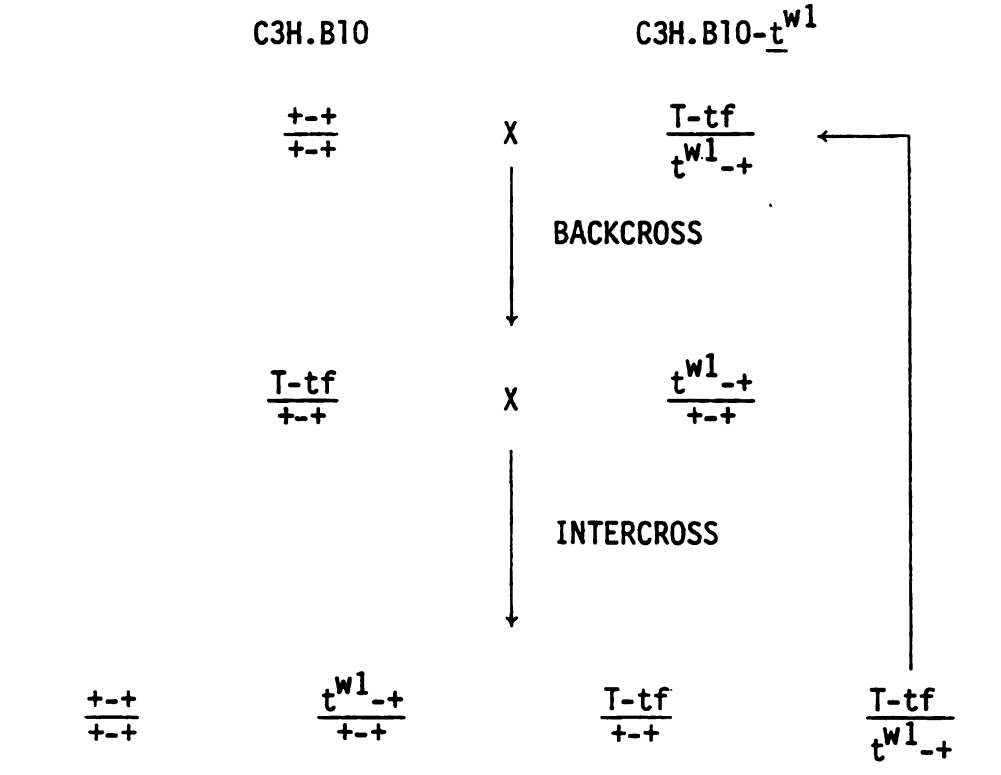
Normal (+/+) mating pairs of C3H.B10 mice having agouti (A/A) coat color were kindly provided by Dr. D. Shreffler, Department of Genetics, University of Michigan. The C3H.B10 strain, carrying the H-2 histocompatibility haplotype of the C57BL/10 strain backcrossed on the C3H background, is maintained by full sib mating and is presently at generation $N_{10}F_{27+17}$.

Three breeding pairs of mice having black and tan coat colors (a^t/a^t) and carrying the mutations T and t^{w1} as balanced lethals (T-tf/t^{w1}-+) were obtained from Dr. H. O. McDevitt, Department of Medicine, Stanford University. In order to overcome the poor reproductive capability of the original stock of T-tf/t^{w1}-+ mice and to reduce the heterogeneous genetic background of the original strain, genes T and t^{w1} were successfully transferred from the balanced lethal "pen bred" stock to the inbred strain of mice, C3H.B10. (Similar crosses with C57BL/10ScSn were not successful; see Results.) Mutant t^{w1} is presently maintained in two ways: as a balanced lethal line, designated as C3H.B10-Tt^{w1} (generation F_{13}; Figure 4A) and as a backcross-intercross line, designated as C3H.B10-t^{w1} (generation N_5F_4; Figure 4B).

Animals were kept on an artificial light-dark cycle (13 hours light, 11 hours dark). Food and water were given ad libitum.



A. Balanced Lethal Line



B. Backcross-Intercross Line

Figure 4. Mating schemes used to maintain mouse mutant $\underline{t}^{w1}$.

Timed Matings

Males and females of appropriate genotypes (C3H.B10 +/+ x C3H.B10 +/+ or C3H.B10-t^{w1} +/t^{w1} x C3H.B10-t^{w1} +/t^{w1}) were mated, and females were examined twice daily for vaginal plugs. At 9, 9½, 10, 10½, 11, 12, 13, and 15 days post-coitus (day of observation of a vaginal plug = day 0), pregnant females were killed by cervical dislocation. Embryos were sterilely removed, placed in sterile calcium-magnesium free phosphate buffered saline (PBS), dissected free from amniotic sacs under a Wild M7A dissection microscope, and transferred to 35 mm sterile culture dishes containing PBS. Embryos were staged according to Grüneberg (1943) and crown-rump (CR) lengths were measured with an ocular micrometer. Tailbud, forelimb bud, or heart tissues were excised and transferred to sterile vials for tissue culture. Prior to fixation in Bouin's solution (Weesner, 1960), embryos were photographed with a Canon F-1 camera using an Olympus 28 mm macrolense.

Preparation of Embryos for Light Microscopy

Embryos fixed in Bouin's solution were dehydrated in graded ethanols, cleared in xylenes, and embedded in paraplast (Table 3; Weesner, 1960). Embryos were serially sectioned at 8 µm on an AO rotary microtome. Sections were mounted on glass slides with albumin, stained with hematoxylin and eosin (Table 4; Luna, 1968), and examined on a Zeiss Universal II microscope with camera attachment at magnifications to x250.

Table 3

Embedding Protocol for Embryonic Tissue

Solutions	Time, min	Solutions	Time, min
30% ethanol	20	100% ethanol	20
50% ethanol	20	100% ethanol	20
70% ethanol	20	100% xylenes	30
80% ethanol	20	100% xylenes	30
95% ethanol	20	Paraplast[1]	30
95% ethanol	20	Paraplast	30

[1]Infiltration of paraplast performed under 15 lbs. of vacuum at 60°C.

Table 4

Harris' Alum Hematoxylin and Eosin Staining Protocol

Solutions	Time, min
Xylenes	4
Xylenes	4
100% ethanol	2
100% ethanol	2
95% ethanol	2
70% ethanol	2
Tap water	3
Harris' alum hematoxylin[1]	10
Tap water	1/3
Tap water	1/3
0.9% hydrochloric acid	3 dips
Tap water	1 dip
Tap water	5
Alcoholic eosin Y[2]	1/2
95% ethanol	3 dips
95% ethanol	3 dips
100% ethanol	2
100% ethanol	2
Xylenes	3
Xylenes	3
Xylenes	3
Coverslip with Permount; dry overnight on warming tray.	

[1]Harris' alum hematoxylin prepared as described in Luna, 1968.

[2]Alcoholic eosin Y prepared as described in Luna, 1968.

Reconstruction of Mouse Embryos from Transverse Sections

In order to reconstruct a composite view of a neural tube from a serially sectioned embryo, every fifth section was projected onto a sheet of paper with a Bausch and Lomb microscope slide projector, and the neural tube, heart, and esophagus were traced. Every fifth section was then examined with a Zeiss Universal II microscope for placement of pycnotic cells within the embryonic tissue on the traced sections. Traced sections were sequentially aligned at every two mm on graph paper, and the extent or width of the neural tube, heart, and esophagus and pycnotic cells were marked with dots. The general outline of a saggital view was constructed by joining the appropriate dots for the neural tube, heart, and esophagus. The width of the embryo was magnified approximately 43 times and the length was slightly distorted by a factor of 1.18.

Growth of Primary Explants

Tailbud, forelimb bud, or heart tissues excised from embryos were transferred under sterile conditions from vials to 35 mm culture dishes containing 0.5 ml of CMRL 1066 medium supplemented with 10% heat inactivated (56°C for 30 minutes) fetal calf serum, 2.0 mM glutamine, 50 units/ml of penicillin, 50 units/ml of streptomycin, and 100 units/ml of mycostatin (CMRL 1066; Oldham and Herberman, 1976; Murrell, 1979) Primary explants were placed in a humidified incubator at 37°C in an atmosphere of 5% CO_2, 95% air. During the first week, explants were observed daily on an Olympus CK inverted stage microscope magnified to x200 for attachment and cellular outgrowth. Subsequent observations were made every three to four days prior to feeding cultures

by replacing old medium with one ml of fresh CMRL 1066. Cultures contaminated with bacteria, yeast, or fungi were discarded.

Subculture of Primary Explants or Cell Lines

CMRL 1066 medium from primary explants or cell lines to be subcultured was removed and the culture was rinsed twice with two mls of sterile PBS. Each culture was incubated with 0.5 mls of 0.05% trypsin (bovine pancrease type III) in PBS containing 50 units/ml each of penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2, 95% air for three minutes. Excess trypsin was removed and the culture was incubated an additional two minutes. Approximately 1 to 3 mls of CMRL 1066 was added to stop the enzymatic disaggregation of cells by trypsin. Cells were dispersed by trituration with a Pasteur pipet. Cell density was determined by counting cells in a drop of medium in a hemacytometer on an American Optical Series 20 microscope magnified to x200. Cells were diluted to the appropriate cell density (2-4 x 10^5 cells/ml) and plated in either 35mm tissue culture dishes at 2-4 x 10^5 cells/dish or wells of MicroTest II plates at 2-4 x 10^4 cells/well. Subcultures were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2, 95% air and fed every three to four days by replacing old medium with fresh CMRL 1066. Subsequent subculturing was generally performed before cells reached confluency.

Mouse Anti-H-2^{tw1} Production

In order to produce mouse alloantisera to antigen 108 (H-2^{tw1}), heterozygous C3H.B10-$\underline{t}^{w1}$ ($\underline{+}/\underline{t}^{w1}$) and normal C3H.B10 ($\underline{+}/\underline{+}$) male mice were used as donors and recipients, respectively. A spleen was sterilely removed from a $\underline{+}/\underline{t}^{w1}$ male mouse, rinsed in sterile PBS,

and placed in a 35 mm sterile culture dish containing two mls of PBS. The spleen sac was punctured with a 25 gauge needle, and cells were squeezed through holes in the spleen sac with forceps. The empty spleen sac was discarded. Cells were dispersed by passing through 25 and 27 gauge needles sequentially, and counted in a hemacytometer on an American Optical Series 20 microscope. The splenic cell concentration was adjusted to 5-6 x 10^7 cells/ml.

Normal C3H.B10 (+/+) male mice five to six weeks old were injected weekly for seven weeks in the intraperitoneal cavity with 0.2 mls of freshly prepared spleen cell suspension containing 2-4 x 10^7 cells (Snell, et al., 1976). One week after the last injection, recipient mice were anesthetized with chloroform and bled via heart puncture with a 25 gauge needle and one ml syringe. Blood was pooled into three conical test tubes and allowed to clot at room temperature for three hours. The clots were freed from the sides of the tubes and allowed to contract overnight at 4°C. Antiserum was removed with a Pasteur pipet from each tube to a clean conical tube, spun at 700 x g for 10 minutes at 4°C in an IEC CRU-5000 centrifuge, aliquoted into 0.5 ml samples, and stored at -70°C. Prior to use, an aliquot was thawed and heat inactivated at 56°C for 30 minutes. Antiserum was subsequently stored at -20°C.

NIH Mouse Alloantisera

Four H-2 typing reagents (D-2, D-23, D-28, and D-33) were received gratus from NIH. Each was reconstituted with one ml of sterile distilled water, aliquoted with a Hamilton syringe into microcentrifuge tubes as 100-200 µl samples, and stored at -70°C. For use, sera was

thawed to room temperature; subsequent storage was at -20°C. The activity of the antisera was not affected by refreezing.

One Stage Lymphocytotoxicity Assay

Lymphocytes were isolated from mouse spleens of appropriate genotypes as described above (Mouse Anti-H-2^{tw1} Production), centrifuged in an IEC CRU-5000 centrifuge at 300 x g for 10 minutes at 4°C, and resuspended in four mls of MEM/Hanks medium. Cells were counted in a hemacytometer and the cell density was adjusted to give a final concentration of 3-5 x 10^7 cells/ml. Appropriate dilutions of antisera were made in MEM/Hanks. Rabbit complement, previously absorbed with agarose, was diluted 1:3, complement:Mem/Hanks medium.

A modification of the Amos assay (Amos, et al., 1969) was used to test for the H-2 antigens found on lymphocytes with mouse alloantisera H-2^{tw1}, H-2.2, and H-2.33. To a clean test tube were added 20 µl of cells, 20 µl of diluted antisera and 20 µl of diluted rabbit complement (C'). The mixture was vortexed and incubated for 60 minutes at 37°C in an atmosphere of 9% CO_2, 91% air. The reaction was stopped by placing the test tube on ice and adding 50 µl of cold MEM/Hanks. Prior to counting, 20 µl of 0.4% trypan blue was added. The number of viable cells, which exclude trypan blue, and the number of dead cells, which take up trypan blue, were counted in a hemacytometer on an American Optical Series 10 Microstar microscope. An experimental design, shown in Table 5, indicates the controls needed for cytotoxicity assays. The percent lysis was calculated as:

$$100\% \left[\frac{\text{\# dead cells in complete assay}}{\text{total \# of cells in complete assay}} - \frac{\text{\# dead cells in C' control}}{\text{total \# of cells in C' control}}\right]$$

Table 5

Experimental Design for Cytotoxicity Assays

Purpose	Test tube	Anti-serum	Rabbit complement	Cells to be tested
Cell control	1	-	-	+
C' control	2	-	+	+
Antiserum control	3	+	-	+
Complete assay	4	+	+	+

Iodination of Fibroblasts

Mouse embryonic fibroblasts from primary explants or cell lines were subcultured as described above (Subculture of Primary Explants or Cell Lines) and dispersed into MicroTest II wells at a concentration of 2×10^4 cells/well to 1×10^2 cells/well. Cultures were incubated 24 hours at 37°C in an atmosphere of 5% CO_2, 95% air. CMRL 1066 medium was removed, cultures were washed once with PBS, and 0.1 ml of ^{125}I-iododeoxyuridine labeling mix (^{125}IUdR; Table 6; LeMevel and Wells, 1973; O'Toole and Clark, 1976) was added to each well with a Clay Adams pipeter. Cells were placed in a well-humidified incubator at 37°C in an atmosphere of 5% CO_2, 95% air for 18 hours. The label was removed by aspiration, cells were washed twice with 0.2 ml of PBS, and cells were detached from the wells by incubation in the presence of 0.25% trypsin in PBS for 30 minutes at 37°C. Released cells from each well were removed to a gamma vial, each well was washed twice with distilled water, and the wash was added

Table 6

^{125}I-iododeoxyuridine Labeling Mix

Stock solutions:	
Labeling MEM	
MEM	85 mls
Heat inactivated fetal calf serum	10 mls
100x Non-essential amino acids	1 ml
Glutamine (3 mg/ml)	1 ml
Asparagine (2 mg/ml)	1 ml
100 mM sodium pyruvate	1 ml
Penicillin and streptomycin, 50 units/ml	1 ml
7.5% sodium bicarbonate	3 mls
	103 mls
Fluorodeoxyuridine (FUdR)	10^{-5} M
^{125}I-iododeoxyuridine	50 µCi/ml
Labeling Mix (^{125}IUdR)	
Labeling MEM stock	4.5 mls
FUdR stock	0.5 mls
^{125}I-iododeoxyuridine stock	0.05 mls
	5.05 mls

to the gamma vial containing freed cells. Vials were counted in a Beckman Biogamma II counter.

