INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road. Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600
Molecular dissection of regions required for postimplantation development of the mouse using radiation-induced deletions

Sharan, Shyam Kishore, Ph.D.

Case Western Reserve University, 1994
MOLECULAR DISSECTION OF REGIONS REQUIRED FOR
POSTIMPLANTATION DEVELOPMENT OF THE MOUSE USING
RADIATION-INDUCED DELETIONS

by

SHYAM KISHORE SHARAN

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisor: Dr. Terry Magnuson

Department of Genetics
CASE WESTERN RESERVE UNIVERSITY
January, 1994
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

SHYAM KISHORE SHARAN

candidate for the PH.D

degree.*

(signed)

(chair)

David Samuel

date 11-23-93

*We also certify that written approval has been obtained for any proprietary material contained therein.
I grant to Case Western Reserve University the right to use this work, irrespective of any copyright, for the University's own purposes without cost to the University or to its students, agents and employees. I further agree that the University may reproduce and provide single copies of the work, in any format other than in or from microforms, to the public for the cost of reproduction.
MOLECULAR DISSECTION OF REGIONS REQUIRED FOR POSTIMPLANTATION DEVELOPMENT OF THE MOUSE USING RADIATION-INDUCED DELETIONS

Abstract
by
SHYAM KISHORE SHARAN

Gastrulation, the process of establishment of the three germ layers as well as the anterior-posterior axis of embryos, is one of the key events during early postimplantation development. To understand the molecular basis of this process it is essential to identify and characterize the genes that are involved. Mutations affecting the process of gastrulation are ideal to identify such genes. Several radiation-induced deletions that remove the albino coat color locus and varying amounts of the surrounding region of chromosome 7 have been generated that result in early postimplantation embryonic lethality. Complementation analysis of these deletions has resulted in identification of at least three distinct regions that are required during gastrulation. Molecular dissection of these regions will help to identify gene(s) that are involved in this process.

The work described here is focused on the molecular characterization of one of these regions, the extraembryonic ectoderm development (exed) region. Molecular access to the region
was gained by cloning the breakpoint-fusion-fragment of the appropriate deletions using DNA markers from the proximal end. The breakpoint defining the proximal end of the *exed* region was cloned by using a localized repeat sequence probe. Breakpoint-fusion-fragment of the deletion that defines the other end was cloned by walking to the proximal breakpoint using a probe obtained from a jumping library. DNA probes isolated from these breakpoints were then used to estimate the size of the region. Search for transcription units resulted in isolation of a probe that detects a transcript in the adult brain. Further characterization of the transcript and its expression pattern in the embryos will provide information to understand its role in development.

In addition, a technique for genetic analysis of single, early postimplantation embryos by Southern blotting is described. It involves embedding individual embryos in agarose and deproteinizing them *in situ*. This procedure was applied to genotype embryos homozygous for a deletion that lacks embryonic ectoderm development (*eed*) region. Like the *exed* region, this region is also required during gastrulation. Further, the technique was used to redefine the distal limit of the *eed* region by mapping the distal breakpoint of this deletion relative to the other breakpoints in the region.
DEDICATED TO MY DEAR PARENTS
ACKNOWLEDGEMENTS

I wish to express my feelings of gratitude to everyone who helped me in my work. I would first like to thank my Guru, my mentor, and my thesis advisor, Dr. Terry Magnuson for his guidance and never ending support. His dedication towards understanding the fundamentals of mammalian development has been the greatest inspiration to me. Discussing about work in his office was as much interesting as was playing darts with him in Toronto Bars. I am indebted to my two labmates and teachers, Lee Niswander and Bernadette Holdener, from whom I learnt various lab techniques in the first few years of my graduate studies. I wish to thank them for tolerating me and my mistakes. I wish to thank Bernadette for the pumpkin pie that helped me pass my qualifying examination. I wish to sincerely thank David Threadgill, "Mr. Trouble Shooter" who provided solutions to all my problems. The help and advice I received from him will always be remembered. I am thankful to Della for working with me and helping me in my race against time to achieve my goals. I know it would not have been possible without her help. Thanks for the desserts too! I am grateful to my other labmates, Tracy for taking care of me and my mice; Henry for proving beyond doubt that every cloud has a silver lining; Joe for the long but interesting scientific and political discussions we had; Cindy for preparing me not only for my final defense but also for the Houston gangs; Jim for introducing me to 'Craw'; Christen for her...
smile and Armin for showing interest in my jokes that were not appreciated by anyone else.

I am also grateful to Ann, Norrine and Jean for always being there to help me tackle my personal problems. I wish to express my gratitude to the members of my thesis committee, Drs. Helen Salz, David Samols, David Setzer, Patricia Hunt, and John Schimenti for their advice and showing keen interest in my project.

I wish to thank all my friends including Barbara Kumerle for her Quarters and candy bars; Ann Riedl for inviting me to her fun parties; Vandana for her 'Gulab Jamuns' and frequent dinner invitations; Juzar for letting me almost drown during white-water rafting; Ashish, Venky, Kumar, and Sanjay for having me as their roommates and eating whatever I cooked or could cook; Raj and Paresh, for having me as their guest when I came to Cleveland in fall '89 and being my friend since then; Sandra for being my little sister who was always concerned more about my wife than me.

I am also thankful to each and every member of my family, specially my parents, for their unending love, support, and encouragement. Lastly but not the least, to Shikha for patiently waiting for our 'reunion' and also for not complaining (too much) even when I started writing my thesis instead of writing letters to her.
PREFACE

The work described in chapters II and III of this thesis has been published in the following research papers:


This work has also been published in following review article:

# TABLE OF CONTENTS

CHAPTER I. INTRODUCTION.................................................................01
   A. Specific Locus Test.................................................................02
   B. Saturation Mutagenesis...........................................................06
   C. Random Insertional Mutagenesis.............................................13
   D. Enhancer and gene Traps.........................................................15
   E. Targeted Mutagenesis..............................................................18
   F. Summary..................................................................................20

CHAPTER II: Molecular Mapping of Deletion Breakpoints
    that define Regions required for Embryonic and Extraembryonic ectoderm Development
   A. Introduction.............................................................................22
   B. Materials and Methods
      1. Mice..................................................................................37
      2. Genomic Clones..................................................................38
   C. Results
      1. Ordering of the Proximal Breakpoint of the Deletions.............40
      2. Cloning of the $c^{2YPSj}$ Deletion Breakpoint-fusion-fragment...43
      3. Ordering of the Distal Breakpoints Relative to $D7Cw2D$...........51
   D. Discussion...............................................................................57
CHAPTER III: Genomic mapping to redefine limit of *eed* region using individual early postimplantation embryos

A. Introduction.................................................................................63

B. Materials and Methods
   1. Embryo Preparation and Southern Analysis.........................64
   2. Estimation of Total DNA Content per Embryo......................66
   3. Genomic Clones.........................................................................66

C. Results and Discussion.................................................................67

CHAPTER IV: Molecular Characterization of the *eed* Region

A. Introduction................................................................................81

B. Materials and Method
   1. Mice..........................................................................................86
   2. Genomic Libraries.......................................................................87
   3. Library Screening.......................................................................87
   4. Phage DNA extraction.................................................................88
   5. Yeast Artificial Chromosome (YAC) library.............................88
   6. Northern Blot.............................................................................90
   7. Genomic Clones.........................................................................91
   8. Cloning of the 15 kb *Sal I/Sma I*
      fragment from *eed* region......................................................93

C. Results
   1. Walking towards the *c6H* proximal breakpoint....................97
   2. Cloning and mapping of the *c6H*
      breakpoint-fusion fragment..................................................101
3. Isolation of YAC clones from the \textit{exed} region ..........104
4. Estimation of size of the \textit{exed} region .......................105
5. Cloning the \textit{exed} region ........................................105
6. Mapping the breakpoints within the 40 kb \textit{Sal I} fragment ..........................................................109
7. Identification of a transcription unit in the \textit{exed} region ..........................................................109
8. Mapping of the cloned portion of \textit{exed} region relative to \textit{c^{4FR60Hd}} ........................................114
D. Discussion .....................................................................119

LITERATURE CITED ................................................................126
LIST OF FIGURES

Figure 01. Breeding protocol used to generate and detect
N-ethyl-N-nitrosourea (EtNU)induced recessive
mutations........................................................................................................08

Figure 02. Complementation map of the albino deletions..........23

Figure 03. Schematic diagram of day 7.5 exed/exed. embryos.......26

Figure 04. Schematic diagram of day 7.5 msd/msd embryos..........28

Figure 05. Schematic diagram of day 7.5 eed/eed embryos..........30

Figure 06. Complementation map of the albino deletions
that define the eed and the exed regions.................................35

Figure 07. Mapping of the repeat sequence probe pTME3a1.4. .......41

Figure 08. Ordering proximal breakpoint of albino deletions........44

Figure 09. Detection of the c2YPSj breakpoint-fusion fragment.......46

Figure 10. Mapping of the c2YPSj breakpoint-fusion fragment. .......49

Figure 11. Deletion breakpoint order relative to the
c2YPSj distal breakpoint...........................................................................52

Figure 12. Proximal and distal breakpoint order of the
deletions defining the eed and the exed regions.........................55

Figure 13. Deletions that define the eed and the exed regions
based on complementation and molecular analyses.................61

Figure 14. Southern blot analysis of Eco RI-digested DNA..........68

Figure 15. Thick-section light micrograph of c202G/c202G embryo....73

Figure 16. Southern blot of Eco RI-digested DNA from embryos ....76

Figure 17. Complementation map of the albino deletions..........80
Figure 18. Deletions that define the *exed* region.............................84
Figure 19. Restriction map of the region around the
proximal breakpoint of *c61H* deletion.....................................95
Figure 20. Detection of the *c61H* breakpoint-fusion-fragment..........99
Figure 21. Mapping of the *c61H* breakpoint-fusion-fragment.........102
Figure 22. Estimation of size of the *exed* region..........................106
Figure 23. Restriction map of the *exed* region.............................110
Figure 24. Distance between p1.3H3 and p1.9H3.............................112
Figure 25. Detection of 5 kb transcript in adult brain......................115
Figure 26. Mapping of p2.2SacI relative to *c4FR60Hd* deletion........117
LIST OF TABLES

Table 1. DNA content of early postimplantation embryos................70
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP-</td>
<td>Cytidine 5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SSC</td>
<td>150mM NaCl, 1.5 mM sodium citrate</td>
</tr>
<tr>
<td>TAE</td>
<td>40mM Tris-acetate, 1mM EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris-Cl (pH 8.0), 1mM EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5'-triphosphate</td>
</tr>
</tbody>
</table>
CHAPTER I. INTRODUCTION

To understand the mechanisms underlying mammalian development, it is essential to identify the genes involved and to determine their role in this complex process. Recent advancements in molecular genetics have opened multiple avenues to identify such genes. One such achievement in mice, which is a model system to study mammalian development, has been the ability to generate germ line mutations (for reviews see Reith and Bernstein 1991; Gossler and Balling 1992; Rossant and Hopkins 1992). These mutations serve as a valuable resource for identifying developmentally significant phenotypes and associating them initially with specific regions of the genome and finally with specific DNA sequences.

In the following sections, various techniques to generate germ line mutations in mice and some of their applications are described. Our present knowledge of embryonic development may not be enough to answer various questions related to this process, but the progress made in the past four decades as a result of the availability of mutations, justifies the efforts being made towards techniques resulting in germ line alterations.
Specific Locus Test

One of the initial efforts directed towards generation of germ line mutations in mice is that associated with inducing chromosomal deletions. Deletions have been widely used in genetic experiments since their discovery in *Drosophila* by Bridges in 1917. A general screen for generating deletions was designed by Mohr in 1927 to uncover recessive mutations of visible markers in *Drosophila*. This deletion-screening technique was utilized by Russell in 1951 to select germ line deletions associated with a visible marker gene which resulted in embryonic lethality in mice (Russell 1951). This procedure of selecting site-specific mutations is known as the specific-locus test (SLT). Deletions were generated by exposure to radiation, and more recently, by treating with chemicals like chlorambucil (Russell and Russell 1959; Russell 1989; Russell et al. 1989). The marker loci used in these experiments included nonagouti (*a*) on chromosome 2, brown (*b*) on chromosome 4, pink-eyed dilution (*p*) and albino (*c*) on chromosome 7, dilute (*d*) and short ear (*se*) on chromosome 9 and piebald (*s*) on chromosome 14. A second stock developed at MRC in Harwell, England included five additional marker loci: fuzzy (*fz*), and leaden (*ln*) on chromosome 1, brachypody (*bp^H*) and pallid (*pa*) on chromosome 2, and pearl (*pe^H*) on chromosome 13 (Lyon and Morris 1966).

The SLT involves mating of irradiated or chemically-treated wild-
type animals to a tester stock homozygous for recessive mutations at each of the marker loci. New mutations involving any one of these loci are detected in the F₁ generation by the appearance of visible recessive phenotypes. Since loss of any of these marker loci leads to viable animals exhibiting a visible phenotype (e.g. albino in case of c), any nonviable mutation must involve other loci in addition to the marker loci. Therefore, the mutation is most likely a multilocus deletion. A detailed complementation analysis of these mutations can result in the identification of functional units in the chromosomal region covered by the deletions. Presence of overlapping deletions greatly facilitates the functional and physical mapping of any region of the genome. It is relatively easy to map other mutations and DNA markers to the region. In addition, the deletion-breakpoints serve as valuable tools to gain molecular access to any functionally important region defined by the deletions.