^{125}I-iododeoxyuridine Fibroblast Microcytotoxicity Assay 1

Cultured embryonic cells were subcultured in MicroTest II wells at 1-2 x 10^4 cells/well and incubated for 24 hours at 37°C in an atmosphere of 5% CO_2, 95% air. CMRL 1066 medium was removed, cells were washed twice with 0.2 ml of PBS, and 0.1 ml of ^{125}IUdR was added to each well. Cells were cultured in a well-humidified incubator

for 18 hours at 37°C in an atmosphere of 5% CO_2, 95% air. The label was removed and cells were washed twice with 0.2 ml of PBS. 30 µl of diluted antisera and 30 µl of rabbit complement diluted 1:9 with labeling MEM supplemented with 5% heat inactivated fetal calf serum were added to cultures and mixed on a vortex. Cells were incubated for six hours at 37°C in an atmosphere of 5% CO_2, 95% air. Antisera and complement were removed, and cells were detached from MicroTest II wells by incubating with 0.25% trypsin. Detached cells and subsequent washes with PBS were placed in gamma vials (as described in Iodination of Fibroblasts) and counted in a Beckman Biogamma II counter. The percent lysis was calculated as:

$$\left[1 - \frac{\text{CPM in viable experimental cells}}{\frac{1}{2}(\text{CPM in C' control} + \text{CPM in Antisera control})} \right] 100\%$$

^{125}I-iododeoxyuridine Fibroblast Microcytotoxicity Assay 2

Cultured embryonic cells were subcultured into MicroTest II wells at 1-2 x 10^4 cells/well and incubated 24 hours at 37°C in an atmosphere of 5% CO_2, 95% air. CMRL 1066 medium was removed and cells were washed three times with 0.2 ml of PBS. 30 µl of antisera, appropriately diluted in labeling MEM supplemented with 5% heat inactivated fetal calf serum, and 30 µl of rabbit complement diluted 1:9 with labeling MEM supplemented with 5% heat inactivated fetal calf serum were added. Cells were incubated for 30 minutes at 37°C in an atmosphere of 5% CO_2, 95% air. The supernatant was removed, each well was washed once with 0.2 ml of PBS, and 0.1 ml of ^{125}IUdR was added to each well. Cultures were incubated for 18 hours at 37°C in an atmosphere of 5% CO_2, 95% air. The label was removed, cells

were washed once with 0.2 ml of PBS, and cells were removed to gamma counting vials after the 0.25% trypsin treatment described above (Iodination of Fibroblasts). Vials were counted in the Beckman Biogamma II counter. The percent lysis was calculated as:

$$\left[1 - \frac{\text{CPM in viable experimental cells}}{\frac{1}{2}(\text{CPM in C' control} + \text{CPM in Antisera control})} \right] 100\%$$

Photography

Embryos were photographed after dissection with a Canon F-1 camera and a 28 mm Olympus macrolense attached to bellows. Cultures were photographed with a camera mounted on a Leitz Diavert inverted phase contrast microscope. Tissue sections were photographed with a camera mounted on a Zeiss Universal II microscope.

Kodak Panatomic X (ASA 32) film was purchased in bulk, rolled in film canisters in appropriate lengths, and used in all photographic work. Panatomic X film was developed at room temperature as follows: 8½ minutes in Kodak Microdol X diluted 1:3, Microdol X: water, with agitation every 30 seconds; 30 seconds in 1.5% acetic acid with constant agitation; 3 minutes in Kodak Acid Fixer with constant agitation; 20 minutes in running tap water; and a rinse in Kodak photo-flo solution. Prints were made on Polycontrast Rapid RC II paper with a Leitz enlarger and developed as follows: 1½ minutes in Kodak Dektol diluted 1:2, Dektol:water; 15 seconds in 1.5% acetic acid; 4 minutes in Kodak Acid Fixer; and 4 minutes in running tap water.

RESULTS

Characteristics of Breeding Lines

The original T-tf/t^{w1}-+ strain obtained from Dr. H. O. McDevitt was characterized by black and tan coat color, poor reproduction (only 33% of all breeders reproduced), small litter size (3 pups per litter), and male segregation distortion factor of 0.8 (H. O. McDevitt, personal communication). Since mice with black and tan coat colors are notoriously poor breeders (J. H. Asher, Jr., personal communication), black and tan mice carrying T-tf/t^{w1}-+ were mated with either C57BL/10ScSn (black coat color, a/a) or C3H.B10 (agouti coat color, A/A) normal (+/+) mice to try to establish more vigorous breeding lines and to eliminate the heterogeneous background of the "pen bred" stock. The resultant progeny were characterized by either short tails (T-tf/+-+) or normal tails (t^{w1}-+/+-+). Short tail and normal tail mice were mated inter se (T-tf/+-+ x t^{w1}-+/+-+) and progeny characterized by taillessness and black (matings involving C57BL/10ScSn mice) or agouti (matings involving C3H.B10 mice) coat were selected as future breeders.

Efforts to establish breeding lines of t^{w1} backcrossed onto C57BL/10ScSn background were unsuccessful. Tailless females with black coat colors were not capable of breeding. An anatomical abnormality in the pelvic region appearing after two generations of

backcrossing prevented copulation. Consequently, attempts to establish $\underline{t}^{w1}$ on C57BL/10ScSn were discontinued.

Efforts to establish breeding lines of $\underline{t}^{w1}$ on C3H.B10 background were successful. Animals were always selected for agouti coat color in order to eliminate the breeding problems associated with the black and tan gene, $\underline{a}^{t}$. Two breeding schemes used to maintain the mutant $\underline{t}^{w1}$ are shown in Figure 4. The balanced lethal line (C3H.B10-$\underline{Tt}^{w1}$; Figure 4A) will only produce viable tailless ($\underline{T}$-$\underline{tf}/\underline{t}^{w1}$-$\underline{+}$) progeny in the absence of recombination since $\underline{T}$-$\underline{tf}/\underline{T}$-$\underline{tf}$ and $\underline{t}^{w1}$-$\underline{+}/\underline{t}^{w1}$-$\underline{+}$ animals die *in utero*. The backcross-intercross line (C3H.B10-$\underline{t}^{w1}$; Figure 4B) is dependent upon maintaining a breeding nucleus of normal C3H.B10 mice to be used in the backcross portion of the breeding scheme. Tailless progeny are backcrossed with normal C3H.B10 mice to produce short tail ($\underline{T}$-$\underline{tf}/\underline{+}$-$\underline{+}$) and normal tail ($\underline{t}^{w1}$-$\underline{+}/\underline{+}$-$\underline{+}$) offspring. Short tail and normal tail animals are intercrossed; the tailless progeny are subsequently backcrossed to continue the breeding line.

The breeding problems described by McDevitt were not as prevalent in animals with a C3H.B10 background. Data are summarized in Table 7 for each mating system with respect to the percent of successfully reproductive females, average litter size at birth, and death rate at weaning. In the balanced lethal line, the percent of females that reproduce has increased and the average litter size has doubled compared to values for McDevitt's stock. The decrease in litter size from F_1 through F_{10} was due to inbreeding depression (Green, 1968, 1981); litter size has subsequently increased. The high death rate detected at weaning in all three strains results from the inability of breeding females to nourish pups. Females stressed by

Table 7

Breeding Data for C3H.B10, C3H.B10-Tt^{w1}, and C3H.B10-t^{w1}

Generation	% Reproductive Females[1]	Avg. Litter Size at Birth	Death Rate at Weaning, %[2]
C3H.B10			
$N_{10}F_{27+1}$	100	6.1	18.2
$N_{10}F_{27+2}$	86	6.2	26.5
$N_{10}F_{27+9}$	100	5.0	25.3
$N_{10}F_{27+10}$	78	5.1	9.3
$N_{10}F_{27+16}$	80	7.4	10.8
$N_{10}F_{27+17}$	---[3]	6.0	11.9
C3H.B10-Tt^{w1}			
F_1	67	6.8	5.9
F_2	88	5.5	9.3
F_9	50	4.2	15.8
F_{10}	33	3.3	15.4
F_{12}	---[3]	6.8	14.7
C3H.B10-t^{w1}			
N_1	100	7.9	4.2
N_2F_1	100	7.4	11.9
N_3F_2	100	6.0	12.3
N_4F_3	100	6.4	15.6

[1]Females giving birth to one or more litters were considered to be fertile.
[2]Based on the number of pups born and the number of pups weaned at three weeks of age.
[3]Data not available since these females are currently being mated.

fluctuating environmental conditions form mammary tumors, thus inhibiting lactation.

The male and female segregation distortion factors were determined in the backcross-intercross line. Phenotypic examinations of progeny from the backcross matings revealed the number of $\underline{T}$ and $\underline{t}^{w1}$ gametes fertilizing ova. Mice carrying $\underline{t}^{w1}$ had normal tails whereas mice carrying $\underline{T}$ had short tails. In matings of $\underline{T}/\underline{t}^{w1}$ males x $\underline{+}/\underline{+}$ females, the percent of segregation distortion was high and increased from generation N_1 to N_4F_3. The current male segregation distortion is 0.9. In matings of $\underline{T}/\underline{t}^{w1}$ females x $\underline{+}/\underline{+}$ males, a slight but insignificant distortion occurred (Table 8). The male to female ratio did not differ from normal Mendelian expectations in either case.

Table 8

Segregation Distortion in Males and Females of the Backcross-Intercross Line

Generation of Progeny	Segregation Distortion							
	T/t^{w1} Males				T/t^{w1} Females			
	$\underline{+}/\underline{t}^{w1}$	$\underline{T}/\underline{+}$	% $\underline{t}^{w1}$	χ^2	$\underline{+}/\underline{t}^{w1}$	$\underline{T}/\underline{+}$	% $\underline{t}^{w1}$	χ^2
N_1	49	13	79.0	20.9[1]	6	4	60.0	0.4
N_2F_1	56	10	84.8	32.1[1]	39	36	52.0	0.1
N_3F_2	35	4	89.7	24.6[1]	61	40	60.4	4.4[2]
N_4F_3	49	5	90.7	35.9[1]	61	49	55.5	1.3

[1] $\chi^2_{0.005\ [1]} = 7.88$

[2] $\chi^2_{0.05\ [1]} = 3.84$

Characteristics of Embryos on the Basis of Gross Morphology

Matings in normal C3H.B10 mice (+/+ x +/+) and heterozygous C3H.B10-t^{w1} mice (+/t^{w1} x +/t^{w1}) were performed. Embryos for histological examinations were collected from pregnant females at days 9, 9½, 10, 10½, 11, 12, 13, and 15 days post-coitus (pc). Information obtained at the time of dissection included the number of implants and embryos in each litter. For each embryo, the crown-rump (CR) length was measured, age based on Grüneberg's description of external embryonic features was determined, presence or absence of a heart beat was noted, and presence of any abnormal morphology was identified at the gross level. The gross morphological data were used to examine the effects of t^{w1} on embryonic development in a new genetic background. In the following discussion, embryos from +/t^{w1} x +/t^{w1} matings could have three possible genotypes. Homozygous normal (+/+) and heterozygous (+/t^{w1}) embryos were indistinguishable and will be considered as normal (+/-); homozygous mutant (t^{w1}/t^{w1}) embryos will be considered as abnormal.

Data on average CR lengths of embryos and range of CR lengths for a given embryonic age are summarized in Table 9. Embryos were staged according to Grüneberg's criteria; the embryonic age as based on external morphology, not the expected age as determined from the day of plug (day 0), was used in summarizing the information. CR lengths for abnormal embryos were excluded if the age of the embryo was found to be retarded as compared to normal litter mates. Comparisons of average sizes and range of sizes in embryos from C3H.B10 or C3H.B10-t^{w1} matings at different embryonic ages showed few discrepancies. The sizes and ranges were comparable in embryos collected

Table 9

Average CR Lengths and Range of CR Lengths of Embryos at Different Stages of Development

Embryonic Age, Day	C3H.B10 Matings		C3H.B10-t^{w1} Matings	
	Average Size, mm	Range in Size, mm	Average Size, mm	Range in Size, mm
9	2.29 (39)[1] ± 0.28[2]	1.85- 3.08	2.38 (34)[1] ± 0.30[2]	1.61- 3.08
9½	3.29 (18) ± 0.34	2.85- 3.85	3.25 (35) ± 0.36	2.54- 4.08
10	4.16 (28) ± 0.29	3.85- 4.62	3.87 (30) ± 0.41	3.31- 4.62
10½	4.75 (44) ± 0.29	4.08- 5.31	4.92 (34) ± 0.31	3.93- 5.53
11	6.15 (25) ± 0.35	4.98- 6.62	6.11 (12) ± 0.50	5.39- 6.93
12	8.18 (19) ± 0.69	6.38- 9.33	7.72 (16) ± 0.67	6.16- 8.47
13	9.86 (26) ± 0.66	8.24-10.73	9.42 (8) ± 0.58	8.47-10.24
15	13.88 (21) ± 0.47	13.22-14.78	15.33 (3) ± 1.53	14.00-17.00

[1]Number of embryos measured with an ocular micrometer on a Wild M7A dissection microscope.

[2]Standard deviation of the mean.

from either mating, indicating that the presence of mutant $\underline{t}^{w1}$ did not affect the development of normal embryos within litters. The values listed are similar to previously published data for other mouse strains (Altman and Katz, 1979).

The expected litter age was used to compare ranges in age and CR lengths within litters from C3H.B10 and C3H.B10-$\underline{t}^{w1}$ matings (Table 10). Prior to 10½ days pc, the range in age and size of embryos from either mating was very similar. Homozygous $\underline{t}^{w1}$ embryos could not be identified. However, at 10½ days pc, discrepancy in range of age appeared in litters derived from C3H.B10 and C3H.B10-$\underline{t}^{w1}$ matings. In litters derived from C3H.B10 matings at 11 to 15 days pc, the range of age decreased, indicating that embryos within a litter were of equivalent ages. Embryos identified as developmentally younger than their littermates at earlier stages of development had grown and attained the same age and developmental features characteristic of its littermates at later stages of development. Conversely, the increased range in age and size of litters derived from C3H.B10-$\underline{t}^{w1}$ matings was indicative of the presence of abnormally developing embryos. Subsequent histological studies showed these "retarded" embryos to be homozygous for $\underline{t}^{w1}$. Contrary to a previous report on mutant $\underline{t}^{w1}$ (Bennett, et al., 1959b), the decreased size of an embryo could be used only after day 11 pc to identify the embryo as a mutant homozygous for $\underline{t}^{w1}$.

The number of implants and aborts in litters derived from C3H.B10 and C3H.B10-$\underline{t}^{w1}$ matings are summarized in Table 11. The number of implants per litter was 8.5 ± 0.84 and 8.7 ± 1.24 for C3H.B10 and C3H.B10-$\underline{t}^{w1}$, respectively. The mutant $\underline{t}^{w1}$ did not appear to affect

Table 10

Variation of Age and CR Length of Embryos Within Litters

Expected Age, Day	C3H.B10 Matings		C3H.B10-t^{w1} Matings	
	Range in Age, Days	Range in Size, mm	Range in Age, Days	Range in Size, mm
9	8½-9	2.00- 2.92	8½-9	1.61- 3.08
9½	9-9½	1.85- 3.77	9-9½	2.16- 3.47
10	9½-10	3.46- 4.62	9½-10	2.62- 4.31
10½	9-10½	3.08- 5.31	8½-10½	3.39- 5.39
11	11	4.98- 6.38	10-11	4.47- 6.93
12	12	6.38- 8.87	9-12	2.31- 8.47
13	12½-13	7.78-10.73	12-13	7.24-10.24
15	15	13.22-14.78	11-15	4.39-17

Table 11

Number of Implants, Aborts, and Abnormal Embryos

Expected Age, Days	C3H.B10 Matings				C3H.B10-t^{w1} Matings				
	Number of Implants	Implants/ Litter	Number of Aborts	Aborts/ Litter	Number of Implants	Implants/ Litter	Number of Aborts	Aborts/ Litter	Number of t^{w1}/t^{w1}
9	51 (6)[1]	8.50	6	1.00	26 (3)[1]	8.67	5	1.67	2[2]
9½	17 (2)	8.50	0	0.00	16 (2)	8.00	2	1.00	0
10	18 (2)	9.00	1	0.50	40 (4)	10.00	3	0.75	4
10½	69 (7)	9.86	4	0.57	29 (3)	9.67	6	2.00	5
11	28 (3)	9.33	3	1.00	20 (3)	6.67	3	1.00	8
12	23 (3)	7.67	4	1.33	28 (3)	9.33	3	1.00	10
13	37 (5)	7.40	9	1.80	15 (2)	7.50	5	2.50	3
15	24 (3)	8.00	3	1.00	10 (1)	10.00	2	2.00	5
		8.53 ± 0.84[3]		0.9 ± 0.55[3]		8.73 ± 1.24[3]		1.49 ± 0.64[3]	

[1]Number of litters given in parentheses.

[2]Homozygotes identified after histological examinations.

[3]Standard deviation of the mean.

the ability of embryos to implant in the uterine wall after fertilization. The average abort rate per litter for C3H.B10 and C3H.B10-$\underline{t}^{w1}$ matings was 0.9 ± 0.55 and 1.49 ± 0.64, respectively. The abort rate for C3H.B10-$\underline{t}^{w1}$ excludes $\underline{t}^{w1}/\underline{t}^{w1}$ embryos identified histologically but includes those homozygotes that could not be identified.

After the embryos derived from C3H.B10-$\underline{t}^{w1}$ matings were examined histologically, the number of homozygous $\underline{t}^{w1}$ embryos for each time period were determined. The percent of abnormal embryos (aborts plus abnormal embryos) was found to increase from 12.5% to 70% with increasing age. The ability to identify homozygous $\underline{t}^{w1}$ embryos and its correlation with the percent of abnormal embryos expected and observed will be discussed later.

Characteristics of Embryos on the Basis of Histology

Histological examinations by light microscopy of serially sectioned embryos derived from C3H.B10-$\underline{t}^{w1}$ matings at different stages of development were performed. Emphasis was placed upon the ability to identify homozygous $\underline{t}^{w1}/\underline{t}^{w1}$ embryos as early as possible and the determination of the effects of the mutant gene, especially in homozygous $\underline{t}^{w1}/\underline{t}^{w1}$ embryos, at different stages of embryonic development. The gross morphological data together with the histological data would provide a description of the effect of mutant $\underline{t}^{w1}$.

Day 9 pc: Homozygous abnormal embryos were impossible to distinguish from normal littermates at day 9 pc on the basis of CR length or gross morphology (Figure 5A,B). No edema, reduction in size, or other gross abnormalities such as an enlarged heart were present. Only 2 of 21 embryos were identified as $\underline{t}^{w1}/\underline{t}^{w1}$ after tissue

Figure 5. Normal and abnormal mouse embryos from days 9 to 10½ pc photographed after dissection. Tailbuds were excised for in vitro culturing.

A. Normal day 9 pc embryo (x30.3).

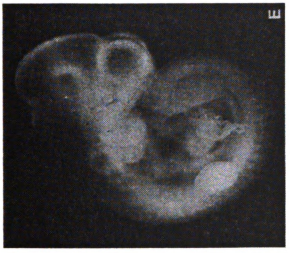
B. Abnormal day 9 pc embryo (x30.3).

C. Normal day 10 pc embryo (x16.2).

D. Abnormal day 10 pc embryo (x16.2).

E. Normal day 10½ pc embryo (x14.4).

F. Abnormal day 10½ pc embryo (x14.4).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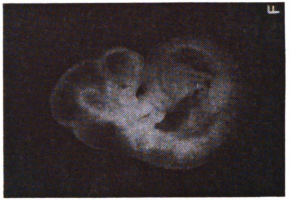
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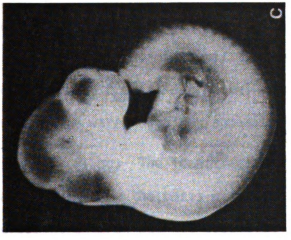
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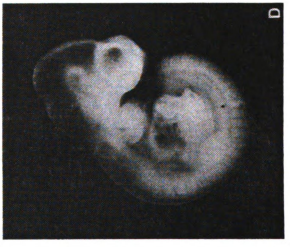
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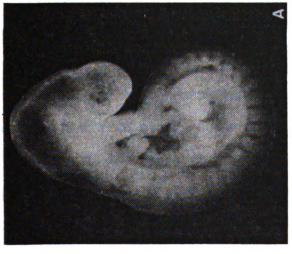
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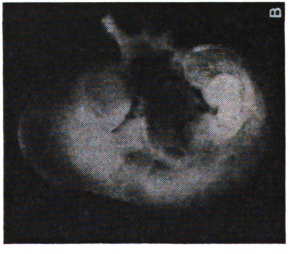
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sections stained with hematoxylin and eosin were examined by light microscopy. Degenerating and pycnotic cells were characterized by the presence of darkly staining chromatin material in the mantle layer of the ventral (basal) neural tube at the rostral border of the rhombencephalon (Figure 6A-C). Nucleated red blood cells, whose nuclei stained with hematoxylin and were centrally located within the cells, were easily distinguished from pycnotic cells. Actively dividing cells of the neural tube were prevalent in all embryos and were confined to the ventricular zone (Boulder Committee, 1970). Pycnoses were absent in the regions rostral and caudal to the degenerating rhombencephalon (Figure 6D-F). The width of the neural tube (measured from the ependymal to marginal layers), neural ectoderm derivatives, cranial and spinal ganglia, somites, notochord, and mesenchyme were normal in appearance.

Day 9½ pc: The identification of normal and abnormal embryos at day 9½ pc on the basis of size differences or abnormalities in gross structures was not possible. Although detection of a 9½ day pc abnormal embryo from an expected 9½ day pc litter was not made histologically, one abnormal 9½ day embryo from a 10 day pc litter was found. The embryo was characterized histologically by the presence of pycnotic cells in the mantle layer of rostral and caudal portions of the ventral rhombencephalon (Figure 7A-B, D-E). Pycnotic cells were absent from other regions of the neural tube (Figure 7C-F). The width of the neural tube was decreased only in regions containing pycnoses. Actively dividing cells were present in the ventricular zone, but the mantle layer of affected neural tube regions was not as wide as the mantle layer in comparable normal neural tube regions.

Figure 6. Transverse sections of normal and abnormal mouse embryos at day 9 pc.

A. Rostral portion of rhombencephalon in the region of the eye from a normal mouse embryo (x73).

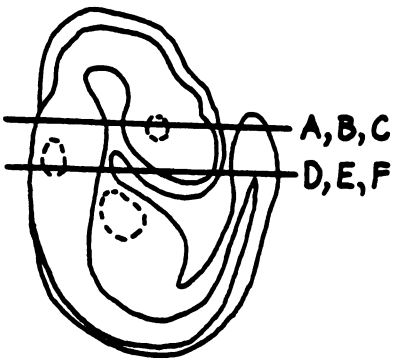
B. Magnification of region outlined in A. Note the presence of mitotically dividing cells in the ventricular zone and the cellular arrangement found in the mantle layer of the neural tube. Nucleoli appear as darkly staining granules within cells of the neural tube (x290).

C. Rostral portion of rhombencephalon in the region of the eye from an abnormal mouse embryo. Note the presence of mitotically dividing cells confined to the ventricular zone and of a few pycnotic cells in the mantle layer of the neural tube (x290).

D. Caudal portion of rhombencephalon in the region of the otic vesicle from a normal mouse embryo (x73).

E. Magnification of region outlined in D. Note the presence of mitotically dividing cells confined to the ventricular zone of the neural tube (x290).

F. Caudal portion of rhombencephalon in the region of the otic vesicle from an abnormal mouse embryo. Note the presence of mitotically dividing cells confined to the ventricular zone and the absence of pycnotic cells in the mantle layer of the neural tube (x290).



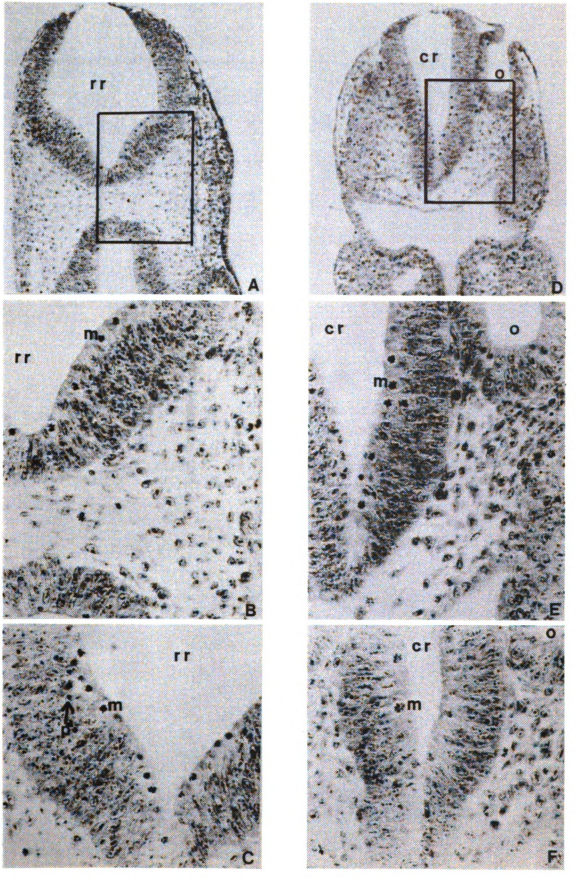

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Figure 7. Transverse sections of normal and abnormal mouse embryos at day 9½ pc.

A. Rostral portion of rhombencephalon in the region of the eye from a normal mouse embryo. Note width of neural tube and absence of pycnotic cells within the mantle layer (x300).

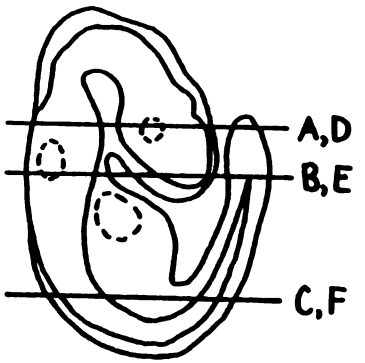
B. Caudal portion of rhombencephalon in the region of the otic vesicle from a normal mouse embryo. Note width of neural tube and absence of pycnotic cells within the mantle layer (x300).

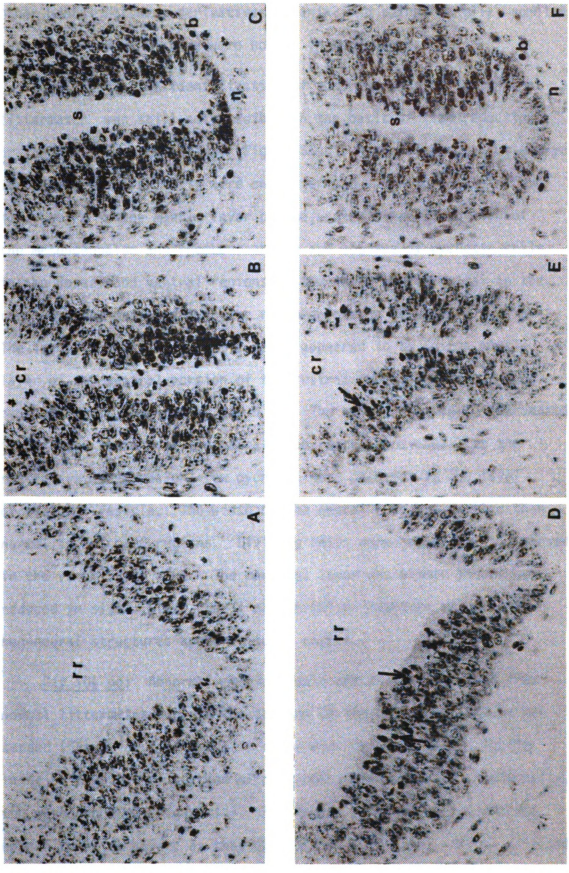
C. Caudal region of spinal cord from a normal mouse embryo. Note width of the neural tube and presence of the notochord (x300).

D. Rostral portion of rhombencephalon in the region of the eye from an abnormal mouse embryo. Note slightly decreased width of the neural tube and presence of pycnotic cells (arrows) within the mantle layer (x300).

E. Caudal portion of rhombencephalon in the region of the otic vesicle from an abnormal mouse embryo. Note slightly decreased width of neural tube and presence of pycnotic cells (arrows) within the mantle layer (x300).

F. Caudal region of spinal cord from an abnormal mouse embryo. Note width of neural tube and absence of pycnotic cells in the mantle layer (x300).

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Other neural ectoderm derivatives, cranial and spinal ganglia, somites, notochord, and mesenchyme were normal in appearance.

Day 10 pc: The identification of abnormal embryos from normal littermates was still not possible on the basis of size reduction or abnormal gross morphology (Figure 5C,D). Several homozygous embryos identified histologically were characterized by the presence of pycnotic cells in the mantle layer of the ventral (basal) portion of the mesencephalon and rhombencephalon and absence of pycnotic cells in the dorsal and ventral regions of the neural tube rostral to the mesencephalon and caudal to the rhombencephalon (Figures 8 and 9). The largest number of pycnotic cells appeared to be in the mantle layer of the ventral portion of the rostral rhombencephalon, where pycnoses first appeared at day 9 pc. The number of pycnoses decreased in affected regions of the neural tube rostral and caudal to the rostral rhombencephalon; few pycnoses were observed at the level of the otic vesicle. The width of the neural tube was smaller than normal in affected regions. Dividing cells were occasionally observed in the ventricular zone. The marginal layer was either absent or reduced in size. Other neural ectodermal derivatives as well as non-neural structures appeared to be normal.

Day 10½ pc: Abnormal embryos could not be distinguished from normal littermates unless the age and CR length were obviously retarded (Figure 5E,F). Pycnotic cells were confined to the mantle layer of the ventral portion of the rhombencephalon and, occasionally, the mesencephalon (Figure 10). The degree of degeneration did not appear to be as severe as that observed at day 10 pc. In the affected regions, width of the neural tube was slightly smaller and the

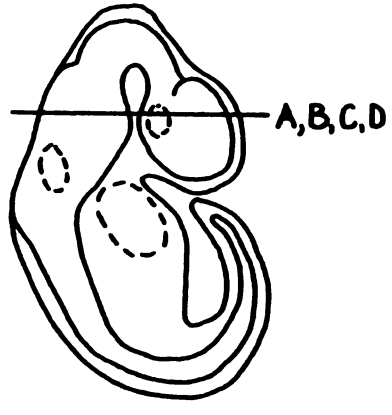
Figure 8. Transverse sections of normal and abnormal mouse embryos at day 10 pc.

A. Rostral portion of rhombencephalon in the region of the eye from a normal mouse embryo. Note the presence of mitotically dividing cells confined to the ventricular zone and the width of the neural tube (x75).

B. Magnification of region outlined in A. Note the presence of three zones in the neural tube: ventricular zone containing mitotically dividing cells; mantle zone containing neuroblasts; and marginal zone containing neuronal fibers (x300).

C. Rostral portion of rhombencephalon in the region of the eye from an abnormal mouse embryo. Note the decreased width of the neural tube and the number of darkly staining pycnotic cells (arrow). Mitotically dividing cells are present in the ventricular zone (x75).

D. Magnification of region outlined in C. Note the number of pycnotic cells confined to the mantle layer, the decreased width of the mantle zone, and the absence of a marginal zone (x300).