Of all the deletion complexes, those associated with d, se and c have been most extensively studied (Rinchik and Russell 1990). Complementation analysis of the deletions associated with the closely linked d and se loci (0.16 cM) has resulted in identification of six regions required for prenatal (pl-1 to pl-6) and two for neonatal (nl-1 and nl-2) development (Russell 1971; Rinchik et al. 1985; Rinchik et al. 1986; Russell and Montgomery 1987). The discovery that the original d mutation was caused by integration of the Env-3 murine leukemia virus into the noncoding region of the gene and the
subsequent cloning of the *Emu*-3 provirus along with the flanking sequences provided molecular access to the region spanned by these deletions (Jenkins *et al.* 1981; Copeland *et al.* 1983; Jenkins *et al.* 1989). These flanking DNA probes were later used to clone the deletion breakpoint of one of the deletions that mapped between pl-3 and nl-1 (Strobel *et al.* 1990).

Recent work in this region includes identification and characterization of a candidate gene for both the *d* and *se* locus. The *dilute* candidate gene codes for a protein that is a member of the myosin I family, a group of proteins presumed to drive the movement of membranes along actin filaments (Mercer *et al.* 1991). The *dilute* mutation produces a lightening of coat color due to abnormal adentritic melanocyte morphology that results in an uneven release of pigment granules into the developing hair shaft. Most alleles (*dilute-lethal*) also produce a neurological defect, suggesting a role for this protein in the elaboration, maintenance and function of cellular processes of melanocytes and neurons. The candidate gene for the *se* locus codes for a TGF-β related protein called bone morphogenetic protein-5 (*Bmp*-5) (Kingsley *et al.* 1992). The true identity between *se* and *Bmp*-5 will be provided by functional complementation of the mutant phenotypes in transgenic *short ear* mice containing *Bmp*-5 gene.

The other well characterized deletion complex is defined by 37
overlapping chromosomal deficiencies that remove the albino coat color (c) locus and in some cases, surrounding genes. These deletions have been classified into 15 complementation groups that define at least six regions containing genes affecting pre- or postnatal development (Gluecksohn-Waelsch 1979; Russell 1979; Russell and Raymer 1979; Russell et al. 1979; Russell et al. 1982). The regions have been named on the basis of phenotypic effects observed in individuals homozygous for the deletions: *pid* (preimplantation development), *exed* (extraembryonic ectoderm development), *msd* (mesoderm deficiency), *eed* (embryonic ectoderm development), *hsdr* (hepatocyte specific developmental regulator-1) and *jdf* (juvenile development and fertility) (for review see Holdener-Kenny et al. 1992).

An extensive molecular analysis of the albino-deletion complex which includes cloning of deletion breakpoints and generation of physical maps is being carried out by several groups. Such studies have recently resulted in the cloning of a candidate gene responsible for the *hsdr-1* phenotype. Mice homozygous for deletions that remove this region die within 12 hours after birth (Gluecksohn-Waelsch, 1979; Gluecksohn-Waelsch, 1987; McKnight et al. 1989; Russell, 1979). Lethality is thought to be the consequence of hypoglycemia resulting from failure to activate liver-specific enzymes involved in gluconeogenesis (Erickson et al. 1968). The *hsdr-1* candidate gene codes for fumarlylacetoacetate hydrolase (FAH) enzyme (Klebig et al. 1992; Ruppert et al. 1992). Interestingly, the
inherited deficiency of FAH in humans which results in a fatal
disorder known as tyrosinemia type-1 shows strong phenotypic
similarity to the effects of loss of hsdr-1 in mice (Kvittingen 1986;
Goldsmith and Laberge 1989).

Deletions associated with the p, b and a loci are now being
exploited to identify potentially important regions surrounding these
loci. The complementation analysis of lethal and sublethal p
deletions has defined several new functional units associated with
prenatal, neonatal and juvenile development (c.f. from Rinchik and
Russell 1990). A detailed phenotypic analysis of the mutant embryos
or pups would help to identify the exact defect associated with various
functional units.

Saturation Mutagenesis

The functional map generated by complementation analysis of the
various deletion complexes can be refined further by initiating a
saturation mutagenesis screen of different regions (Rinchik 1991).
This work has been undertaken for the albino region and the
mutagen used was N-ethyl-N-nitrosourea (ENU), (Russell et al. 1979;
Rinchik et al. 1990; Rinchik et al. 1990). Its high efficiency
mutagenesis rate (mean per-locus mutation rate of nearly 1.5 X 10^{-3}),
which is a prerequisite for such experiments, has made ENU an ideal
choice (Hitotsumachi et al. 1985). In addition, ENU has been shown
to produce small lesions mutating a single gene with each mutation event (Popp et al. 1983; Zdarsky et al. 1990). Such putative point mutants can be used to determine whether the phenotype observed in deletion homozygotes is due to loss of a single gene, or alternatively, whether it represents a combined effect of loss of multiple genes (Rinchik et al. 1990). Further, saturation mutagenesis experiments are valuable in determining total numbers of genes present within a particular region as well as producing multiple alleles of specific genes. Phenotypic analysis of different alleles is beneficial in understanding the biological role of a particular gene.

A two-step screening procedure has been used to recover albino-region mutants (Rinchik et al. 1990). This procedure is based on deletion-associated hemizygosity (see breeding protocol in Fig. 1). Briefly, ENU-mutagenized chromosomes marked with nonlethal albino (c) mutation are isolated in G₁ females. These females are crossed to c<sup>ch</sup>/c<sup>Fp1</sup> males, which are heterozygous for the Fp1 "tester" deletion [Df(c Mod-2 sh-1)]. Absence of albino offspring in G₂ would be evidence that the G₁ female carried a newly induced prenatally lethal mutation that is responsible for the embryonic or fetal death when hemizygous. The protocol allows for retrieval of all new mutations, lethal or viable, from the light chinchilla (c<sup>ch</sup>/c) G₂ siblings for subsequent maintenance in breeding stocks.

In a screen to test over 3500 gametes exposed to ENU, several
Figure 1. Breeding protocol used to generate and detect $N$-ethyl-$N$-nitrosourea (EtNU) induced recessive mutations within the region corresponding to the 6-to-11 cM $c^{Fp1}$ deletion (Rinchick, Carpenter and Shelby, 1990). The heavily outlined box highlights the $G_2$ albino class ($c/\bar{c}$), which is the test class for new mutations in this screening protocol. The lightly outlined box highlights the light $G_2$ chinchilla ($c/c^{ch}$) carrier class, from which new induced mutations can be recovered and propagated. $m$ is a mutation induced by EtNU.
$G_0$  
♀  
+ +  

$G_1$  
♀  
+ +  
$cm$  

$G_2$  
+ +  
$c^{ch}+$  

$X$  

$c$  

$c^{ch}+$  

$EtNU$  

$X$  

$c^{ch}$  

$cm$  

$cm$  

Wild type  Wild type  Light Chinchilla (carriers)  Albino (test)
mutations have been identified including those that define new loci (Rinchik et al. 1990). These represent three broad phenotypic groups: prenatal lethality (six mutations), a fitness/runting syndrome (five alleles, designated as fitness (fit) variants), and a neurological/balance-defect [four alleles, designated as variants of shaker-1 (sh-1)] locus. Of the six prenatal lethal mutations, four have been mapped between pid (preimplantation development) and sh-1, a region that was previously not associated with any lethal mutation based on the deletion analysis. The other two lethal mutations that map to the eed (embryonic ectoderm development) region have been shown to complement each other in trans, but could not be complemented by c deletions known to include eed region (Rinchik and carpenter 1993). This result provides evidence for at least two genes necessary for normal prenatal development. This is in accordance with studies describing presence of two distinct abnormalities associated with lack of eed region, namely, defective anterior organizing region of the primitive streak and inability to establish stem cell lines (Niswander et al. 1988). Therefore, the phenotypic studies and saturation mutagenesis results suggest the presence of at least two genes in the eed region, rather than one as was defined on the basis of complementation studies of the albino deletions. These findings demonstrate the usefulness of generating fine structure map of the genome using saturation mutagenesis.

Once such developmentally important genes are identified, the
next goal is to clone the gene(s) involved. To achieve this, first the size of the region containing the gene is estimated using markers that define its proximal and distal limits. This involves generation of a long-range restriction map using rare cutting restriction enzymes (Barlow and Lehrach 1986; Smith et al. 1988). Presence of DNA rearrangements, especially deletions and translocations, that define the limits of the region containing the gene are extremely beneficial. Cloning of the breakpoints involved in these rearrangements provides ideal markers for such physical mapping studies. For example, the breakpoints of two independent translocation mutations defined the borders of 60 kb segment containing part of the Type1-Neurofibromatosis (NF1, Wallace et al. 1990) and overlapping deletions narrowed down the location of Brachyury (T, Herrman et al. 1990) genes.

Once the size is determined, a molecular analysis of the region is initiated using the nearest cloned DNA marker as a starting point. Using chromosome walking (Steinmetz et al. 1982) and jumping (Collins and Weissman 1984; Poustka and Lehrach 1986; Collins et al. 1987; Poustka et al. 1987), DNA sequences isolated from the region are tested for evolutionary conservation (Monaco et al. 1986), presence of CpG islands (Lindsay and Bird 1987; Larsen et al. 1992), or for presence of coding sequences by the "exon-trapping" (Duyk et al. 1991; Hamaguchi et al. 1992) and "exon amplification" (Buckler et al. 1991) method. The technique of positional cloning has been used to clone
several genes involved in human diseases, examples of which include cystic fibrosis (Rommens et al. 1989; Riordan et al. 1990), Type-1 Neurofibromatosis (Viskochil et al. 1990; Wallace et al. 1990) and Wilm's tumor (Call et al. 1990; Gessler et al. 1990; Rose et al. 1990). In mice, cloning of the Brachyury (T) gene is an ideal example of the application of this procedure. Mutations at the T locus induce mild dominant defects in vertebrae development (Dobrovolskai-Zavadskaia 1927). T/T homozygotes die at ~ 10 days of gestation, a consequence of a primary failure to produce sufficient mesoderm during primitive streak formation at ~ 7.5- 8.5 days of gestation (Chelsey 1935; Yanagisawa et al. 1981). Using deletion breakpoints, the gene was mapped to a 75 kb region (Herrman et al. 1990). A gene coding for a novel protein, pme75 was isolated from the region and found to be expressed during early stages of gastrulation, which coincides with the mutant phenotype (Herrman et al. 1990; Wilkinson et al. 1990). The gene has been successfully used to rescue the mutant phenotype (Stott et al. 1993). Recently, the Brachyury gene product was shown to be a DNA binding protein which may be involved in tissue-specific control of gene expression (Kispert and Herrmann 1993).

Though the cloning of genes by positional cloning is extremely valuable, absence of cloned markers from the desired region greatly increases the time involved. Recently, this limitation has been overcome by the availability of a large number of markers defining
simple sequence length polymorphisms (SSLPs) (Dietrich et al. 1992). These SSLP markers consist of polymerase chain reaction (PCR) primers that amplify simple sequence repeats that are polymorphic between different mouse species. Currently, such SSLP markers are available every 4.3 cM of the mouse genome and are soon expected to be present every 1000 kb. The PCR primers that define various SSLPs are ideal for screening and isolating mouse genomic sequences cloned into Yeast Artificial Chromosome (YAC) (Burke et al. 1987) or P1 bacteriophage vectors (Steinberg 1990), thus allowing rapid cloning of any region of interest.

**Random Insertional Mutagenesis**

Techniques to generate transgenic mice have opened doors to a novel way to induce germ line mutations. The fact that foreign DNA, when introduced into mice, would occasionally generate recessive mutations has been exploited to develop a powerful technique of insertional mutagenesis (Gridley et al. 1987; Jaenisch 1988). It involves integration of known DNA sequences to disrupt and in addition, tag the target gene. Tagging of the gene by a known DNA fragment greatly facilitates its cloning.

Initially, insertional mutants were produced by exposure of either pre- or post implantation embryos to replication incompetent retrovirus (Jaenisch 1976; Jaenisch 1977). These experiments
involved coculturing 8-16 cell embryos with a layer of virus producing cells or by microinjection of concentrated retrovirus into the blastocoelic cavity of embryos prior to their being transplanted into the uterus of pseudopregnant mice. Germ-line integration of retroviruses has yielded several mutations all of which have been recessive and lethal. Examples of such mutations are Mov13 which results in midgestation embryonic lethality (Jaenisch et al. 1983), Mov34, which is postimplantation embryonic lethal (Soriano et al. 1987; Gridley et al. 1991); and MpV17 which causes adult lethal nephrotic syndrome (Weiher et al. 1990). Since retroviral insertions are usually simple and cause little or no alteration to surrounding host DNA, the mutagenic lesion is likely to be a direct consequence of the viral integration into or near an endogenous gene. This property of viral integration has been beneficial in cloning sequences flanking the integration site and molecular characterization of most of the mutations generated by this method. However, the integration of retroviruses appears to occur preferentially near DNAse-I-hypersensitive sites, transcriptionally active regions in the host chromatin (Vijaya et al. 1986; Rohdewohld et al. 1987; Shih et al. 1988). This suggests that only a subset of all genes, those that are transcribed in embryonic cells, may be potential targets for viral integration.

The problem of preferential insertion can be overcome by microinjection of DNA sequences into the pronucleus of fertilized
mouse oocytes (Gorden et al. 1980; Constantini and Lacy 1981; Gordon and Ruddle 1981). One of the best characterized mutations generated by this method is limb deformity (ld) (Woychik et al. 1985). The ld gene encodes a novel set of proteins, the formins, which are involved in patterning of limb development (Woychik et al. 1990). Although several other recessive lethal mutants have been generated by this method, the molecular characterization of these mutations has been slow. The main reason for this seems to be that transgenic insertions generated by microinjection often cause deletions and other rearrangements that can complicate association of a particular transcriptional unit with a particular phenotype (Covarrubias et al. 1986; Covarrubias et al. 1987; Mahon et al. 1988). However, progress is being made in a few other cases e.g. legless (McNeish et al. 1988) where genomic locus has been cloned and a few conserved sequences have been identified which are being used to isolate cDNA clones (Singh et al. 1991).

**Enhancer and gene Traps**

Apart from the inherent problems associated with insertional mutagenesis using retroviruses or DNA microinjection into embryos described above, one of the biggest difficulties is that only 5-10 % of transgenic strains generated carry a recessive mutation causing lethality or a visible phenotype (Palmiter and Brinster 1986). This means that to have a mutation in all the genes that would yield a
visible phenotype when mutated, a very large number of transgenic mice would have to be generated and maintained. In addition, since many of the transgenic mice must be bred to homozygosity to analyze the phenotypic effect, this procedure is extremely time consuming, laborious and expensive. To avoid the above problems, a method to select desirable mutations in vitro would be beneficial. The establishment of embryonic stem cell lines (Evan and Kaufman 1981; Martin 1981), which can contribute to all cell lineages including the germ line when microinjected into blastocyst-stage embryos (Bradley et al. 1984), has made such in vitro manipulations and selection possible. In addition, to make the insertional mutagenesis more efficient, vectors have been designed that select for insertions that lie within coding sequences.

This technique has been utilized in two approaches. One approach takes advantage of the presence of enhancer sequences in the genome (Gossler et al. 1989). It involves insertion of enhancer-trap vectors containing a reporter transgene, lacZ, under the control of a weak promoter into ES cells. Successful insertion of the vector proximal to an enhancer is marked by the pattern of expression of lacZ in a temporal and tissue specific manner in chimeric embryos (Allen et al. 1988; Kothary et al. 1988; Gossler et al. 1989). Such enhancer-trap vectors have been successfully used in Drosophila in screens for genes expressed at particular developmental stages (O’Kane and Gehring 1987; Bellen et al. 1989; Bier et al. 1989). Unfortunately, in mouse, so
far this approach has not been very successful, as the expression pattern of the transgene so far does not mimic the endogenous gene. Another drawback of this approach is its inability to mutate the tagged gene, which makes the phenotypic analysis difficult. In contrast, a second approach using another entrapment vector, the promoter- or gene-trap vector, has been designed to overcome these problems (Friedrich and Soriano 1991; Skarnes et al. 1992; von Melchner et al. 1992). The vector consists of a promoterless lacZ gene with an upstream splice acceptor site. The rationale for this vector is that the lacZ would be activated and expressed as a spliced fusion transcript if integrated into the intron of a gene, allowing for rapid cloning of the upstream endogenous transcribed sequences from lacZ containing cDNAs. In addition, the activity of the tagged gene can be assessed in a heterozygous animal by staining for presence of lacZ gene product. Further, the functional analysis can be conducted in homozygotes as the insertion within the gene should be mutagenic.

To date, approximately 30 ES cell lines with promoter- or gene-trap vector insertions have been used to generate germ line chimeras (Friedrich and Soriano 1991; Skarnes et al. 1992; von Melchner et al. 1992). At least 1 in 4 of these insertions resulted in a recessive embryonic lethal or visible phenotype when homozygous. In addition, a close correlation was found between the endogenous gene and the lacZ expression in two lines that were analysed. Though the number of lines analysed is very small, results obtained so far are
quite promising.

**Targeted Mutagenesis**

The technique of insertional mutagenesis can not only be used to identify novel genes by generating random mutations but can also be used to mutate specifically any cloned gene. This is possible by targeting recessive mutations in ES cells by homologous recombination into the gene of interest (Capecci 1989). Targeted mutations have been reported for several genes that have been thought to be developmentally important. Several interesting results have been obtained from these studies. The inactivation of a homeotic box-containing gene, *Hox3.1*, provided evidence for a homeotic transformation produced by a putative loss-of-function allele in mice (Mouellic et al. 1992). In the trunk region of homozygous mutant animals, several skeletal segments were transformed into the likeness of more anterior ones, as observed in homeotic mutations in *Drosophila* (Lewis 1978; Bender et al. 1983). Recently, homeotic transformation was also reported in *Hox 2.6* mutant mice (Ramirez-Solis et al. 1993). Mutation in *Hox 2.6* caused anterior homeotic transformation of the second cervical vertebra or axis to the first one, the atlas. The result supports a direct role for *Hox 2.6* in specifying regional identity in the axial skeleton which was initially proposed based on its expression pattern during midgestation (Graham et al. 1988; Hunt et al. 1991).
However, not all homeobox-containing genes result in homeotic transformation when mutated. For example, mice homozygous for a mutation in Hox 1.5 die at birth with no obvious homeotic transformation (Chisaka and Capechi 1991). Instead they show abnormalities associated with defective development of neural crest cells. No abnormalities could be detected in several other tissues that express this gene. The fact that the mutant phenotype is restricted to only a subset of cells expressing the gene has been observed in several other cases. A mutation in the homeobox of the engrailed-like gene En-2 led to a defect in postnatal patterning of the cerebellum, rather than a defect in the embryonic development of the engrailed expressing domain of neural tube (Joyner et al. 1991). Several non-homeobox containing genes have shown similar phenotypic effects. Mice homozygous for a mutation in c-src die within a few weeks of birth due to osteopetrosis although the gene is expressed in all cells (Soriano et al. 1991). Similarly, mice lacking transforming growth factor a (TGF-α) are healthy and fertile (Luetteke et al. 1993; Mann et al. 1993). The only phenotype that distinguishes TGF-α -/- animals from heterozygotes or normal littermates is the pronounced waviness of the coat, though the gene is normally expressed in the unfertilized oocyte, the preimplantation embryo and several tissues of developing and adult mouse.
In summary, the mutant phenotypes obtained from targeted mutagenesis experiments of these and several other genes do not correlate well with their expression pattern. These results may suggest a functional redundancy between genes and genetic pathways or nonfunctional expression of genes in certain tissues (Schwartzberg et al. 1991; Soriano et al. 1991; Tybulewicz et al. 1991). In at least one case, the Wnt gene family, evidence has been found to suggest that Wnt-1 and Wnt-3a may be functionally redundant in tissues where they are coexpressed (McMahon and Bradley 1990; McMahon et al. 1992). Further studies involving phenotype analysis of double mutants may help to clarify if there exists any functional redundancy between these genes. Genes with redundant function have also been identified in organisms like Caenorhabditis elegans and Drosophila (Lambie and Kimble 1991; Elkin et al. 1990).

Summary

Application of all these techniques to generate germ line mutations in mice, as described above, has resulted in identification of a large number of developmentally important genes and a better understanding of their function. These studies are extremely valuable in achieving our goal of understanding the regulation of mammalian development. The work described in the following chapters has been focused on some of the mutations that have resulted from the specific locus test around the albino coat color locus.
The work describes a positional cloning strategy to isolate a gene or genes required during early postimplantation stages.
CHAPTER II: Molecular Mapping of Deletion Breakpoints that define Regions required for Embryonic and Extraembryonic ectoderm Development.

A. INTRODUCTION

One of the key events during early postimplantation development of mouse embryos is the process of gastrulation. It is defined as a process of extensive cell movement whereby a population of embryonic cells, known as the epiblast, is transformed into a three (Lyon and Morris 1966; Russell et al. 1982; Niswander et al. 1989) layered structure consisting of ectoderm, endoderm and a middle layer of mesoderm (Stern 1992). The three germ layers give rise to the entire fetus.

The albino-deletion complex is an ideal system to study genes involved in gastrulation. As described in the previous chapter, this complex is defined by more than 37 overlapping deletions around the albino coat locus (c) that are grouped into 14 complementation groups (Fig. 2) (Lyon and Morris 1966; Russell et al. 1982; Niswander et al. 1989). At least three regions have been identified that are required during the process of gastrulation. These regions are *exed* (extraembryonic ectoderm development), *msd* (mesoderm deficiency) and *eed* (embryonic ectoderm development) (Niswander et al. 1988; Niswander et al. 1989; Holdener, Faust and Magnuson unpublished
Figure 2. : Complementation map of the albino deletions (Holdener et al, 1992). Marker loci include \textit{tp} (taupe), \textit{c} (albino), \textit{Mod-2} (mitochondrial form of malic enzyme), \textit{sh-1} (shaker-1). \textit{cM} = centiMorgan. Regions identified by complementation analysis are indicated below the genetic map. Boxes indicate the deletion interval to which they map. No correlation with genetic or physical distance is implied; \textit{msd}: mesoderm deficiency; \textit{hsdr-1}: hepatocyte-specific developmental regulator-1; \textit{jdf}: juvenile development and fertility; \textit{eed}, embryonic ectoderm development; \textit{exed}: extraembryonic ectoderm development; \textit{pid}: preimplantation development. The actual phenotype resulting from the deletion of any of these loci may be due to the removal of more than one gene. Deletion chromosomes are illustrated below the genetic map as complementation groups. Deleted regions are represented by gaps. Symbols within the deleted region indicate the name of the complementation group, and the number in the parentheses represents the number of individual deletion chromosomes assigned to each group. Dotted lines indicate that the exact position of the breakpoints with respect to the regions shown in the boxes is not known.
result). Embryos homozygous for deletions that extend beyond the *exed* region develop normally until day 6.5 of gestation. At day 7.5, the mutant phenotype is visible and is marked by the presence of a small egg cylinder with no primitive streak or mesoderm formation (Fig. 3) (Niswander et al. 1988). The extraembryonic ectoderm exists as an undifferentiated mass of pycnotic cells. Wild-type embryos at this stage have already initiated primitive streak formation and mesoderm production. Extraembryonic structures like amnion, chorion and allantois are also beginning to form during this time.

*msd*, the second region containing gene(s) required during gastrulation, manifests its phenotype at day 7.5 of development (Fig. 4) (Holdener, Faust and Magnuson unpublished result). Although *msd* homozygotes grow beyond the stage at which *exed* homozygotes stop, they fail to form a primitive streak and extraembryonic structures do not differentiate. In fact, the embryos continue to increase in size with no further differentiation until day 9.5, after which they are resorbed. A characteristic feature of these embryos is the disproportionate increase in size of the parietal endoderm which can be three-to-five times the size of the mutant embryo.

The third region containing gene(s) required during gastrulation is *eed*. Embryos deficient for this region progress further in development than *exed* or *msd* homozygotes and are recognized as being abnormal at day 8.5 of gestation (Fig. 5) (Niswander et al. 1988;
Figure 3. Schematic diagram of day 7.5 embryos. (A) wild type, (B) exed/exed. Al: allantois; Am: amnion; Ch: chorion;
EC: ectoplacental cone, ECa: ectoplacental cavity,
EE: embryonic ectoderm; Exo: exocoelom;
Me: embryonic mesoderm;
PEn: parietal endoderm,
VEn: visceral endoderm; Magnification: 60X.
Figure 4. Schematic diagram of day 7.5 embryos.

(A) wild type, (B) msd/msd. Al: allantois; Am: amnion; Ch: chorion; EC: ectoplacental cone, ECa : ectoplacental cavity, EE: embryonic ectoderm; ExE: extraembryonic ectoderm; Exo: exocoelom; Me: embryonic mesoderm; PEN: parietal endoderm, VEn: visceral endoderm; Magnification: 60X.
Figure 5. Schematic diagram of day 7.5 embryos.

(A) wild type, (B) eed/eed. Al: allantois; Am: amnion;
Ch: chorion; EE: embryonic ectoderm; ExE: extraembryonic ectoderm; Me: embryonic mesoderm; Pen: parietal endoderm,
So: somite; VEn: visceral endoderm; Magnification: 50X.
Niswander et al. 1989). The primitive streak has formed and mesoderm production has occurred. In addition, extensive development of the extraembryonic structures, allantois, amnion and chorion is seen. However, unlike wild-type embryos at this stage, there is no organization of mesodermal cells into somites, or the formation of notochord or induction of neural axis. Absence of these structures, all of which are derivatives of the primitive node suggests a defect in the anterior organizing region of the primitive streak. The presence of extensive extraembryonic mesoderm indicated that structures originating from the posterior end of the streak are unaffected (Niswander et al. 1988; Niswander et al. 1989; Holdener, Faust and Magnuson unpublished result).