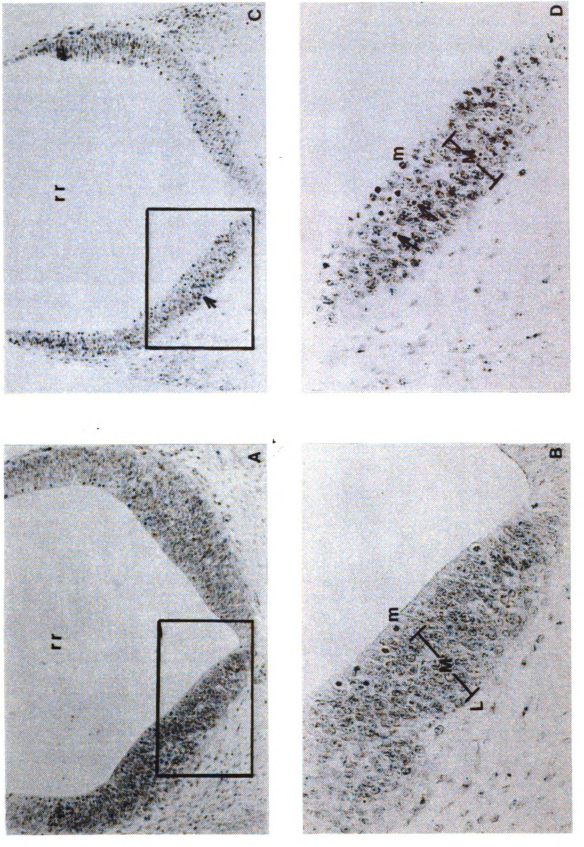

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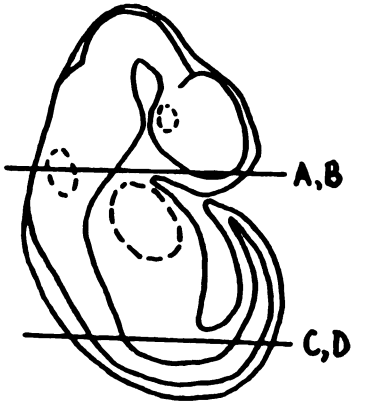
Figure 9. Transverse sections of normal and abnormal mouse embryos at day 10 pc.

A. Caudal portion of rhombencephalon in the region of the otic vesicle from a normal mouse embryo (x300).

B. Caudal portion of rhombencephalon in the region of the otic vesicle from an abnormal mouse embryo. The width of the neural tube is decreased slightly compared to that observed in A (x300).

C. Caudal portion of the spinal cord from a normal mouse embryo. Note the presence of mitotically dividing cells in the ventricular zone (x300).

D. Caudal portion of the spinal cord from an abnormal mouse embryo. Mitotically dividing cells are confined to the ventricular zone and the cell density in the mantle layer is comparable to that observed in a normal embryo (x300).



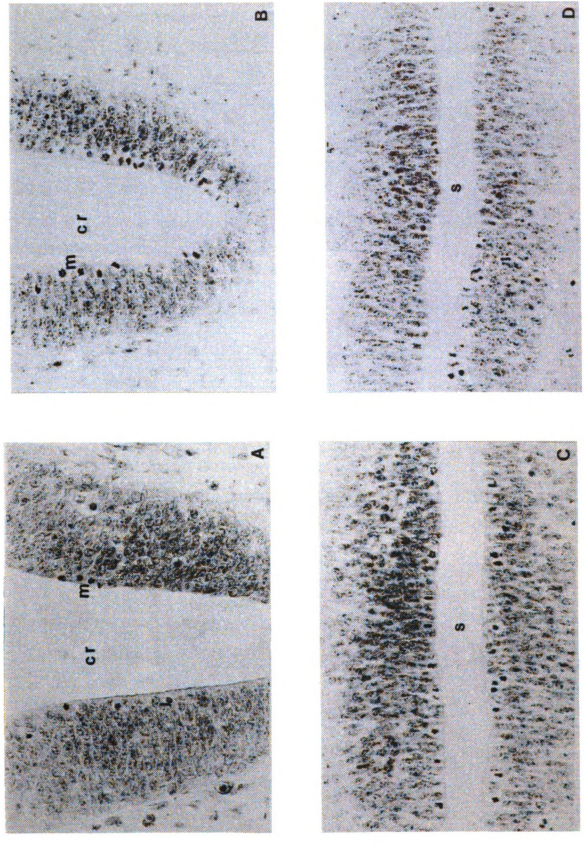

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Figure 10. Transverse sections of normal and abnormal mouse embryos at day 10½ pc.

A. Rostral portion of rhombencephalon in the region of the eye from a normal mouse embryo. Note the presence of the three distinct zones of the neural tube (x75).

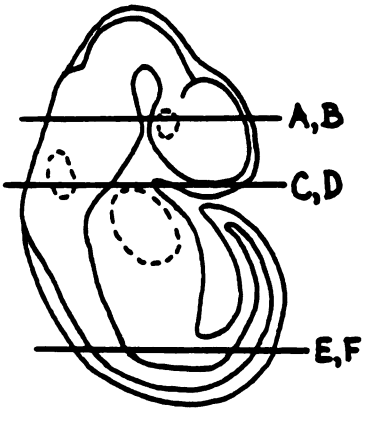
B. Rostral portion of rhombencephalon in the region of the eye from an abnormal mouse embryo. Note the presence of the ventricular and mantle zones and the absence of the marginal zone (x75).

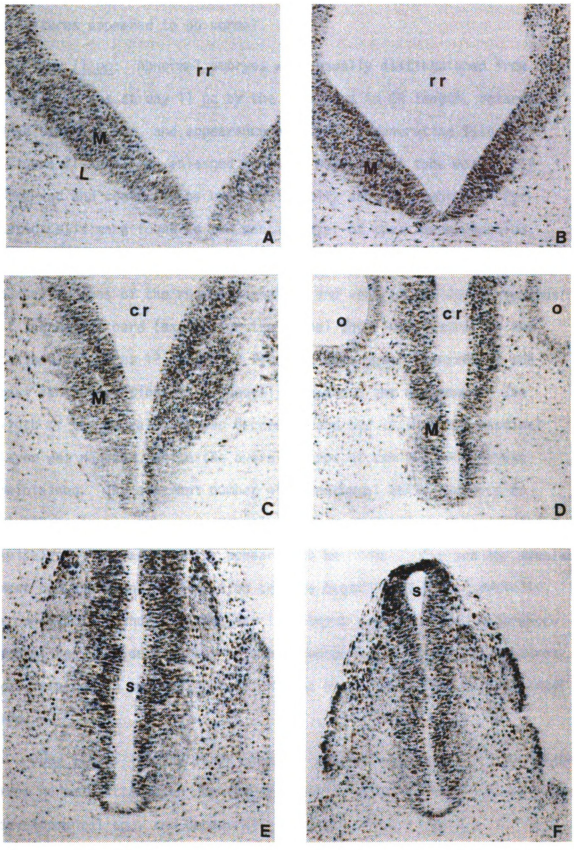
C. Caudal portion of the rhombencephalon in the region of the otic vesicle of a normal mouse embryo. Note the width of the neural tube and the presence of three zones (x75).

D. Caudal portion of the rhombencephalon in the region of the otic vesicle from an abnormal mouse embryo. Note the decreased width of the neural tube and the absence of the marginal zone (x75).

E. Caudal portion of the spinal cord from a normal mouse embryo. Note the presence of mitotically dividing cells in the ventricular zones and neuroblasts in the mantle zone (x75).

F. Caudal portion of the spinal cord from an abnormal mouse embryo. Note the presence of mitotically dividing cells in the ventricular zone. The width of the neural tube appears to be smaller than that observed in E (x75).

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marginal layer was not as prevalent. Other neural and non-neural structures appeared to be normal.

Day 11 pc: Abnormal embryos were usually distinguished from normal embryos at day 11 pc by the reduction in CR length, retarded developmental age, and appearance of opaque degenerating tissues (Figure 11 A-D). An enlarged heart or wavy neural tube was rarely observed and could not be used to identify abnormal embryos. Pycnotic cells were found in the mantle layer of the: mesencephalon, ventral regions of the diencephalon and telencephalon, ventral and dorsal regions of the rhombencephalon, and ventral and dorsal regions of the spinal cord (except for the spinal cord just rostral to the tailbud) (Figures 12 and 13). Pycnoses were never observed in the most ventral portion of the neural tube above the notochord. The width of the neural tube was decreased and the size of the marginal layer was reduced, while the overall shape of the neural tube was maintained. The apparent number of mesenchymal cells appeared to fluctuate in all embryos. Neural ectoderm derivatives, cranial and spinal ganglia, and somites appeared to be normal. Eye and ear development continued and ossification centers began to appear, especially around the notochord. Since day 11 embryos were retarded in developmental age, the development of other neural and non-neural structures was compared to younger, normal embryos of an equivalent developmental age.

Day 12 pc: By day 12 pc abnormal embryos were easily distinguished from normal littermates by the presence of reduced CR length, retarded developmental age, and degenerating tissues (Figure 11E-G). In the

Figure 11. Embryos from days 11 and 12 pc photographed after dissection. Tailbuds were excised for in vitro culturing.

A. Day 11 pc normal embryo (x11.5).

B. Day 11 pc abnormal embryo, littermate to embryo in A (x12.4).

C. Day 11 pc abnormal embryo (x11.5).

D. Day 11 pc abnormal embryo, littermate to embryo in C (x11.0).

E. Day 12 pc normal embryo (x14.3).

F. Day 12 pc abnormal embryo, littermate to embryo in E (x17.8).

G. Day 12 pc abnormal embryo, littermate to embryo in E (x14.1).

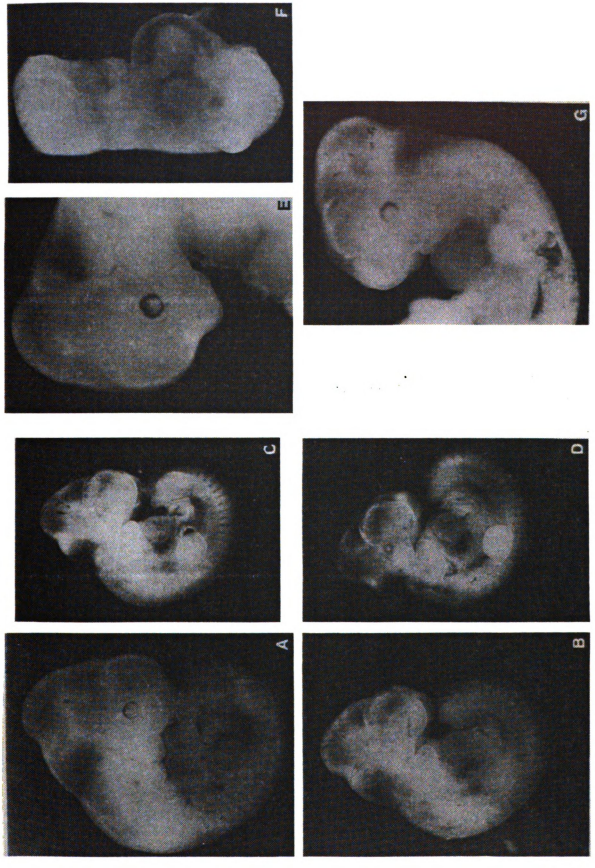
A
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Figure 12. Transverse sections of normal and abnormal mouse embryos at day 11 pc.

A. Caudal portion of rhombencephalon in the region of the otic vesicle from a normal embryo. Note the cell density and width of the mantle layer. The marginal layer is quite distinct (x75).

B. Rostral portion of rhombencephalon in the region of the eye from an abnormal embryo. Note the increased number of pycnotic cells found in the mantle layer and the absence of a marginal layer (x75).

C. Rostral portion of rhombencephalon in the region of the eye from an abnormal mouse embryo. Note the presence of pycnotic cells confined to the mantle layer (x300).

D. Caudal portion of rhombencephalon in the region of the otic vesicle from an abnormal embryo. Note that pycnotic cells are confined to the ventral half of the neural tube and absent in the region of the neural tube directly above the notochord (x75).

E. Caudal portion of rhombencephalon in the region of the otic vesicle from an abnormal embryo. Note that pycnotic cells are found in the ventral half of the mantle layer but absent in the region directly above the notochord (x75).

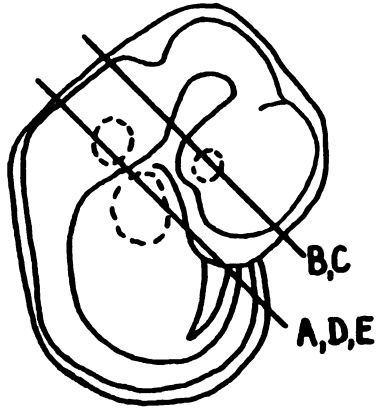

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Figure 13. Transverse sections of normal and abnormal mouse embryos at day 11 pc.

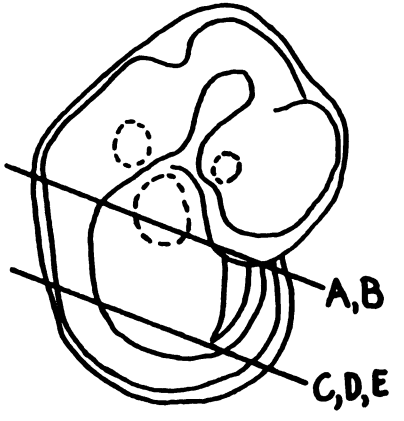
A. Spinal cord in the region of the heart from a normal mouse embryo. Note the cell density of the mantle layer and distinct marginal layer (x75).

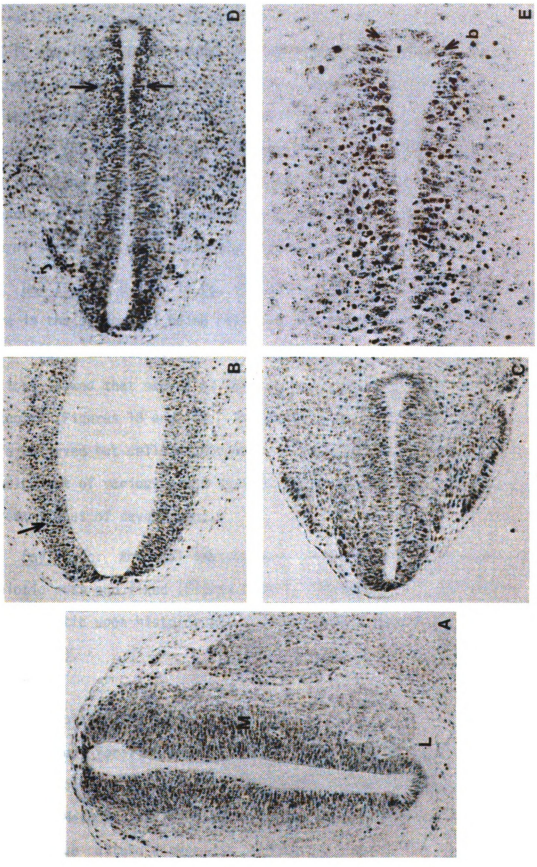
B. Spinal cord in the region of the heart from an abnormal mouse embryo. Note the decreased width of the neural tube and the presence of a few pycnotic cells in the mantle layer (x75).

C. Caudal region of the spinal cord from an abnormal mouse embryo. Note the general shape of the spinal cord and the absence of pycnotic cells (x75).

D. Caudal region of the spinal cord from an abnormal mouse embryo. Note the confinement of the pycnotic cells in the mantle layer of the ventral portion of the neural tube (x75).

E. Magnification of the ventral half of the neural tube shown in D. Note the presence of pycnotic cells in the mantle layer except in the region above the notochord (arrows) (x300).

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most extreme cases, the neural tube was completely pycnotic and the overall shape was generally discernible but disorganized (Figures 14C, 15C-D, and 16C). Other neural and non-neural structures displayed pycnotic cells. In less extreme cases, pycnoses were still confined to the neural tube (Figures 14B, 15B, 16B). The mantle and marginal layers of the neural tube were decreased in size. In either case, eye, ear, and cartilage development had progressed to the same stage found in a comparable littermate even though other neural or non-neural structures were obviously degenerating.

Day 13 pc: By day 13 pc, the easily identified abnormal embryos were in the process of being resorbed (Figure 17A-C). The tissues were fragile and easily torn during dissection. Histological examinations showed that neural as well as non-neural structures were pycnotic (Figures 18 and 19). The general outlines of structures were observed but cells within were disorganized and pycnotic. The development of various organ systems appeared to be arrested at different stages of development.

Day 15 pc: Abnormal embryos were carefully dissected free from amniotic sacs and fixed (Figure 17D-E). Obviously, all structures were pycnotic upon histological examination by light microscopy (Figure 20).