Embryological and complementation analyses of a subset of the albino deletions ($c^{11DSD}, c^{5FR60Hg}, c^{2YPSj}, c^{4FR60Hd}$ and $c^{6H}$) showed that all five remove the eed region (Lewis et al. 1976; Niswander et al. 1988; Niswander et al. 1989). Embryos homozygous for $c^{11DSD}$, $c^{5FR60Hg}$ and $c^{2YPSj}$ deletions showed the phenotype associated with lack of this region as described above. Further, deficiency of this region also resulted in the inability to establish embryonic stem cell lines from the mutant embryos. The two other deletions, $c^{4FR60Hd}$ and $c^{6H}$ appear to remove the exed region in addition to the eed region (Lewis et al. 1976; Niswander et al. 1988; Niswander et al. 1989). This was determined by a complete lack of extraembryonic structures
in c^{4FR60Hd} and c^{6H} homozygotes, but extensive development of these structures in c^{11DS}, c^{5FR60Hg} and c^{2YP5j} homozygotes. Furthermore, the c^{11DS}, c^{5FR60Hg} and c^{2YP5j} deletion chromosomes were capable of complementing the c^{4FR60Hd} and c^{6H} chromosomes by allowing for development of the extraembryonic structures in the compound heterozygotes (Niswander et al. 1988; Niswander et al. 1989).

Thus, based on genetic data, it was proposed that the c^{11DS}, c^{5FR60Hg} and c^{2YP5j} distal breakpoints lie more proximal than those of the c^{4FR60Hd} and c^{6H} deletions. The area of overlap between the two sets of deletions distal to the c^3H breakpoint would thus define the region containing eed, whereas the area of non-overlap between the two sets of deletions would define the region containing exed (see Fig. 6). Each of these mutations is defined by the phenotype exhibited by deletion homozygotes. In reality, these phenotypes could be the result of deletion of one important gene or a combinatorial effect of the loss of more than one gene.

To obtain molecular markers within the albino region, a partial genomic library of the distal region of chromosome 7 was produced using the techniques of chromosome microdissection and microcloning (Niswander et al. 1991). One microclone (palb18) was shown to define a locus, D7Cw18, mapping to a region of chromosome 7 proximal to c that is removed by c^{11DS} deletion but
not by the $c^{5FR60Hg}$, $c^{2PYSj}$, $c^{4FR60Hd}$ or $c^{6H}$ deletions. Thus, palb18 and eed /exed map to opposite sides of the deleted region of chromosomes 7 (see Fig. 6), and it was clear that cloning of one or more of the deletion breakpoint-fusion fragments would be needed to provide molecular access to the region of chromosome 7 containing eed and exed.

Using the clone palb18, an albino-region-specific (ars) repeat sequence was identified. The present work describes the mapping studies that localized the repeat sequence primarily to the region of chromosome 7 covered by the proximal portion of the $c^{11DSD}$ deletion. Because the albino-region-specific (ars) repeat probe hybridized differentially to $c^{5FR60Hg}$, $c^{2PYSj}$, $c^{4FR60Hd}$ and $c^{6H}$ deletion DNA, it was possible to define the order of the proximal breakpoints by examining Southern blot banding patterns. This order was subsequently confirmed with single-copy sequence probes. In addition, cloning of the $c^{2PYSj}$ breakpoint-fusion fragment by isolating a unique restriction fragment associated with this deletion detected by the ars repeat probe is also described. Isolation of this breakpoint provided molecular access to the distal side of the deletions and has allowed us to define more precisely the proximal and distal limits of the region of chromosome 7 containing exed and eed.
Figure 6. Complementation map of the albino deletions that define the *eed* and the *exed* regions. The names of the marker loci are indicated in legend to Figure 2. Position of the microclone palb18 which defines the *D7Cw18* locus is also shown on the map. It is deleted from the *c3H* and *c11DSD* deletions but is present in others.
B. MATERIALS AND METHODS

1. Mice

   a. Deletion Mice: Seven albino-deletion stocks (designated Df(c)) were used in these experiments. The c6H and c3H mice originated at the MRC Radiobiology Unit, Harwell, England, and were obtained from Dr. S. Gluecksohn-Waelsch (Albert Einstein College of Medicine) (Gluecksohn-Waelsch et al. 1974). The c11DSD, c5FR60Hg, c2YPSj, c4FR60Hd, and c14CoS mice originated at the Oak Ridge National Laboratory (Russell et al. 1979). All mice have been maintained as closed colony, heterozygous stocks with chinchilla (cch). All Df(c)/cch stocks are a dilute chinchilla coat color as compared to a full chinchilla color evident in cch/cch mice.

   b. Mus spretus/Mus musculus interspecies cross. Wild-type (non-deletion) M. spretus males were crossed with Df(c)/cch M. musculus females. To determine which of the phenotypically wild-type F1 progeny carried the deletion chromosome rather than the cch chromosome, progeny testing was carried out as described by Johnson et al. (1989). High-molecular-weight DNA was prepared from spleen or liver of appropriate F1 progeny along with their Df(c)/cch dams and M. spretus sires as previously described (Johnson et al. 1989).

   c. Homozygous Mice: Mice homozygous for the c14CoS or c3H
deletions were produced by crossing appropriate $Df(c)/c^{ch}$ heterozygotes. The homozygous pups were identified prior to death (6-12 hrs after birth) by lack of eye pigmentation. The newborn pups were decapitated and intestines removed. The remainder of the carcass was used for isolation of high molecular weight DNA. $c^{ch}/c^{ch}$ littermates were used for non-deletion DNA.

d. Double Heterozygous Mice: Mice doubly heterozygous for the $c^{3}H/-$ or the $c^{14}CoS/c^{11}DSD, c^{4}FR60Hd, c^{2}YPSj, c^{5}FR60Hg$ or $c^{6}H$ deletions were produced by crossing appropriate $Df(c)/c^{ch}$ heterozygotes. The double heterozygotes were distinguished from $c^{ch}/c^{ch}$ or $Df(c)/c^{ch}$ littermates by the presence of albinocoat color. DNA was prepared from 3-4 week-old mice.

2. Genomic Clones

pTME3a is a 2.8 kb Eco R1 fragment that was subcloned from a lambda genomic clone (TM9) isolated from a library in lambda Dash (Ruppert et al. 1988) using palb18 as the probe (Niswander et al. 1991). The genomic fragment was subcloned into the plasmid vector Bluescript M13+.

$\lambda^{14}R1c2$ represents a 14-kb Eco RI fragment isolated from a $c^{2}YPSj/c^{3}H$ subgenomic library cloned into lambda Dash (Stratagene).
The library was prepared from 60 μg of DNA digested to completion with Eco RI followed by size fractionation on a sucrose gradient (Ausubel et al. 1989). DNA in the 12-15-kb range was collected and concentrated (Centricon 30 filter, Amicon). Insert DNA (1 μg) was ligated (15°C for 12 hrs) to 1.5 μg lambda Dash vector arms using T4 DNA ligase (Boehringer Mannheim) and then packaged in vitro (Promega packaging extracts). E. coli LE392 was infected with packaged phage. To identify positive clones, plaque lifts were hybridized at 65°C in Church buffer (Church 1984) with 32P-labeled 1.4-kb Eco RI-Xba I fragment of pTME3a containing the albino-region-specific repeat sequence (Niswander et al. 1991). Washes were done in 40 mM Sodium Phosphate Buffer, pH 7.2, 1 mM EDTA, 100 mM NaCl, and 1% SDS at 63°C, 4 times for 15 min each. For mapping purposes, Rsa I- or Hae III-digested phage DNA was used as template to generate 32P-labeled transcripts with T3 and T7 RNA polymerases (Boehringer Mannheim), respectively, according to the procedures outlined by Stratagene.

**p7.5(X/RI)** represents a 7.5-kb Xba I-Eco RI fragment from λ14RIc2 subcloned into pBS +/- (Stratagene). T7 riboprobes were generated from Rsa I-linearized plasmid DNA.

**p77-2** represents a 1.0-kb Eco RI fragment isolated from a partial genomic library generated by microdissection of chromosome 7 (Niswander et al. 1991). This insert was shown to detect a genomic
sequence that maps outside of the region of chromosome 7 removed by the albino deletions.

C. RESULTS

1. Ordering of the Proximal Breakpoint of the Deletions

_palb18_ was used to screen a genomic _lambda_ library. A 1.2 kb Xba I/Eco RI fragment (pTME3a1.4) located approximately 1.2 kb from _D7TM18_ was isolated from one of these clones. When hybridized to _Dra_ I digested wild-type DNA under low stringency, this Xba I/Eco RI fragment detected several fragments predominantly less than 4 kb in size (Fig. 7, lane 1). When hybridized to _c3H/c3H_ (Fig. 7, lane 2), _c11DSD/c14CoS_ (Fig. 7, lane 7) or to _c14CoS/c14CoS_ (Fig. 7, lane 8) DNA, the majority of the _Dra_ I fragments were missing. In contrast when _c6H/c3H_ DNA was used (Fig. 7, lane 3) all of the fragments were present. These results indicate that the repeat sequence is located primarily in the region of chromosome 7 removed by the _c11DSD_ deletion but proximal to the _c6H_ breakpoint. It became immediately obvious that the banding pattern resulting from hybridization with such a repeat probe could be used to order the remaining four breakpoints. DNA from _c5FR60Hg/c3H_ and _c4FR60Hd/c14CoS_ showed all but three of the _Dra_ I fragments seen with the _c6H_ chromosome (Fig. 7, lanes 4 & 5, respectively, bands marked A). These three fragments as well as six others (marked B) were also missing
Figure 7. Mapping of the repeat sequence probe pTME3a1.4. Southern blot analysis of Dra I digested homozygous and double heterozygous deletion DNA hybridized with pTME3a1.4. Lanes 1-7 represent DNA from the following animals: (1) wild-type cch/cch; (2) c3H/c3H; (3) c6H/c3H; (4) c5FR60Hg/c3H; (5) c4FR60Hd/c14CoS; (6) c2YPSj/c3H; (7) c11DSD/c14CoS; (8) c14CoS/c14CoS.

'A' and 'B' show the diagnostic bands described in the text that define the relative order of the deletions.
from $c^2YPSj/c^3H$ DNA (Fig. 7, lane 6). Based on the presence or absence of $Dra\ I$ fragments detected by the dispersed repeat sequences, the order of the proximal chromosome breakpoints was determined to be $c^{11DSD}$ more proximal than $c^2YPSj$ which is more proximal than $c^5FR60Hg$ or $c^4FR60Hd$ which are more proximal than $c^6H$ (Fig. 8). Utilizing this strategy, the order of the $c^5FR60Hg$ relative to the $c^4FR60Hd$ breakpoint could not be determined.

2. Cloning of the $c^2YPSj$ Deletion Breakpoint-fusion fragment

Subsequent to the work of defining the breakpoint order, the albino-region-specific repeat probe was hybridized to a panel of $Eco\ RI$-digested deletion DNAs. A 14-kb fragment was detected in $c^2YPSj/c^3H$ DNA (Fig. 9, lane 6, band marked A). This fragment was not detected in $c^{ch}/c^{ch}$ (lane 1), $c^3H/c^3H$ (lane 2), $c^6H/c^3H$ (lane 3), $c^5FR60Hg/c^3H$ (lane 4), $c^4FR60Hd/c^3H$ (lane 5), $c^{11DSD}/c^{14CoS}$ (lane 7), or $c^{14CoS}/c^{14CoS}$ (lane 8) DNA. This aberrant fragment is not the result of a single $Eco\ RI$ polymorphism associated with the $c^2YPSj$ chromosome since several other restriction enzymes ($Hind\ III$, $Xba\ I$, $Pst\ I$, $Sac\ I$) also produced an altered fragment (data not shown). Given the proposed relative order of the proximal breakpoints, it should not be possible to detect a fragment present in $c^2YPSj$ DNA that is absent in $c^4FR60Hd$, $c^5FR60Hg$, $c^6H$, or $c^{ch}$ DNA, provided the deletions are linear. For this reason, we hypothesized that the altered fragment contained the $c^2YPSj$ deletion breakpoint-fusion fragment.
Figure 8. Ordering proximal breakpoint of albino deletions ($c^{11DSD}$, $c^{2YPSj}$, $c^{5FR60Hg}$, $c^{4FR60Hd}$, and $c^{6H}$) that define the *eed* and the *exed* regions using albino region specific repeat probe. The relative order of $c^{5FR60Hg}$ and $c^{4FR60Hd}$ deletions proximal breakpoints are not resolved. Distal breakpoints of these deletions are assigned based on complementation analysis (Niswander et al. 1988, 1989)
Figure 9. Detection of the $c^{2YPSj}$ breakpoint-fusion fragment.