Primary Explants and Subcultures

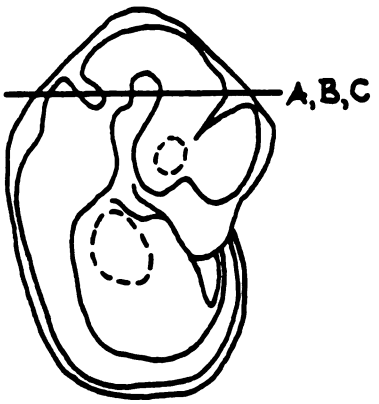
Tailbud, forelimb bud, or heart tissues were excised from 207 embryos representing days 9 to 15 pc and cultured in vitro as described in Methods. Primary explants were transferred to untreated 35 mm tissue culture dishes and usually attached to the plastic

Figure 14. Transverse sections of normal and abnormal mouse embryos at day 12 pc.

A. A portion of the basal neural tube in the region of the isthmus from a normal mouse embryo. Mitotically dividing cells are present in the ventricular zone. A large number of cells are present in the mantle zone (x300).

B. A portion of the basal neural tube in the region of the isthmus from an abnormal mouse embryo. Note the large number of pycnotic cells and decreased cell density in the mantle zone (x75).

C. A portion of the basal neural tube in the region of the isthmus from an abnormal mouse embryo. Note the loss of structural integrity, cellular disorganization and cellular pycnosis in the neural tube (x75).



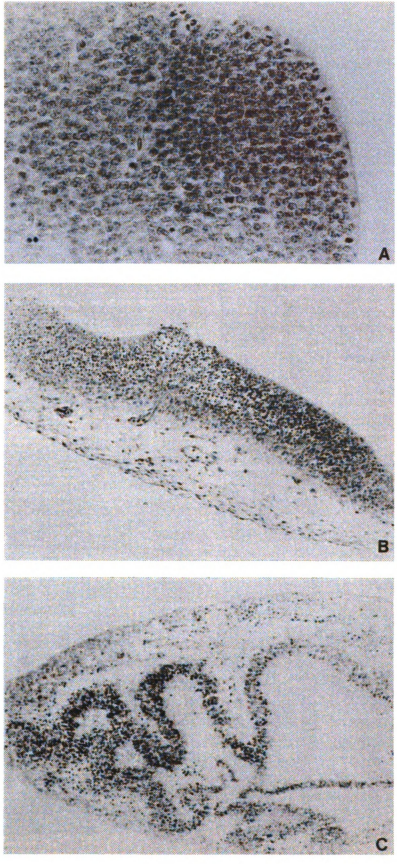

A
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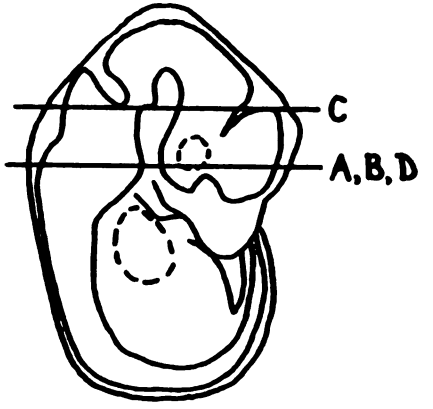
Figure 15. Transverse sections of normal and abnormal mouse embryos at day 12 pc.

A. Caudal portion of the rhombencephalon in the region of the eye/ear from a normal mouse embryo. Note the cell density of the mantle zone and the distinct marginal zone (x75).

B. Caudal portion of the rhombencephalon in the region of the eye/ear from an abnormal embryo. Note the number of pycnotic cells confined to the mantle zone and lack of a marginal zone (x75).

C. A portion of the metencephalon in the region of the eye from an abnormal mouse embryo. Note the lack of structural integrity and cellular disorganization of the neural tube (x75).

D. Caudal portion of the rhombencephalon in the region of the eye/ear from an abnormal embryo. Note the lack of structural integrity and cellular disorganization of the neural tube and surrounding cells (x75).



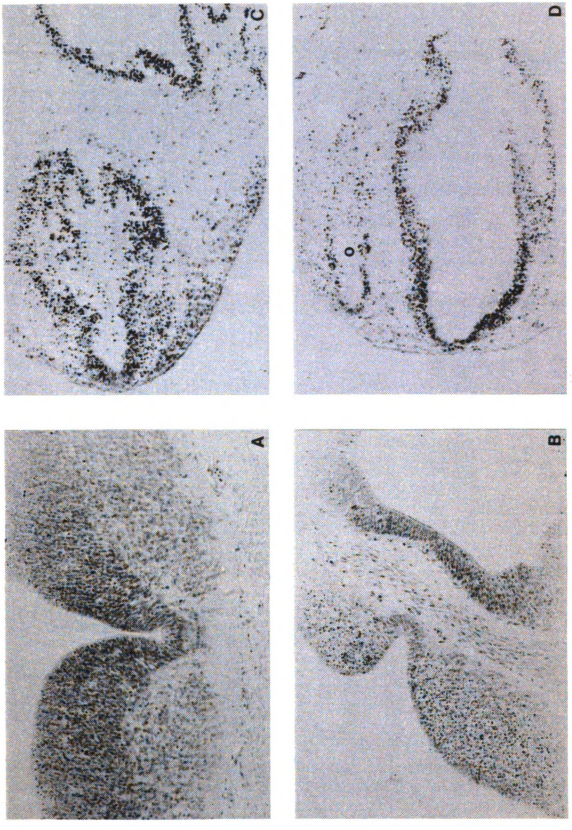

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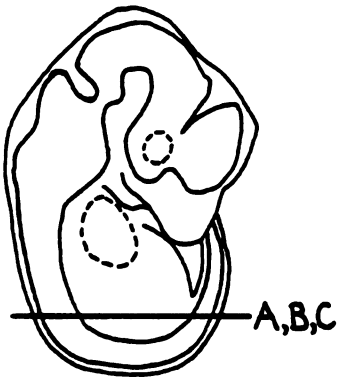
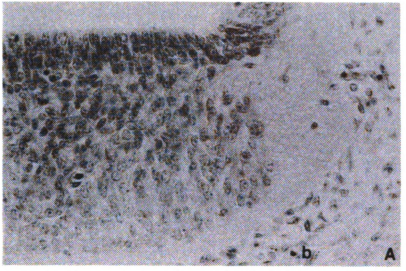
Figure 16. Transverse sections of normal and abnormal mouse embryos at day 12 pc.

A. Caudal portion of spinal cord from a normal mouse embryo (x300).

B. Caudal portion of spinal cord from an abnormal mouse embryo. Note the presence of pycnotic cells in the alar and basal regions of the mantle layer and absence of pycnotic cells above the notochord (x75).

C. Caudal portion of spinal cord from an abnormal mouse embryo. Note the lack of structural integrity and general cellular disorganization of the neural tube (x75).

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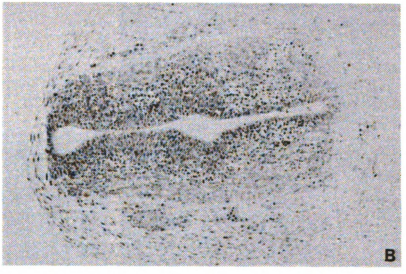
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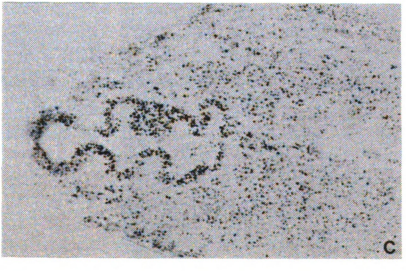

C

Figure 17. Embryos from days 13 and 15 pc photographed after dissection. Tailbuds were excised for in vitro culturing.

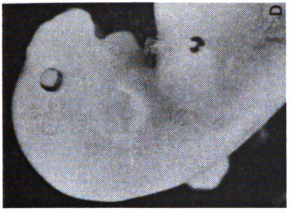
A. Day 13 pc normal embryo (x16.0).

B. Day 13 pc abnormal embryo (x15.3).

C. Day 13 pc abnormal embryo (x15.2).

D. Day 15 pc abnormal embryo (x16.0).

E. Day 15 pc abnormal embryo (x11.4).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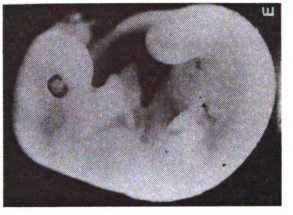
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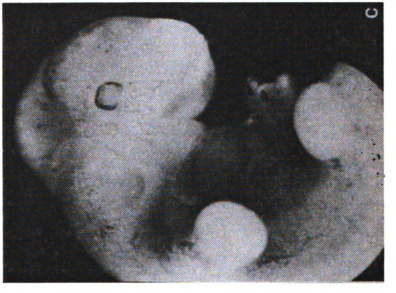
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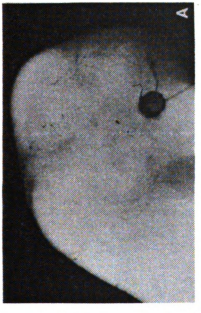
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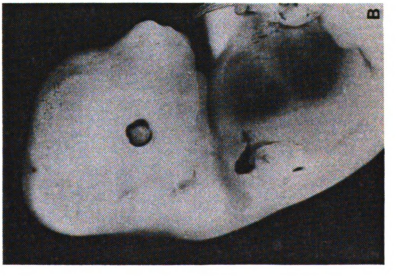

B

Figure 18. Transverse sections of normal and abnormal mouse embryos at day 13 pc.

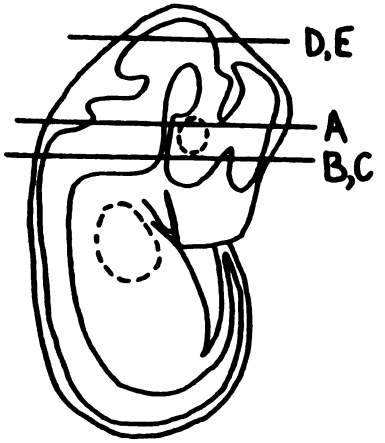
A. A portion of the metencephalon in the region of the eye/ear from a normal mouse embryo. Note the presence of normal mantle and marginal zones (x30).

B. Caudal portion of the rhombencephalon in the region of the otic vesicle from an abnormal mouse embryo. Note the presence of pycnotic cells in the mantle layer of the neural tube (x30).

C. Magnification of ventral portion of the neural tube shown in B. Note the presence of pycnotic cells in the neural tube and of nerve fiber leaving the neural tube (x75).

D. A portion of the mesencephalon from an abnormal mouse embryo. Note the large numbers of pycnotic cells in the neural tube and absence of any well-defined cellular organization (x30).

E. A portion of the mesencephalon from an abnormal mouse embryo. Note the lack of integrity and cellular disorganization in the neural tube as well as surrounding tissue (x75).



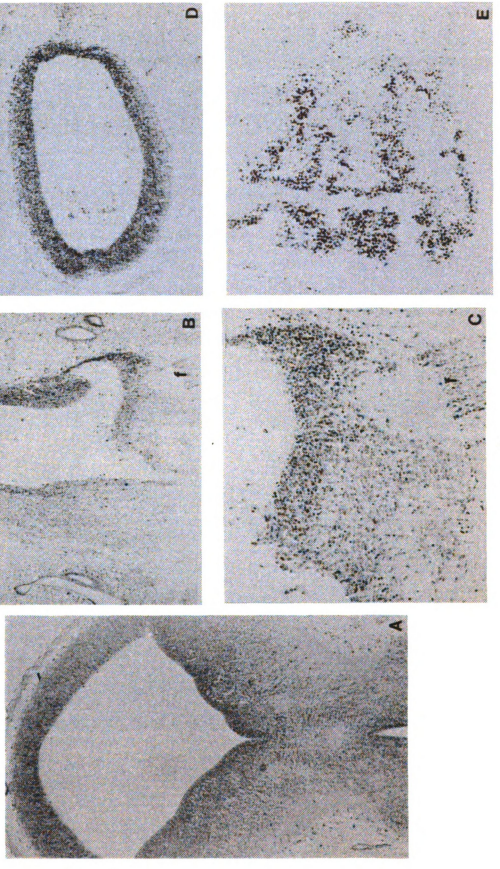

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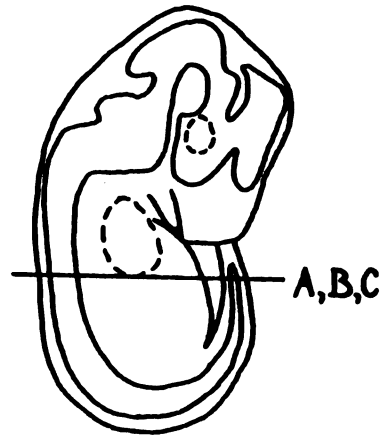
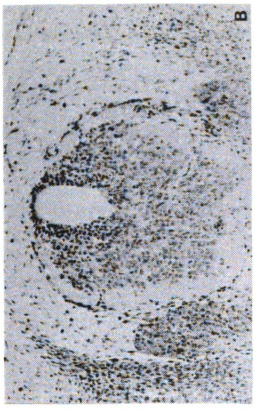
Figure 19. Transverse sections of normal and abnormal mouse embryos at day 13 pc.

A. Caudal portion of the spinal cord from a normal mouse embryo (x30).

B. Caudal portion of the spinal cord from an abnormal mouse embryo. Note the deceased width and cell density of the mantle layer. A few pycnotic cells are present (x75).

C. Caudal portion of the spinal cord from an abnormal mouse embryo. Note the lack of structural integrity and cellular disorganization (x75).

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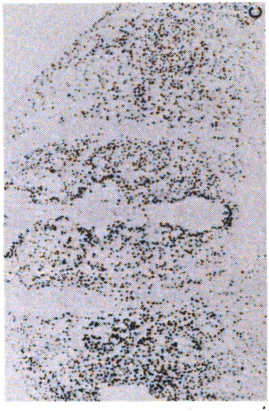
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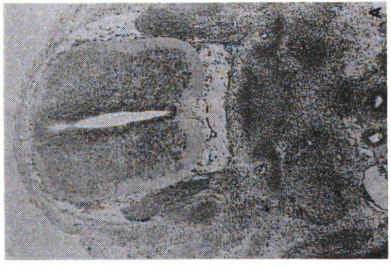


Figure 20. Transverse sections of normal and abnormal mouse embryos at day 15 pc.

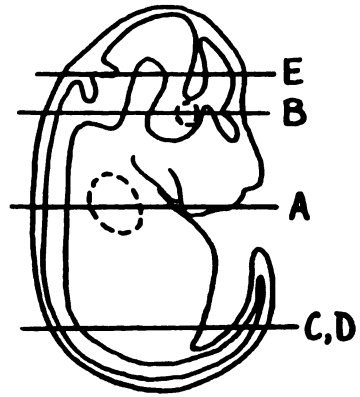
A. Spinal cord in the region of the heart from an abnormal mouse embryo. Note the pycnoses of all cells and the loss of cellular organization (x30).

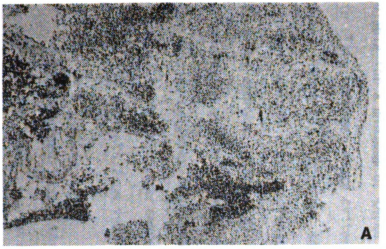
B. A portion of the diencephalon in the region of the eye from an abnormal mouse embryo. Note the general outline of where the diencephalon should be and presence of pycnotic cells in all tissues (x30).

C. Caudal portion of the spinal cord from an abnormal mouse embryo. Note the presence of the notochord and formation of vertebrae in the midst of pycnotic cells (x30).

D. A portion of the spinal cord from an abnormal mouse embryo. Note the outline of structures are discernable even though all cells are pycnotic (x30).