Southern blot analysis of Eco RI-digested DNA hybridized with pTME3a1.4. Lanes 1-8 represent DNA from the following animals: (1) non-deleted $c^{ch}/c^{ch}$ (originated from the Oak Ridge colony) (2) $c^{3H}/c^{3H}$; (3) $c^{6H}/c^{3H}$; (4) $c^{5FR60Hg}/c^{3H}$; (5) $c^{4FR60Hd}/c^{3H}$; (6) $c^{2YPSj}/c^{3H}$; (7) $c^{11DSd}/c^{14CoS}$; (8) $c^{14CoS}/c^{14CoS}$. The relative position of molecular weight markers (kb) is designated on the left. The symbol "A" denotes the 14 kb Eco RI fragment detected specifically in $c^{2YPSj}/c^{3H}$ DNA.
To determine if the aberrant fragment contains the \( c^{2YPSj} \) deletion junction, a sub-genomic library was generated from 12-15 kb size-selected \( c^{2YPSj}/c^3H \) \textit{Eco} RI-digested DNA. 1 \times 10^5 plaques were screened with the albino-region-specific-repeat probe and two of the recombinant clones that hybridized with the repeat probe were analyzed. The first clone was rearranged and was not analyzed further. The second clone, \( \lambda 14\text{Ric2} \), contained a 14-kb \textit{Eco} RI insert. End-specific riboprobes were hybridized to \( c^{ch}/c^{ch}, c^3H/c^3H \) and \( c^{2YPSj}/c^3H \) DNA (Fig. 10B, 10D). If the 14-kb fragment carried the deletion breakpoint-fusion fragment, we expected the proximal end to be present in the non-deleted and \( c^{2YPSj} \) DNAs and deleted from the \( c^3H \) DNA. In contrast, the distal end should be present in all three chromosomes (Fig. 10A). The T7 riboprobe detected a 6-kb band when hybridized with \( c^{ch}/c^{ch} \) (non-deleted) \textit{Eco} RI-digested DNA (Fig. 10B, lane 1), and the aberrant 14-kb band when hybridized with \( c^{2YPSj}/c^3H \) \textit{Eco} RI-digested DNA (Fig. 10B, lane 3). No signal was detected when the T7 riboprobe was hybridized with \( c^3H/c^3H \) \textit{Eco} RI-digested DNA (Fig. 10B, lane 2). In contrast, the T3 riboprobe hybridized to a 9-kb band in non-deleted (\( c^{ch}/c^{ch} \)) (Fig. 10D, lane 1) and \( c^3H/c^3H \) (Fig. 10D, lane 2) \textit{Eco} RI-digested DNAs, and to both the 9-kb and the aberrant 14-kb band in \( c^{2YPSj}/c^3H \) \textit{Eco} RI-digested DNA (Fig. 10D, lane 3). Rehybridization of the blots with a probe (77-2) located outside of the deletion complex served as a loading control (Figs. 10C and 10E). These results are consistent with the hypothesis that \( \lambda 14\text{Ric2} \) contains the \( c^{2YPSj} \) deletion breakpoint-fusion fragment. From these data, it
Figure 10. Mapping of the $c^{2YPSj}$ breakpoint-fusion fragment.
Southern blot analysis of *Eco* RI-digested DNA hybridized with end-specific RNA probes generated from clone l14RIc2. (A)
Complementation map of the relevant chromosomes. The names of the marker loci are indicated in legend to Fig. 2. $c^{ch}$ represents a non-deleted chromosome. $c^{3H}$ and $c^{2YPSj}$ represent deletion chromosomes. Deleted regions are represented by gaps. The vertical lines are schematic representations of the regions from each chromosome to which the T7 or T3 riboprobe would hybridize. (B) DNA hybridized with T7 riboprobe of λ14RIc2. (C) Rehybridization of (B) with the non-albino region probe p77-2. (D) DNA hybridized with T3 riboprobe of λ14RIc2. (E) Rehybridization of blot in (D) with p77-2. In all cases, lanes 1-3 represent DNA from the following animals; (1) non-deleted $c^{ch}/c^{ch}$ (originated from the Albert Einstein colony) (2) $c^{3H}/c^{3H}$; (3) $c^{2YPSj}/c^{3H}$. 
can be concluded that the \( \lambda 14R1c2 \) DNA sequence included in the T7 riboprobe is homologous to a genomic locus \( (D7Cw2P) \) that lies on the proximal side of the \( c^{2YPSj} \) proximal breakpoint (Fig. 10A). In contrast, the \( \lambda 14R1c2 \) DNA sequence included in the T3 riboprobe is homologous to a genomic locus \( (D7Cw2D) \) located on the distal side of the \( c^{2YPSj} \) distal breakpoint (Fig. 10A).

3. Ordering of the Distal Breakpoints Relative to \( D7Cw2D \).

\( D7Cw2D \) provides molecular access to the general region of chromosome 7 containing \( eed \) and \( exed \). To determine the molecular limits of the regions containing these loci, it was necessary to map the distal breakpoints of the \( c^{11DSD}, c^{5FR60Hg}, c^{4FR60Hd} \) and \( c^{6H} \) deletions relative to \( D7Cw2D \). A 7.5-kb Xba I-Eco RI fragment from the distal side of clone \( \lambda 14R1c2 \) was subcloned into pBS +/-. A T7 riboprobe prepared from this template detects an Eco RI polymorphism in non-deleted DNA of different backgrounds suitable for mapping the position of the deletion breakpoints relative to \( D7Cw2D \). For example, a 9 kb fragment (Fig. 10D, lane 1) was detected with non-deleted DNA from \( c^{ch}/c^{ch} \) mice obtained from the Albert Einstein colony. In contrast, a 16-kb fragment was detected (Fig. 11, lane 1) with non-deleted DNA from \( c^{ch}/c^{ch} \) mice obtained from the Oak Ridge colony. The probe detects a 9-kb fragment with \( c^{14CoS}/c^{14CoS} \) DNA (Fig. 11, lane 2). With DNA from \( c^{11DSD}/c^{14CoS} \) compound heterozygotes, both the \( c^{14CoS} \)-associated 9-kb
Figure 11. Deletion breakpoint order relative to the $c^{2YPSj}$ distal breakpoint. Southern Blot analysis of Eco RI-digested DNA hybridized with T7 end-specific riboprobe generated from p7.5(X/RI). Lanes 1-8 represent DNA from the following animals: (1) non-deleted $c^{ch}/c^{ch}$ (originated from the Oak Ridge Colony) (2) $c^{14CoS}/c^{14CoS}$, (3) $c^{11DSd}/c^{14CoS}$, (4) $c^{5FR60Hg}/c^{ch}$, (5) $c^{6H}/c^{ch}$, (6) $c^{6H}/c^{14CoS}$, (7) $c^{4FR60Hd}/c^{ch}$. 
fragment as well as a 16-kb fragment derived from the \(c^{11DSD}\) chromosome was detected (Fig. 11, lane 3). The presence of the \(c^{11DSD}\)-associated fragment indicates that \(D7Cw2D\) lies distal to the \(c^{11DSD}\) deletion. Likewise, the presence of both a 16-kb \(c^{ch}\)-associated and a 9-kb \(c^{5FR60Hg}\)-associated fragment in \(c^{ch}/c^{5FR60Hg}\) (Fig. 11, lane 4), and in \(c^{ch}/c^{4FR60Hd}\) (Fig. 11, lane 7) DNAs, indicates that \(D7Cw2D\) also lies distal to the \(c^{5FR60Hg}\) and \(c^{4FR60Hd}\) deletions. In contrast, the presence of only the \(c^{ch}\)-associated 16-kb fragment in \(c^{6H}/c^{ch}\) DNA (Fig. 11, lane 5) and the \(c^{14CoS}\)-associated 9-kb fragment in \(c^{6H}/c^{14CoS}\) DNA (Fig. 11, lane 6) indicates that \(D7Cw2D\) is deleted from the \(c^{6H}\) chromosome. These results were confirmed with restriction fragment length variant mapping using \(Mus\ spretus\)-balanced \(Df(c)\) chromosomes (data not shown).

From these data, it can be concluded that the \(c^{2YP5j}\) distal breakpoint lies distal to \(c^{11DSD}, c^{5FR60Hg}\) and \(c^{4FR60Hd}\) but proximal to \(c^{6H}\) (Fig. 12). Furthermore, they establish the \(c^{2YP5j}\) and \(c^{6H}\) distal breakpoints as the proximal and distal limits of the region of chromosome 7 containing the \(exed\) gene. Placement of the \(c^{2YP5j}\) distal breakpoint distal to that of \(c^{4FR60Hd}\) is in direct contrast to the order suggested by our genetic data (Niswander et al. 1988; Niswander et al. 1989). One possible explanation to explain the discrepancy is that the \(c^{4FR60Hd}\) deletion is not linear.
Figure 12. Proximal and distal breakpoint order of the deletions defining the *eed* and the *exed* regions. Deleted regions are represented by gaps and non-deleted regions are represented by solid lines. Hatched box in $c^{4FR60Hd}$ deletion represents non-deleted region with additional rearrangements. The exact nature of this chromosomal aberration is unknown. $D7Cw2D$ is the locus defined by the distal breakpoint of the $c^{2YPSj}$ deletion.
D. DISCUSSION

The microclone *pall*b18 has led to the isolation of a repeat sequence which is localized to a region proximal to the albino coat color (*c*) locus on chromosome 7. This albino region specific (ars) repeat is one of few repeat sequences so far identified in the mouse genome that are localized to a particular chromosome. An X-specific repeat has been found to span approximately 1 Mb and consists of 50 copies of a 14 kb long complex repeat unit (LCRU) (Disteche *et al.* 1985; Nasir *et al.* 1990). Recently, a chromosome 8-specific repeat sequence has been discovered which exceeds 15 kb in size (Boyle and Ward 1992). At present, the biological function and the evolution of these repeats is not well understood.

The characteristic hybridization pattern exhibited by the chromosome 7-specific ars repeat probe has been utilized in the present study to determine the relative proximal breakpoint order of the five deletions (*c*11Dsd, *c*5FR60Hg, *c*2Ypsj, *c*4FR60Hd and *c*6H) that define the limits of *eed* and *exed* regions. The most distal of proximal breakpoints contains a higher number of repetitive elements, whereas breakpoints more proximal will contain fewer repeats. The breakpoints were found in the following proximal to distal order: *c*11Dsd _ *c*2Ypsj _ *c*5FR60Hg/c4FR60Hd _ *c*6H (Fig. 8). The relative order of *c*5FR60Hg and *c*4FR60Hd deletions could not be determined since they exhibit exactly the same hybridization pattern. The order of the five
deletions thus established was further confirmed by single copy probes that were obtained from genomic clones containing the ars repeat sequence.

The ars repeat probe has also been utilized to gain molecular access to the eed and the exed regions. The probe detects a 14 kb Eco RI fragment that is unique to $c^{2YPSj}$ DNA. Cloning and mapping studies shown here have proved that this unique Eco RI fragment contains the $c^{2YPSj}$-breakpoint-fusion-fragment. The locus defined by the distal side of the $c^{2YPSj}$ breakpoint is called $D7Cw2D$. Based on the breakpoint order predicted by earlier genetic analyses (Niswander et al. 1988; Niswander et al. 1989), $D7Cw2D$ should map to a position that lies between eed and exed regions (Fig. 12). To confirm these predictions, the distal breakpoints of the $c^{11DSD}$, $c^{5FR60Hg}$, $c^{4FR60Hd}$, $c^{2YPSj}$ and $c^{6H}$ deletions were mapped relative to $D7Cw2D$. $D7Cw2D$ was found to be deleted from $c^{6H}$ but not from $c^{11DSD}$, $c^{5FR60Hg}$ or $c^{4FR60Hd}$.

Placement of the $c^{4FR60Hd}$ distal breakpoint proximal to that of $c^{2YPSj}$ is in direct contrast to the order suggested by our genetic data (Niswander et al. 1988; Niswander et al. 1989). Embryos homozygous for the $c^{2YPSj}$ deletion show the embryonic-ectoderm defect but not the extraembryonic-ectoderm defect. In contrast, $c^{4FR60Hd}$ homozygotes show a phenotype consistent with both eed and exed being deleted or inactivated. Furthermore, $c^{2YPSj}$ can complement the
extraembryonic defect by providing the wild-type copy of exed in c^{2}YPSj/c^{4}FR60Hd compound heterozygotes. All of the genetic and embryological data are consistent with the c^{4}FR60Hd distal breakpoint lying distal to the c^{2}YPSj distal breakpoint. Yet, our molecular data indicate that the c^{4}FR60Hd breakpoint lies proximal to that of c^{2}YPSj.

One possible explanation for the discrepancy between the genetic and molecular data is that the c^{4}FR60Hd deletion is discontinuous, thereby 'skipping' and not deleting the region containing D7Cw2D. Precedent for radiation-induced, non-continuous or 'skipping' mutations can be found within the dilute-short ear-deletion complex of mouse chromosome 9 (Russell 1971; Rinchik et al. 1986). At least five of the dilute-short ear deletions appear to 'skip' and inactivate genes on both sides of an active functional group. One possibility to explain the 'skipping' is that exed is deleted. Alternatively, exed could be inactivated because of a position effect imposed upon the locus by the c^{4}FR60Hd deletion. For example, the large chromosomal region between hsdrt-1 and c which is proximal to the c^{4}FR60Hd deletion is known to be devoid of CpG islands and, therefore, might represent particularly inactive DNA (Kelsey et al. 1992). Thus, it is possible that the deletion could juxtapose some heterochromatin or silencing region next to exed. Another possibility is that the distal side of the c^{4}FR60Hd deletion is associated with some sort of chromosomal rearrangement such as an inversion or translocation which breaks within exed, and thereby inactivates the locus. In any case, the
molecular basis for the inactivation of \textit{exed} by the \textit{c4FR60Hd} deletion will only be resolved when the breakpoint-fusion fragment has been cloned and a physical map of the region is completed.