E. A portion of the metencephalon from an abnormal mouse embryo. Note that all cells are pycnotic (x75).

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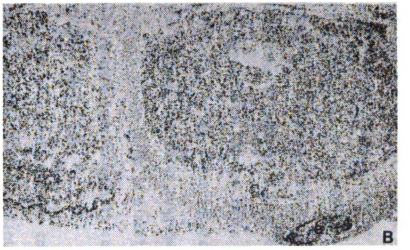
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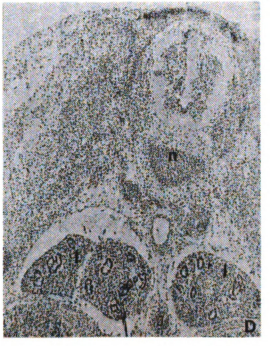
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substratum within 24 hours. Within 48 hours, cellular outgrowth appeared from the cut edge of the primary explant. Cells were either fibroblast-like or epithelial-like and surrounded the primary explant (Penso and Balducci, 1963). After at least a week in culture, cells preparing to undergo mitotic division detached from the substrate and appeared as rounded cells on the substrate surface. Continued growth was noted by the increase in circumference of cells surrounding the explant. Cellular outgrowth was usually limited to a circular focus of 10 to 15 mm in diameter. By this stage, cell confluency was reached and the culture could be used for subculturing and establishing cell lines.

For an explant culture to be considered successful, three criteria had to be met: 1) the explant had to attach to the substratum; 2) cellular outgrowth had to be observed; and 3) cells had to be viable for a minimum of three weeks. Of the 207 explants cultured in vitro, 86% met these criteria and were considered successful cultures (Table 12). However, this value is an underestimate of the rate of success since explants were cultured from living as well as dying embryos. Obviously, tissue excised for in vitro culture from a dying embryo may not be expected to attach to a substratum and display cellular outgrowth. Of the 207 explants, 17 (8%) were known to be derived from embryos dying in utero (Table 12). Therefore, 176 of 190 explants from viable embryos (94%) were successfully cultured.

Tissue excised from either normal or abnormal embryos produced four different types of primary cultures when observed by light microscopy. Most cultures from tailbud or forelimb bud tissues were composed

Table 12

Primary Explants From Embryos Representing Days 9 to 15 pc

Expected Embryo Age, Days	Number of Explants[1]	No. Successful/ No. Failed
9	29 (4)[2]	28/1 (1)[3]
9½	29 (4)	27/2
10	36 (4)	33/3
10½	42 (5)	37/5 (1)
11	28 (4)	25/3
12	25 (3)	18/7 (7)
13	10 (2)	7/3 (3)
15	8 (1)	3/5 (5)

[1]One explant per embryo.

[2]Number in parentheses refers to the total number of litters.

[3]Number in parentheses refers to culture of tissue from resorbing embryos.

of fibroblast-like cells (Figure 21A). These Type I cultures were composed of very narrow, elongated and spindly cells arranged in parallel fashion. In these cultures, a state of confluency was reached very quickly. Cell boundaries were barely discernible. Monolayers of cells were only observed at the peripheral edges of the culture. Rounded, birefringent cells, probably representing cells undergoing mitotic division, were prominent. At the periphery, cells having more than two long processes were occasionally observed. These stellate or spider shaped cells were usually overrun by fibroblasts as the age of the culture increased.

In Type II cultures, tailbud or forelimb bud explants gave rise to cells identified as fibroblasts, characterized by long, spindly shapes (Figure 21B). Very few rounded cells were present. Instead

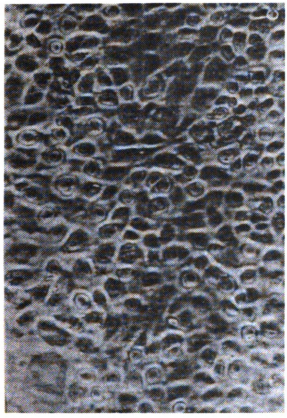
Figure 21. Inverted phase contrast photographs of outgrowths from primary embryonic explants (x370).

A. Type I culture. Note the number of rounded, birefringent cells (arrows) on a dense fibroblast mat.

B. Type II culture. Note the long, spindly shape of the fibroblasts and the presence of very few rounded cells.

C. Type III culture. Note the presence of flattened, epithelial-like cells with polygonal cell boundaries.

D. Type III culture.



of covering a flat surface, the cells tended to form circular mounds with little or no contact between mounds. In regions where fibroblast cells were absent, large amorphous cells with transparent cytoplasm and "fringed" edges were present. Confluency of cells was not reached as quickly as in cultures of Type I.

In Type III cultures, forelimb bud or heart explants were the source of epithelial-like cells (Figure 21C,D). Long, spindly shaped fibroblasts were absent. The flattened cells were characterized by their uniform size and polygonal cell boundaries. Cells in Type III cultures were slower to grow than cells in Type I or Type II cultures.

Cells prevalent in Type IV cultures were neither fibroblast-like nor epithelial-like (Figure 22A,B). Cells of different shapes and sizes were present and were extremely difficult to observe because of their transparency. Vacuolated and granulated cells accumulated with increasing age of the culture. In addition, large clear cells with highly refractive membranes appeared. Extra-cellular "debris", presumably formed by cultured cells, was suspended in culture media and increased in concentration between media changes. In contrast to fibroblasts found in Type I cultures, fibroblasts in Type IV cultures appeared at the peripheral edges of the cultures and never obliterated the unusual cells. Trypsinization of Type IV cultures was extremely difficult. Large areas of cells were often left attached to the substratum, and extra-cellular debris continued to be produced by cells subsequent to the trypsinization treatment.

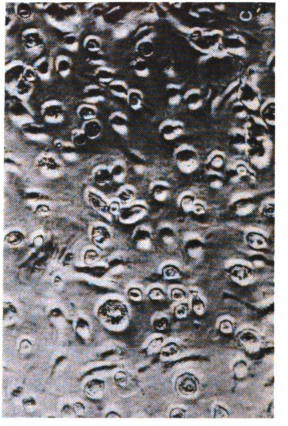
Cells prevalent in cultures derived from heart explants of $\underline{T}/\underline{T}$ embryos usually had two or more processes and were highly refractive

Figure 22. Inverted phase contrast photographs of outgrowths from primary embryonic explants (x370).

A. Type IV culture. Note the presence of cells of different shapes and vacuolated and granulated cells.

B. Type IV culture.

C. Culture from T/T embryos. Note the absence of cellular contact among cells.



(Figure 22C). The cells were scattered around the primary explant and rarely contacted each other. The general features of fibroblast-like or epithelial-like cells observed in culture types I-IV described above rarely appeared in the few (7) T/T cultures examined.

Several primary cultures were subcultured for the production of cell lines and to keep a viable representative example of the original cell outgrowth from the primary explant. The cells were passed either before reaching confluency, or shortly thereafter. Usually the cell type found as the predominant form in the primary culture was established in the subculture. Once a cell line appeared to be established, subculturing could be performed every four to five days.

Histocompatibility Typing

Homozygous lethal t^{w1} embryos cannot be distinguished from normal littermates prior to day 11 pc of development since there is no reduction in CR length and/or abnormalities in gross structures. If mutant embryos can only be determined after histological examinations, then biochemical and other histochemical studies cannot be performed simultaneously. In an attempt to distinguish normal and abnormal embryos without sacrificing all of the embryonic tissues, fibroblast-like cells grown from in vitro explant cultures described above were assayed for the presence of the H-2 antigens. The H-2 haplotype of fibroblasts is representative of the genotype of an embryo. Production of mouse alloantisera to H-2^{tw1}, determination of the specificity of mouse alloantisera to H-2 antigens, and

examination of different assays for detection of H-2 antigens using mouse alloantisera were performed.

Anti-H-2^{tw1} was produced by injection of normal (+/+) C3H.B10 male mice with spleen cells isolated from +/t^{w1} mice (+,H-2^{b}/t^{w1}, H-2^{tw1}). An antibody response to H-2^{tw1} as well as other antigens not shared by the two strains was elicited. Blood samples from twelve mice were pooled to form three samples of H-2^{tw1}: H-2^{tw1}-1, H-2^{tw1}-2, and H-2^{tw1}-3. Specificity and titer of each antiserum were determined in a complement-mediated lymphocytotoxicity assay using spleen cells isolated from C3H.B10 +/+ and C3H.B10-t^{w1} mice (Amos, et al., 1969). The results, shown in Table 13, indicated that all three antisera were specific for the H-2^{tw1} haplotype, even though the antibody titer (reciprocal titer of 10) was relatively low. Antiserum to H-2.33, one of the private antigens for the H-2^{b} haplotype, was also tested. As expected, +/+ cells were lysed only by the anti-H-2.33 antiserum and +/t^{w1} cells were lysed by both anti-H-2.33 and anti-H-2^{tw1}. The three antisera (H-2^{tw1}-1, H-2^{tw1}-2, and H-2^{tw1}-3) were considered to be equivalent and used interchangeably.

The H-2 haplotypes of four strains of mice, C3H.B10 (+/+), C3H.B10-t^{w1} (+/t^{w1}), C3H.B10-Tt^{w1} (T/t^{w1}), and C3H (+/+) were tested with mouse alloanti-H-2.2, a private H-2^{b} antigen that had been previously tested, and mouse alloanti-H-2^{tw1} in a complement-mediated lymphocytotoxicity assay. The known H-2 haplotypes for each donor strain and results of the assay are summarized in Table 14.

The results indicated that C3H.B10 (+/+) cells were lysed only by anti-H-2.2. The +/t^{w1} cells were lysed by both anti-H-2.2 and anti-H-2^{tw1}. The T/t^{w1} cells were lysed by anti-H-2^{tw1} but not by

Table 13

Specificity of Three Samples of Anti-H-2^{tw1} Using $+/+$ and $+/t^{w1}$ Spleen Cells

Antiserum	% Lysis	
	$+/+$	$+/t^{w1}$
Anti-H-2^{tw1}-1		
1/10 dilution	6	46
1/20 dilution	6	32
Anti-H-2^{tw1}-2		
1/10 dilution	6	31
1/20 dilution	6	27
Anti-H-2^{tw1}-3		
1/10 dilution	6	44
1/20 dilution	6	32
Anti-H-2.33		
1/80 dilution	76	53

Table 14

Specificity of Anti-H-2.2 and Anti-H-2^{tw1} Using Spleen Cells Isolated From Four Strains of Mice

Donor Spleen Cells	Known H-2 Type		% Lysis with	
	H-2^b	H-2^{tw1}	Anti-H-2.2[1]	Anti-H-2^{tw1}[2]
C3H.B10 ($+/+$)	+	-	20.3	0
C3H.B10 ($+/t^{w1}$)[3]	+	+	21.1	31.9
C3H.B10 (T/t^{w1})[4]	?[5]	+	0	46.2
C3H ($+/+$)	-	-	0	12.2

[1]Antisera diluted 1/50.

[2]Antisera diluted 1/10.

[3]Cells from mice of the C3H.B10-t^{w1} strain.

[4]Cells from mice of the C3H.B10-Tt^{w1} strain.

[5]H-2 haplotype associated with the T mutation is unknown.

anti-H-2.2, indicating that the H-2.2 antigens associated with T did not include antigen H-2.2. The C3H +/+ cells were not lysed by anti-H-2.2 and cross-reacted with anti-H-2^{tw1}. Since the antisera were specific for a given H-2 haplotype, a complement-mediated cytotoxicity assay was used to determine the H-2 types (and genotypes) of fibroblast-like cells grown from embryonic explants in vitro.

Prior to the determination of H-2 types on fibroblast-like cells in a complement-mediated fibroblast cytotoxicity assay, the ability of fibroblasts to incorporate ^{125}I-iododeoxyuridine (^{125}IUdR) and the cell concentration at which maximum incorporation would occur were examined. ^{125}IUdR, a thymidine analog, is inserted into DNA during DNA biosynthesis prior to mitotic cell division. A constant amount of ^{125}IUdR was added to varying concentrations of normal (+/+) cells (1×10^2 cells/well to 2×10^4 cells/well). The results, shown in Figure 23, indicated that: 1) cells were actively dividing and would incorporate ^{125}IUdR; 2) incorporation of label was dependent upon cell concentration; and 3) maximum ^{125}IUdR incorporation occurred at a cell concentration of 1×10^4 cells/well.

To determine the sensitivity of two different assay protocols and to test for the presence of H-2 antigens of fibroblast-like cells from a primary explant derived from a 10 day pc +/+ embryo subcultured twice in vitro, two different complement-mediated fibroblast cytotoxicity assays utilizing ^{125}IUdR were examined. In assay 1, ^{125}IUdR was incorporated into DNA of actively dividing cells prior to antibody and complement exposure. In assay 2, complement and antisera were added prior to incorporation of ^{125}IUdR into DNA of the remaining viable cells.

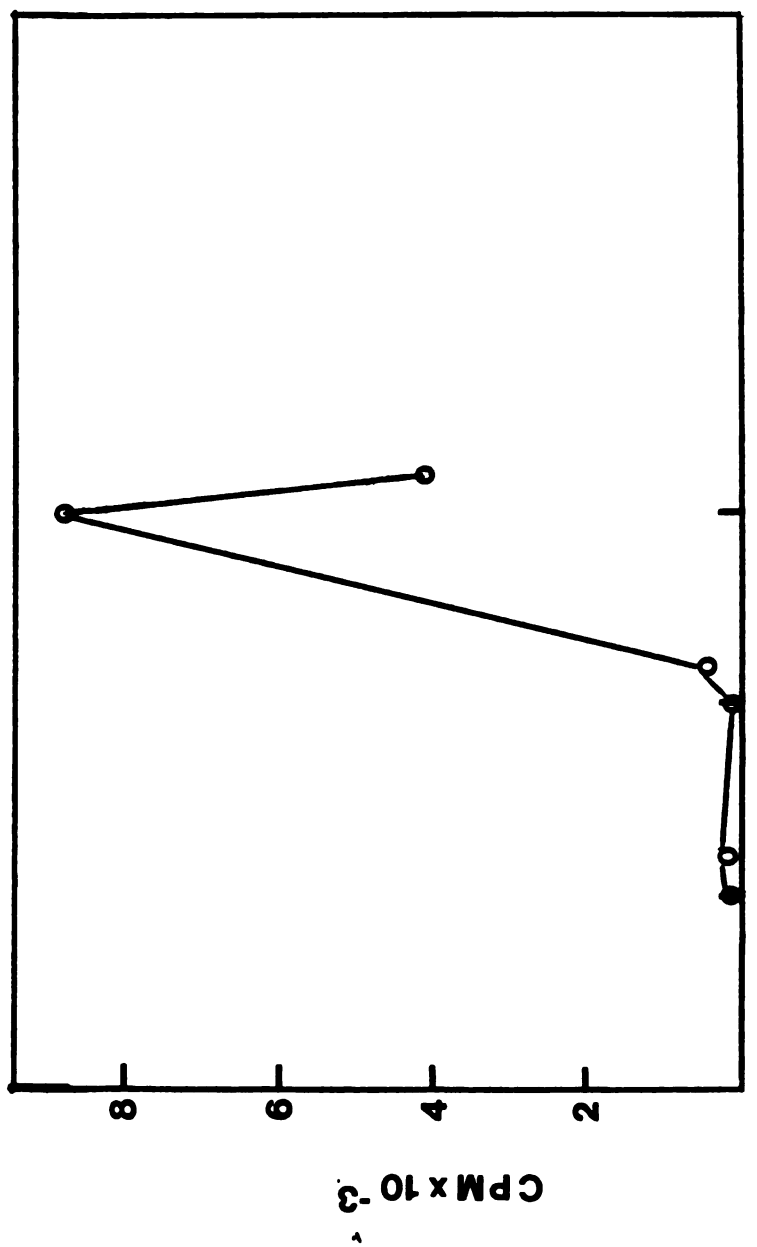


Figure 23. Incorporation of ^{125}IUdR into actively dividing fibroblast cells.

The antisera used in assay 1 were specific for two private antigens of the H-2^b haplotype, H-2.2 and H-2.33. The data, shown in Table 15, indicated that both antigens were present on fibroblast-like cells. More cells were lysed with anti-H-2.2 than with anti-H-2.33. This difference in the degree of cytotoxicity may result from differential expression of H-2.2 and H-2.33 on fibroblast-like cells, differential exposure of each antigen on the cell surface, and/or differential antibody titers to the antigens. Temporal differences of H-2 expression have been noted on embryonic tissues and may explain the variation of expression of the antigens (Ostrand-Rosenberg, et al., 1977; Kirkwood and Billington, 1981). Preferential loss of antigen expression has also been observed on cells grown in vitro and subcultured several times (Ostrand-Rosenberg, et al., 1977). Regardless of the reasons for differences using the two antisera, both were expressed on the cell surface.