Ordering of the \textit{c11DSD}, \textit{c5FR60Hg}, \textit{c2YPSj} and \textit{c6H} distal breakpoints established the molecular limits of the \textit{eed} and \textit{exed} genes. The region containing the \textit{exed} gene is delimited by the \textit{c2YPSj} deletion on the proximal side and the \textit{c6H} deletion on the distal side. The region of chromosome 7 containing the \textit{eed} gene is bounded on the proximal side by the \textit{c3H} distal breakpoint and on the distal side by either the \textit{c11DSD} or the \textit{c5FR60Hg} distal breakpoints.

Using a procedure similar to that described here to clone the \textit{c2YPSj} breakpoint-fusion fragment, the \textit{c11DSD} breakpoint-fusion fragment has now been cloned in our laboratory by Dr. Bernadette Holdener. The probe used to detect a unique restriction fragment associated with the \textit{c11DSD} deletion was pv0.4AP. It was isolated from a lambda phage which was obtained after walking and jumping from the \textit{D7Cw18} locus (Schedl et al. 1992). Mapping of the distal breakpoint, \textit{D7Cw11D}, with respect to the other deletions showed that it was deleted from \textit{c5FR60Hg}, \textit{c4FR60Hd}, \textit{c2YPSj} and \textit{c6H}. This result, therefore, suggests that the distal limit of the \textit{eed} region is defined by the distal breakpoint of the \textit{c11DSD} and not by the \textit{c5FR60Hg} distal breakpoint (Fig. 13).
Figure 13. Deletions that define the eed and the exed regions based on complementation and molecular analyses. eed is defined by the distal breakpoints of $c^3H$ and $c^{11DSD}$ deletions. exed is defined by the $c^{2YPSj}$ and $c^6H$ distal breakpoints. $D7Cw2D$ and $D7Cw11D$ represent the loci defined by the distal breakpoints of $c^{2YPSj}$ and $c^{11DSD}$ deletions, respectively. pv0.4AP represents a 0.4 kb Asp 718-Pst I fragment subcloned into Bluescript. The insert was derived from a phage $(\lambda V31)$ which was obtained after walking and jumping from $D7Cw18$ locus using palb18 (Schedl et al. 1992).
CHAPTER III: GENOMIC MAPPING TO REDEFINE LIMITS OF *eed*
REGION USING INDIVIDUAL EARLY POSTIMPLANTATION EMBRYOS

A. INTRODUCTION

Molecular analyses of DNA obtained from small amounts of cells or tissues, such as early mouse embryos, can be achieved by a limited number of techniques. One approach is the polymerase chain reaction (PCR) (Rappolee *et al.* 1988). Although the technique is widely used, the results may not always be reliable. Due to its extreme sensitivity, false positives or mistyping may occur because of the presence of small amounts of exogenous DNA molecules in the sample (Kwok and Higuchi 1989). Maternal contamination can be a severe problem in the analysis of implanted mouse embryos. To overcome this problem, the use of Southern blotting is described here that is sensitive enough to detect single-copy sequences in individual early postimplantation embryos but is not affected by the few contaminating maternal cells.

Previously, it has not been possible to use Southern blotting to analyze individual early postimplantation mouse embryos due to the limited amount of DNA obtained from standard phenol/chloroform extraction procedures. The loss of DNA at each extraction step often results in poor yields. A method to analyze human hair-root cells has been described that solves this problem (Boultonwood *et al.* 1990).
procedure involved embedding the hair root in agarose before lysing and deproteinizing in situ. The genomic DNA was then digested with restriction enzymes and analyzed by Southern blotting and hybridization. Here the application of this technique is described for the genetic analysis of single, early postimplantation mouse embryos which, at day 6.5, have approximately 115 ng of DNA. This amount represents 4.5-fold less DNA than that reported for the single hair root (Higuchi et al. 1988). Using this methodology, we have genotyped individual embryos homozygous for one of the albino deletions, c202G, which results in early postimplantation lethality and were able to map molecular markers relative to this deletion. These data further refine the albino-deletion complex map by re-defining the limits of the eed region of chromosome 7 containing a gene(s) needed for development of the embryonic ectoderm (Niswander et al. 1988; Niswander et al. 1989)

B. MATERIALS AND METHODS

1. Embryo Preparation and Southern Analysis

CF-1 or c202G/c females were naturally mated to males of the same genotype to obtain embryos which were dissected from the uterine horns at days 6.5, 7.5 and 8.5 of development (the day of vaginal plug was considered day 0.5). c202G is a deletion which removes a region of chromosome 7 surrounding and including the albino-coat-color locus
(c) (Russell et al. 1982). Individual embryos at day 6.5 and 7.5 of development were embedded along with their extraembryonic membranes in 60µl of 0.5% molten (65°C) InCert agarose (FMC) prepared in L Buffer (0.1M EDTA, 0.01M Tris pH7.5, and 0.02M NaCl) using an Insert mould (LKB Produkter, Sweden). The extraembryonic tissues were removed from day 8.5 embryos prior to embedding. The plugs were incubated in ESP (0.5M ETDA pH 9.5, 1% N-Lauroylsarcosine, 1mg/ml proteinase K) at 50°C for 24 hours. Plugs were incubated for another 24 hours in fresh ESP at 50°C before dialysing against 100 vol. of 1mM phenylmethylsulfonylfluoride at 4°C overnight. After rinsing, the plugs were stored in sterile distilled water at 4°C until use.

The embedded DNA was equilibrated in 500 µl of SuRE/cut Buffer H (Boehringer Mannheim Biochemicals) with 100 µg/ml BSA at room temperature for 2 hours, and then digested with 10 units of Eco RI (Boehringer Mannheim Biochemicals) in 50µl of Buffer H containing 100µg/ml BSA at 37°C for 5 hours. Individual plugs were loaded into a well of an 0.8% agarose minigel (5.5cm x 9cm) and sealed in place with 0.5% molten (65°C) LMT agarose (FMC) in 1X TBE buffer (0.09M Tris-borate, and 0.002M EDTA). Following electrophoresis at 50 volts for 3 hours, the DNA was transferred to Nytran membrane (Schleicher & Schuell) using standard procedures (Maniatis et al. 1982). The transferred DNA was crosslinked to the membranes using UV Stratalinker 1800 (Stratagene) and baked at 80°C.
for 1 hr. The membranes were hybridized with $^{32}$P-labeled probes at 65°C in Church Buffer (Church 1984) for 16 hours and washed three times for 15 minutes in a 0.2X SSC, 0.1% SDS solution at 65°C. Exposure times were typically 2-5 days.

2. Estimation of Total DNA Content per Embryo

To estimate total DNA content in embedded embryos, the agarose plugs containing individual embryos were treated with ESP and then melted at 65°C in 0.5 ml tubes. The molten solution was mixed thoroughly and cooled to 37°C. The concentration of DNA in the solution was determined using a DNA Fluorometer (Hoefer Scientific Instruments) following the procedure described by the manufacturer.

3. Genomic Clones

pH2.2H contains a 2.2-kb-Hind III-DNA fragment subcloned from p186+ (obtained from Dr. C. Goridis, Centre d'immunologie de Marseille-Luminy). p186+ carries a portion of the gene which encodes the neural-cell-adhesion molecule (NCAM). The NCAM gene has been mapped to mouse chromosome 9 (D'Eustachio 1985; Nguyen et al. 1986). The insert from pH2.2H was labeled with $^{32}$P-dCTP by random priming (Feinberg and Vogelstein 1984).

12A contains a 5.2-kb Eco RI fragment (Diesteche and Alder 1984)
that maps to chromosome 7 between c and Mod-2 (Saunders and Seldin 1990; Klebig et al. 1992). By genetic analysis, this region is predicted to be deleted by the c<sup>202G</sup>-deletion chromosome (Russell et al. 1982). 12A was labeled by random priming and then used as a probe.

p1.3(BII/P1) represents a 1.3-kb Bgl II-Pst I fragment subcloned from a 7.5-kb Xba I-Eco RI fragment containing the distal breakpoint of the c<sup>2YSj</sup> deletion. The 1.3-kb fragment was labeled by random priming and then used as probe.

pA4.2c11 represents a 4.2-kb Asp 718 fragment containing the distal breakpoint of the c<sup>11DSD</sup> deletion. A 2.9-kb Asp 718-Sph I fragment was labeled by random priming and then used as a probe.

C. RESULTS AND DISCUSSION

To determine the feasibility of Southern analysis on early postimplantation mouse embryos, wild-type embryos at day 6.5, 7.5, and 8.5 of gestation were tested. An autoradiograph of a Southern blot of Eco RI-digested DNA from wild-type CF-1 embryos hybridized with the pH2.2H insert shows a 5.9-kb band in all lanes (Fig. 14). The variation in intensity corresponds to the amount of DNA present at each embryonic stage (Table 1). The pH2.2H probe failed to detect any signal when individual blastocysts were used for analysis (data not
Figure 14. Southern blot analysis of *Eco* RI-digested DNA from individual early postimplantation mouse embryos hybridized with pH2.2H. Embryos were removed from uteri of pregnant females at day 6.5 (lanes 1-2), day 7.5 (lanes 3-4) and day 8.5 (lanes 5-6) of pregnancy and embedded in 0.5% InCert agarose. The agarose plugs were processed as outlined in the text.
Table 1: DNA Content of Early Postimplantation Embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>DNA content (ng)</th>
<th>± S.D.</th>
<th># Embryos Examineed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6.5</td>
<td>115</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td>Day 7.5</td>
<td>185</td>
<td>89</td>
<td>14</td>
</tr>
<tr>
<td>Day 8.5</td>
<td>834</td>
<td>279</td>
<td>13</td>
</tr>
</tbody>
</table>
shown). These observations indicate that Southern analysis of individual embryos can be done with embryos as early as day 6.5 in development.

The major factor responsible for optimizing hybridization signal on the embryo blots is concentration of DNA per unit area of the gel. Care must be taken to keep the embryos intact during the embedding procedure. If disrupted, individual cells disperse throughout the plug, and DNA is neither visible on ethidium bromide-stained gels nor is hybridization signal detected on Southern blots. The type of agarose used for embedding the embryos is also an important factor. For example, pulsed-field gel grade agarose (Boehringer Mannheim Biochemical) has greater tensile strength than either InCert or Sea Plaque (FMC) agarose. Consequently, the pulsed-field gel grade agarose is more convenient for positioning the embryos in the plugs. However, DNA obtained from embryos in these plugs could not be completely digested by restriction enzymes, and therefore, was unsuitable for use even at concentrations as low as 0.5%. For similar reasons, 1.0% SeaPlaque or InCert agarose plugs could not be used. Optimum results with respect to enzyme digestion and embryo positioning were found with 0.5-0.8% InCert agarose.

The above results demonstrate that this technique can be used to genotype early postimplantation mouse embryos possessing mutations with detectable DNA alterations. Such mutations are
normally identified by a retrospective analysis of abnormal embryos produced from a heterozygous cross as compared to those produced from a control cross. However, using agarose plugs, the mutant embryos can be genotyped directly by Southern blot analysis if a probe specific for the locus is available. We have used this technique to determine which embryos are homozygous for the $c^{202G}$-albino deletion. These embryos are known to die between implantation and midgestation; however, the exact period of lethality has not been defined (Russell et al. 1982). To determine the time at which abnormalities first appear, embryos at various stages of development were dissected from heterozygous females that had been mated to males of the same genotype. At day 7.5 of development, no mutant class of embryos could be identified by morphological differences from the pool of 51 examined. In contrast, at day 8.5, approximately 25% of the 205 embryos analyzed showed a distinct mutant phenotype (Figure 15B). The abnormal embryos progressed to the point where both embryonic and extraembryonic mesoderm were produced; however, unlike the wild-type littermate (Fig. 15A) there was no organization of the mesoderm into somites or notochord and no induction of the neural plate. The extraembryonic structures developed normally. This phenotype is identical to that described for embryos homozygous for either for the $c^{11DSD}$ or the $c^{2YPSj}$ deletion (Niswander et al. 1988; Niswander et al. 1989). $c^{202G}/c^{11DSD}$ double heterozygotes were phenotypically similar (data not shown).
Figure 15. Thick-section light micrograph of (a) day 8.5 wild type embryo and (b) littermate homozygous for the c^0^2G deletion. a: amnion cavity; am: amnion, eo: exocoelom; ee: embryonic ectoderm; ex: extraembryonic ectoderm; m: mesoderm; nt: neural tube; o: optic vesicle; p: parietal endoderm, s: somite; v: visceral endoderm; ys: yolk sac. Magnification: x40. Embryos were prepared for histological analysis as described by Niswander et al. (1988; 1989).
To genotype $c^{202G}$ homozygous embryos showing the abnormal phenotype, a Southern blot containing DNA of individual abnormal and normal embryos at day 8.5 of development was hybridized with the 12A probe which maps to the region of chromosome 7 covered by the deletion. A 5.2-kb Eco RI fragment was detected in DNA from a normal embryo (Fig. 16a, lane 1) but not in DNA from an abnormal embryo (Fig. 16a, lane 2). Figs. 16b and 16c, which are described below, represent rehybridizations of the same blots with probes that map outside the region of chromosome 7 covered by the $c^{202G}$ deletion. The presence of hybridization signal serves as positive control for DNA in both lanes. These results demonstrate that DNA from the embryo in lane 2 of Figure 16a is homozygous for the $c^{202G}$ deletion whereas DNA from lane 1 of the same figure is from a $c^{202G}/c$ or $c/c$ embryo. These data also show that individual early postimplantation mouse embryos carrying mutations with detectable DNA alterations can be genotyped by Southern blot analysis.