Only anti-H-2.2 was used in assay 2 to determine if the method was more sensitive than assay 1 to detect the presence of H-2 antigens on the cell surface. The degree of cytotoxicity was approximately twice that observed in assay 1. The apparent enhanced sensitivity

Table 15

Determination of H-2 Antigens of Normal (+/+) C3H.B10 Fibroblasts Using Two Different Assays

Anti-Serum	% Lysis in Assay 1	% Lysis in Assay 2
Anti-H-2.2, diluted 1/10	28.7	54.3
Anti-H-2.33, diluted 1/10	9.3	NT[1]

[1]NT - not tested.

of assay 2 may result from exposure of cells to complement and antisera prior to ^{125}IUdR incorporation into DNA of dividing cells. If cells react to complement and antisera, few viable cells remain that will become labeled prior to mitotic division. Since label is incorporated into viable cells prior to antibody and complement treatment in assay 1, cell overgrowth may inhibit complement and antibody complex formation on most of the cell surfaces and yield a low cytotoxic value. On the basis of this information, assay 2 was used in subsequent experiments.

The antibody titer of anti-H-2.2 used in assay 2 was determined with normal (+/+) fibroblast-like cells subcultured three times *in vitro*. The data, shown in Figure 24, indicated that maximum cell lysis occurred at an antibody reciprocal titer of 80. The degree of cell lysis decreased in either apparent antibody or antigen excess. At all antibody dilutions the degree of lysis exceeded 20%.

The results described above for one normal (+/+) cell line were encouraging because the cell line did express H-2 antigens that could be detected via complement-mediated cytotoxicity measuring ^{125}IUdR incorporation. Subsequently two cultures whose haplotypes were unknown as well as the cell line previously tested were assayed for H-2 antigens. Unfortunately, the detection of H-2 antigens on normal (+/+) cells became erratic; the results described above could not be reproduced. Results from assays on the two unknown lines were ambiguous; consequently, determination of H-2 types and genotypes could not be made.

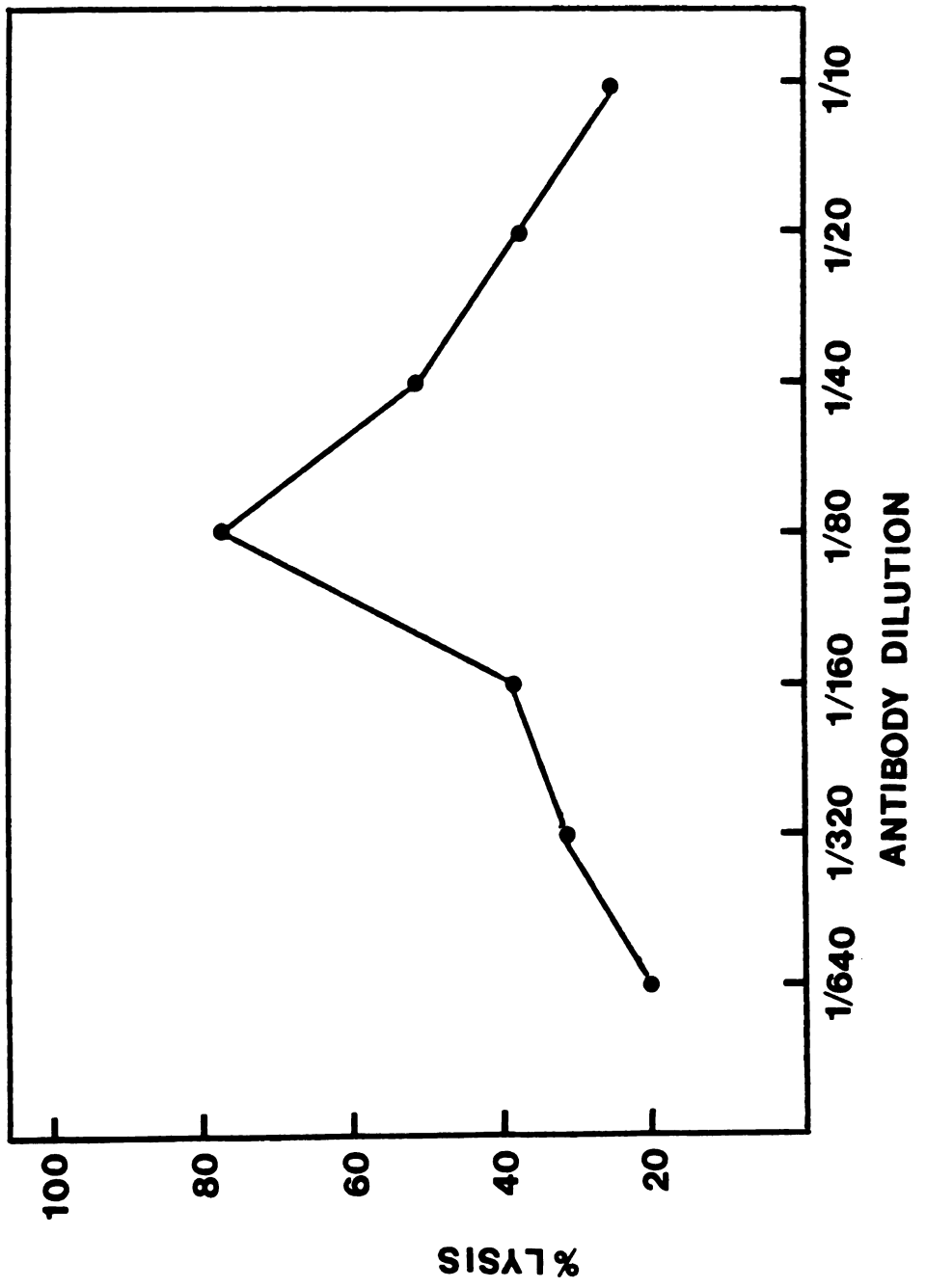


Figure 24. Determination of antibody titer of anti-H-2.2

DISCUSSION

In the original papers describing the effects of four *t* mutations from the t^{w1} complementation group (t^{w1}, t^{w3}, t^{w12}, and t^{w20}) on embryonic development, the mutations were considered to be allelic and identical with respect to the time and expression of gene action (Bennett, *et al.*, 1959a,b). Consequently, crosses were made at random to produce embryos of homozygous (t^x/t^x) or compound heterozygous (t^x/t^y, where x and y represent two non-complementing alleles within the t^{w1} group) genotypes. Information obtained from histological examinations of embryos at days 9, 10 to 11, 12, 13, 14, and 15 to 20 of gestation was thought to represent the morphological effects of the *t* mutations in the t^{w1} complementation group as a whole, but the pooled data obliterated any subtle differences that might have been observed for each homozygous class, i.e., t^{w1}/t^{w1}, t^{w3}/t^{w3}, t^{w12}/t^{w12}, and t^{w20}/t^{w20}. Other studies on *t* mutations within the same complementation group have demonstrated that *t* "alleles" do not have the same effects upon embryonic development (Hillman and Hillman, 1975; Sherman and Wudl, 1977). Thus, the value of information obtained from pooled observations on homozygous and compound heterozygous embryos is questionable for several reasons.

First, the mutations were derived from wild populations of mice located in different geographical locales (t^{w1} - New York: t^{w3} - Connecticut; t^{w12} - California) and an exception in a balanced laboratory

stock carrying the mutation $\underline{t}^{w15}$ (an allele of the $\underline{t}^{w5}$ complementation group; Bennett, et al., 1959a). The mutants were crossed to an inbred Brachyury laboratory stock for 2 to 3 generations and subsequently inbred for 3 to 5 generations. Although each mutant had been crossed to a common laboratory stock and inbred several generations, the genetic background of each mutant was still heterogeneous, especially for linked genes, and not comparable to one another. For example, the genetic background of $\underline{T}$-$\underline{tf}/\underline{t}^{w1}$-$\underline{+}$ would have been different from the genetic background of $\underline{T}$-$\underline{tf}/\underline{t}^{w20}$-$\underline{+}$. The presence of different modifier genes contributed by the diverse genetic background in each mutant could alter the gene action of the mutation so that phenotypic expression of each might appear to be similar, but the cellular processes affected would be different.

Second, in matings between mice heterozygous for $\underline{t}$ ($\underline{T}$-$\underline{tf}/\underline{t}^{x}$-$\underline{+}$ x $\underline{T}$-$\underline{tf}/\underline{t}^{x}$-$\underline{+}$), the number of dead embryos (presumably representing the homozygous mutant genotypes) observed from days 10 to 12 pc were drastically different for each non-complementing $\underline{t}^{w1}$ mutation. In the $\underline{t}^{w1}$ complementation group, the percent moles observed ranged from 47% for $\underline{t}^{w1}/\underline{t}^{w1}$ to 96% for $\underline{t}^{w20}/\underline{t}^{w20}$ (Bennett, et al., 1959a). The variation in numbers of dead embryos observed for each of the four homozygous genotypes may have resulted from differences in content of genetic modifiers associated with each mutation or may have been a reliable index for expressing differences in gene action for each mutation. For example, studies of two alleles in the $\underline{t}^{12}$ complementation group, $\underline{t}^{12}$ and $\underline{t}^{w32}$, have shown that $\underline{t}^{12}/\underline{t}^{12}$ and $\underline{t}^{w32}/\underline{t}^{w32}$ embryos differ morphologically from each other (Hillman and Hillman, 1975). Although both mutations arrest development at the morula

stage, $\underline{t}^{w32}$ alters cells in the early morula stage and $\underline{t}^{12}$ alters cells at the late morula stage. Since the mutations were not on isogenic backgrounds, it is difficult to ascertain if the variation of expression is due to differences in genetic background or to intrinsic differences inherent to each of the two $\underline{t}$ mutations.

Third, results from genetic complementation tests involving alleles of the $\underline{t}^{w1}$ complementation group with the $\underline{t}^{0}$ mutation ($\underline{t}^{0}$ complementation group) show that the number of viable offspring varies with each allele in the $\underline{t}^{w1}$ complementation group (Bennett and Dunn, 1964). The relative viability of offspring heterozygous for $\underline{t}^{0}$ and the $\underline{t}^{w1}$ mutations were: $\underline{t}^{w1}$, 85%, $\underline{t}^{w3}$, 90% (strongly complements); $\underline{t}^{w12}$, 59%; and $\underline{t}^{w20}$, 1.2% (weakly complements). Similar observations have been made in genetic complementation tests involving other $\underline{t}$ mutations (Lyon, et al., 1979b). The differences in magnitude of degree of viability is not thought to result from variations in genetic background (Bennett and Dunn, 1964). Chromosomal structural differences among the $\underline{t}^{w1}$ mutations have been proposed to explain the variation of viability observed in complementation tests involving the $\underline{t}^{0}$ mutation and $\underline{t}$ mutations in the $\underline{t}^{w1}$ complementation group (Bennett and Dunn, 1964). Existence of such chromosomal structural differences would imply that members of the $\underline{t}^{w1}$ complementation group are not truly allelic.

The variation of degrees in complementation of $\underline{t}^{w1}$ mutations with $\underline{t}^{0}$ provides a strong argument for differences of gene action in each $\underline{t}^{w1}$ mutation. Each mutation may alter different cellular processes and interactions, even though the end result of the gene action encoded by these mutations is manifested as effects on embryonic

central nervous system development acting at similar time periods. Until the genes are placed on the same genetic background and homozygous mutant embryos are re-examined to determine the mutant effect, the mutations and morphological anomalies caused by the mutations should be considered as separate entities altering similar stages of embryonic development.

Since little information was readily available on the gene action of one mutation, $\underline{t}^{w1}$, as expressed in homozygous embryos at 9 to 15 days of gestation, the current study was undertaken. Significant differences were noted in descriptions of embryos homozygous for $\underline{t}^{w1}$ given in this thesis and those made by Bennett, et al. (1959b). Whether the observed differences result from the various genetic backgrounds in which the $\underline{t}^{w1}$ mutation resides, the methods from which data was collected (pooled homozygous and compound heterozygous embryos versus single $\underline{t}^{w1}/\underline{t}^{w1}$ embryo samples), or the influence of the Brachyury (T) mutation upon the $\underline{t}^{w1}$ mutation (homozygous embryos collected from $\underline{T}$-$\underline{tf}/\underline{t}^{w1}$-$\underline{+}$ x $\underline{T}$-$\underline{tf}/\underline{t}^{w1}$-$\underline{+}$ matings versus $\underline{+}/\underline{t}^{w1}$ x $\underline{+}/\underline{t}^{w1}$ matings) cannot presently be evaluated. (In the ensuing discussion, references made to Bennett refer to the work performed by Bennett, et al., 1959b.)

Differences in Embryonic Observations

A summary of the number of embryos examined at different stages of development and the number of homozygous $\underline{t}^{w1}$ embryos identified after histological examination by light microscopy is given in Table 16. The number of homozygous $\underline{t}^{w1}$ embryos expected were calculated by using a male segregation distortion factor of 0.9. Since 90% $\underline{t}^{w1}$

Table 16

Number of Expected and Observed Embryos Homozygous for $\underline{t}^{w1}$

Expected Age, Days	Number of Implants	Number of Aborts	Number of $\underline{t}^{w1}/\underline{t}^{w1}$	Total Number of Abnormal Embryos	Other Genotypes	Number of Expected $\underline{t}^{w1}/\underline{t}^{w1}$	χ^2[4]
9	24 (2)[1]	5	2	7[2]	17	10.8[3]	2.43
9½	16	2	0	2	14	17.2	6.82
10	37 (3)	3	4	7	30	16.7	10.17
10½	28 (1)	6	5	11	17	12.6	0.37
11	20	3	8	11	9	9.0	0.80
12	28	3	10	13	15	12.6	0.21
13	15	5	3	8	7	6.7	0.42
15	10	2	5	7	3	4.5	2.53

[1]Number of embryos lost in the embedding procedure were omitted from the total number of implants; number lost given in parentheses.

[2]Sum of the number of aborts and number of $\underline{t}^{w1}$ homozygotes.

[3]Based on segregation distortion factor of 0.9.

[4]$\chi^2_{0.05\ [1]} = 3.84$

bearing sperm and 10% +-bearing sperm will fertilize ova, 45% of the embryos in a litter are expected to be homozygous for t^{w1}. From days 9 to 10½ pc, the number of observed homozygous embryos were deficient. From days 11 to 15 pc, however, the number of observed and expected homozygous t^{w1} embryos coincided, indicating that embryos homozygous for t^{w1} were identified at later embryonic stages of development. In contrast, Bennett was able to identify 74% of the expected number of abnormal embryos at day 9 pc and 100% of the affected embryos subsequent to day 10 pc.

At day 9 pc, the characteristics observed by Bennett in abnormal embryos included: 1) reduction in size to 1/2 - 3/4 that of normal littermates; 2) presence of microcephaly; and 3) pycnotic cells located in the mantle layer of the ventral portion of the hindbrain, midbrain, and anterior regions of the spinal cord. In the two embryos identified after histological examination in this thesis, the characteristics observed for each were that: 1) the size of the embryo was similar to a "normal" littermate; 2) microcephaly was not evident; and 3) pycnotic cells were confined to the mantle layer of the ventral region of the rostral rhombencephalon. The embryos described by Bennett may possibly represent the embryos homozygous for t^{w20} or compound heterozygous for t^{w20} and $t^{w1,3, \text{ or } 12}$. Approximately 95% of the embryos homozygous for t^{w20} died early and were represented by moles (Bennett, et al., 1959a); only 47% of the embryos homozygous for t^{w1} died at the same embryonic age.