In addition to genotyping mutant embryos, we have used the embryo-blotting technique to map two cloned markers relative to the $c^{202G}$ deletion. Conventional mapping procedures rely on the ability to detect restriction-fragment-length-polymorphisms (RFLPs) in $M.\space musculus/M.\space spretus$ interspecies hybrids (for example, see (Johnson et al. 1989; Niswander et al. 1991)). Presence or absence of the RFLP associated with the $M.\space musculus$ deletion chromosome is used to map any probe outside or within the region covered by the deletion.
Figure 16. Southern blot of Eco RI-digested DNA from embryos obtained from c\textsuperscript{202G}/c females mated to males of the same genotype. Lane 1 contains DNA from a phenotypically normal embryo. Lane 2 contains DNA from a phenotypically abnormal embryo. (a) hybridized with random-primer labeled 12A. (b) same blot stripped and re-hybridized with random-primer labeled pA4.2c11. (c) same blot stripped and re-hybridized with random-primer labeled p1.3(BII/PI).
However, obtaining the desirable hybrid offspring and screening for RFLPs are time consuming. In contrast, by using DNA obtained from embyros homozygous for the deletion, the probe can be mapped directly. To illustrate this approach, the Southern blot presented in Figure 16a was stripped and rehybridized first with random-primer labeled pA4.2c11 (Fig. 16b) followed by a second stripping and rehybridization with random-primer labeled probe from p1.3(BII/PI) (Fig. 16c). The former represents the distal breakpoint of the c11DSD deletion which defines the D7Cw11D locus, whereas the latter represents the c2YPSj distal breakpoint which defines the D7Cw2D locus (see Fig. 17 for map). When hybridized with pA4.2c11, a 7.8-kb band was present in non-deleted DNA (Fig. 16b, lane 1) and in c202G homozygous deletion DNA (Fig. 16b, lane 2). Similarly, p1.3(BII/PI) detects a 16-kb band present in non-deleted (Fig. 16, lane 1) and in c202G -homozygous deletion DNA (Fig. 16c, lane 2). These results demonstrate that both D7Cw11D and D7Cw2D map outside the region covered by the c202G deletion.

The limits of eed were originally defined on the basis of genetic complementation analyses (Niswander et al. 1988; Niswander et al. 1989) and further refined as a result of the cloning and molecular mapping of the c11DSD and c2YPSj distal breakpoints as described in the previous chapter. These studies indicated that the D7Cw11D represented the closest molecular marker to eed and that D7Cw2D mapped distal to D7Cw11D. However, the mapping experiments
reported there were time consuming as they relied on the ability to detect RFLPs in appropriate intra- and/or interspecific hybrids. As a more direct approach, we have developed a method whereby embryos homozygous for the deletion of interest can be used directly for Southern analysis. In this study, data are presented on the molecular mapping of $D7Cw2D$ and $D7Cw11D$ relative to the $c^{202G}$ deletion using individual early postimplantation embryos. The molecular data indicate that the $c^{202G}$ distal breakpoint lies proximal to $D7Cw11D$ and $D7Cw2D$. Furthermore, the embryological analysis establishes that the $c^{202G}$ deletion removes $eed$. Together, these data define the $c^{202G}$ distal breakpoint as the new distal limit of the region of chromosome 7 containing $eed$ (Figure 17).
Figure 17. Complementation map of the albino deletions used in the present work. This map is modified from the map described in chapter II. Deleted regions are represented by gaps. Marker loci include \textit{tp} (taupe), \textit{c} (albino), \textit{Mod-2} (mitochondrial form of malic enzyme), \textit{sh-1} (shaker-1). \textit{D7Cw11D} and \textit{D7Cw2D} are genomic loci defined by cloned deletion-breakpoint-fusion fragments. The following loci are defined by complementation and embryological analyses using the various albino deletions: \textit{msd}: mesoderm deficient; \textit{hsdr-1}: hepatocyte-specific developmental regulator; \textit{jdf}: juvenile development and fertility; \textit{eed}: embryonic ectoderm development; \textit{exed}: extraembryonic ectoderm development; \textit{pid}: preimplantation development. The actual phenotype resulting from the deletion of any of these loci may be due to the removal of more than one gene. Symbols in the deleted region represent the name of the deletion chromosomes.
CHAPTER IV: MOLECULAR CHARACTERIZATION OF THE *exed* REGION.

A. INTRODUCTION

The extraembryonic ectoderm development (*exed*) region on mouse chromosome 7, 3-4 cM distal to the albino coat color locus (*c*), has been shown by embryological and complementation studies to be important for early postimplantation development (Niswander *et al.* 1988; Niswander *et al.* 1989). Embryos lacking this region develop normally until day 6.5 of development at which time they are indistinguishable from their wild-type littermates. In contrast, at day 7.5, unlike wild-type embryos undergoing the process of gastrulation, the embryonic ectoderm of mutant embryos resembles a 6.5 day egg cylinder showing no primitive streak formation or mesoderm induction (see fig. 3). In addition, the extraembryonic ectoderm fails to differentiate and begins to die. The mutant embryos will be resorbed within 24 hours.

The *exed* region was first identified and mapped on the basis of genetic analysis of five albino deletions (*c*11D5D, *c*5FR60Hg, *c*2YP5j, *c*4FR60Hd and *c*6H) known to result in early postimplantation lethality (Lewis *et al.* 1976; Russell 1979; Russell *et al.* 1982; Niswander *et al.* 1988; Niswander *et al.* 1989). Two of these deletions (*c*4FR60Hd, *c*6H), when homozygous, were found to result in the *exed* phenotype. In
contrast, embryos homozygous for the c11DSD, c5FR60Hg and c2YPSj deletions developed normally at this stage. Furthermore, these three deletions were capable of complementing the c4FR60Hd and c6H chromosomes, allowing for development of the extraembryonic structures in the double heterozygotes (Niswander et al. 1988; Niswander et al. 1989). Thus, the area of non-overlap between the two groups of deletions, i.e. the region between the distal breakpoint of c11DSD, c5FR60Hg and c2YPSj and the distal breakpoint of c4FR60Hd and c6H deletions defines the exed region (Fig. 8).

To understand the molecular basis of the exed phenotype, it is essential to identify, clone and characterize the gene(s) present in this region. The first step towards achieving this is goal is to identify the deletion breakpoints that define the limits of the region, clone these breakpoints and use them to estimate the size of the region. This is important for determining the most appropriate strategy for cloning the exed region. As described in Chapter II, this work was initiated by cloning the distal breakpoint of the c2YPSj deletion which was found to define the proximal limit of the region (Fig. 18).

To gain molecular access to the distal end of the exed region, cloning of the c6H distal breakpoint is necessary. This chapter describes the approach used to clone this breakpoint. Once cloned, the c2YPSj and c6H distal breakpoints were used to establish a YAC contig and generate a physical map of the exed region. A search for transcription
Figure 18. Deletions that define the *exed* region. Deleted regions are represented by gaps. Symbols in the deleted region represent the name of the deletion chromosomes. Marker loci include *tp* (taupe), *c* (albino), *Mod-2* (mitochondrial form of malic enzyme), *sh-1* (shaker-1). *D7Cw2D* is the genomic locus defined by distal breakpoint of *c2YP5j* deletion. The following loci are defined by complementation and embryological analyses using the various albino deletions: *msd*: mesoderm deficient; *hsdr-1*: hepatocyte-specific developmental regulator; *jdf*: juvenile development and fertility; *eed*: embryonic ectoderm development; *exed*: extraembryonic ectoderm development; *pid*: preimplantation development. The actual phenotype resulting from the deletion of any of these loci may be due to the removal of more than one gene.
units in the region resulted in isolation of a genomic sequence that
detects a 5.0 kb transcript in the adult brain.

B. MATERIALS AND METHODS

1. Mice:

   a. Deletion mice: Four albino-deletion stocks [designated \( Df(c) \) ]
   were used in the present work. The \( c^6H \) mice originated at the MRC
   Radiobiology Unit, Harwell, England, and were obtained from Dr. S.
   Waelsch (Albert Einstein College of Medicine). The \( c^2YPSI \), \( c^{AFR60Hd} \),
   and \( c^{14CoS} \) mice originated at the Oak Ridge National Laboratory and
   were provided by Dr. Eugene M. Rinchick. All mice have been
   maintained as closed colony, heterozygous stocks with chinchilla
   \( (c^{ch}) \). All \( Df(c)/c^{ch} \) stocks have a dilute chinchilla coat color as
   compared to chinchilla color evident in \( c^{ch}/c^{ch} \) mice.

   b. Mus spretus/Mus musculus interspecies cross: Wild-type (non-
      deletion) \( M. \ spretus \) males were crossed with \( c^{AFR60Hd}/c^{ch} M. \)
      musculus females. To determine which of the phenotypically wild-
      type \( F_1 \) progeny carried the deletion chromosome rather than the \( c^{ch} \)
      chromosome, progeny testing was carried out as described by Johnson
      et al. (1989).

   c. Genomic DNA extraction: High molecular weight genomic
      DNA was extracted from different organs (heart, brain, lung, kidney
and spleen) following the procedure described by Johnson et al. (1989).

2. Genomic libraries

*Lambda library:* A mouse 129SV genomic library (Stratagene) in lambda Fix II vector was used to isolate overlapping clones for chromosome walking.

3. Library screening

Each library screening included approximately $6 \times 10^5$ phages. Plaques were transferred to Hybond membrane (Amersham) and the filters were treated using standard protocols. Prehybridization and hybridization with radiolabeled probe ($2 \times 10^6 \text{cpm/ml}$) were performed at $65^0\text{C}$ in Church buffer (Church 1984). DNA fragments were radiolabeled with $\alpha^{32}\text{P}-\text{dCTP}$ by random priming method (Feinberg and Vogelstein 1984). Alternatively, end probes from lambda clones were made by using *Rsa I* or *Hae III* digested DNA as templates. $\alpha^{32}\text{P}-\text{UTP}$-labeled riboprobes were synthesized *in vitro* from the T3 and T7 promoters using the respective RNA polymerases. Background hybridization due to the presence of repetitive elements in the template DNA was suppressed by using cold mouse Cot-1 DNA (Gibco BRL). Filters were washed in $0.2 \times \text{SSC}$ and $0.1\% \text{SDS}$ at $65^0\text{C}$ and exposed to X-ray film for 16-20 hours at $-70^0\text{C}$ using intensifying screens. Generally two or three rounds of plaque
purification were required to obtain isolated positive plaques.

4. Phage DNA extraction

DNA from the isolated lambda clones was extracted following the procedure described by Ausubel et al. (1989). To increase the yield, NZC media (NZ amine A 10g, NaCl 5g, MgCl\textsubscript{2}.6H\textsubscript{2}O 2g and 20% Casamino acids 5 ml per 1L of water) was replaced by LB10 (Trypton 10g, NaCl 10g, Yeast Extract 5g and 10ml of 1M MgSO\textsubscript{4} per 1L of water).

5. Yeast Artificial Chromosome (YAC) Library

A large insert (average insert size is 650 kb) mouse YAC library at Princeton University was screened (Kusumi et al. 1993). The library was made with C57BL/6J female mouse DNA. Genomic DNA was cloned into the Eco RI site of pYAC4 vector and transformed into the host yeast strain AB1380.

a. Library screening: 19,200 YAC clones stored in 96 well microtiter plates were screened using a Polymerase Chain Reaction- (PCR) based assay (Green and Olson 1990), providing the equivalent of approximately 4 haploid mouse genomes. The entire library has been grouped together in 25 blocks or ‘super-pools’ of 8 microtiter plates each. The first level of screening identifies the positive super pool.
Each super pool is further grouped into 28 sub-pools which includes: 8 microtiter plate pools, 8 row pools, and 12 column pools. PCR assays of 28 sub-pools identify the 96-well array as well as the coordinates of the positive clone within the array.

b. PCR primers: PCR analysis was performed on 10 μl of each super-pool and sub-pool DNA sample. PCR was carried out in a 50 μl final volume using 0.25 mM of each primer, 0.2 mM nucleotides and 2.5 units of Taq DNA polymerase (Boehringer Mannheim) in 1 X Taq Buffer. Following an initial denaturation step (94°C for 5 min.), amplification was achieved through 30 cycles at 94°C for 30 sec., 55°C for 30 sec., and 72°C for 1 min. The following set of primers from p1.4SacI (see 'genomic clones' section for description) were used for amplification: 5'-dGTCAGCACACATCAATACAT-3' and 5'-dCCCATAATCCACTCATAAG-3'. The PCR products were resolved on a 1.0% agarose gel. The results were confirmed by hybridizing the amplified product with radiolabeled probe on Southern blot.

c. Characterization of YAC clones: High molecular weight DNA from YAC strains was prepared in agarose plugs (Smith et al. 1988) and separated by pulsed-field gel electrophoresis in a 0.7% agarose gel (Seakem FMC.) using contour-clamped homogeneous electric field (CHEF) gel electrophoresis (DRII, Biorad). The gel was run in 0.5 X TBE Buffer (45 mM Tris Borate, 1 mM EDTA) for 24 hours at 200 volts with switching time ramped from 80 to 90 seconds.
d. Restriction Digest analysis of YACs: Agarose plugs containing approximately 3-4 mg of yeast chromosomal DNA were equilibrated in 500 µl of appropriate 1 X restriction enzyme buffer (New England Biolabs) with 100 µg/ml BSA at room temperature for 2 hours. After equilibration, the digestion was performed in 50 µl of 1X restriction enzyme buffer with 100 µg/ml BSA and 5 units of restriction enzyme per µg of DNA at $37^0$C ($Sma$ I digests were performed at $25^0$C) for 10-12 hours. Plugs were then loaded on 1.0% agarose gel in 0.5 X TBE buffer and DNA was separated on CHEF gel electrophoretic apparatus. The gel was run at 200 volts with a switching time ramped from 40 to 70 seconds for 24 hours. After staining with ethidium bromide, the DNA was nicked by exposing the gel to UV light (312 nm) for 5 minutes. The DNA was then transferred to Nytran membrane (Schleicher & Schuell) by standard Southern blotting. Hybridization and washing conditions were the same as described for the library filters.