At days 10 to 11 pc, the characteristics observed in abnormal embryos by Bennett were: 1) retarded (younger developmental age than normal littermates) growth and reduced size; 2) presence of

pycnotic cells extending from the hindbrain rostrally in the brain and caudally in the spinal cord; and 3) presence of pycnoses with widespread necrosis and loss of structural integrity in the ventral half of the neural tube. The features described by Bennett were not observed in abnormal embryos described in this thesis until day 11 pc. Few homozygous t^{w1} embryos were identified at days 10 or 10½ pc. Of those observed at day 10 pc, pycnotic cells were confined to the mantle layer of the ventral portion of the mesencephalon and rhombencephalon. The focus of degeneration corresponded to that observed at day 9 pc. The decreased width of the neural tube in affected regions may correspond to a decrease in the number of neuroepithelial cells undergoing mitotic division. Of those observed at day 10½ pc, the degree of degeneration did not seem as severe, but the width of the neural tube was decreased and the marginal layer was not prevalent. At day 11 pc, however, abnormal embryos were recognized and the characteristics observed by Bennett, except number 3, were also noted. The loss of structural integrity of the ventral half of the neural tube was rarely present at this stage of development.

At day 12 pc Bennett observed that the degree of necrosis and structural degeneration was never as severe as that observed at day 11 pc; in fact, considerable areas were almost free of pycnosis but deficient in the number of typical neural cells. In embryos described herein, the degree of pycnosis and degeneration in the neural tube was severe and the neural tube was beginning to lose its structural integrity. "Recovery" of the neural tube in abnormal embryos was not present.

At day 13 pc, general external features described by Bennett included: edema, microcephaly, and enlarged hearts. Histologically, few pycnotic cells were observed in the brain and spinal cord; spinal ganglia were normal, and vertebral cartilage formation had begun. In abnormal embryos described in this thesis, external features of abnormal embryos included retarded size and occasionally, microcephaly and enlarged hearts. Histologically, the neural tube was disorganized and had lost structural integrity. Although spinal ganglia and ossification centers were present, non-neural as well as neural structures were degenerating. Structures in older embryos (day 15 pc) were almost impossible to identify since tissue was being resorbed.

In summary, from observations given herein, the mutation t^{w1} acts prior to day 11 pc, but its effects are not overtly noticeable until day 11 pc. The degree of pycnosis and degeneration have been summarized for various embryonic stages of development in Figure 25. The initial action of the gene appears to be on neuroepithelial cells or neuroblasts found in the mantle layer of the neural tube. The focus is in the ventral portion of the neural tube at the rostral border of the rhombencephalon (Figure 25A). The presence of pycnotic cells follows a specific pattern of development, spreading rostrally and caudally in the ventral portion of the neural tube and caudally in the dorsal portion of the neural tube (Figure 25A-E). Pycnotic cells were never observed in the most basal portion of the neural tube in the region above the notochord.

The biochemical basis of this defect is presently unknown but may correlate with the inability of neuroepithelial cells to divide and/or neuroblast cells to migrate within the neural tube. Both

Figure 25. Reconstructed composite view of mouse embryos from serially sectioned material. The neural tube is depicted by a heavy black line. Dotted lines represent the developing eye and otic vesicle. Pycnotic cells found in sections of the developing neural tube are shown by dots (x12).

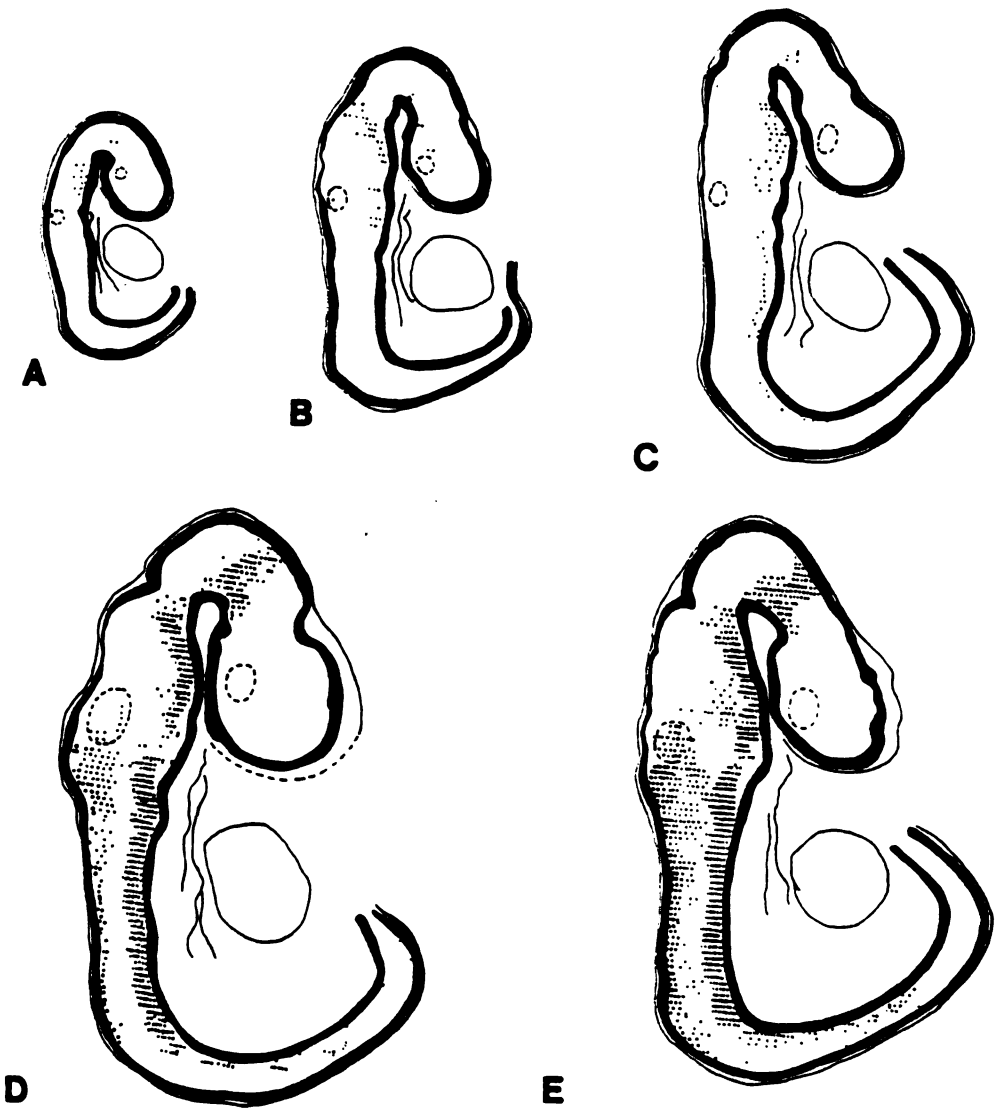
A. Abnormal day 9 embryo showing restriction of pycnotic cells to the rostral portion of the rhombencephalon.

B. Abnormal day 9½ embryo showing the increased number of pycnotic cells in the ventral portion of the neural tube.

C. Abnormal day 10 embryo showing the continued increase in numbers of pycnotic cells and confinement of pycnoses to the ventral neural tube.

D. Abnormal day 10½ embryo showing pycnotic cells in dorsal and ventral regions of the neural tube.

E. Abnormal day 10 embryo from litter dissected at 11 days. Non-neural as well as neural structures are degenerating (only pycnotic cells in the nueral tube are shown).


A
B
C
D
E

mitotic and migratory processes may rely upon the synthesis of specific classes of glycosaminoglycans, a group of highly branched carbohydrates composed of repeating dissaccharide units and usually linked to a protein core (proteoglycans; Margolis and Margolis, 1979; Lennarz, 1980). Glycosaminoglycans (GAG) are important components of the extracellular matrix (ECM) and may play a role in cellular migration (Toole, 1972, 1973; Pratt, et al., 1975), cellular differentiation (Shur and Roth, 1973), and cellular morphology (Cohn, et al., 1977; Solursh and Morriss, 1977). A correlation exists in synthesis of GAG by the neural tube and notochord, accumulation of GAG into the basal laminae and ECM, and changes in neural tube and mesenchymal cell morphology (Hay and Meier, 1974; Morriss and Solursh, 1978). If abnormal synthesis of GAG occurs in embryos homozygous for $\underline{t}^{w1}$, then neural tube cell morphology may be altered and cause the secondary defects observed in abnormal embryos.

One class of enzymes, glycosyltransferases, are required for GAG synthesis (Margolis and Margolis, 1979) and may be involved in cell recognition and migration (Shur and Roth, 1973). One model has suggested that cells migrate over carbohydrate chains (Shur, 1977a). Studies supporting this hypothesis have been performed on different aged chick embryos with various glycosyltransferases (Shur, 1977a,b; Roth, 1979). Of particular importance is the presence of galactosyltransferases in neural tube regions of developing chick embryos (Shur, 1977a), and the teratogenic effects of excess UDP-galactose and UDP-N-acetylglucosamine during gastrulation (Roth, 1979). Since abnormalities in glycosyltransferase activities have been reported in the t mutants (Shur and Bennett, 1979; Shur, et al.,

1979), it is possible that proteins involved in synthesis of carbohydrate containing macromolecules may represent a primary target of the mutant gene $\underline{t}^{w1}$ during embryonic development.

In Vitro Growth of Primary Explants from t^{w1}/t^{w1} Embryos

The $\underline{t}$ mutations can be divided into two groups on the basis of time of gene expression: those mutants acting early during embryonic development and causing death prior to organogenesis and those mutants acting shortly after the beginning of organogenesis and causing death prior to birth (Fujimoto and Yanagisawa, 1979). Studies have shown that primary explants of embryonic tissue from early acting $\underline{t}$ mutations $\underline{t}^{6}$, $\underline{t}^{w5}$, and $\underline{t}^{12}$ do not grow in culture (Wudl and Sherman, 1976; Wudl, et al., 1977), while primary explants from $\underline{T}/\underline{T}$ embryos, a "late" acting $\underline{T}$ mutation, do grow (Ephrussi, 1935; Yanagisawa and Fujimoto, 1977; Yanagisawa, et al., 1980). $\underline{t}^{w1}$ is classified as a late acting mutation, and primary explants from mouse embryos of all three genotypes ($\underline{+}/\underline{+}$, $\underline{+}/\underline{t}^{w1}$, and $\underline{t}^{w1}/\underline{t}^{w1}$) were cultured in vitro. The data given in this thesis represents the first report on in vitro culture of a "late" acting recessive $\underline{t}$ mutation.

Since the explants originated from embryos in matings between heterozygous ($\underline{+}/\underline{t}^{w1}$) males and females, the number of explants from embryos homozygous for $\underline{t}^{w1}$ was expected to be greater than 25%. Segregation distortion, a phenomenon that occurs in heterozygous males, alters the expected genotypic ratios from 25% $\underline{t}^{w1}/\underline{t}^{w1}$: 50% $\underline{+}/\underline{t}^{w1}$: 25% $\underline{+}/\underline{+}$ to 45% $\underline{t}^{w1}/\underline{t}^{w1}$: 50% $\underline{+}/\underline{t}^{w1}$: 5% $\underline{+}/\underline{+}$ because 90% of the sperm carrying $\underline{t}^{w1}$ will fertilize ova (see Figure 2). Of the 190 primary explants studied, 85 were expected to be $\underline{t}^{w1}/\underline{t}^{w1}$. Since homozygous $\underline{t}^{w1}$

embryos die by day 12 pc in utero, tissue excised from these embryos might also die in vitro. If explants from t^{w1}/t^{w1} embryos do die in vitro, then only 55% of the cultures would be expected to succeed. The number of successful cultures (94%) far exceeded the number predicted (55%). Hence, homozygous t^{w1} explants have been shown to survive in vitro long past the time that they would normally die in vivo.

The length of time that cells survived in vitro was important. If explants and cells derived from explants of t^{w1}/t^{w1} embryos did not live past their in utero equivalent gestational age of death, usually 12 days pc, then the results would support the conclusion that all cells of t^{w1}/t^{w1} embryos were "programmed" for cell death, an observation that has been made to t^{6}/t^{6} and t^{w5}/t^{w5} embryos (Wudl and Sherman, 1978; Hogan, et al., 1980). However, 94% of the explants survived for at least three weeks. Many lasted for three to four months, when the study ended.

Primary explants from embryos which had been identified as t^{w1}/t^{w1} on the basis of gross and histological examinations of embryos were as viable as primary explants from normal ($+/+$, $+/t^{w1}$) littermates. Provided that the embryo from which the explants were taken was not dead (as defined by the presence of a beating embryonic heart), the mutant explants had similar cell morphologies, survived in vitro for extended periods of time, and were successfully subcultured. The significance of these studies is that the gene does not appear to affect all cells of the mutant embryo; otherwise in vitro cell growth would not be possible. The gene appears to affect specific cells

within the developing neural tube and would, therefore, appear to be an intrinsic cell specific lethal.

Determination of Embryonic Genotypes Based on H-2 Typing

One property of the t mutations that can be advantageous is cross-over suppression. Suppression of recombination extends from the T locus to the distal end of the H-2 locus and includes the t^{w1} mutation (Klein and Hammerberg, 1977). As a result, the t^{w1} mutation is tightly linked to the H-2 locus (1% recombination), and the two loci are inherited as a unit. Since the H-2 haplotype, $H\text{-}2^{tw1}$, associated with the t^{w1} mutation is distinctly different from the H-2 haplotype, $H\text{-}2^{b}$, associated with the normal allele (Klein, et al., 1978; Klein, 1975), the different H-2 haplotypes expressed in cell lines would correlate with the genotypes of the embryos from which the cultured tissue was taken. Three possible embryonic genotypes derived from $+/t^{w1}$ x $+/t^{w1}$ matings and their H-2 haplotypes are: $+$, $H\text{-}2^{b}/+$, $H\text{-}2^{b}$; $+$, $H\text{-}2^{b}/t^{w1}$, $H\text{-}2^{tw1}$; and t^{w1}, $H\text{-}2^{tw1}/t^{w1}$, $H\text{-}2^{tw1}$. Thus experiments were performed in an attempt to identify haplotypes of fibroblasts growing in tissue culture and derived from primary embryonic explants. Determination of haplotypes would be representative of the genotypes of the embryos.

Initial results on the determination of the $H\text{-}2^{b}$ haplotype associated with $+/+$ fibroblasts implied that the genotypes of embryos could be ascertained from H-2 types of fibroblasts derived from embryonic explants. Results from subsequent studies were, however, erratic and might be explained by altered expression of H-2 antigens during

culturing and/or low concentrations of the H-2 antigens on the cell surface.

Several reports have indicated that the detection of H-2 antigen expression is dependent upon the age of the culture, the number of times that cells were subcultured, the specific haplotype under examination, the tissue source of cultured cells, and the antiserum batch employed (Klein, 1965; Edidin, 1976; Holtkamp, et al., 1979; Ostrand-Rosenberg, et al., 1980; Kirkwood and Billington, 1981). Utilization of a more sensitive method, such as combined absorption and cytotoxic assays, may alleviate problems of sensitivity and reproducability (Klein, 1975). In addition, the effects of culturing cells derived from primary embryonic explants on H-2 expression should be investigated to determine if and how expression of H-2 antigens on cultured cells grown in vitro change with time and subculturing.

SUMMARY AND PROSPECTIVE RESEARCH

Experiments and observations described herein on mouse mutant $\underline{t}^{w1}$ indicate that the action of the gene affects only some cell types within the neural tube at specific stages of development. Embryonic tissue derived from homozygous embryos can be grown *in vitro* and will survive beyond the lethal period observed for homozygous embryos *in utero*. Although the primary action of the gene is unknown, the growth and maintenance of the neural tube in homozygous $\underline{t}^{w1}$ embryos appears to be inhibited by the $\underline{t}^{w1}$ mutation. One possible explanation is that the $\underline{t}^{w1}$ mutation affects the activity of a protein required for the synthesis of carbohydrate containing macromolecules, and thereby alters the synthesis, composition, and distribution of glycosaminoglycans in the neural tube and surrounding regions.

The hypothesis that the neural tube growth and maintenance are inhibited by the $\underline{t}^{w1}$ mutation will be examined in future studies. Mutant embryos will be analyzed with respect to: 1) developmental interrelationships between the processes of proliferation and degeneration in the developing CNS, 2) the nature of prenatal necrosis and its cellular consequences at the fine structural level, and 3) the status of GAG within the basal laminae of the neural tube and extracellular matrix of nearby mesenchymal cells.

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