6. Northern blot

A Northern blot containing polyA+ RNA from various adult tissues was purchased from Clontech Inc. The blot was prehybridized and hybridized with radiolabeled probes and then washed under conditions similar to that described above for genomic library filters.
7. Genomic Clones

(Map positions of all the genomic clones used in this study are shown in figures 19 and 23.)

\textbf{p2.4XI/H3} represents a 2.4 kb \textit{Xba I/Hind} III fragment cloned into \textbf{pBS+/-} (Stratagene) from the \textit{D7Hd1} locus (Holdener and Magnuson, unpublished result). The fragment was obtained from a \textit{lambda} genomic clone isolated from the \textit{D7Hd1} locus on chromosome 7 which is defined by pJLE1.7 (Kelsey \textit{et al.} 1992)

\textbf{\lambda6P-1/1} is a \textit{lambda} clone with an insert (15 kb) isolated from the genomic library using probe p2.4XI/H3 from the \textit{D7Hd1} locus. The end probes were made from \textit{Hae} III-digested DNA using T3 and T7 RNA polymerase (Boehringer Mannheim).

\textbf{\lambda6P-2/3} represents a 12 kb genomic fragment isolated from the genomic library using riboprobe generated from T3 end of \textbf{\lambda6P-1/1}.

\textbf{700H3} represents a 700 bp \textit{Hind} III fragment that was obtained from \textbf{\lambda6P-2/3}. It maps 18-20 kb distal to p2.4XI/H3 and detects the \textit{c6H} breakpoint-fusion-fragment. The 700 bp fragment was used as a probe after random primer labeling.

\textbf{p3Bc6j} represents a 3.0 kb \textit{Bam HI} fragment containing the \textit{c6H} breakpoint-fusion fragment. It was isolated from a subgenomic
library generated in λ Zap II vector (Stratagene). The insert DNA was prepared from 50 μg of genomic DNA extracted from c6H/c14Co5 mice and digested to completion with Bam HI restriction enzyme. The digested fragments were separated on a 0.8% agarose gel (SeaPlaque, FMC) in 1X TAE (40 mM Tris-acetate and 1 mM EDTA) buffer. DNA fragments in the 2.8 - 3.2 kb approximate size range were extracted from the gel using the QIAEX extraction system (Qiagen) following the procedure provided. The λ Zap II vector DNA was digested with Xho I restriction enzyme after the cos sites were ligated. The Bam HI ends of insert DNA were partially filled with dATP and dGTP while the Xho I ends of vector arms were filled with dTTP and dCTP using Klenow enzyme (Boehringer Mannheim) to generate compatible ends. Approximately 50 ng of insert DNA was ligated to 680 ng of vector DNA using T4 DNA ligase at 4°C for 15 hours and then packaged in vitro using phage packaging extract (Promega) at room temperature. The packaged phage particles were used to infect XL1-Blue (Stratagene) host cells. The plaques were screened with random primer labeled 700H3 fragment following standard procedures described above for genomic library screening. The positive plaques were isolated after two rounds of purification. The Bluescript SK plasmids, containing the inserts, were excised using an in vivo excision protocol described by Stratagene and then transformed into Escherichia coli DH5α cells.

1.9H3 is a 1.9 kb Hind III fragment from the distal end of p3Bc6j
which contains the distal breakpoint of $c^6H$ deletion. Due to the presence of repetitive elements, the radiolabeled probes were soaked with cold mouse Cot-1 DNA (Gibco BRL) prior to hybridization with genomic DNA.

p1.3BII/PI represents a 1.3 kb Bgl II/Pst I fragment subcloned from p7.5(X/RJ) into pBS +/- (Stratagene) and defines the D7Cw2D locus, the distal breakpoint of $c^2YPSj$ deletion.

$\lambda$2D-1/2 and $\lambda$2D-1/4a represent two lambda clones with 15 kb and 19 kb inserts, respectively. The two clones were isolated from the 129SV mouse genomic library using the probe p1.3BII/PI.

p1.4SacI and p2.2SacI represent Sac I fragments, 1.4 kb and 2.2 kb in size, respectively, subcloned from $\lambda$2D-1/4a into pBSIISK (Stratagene).

p1.3H3 represents a 1.3 kb Hind III fragment that was subcloned into pBSIISK (Stratagene) from the genomic clone $\lambda$2D-1/2.

8. Cloning of the 15 kb Sal I/Sma I fragment from the exed region

To clone a 15 kb Sal I/Sma I fragment from the exed region which could not be isolated from the genomic library, a subgenomic library was generated from YAC clone D7YACB3. 50 µg of total DNA was digested with Sal I and then with Sma I restriction enzymes. The
digested DNA fragments were separated on a 0.5% agarose gel (Pulsed-field gel grade, Boehringer Mannheim) in 1 X TAE buffer. DNA fragments in the size range of 15 kb were extracted from the gel using β-Agarase I (New England Biolabs). Phosphorylated Sal I linkers were ligated to the 15 kb DNA fragments and then digested with Sal I. The digested linkers were separated from the 15 kb insert DNA on a 0.7% agarose gel (SeaPlaque, FMC) and eluted from the gel using β-Agarase I (New England Biolabs). The Insert DNA was then dephosphorylated by treating with Shrimp alkaline phosphatase (US Biochemicals). The λ Dash (Stratagene) vector DNA was digested with Sal I restriction enzyme and then with Eco RI to generate the vector arms with Sal I ends and stuffer region with Eco RI ends. Approximately 25 ng of the insert DNA was ligated to 135 ng of the vector DNA using T4 ligase (Boehringer Mannheim) at 40°C for 12 hours. The ligated DNA was packaged at room temperature for 2 hours using in vitro packaging extract (Promega). Approximately 10^5 recombinant phages were screened with random primer labeled p1.3H3, following the procedure described for genomic library screening.
Figure 19. Restriction map of the region around the proximal breakpoint of $c^{6H}$ deletion. Restriction sites are shown on wild-type chromosome at the top (H3 is Hind III; S is Sal I). Stippled boxes at the bottom of the wild-type chromosome show the location of some of the genomic clones used in the present work. jLE1.7 represents a genomic clone isolated from a Sma I jumping library (Schedl et al. 19992). 2.4XI/H3 is the 2.4 kb Xba I-Hind III fragment. It was used to initiate a chromosome walk towards the proximal breakpoint of the $c^{6H}$ deletion. $\lambda$6P-1/1 and $\lambda$6P-2/3 represent the two lambda clones that were isolated to reach this breakpoint. 700H3 represents a 700 bp Hind III fragment, subcloned from $\lambda$6P-2/3, that detects the $c^{6H}$ breakpoint-fusion-fragment as shown in Figure 20.
C. RESULTS

1. Walking towards the $c^{6H}$ proximal breakpoint:

Molecular mapping results described in Chapter II show that the distal limit of the $exed$ region is defined by the distal breakpoint of the $c^{6H}$ deletion. No DNA probe is available close to this breakpoint. However, a DNA marker from the $D7Hd1$ locus, pJLE1.7, that maps within 50 kb of the proximal breakpoint of $c^{6H}$ deletion can be used to walk towards the breakpoint-fusion-fragment (Fig. 19) (Niswander et al. 1991; Kelsey et al. 1992). Cloning of this fusion fragment containing portions of genomic sequences from proximal and distal ends of the $c^{6H}$ deletion will provide molecular access to the distal end of the $exed$ region. Therefore, a chromosome walk was initiated from the $D7Hd1$ locus using a genomic clone, p2.4Xi/H3 which was found to be a more suitable probe than pJLE1.7 (Holdener and Magnuson, unpublished result). Overlapping clones were isolated from a mouse 129SV genomic library (Stratagene). Four clones were obtained from the first step using p2.4Xi/H3 as a probe. The inserts were oriented relative to the chromosome by comparing their restriction maps with the genomic restriction map generated by Kelsey et al. (1992) and Schedl et al. (1992) (Fig. 19). The clone containing the most distal fragment was identified by generating riboprobes from the two ends of each clone and hybridizing them individually to Southern blots containing DNA from all four lambda
clones. The most distal end should hybridize only to the clone from which it is generated. On this basis, the T3 end of the genomic clone, λ6P-1/1 with a 15 kb insert was found to be the most distal end (data not shown, Fig. 19). It was then hybridized against $c^{6H}/c^{ch}$, $c^{6H}/c^{14CoS}$ and $c^{14CoS}/c^{14CoS}$ DNAs to see if it detected the $c^{6H}$ breakpoint. Since the probe did not show any aberrant fragment compared with $c^{6H}$ deletion DNA (which would mark the presence of a DNA rearrangement as compared to wild-type DNA), it was used to take a second step by rescreening the genomic library. A genomic clone, λ6P-2/3, with a 16 kb insert was subsequently isolated (Fig. 19). The insert was oriented by hybridizing riboprobes generated from the two ends to λ 6P-1/1. The T3 end was found to be distal, because, unlike the T7 probe, it failed to hybridize to λ 6P-1/1 DNA. However, presence of repetitive elements made this riboprobe unsuitable for subsequent work, therefore, a 700 bp Hind III fragment, 700H3, containing single copy sequence was isolated from the T3 end of the λ6P-2/3 clone. When hybridized to Bam HI digested $c^{6H}/c^{ch}$, $c^{6H}/c^{14CoS}$ and $c^{14CoS}/c^{14CoS}$ genomic DNAs, the 700H3 probe detected a 1.5 kb fragment in non-deleted $c^{ch}$ DNA (Fig. 20B, lane 1) where as in $c^{6H}$ deletion DNA a 3.0 kb fragment was observed (Fig. 20B, lane 2). Since this region of the genome is deleted from the $c^{14CoS}$ chromosome, no signal was obtained in $c^{14CoS}/c^{14CoS}$ genomic DNA (Fig. 20B, lane 3). Furthermore, an altered fragment associated with $c^{6H}$ DNA was also detected when digested with other restriction enzymes (data not shown). These observations suggested that the
Figure 20. Detection of the \( c^{6H} \) breakpoint-fusion-fragment. (A) Complementation map of the relevant deletion chromosomes. \( c^{ch} \) represents the non-deleted chromosome, \( c^{14CoS} \) and \( c^{6H} \) are the deletion chromosomes. (B) Southern blot analysis of \( \text{Bam HI} \)-digested DNA hybridized with random primer labeled 700H3. The 3.0 kb band represents the putative \( c^{6H} \) breakpoint-fusion-fragment. Lanes 1-3 represent DNA from the following animals: (1) \( c^{14CoS}/c^{6H} \); (2) \( c^{ch}/c^{6H} \); (3) \( c^{14CoS}/c^{14CoS} \). Presence of DNA in lane 3 is shown in fig. 21 B where the same blot was stripped and reprobed.
altered fragment detected in \( c^{6H} \) DNA could be due to a DNA rearrangement rather than a restriction fragment length polymorphism, and therefore, may contain the \( c^{6H} \) breakpoint-fusion fragment.

2. Cloning and Mapping of the \( c^{6H} \) breakpoint fusion fragment:

To determine whether the 3.0 kb \textit{Bam HI} fragment detected by 700H3 contains the \( c^{6H} \) breakpoint-fusion fragment, a subgenomic library in \textit{lambda} Zap vector (Stratagene) was generated from 2.8 - 3.2 kb \textit{Bam HI} fragments of \( c^{6H}/c^{14CoS} \) genomic DNA. Approximately 3 \( \times 10^5 \) plaques were screened and five positive clones were isolated. Three of the five clones successfully excised out the Bluescript SK-plasmid containing the insert from the \( \lambda \) Zap II vector. The plasmid was transformed into \textit{E. coli} DH5\( \alpha \) cells and used for further analysis.

To characterize the cloned fragment, the insert was random-primer labeled and hybridized to \textit{Bam HI} digested \( c^{6H}/c^{ch} \), \( c^{6H}/c^{14CoS} \) and \( c^{14CoS}/c^{14CoS} \) genomic DNAs (Fig. 21B). In \( c^{14CoS}/c^{14CoS} \) DNA, a 2.3 kb fragment was observed (Fig. 21B, lane 3) while in \( c^{6H}/c^{14CoS} \) DNA two fragments, 2.3 kb and 3.0 kb in size were seen (Fig. 21B, lane 1). In \( c^{6H}/c^{ch} \) DNA three fragments that were 1.5 kb, 2.3 kb and 3.0 kb in size were detected (Fig. 21B, lane 2). The fact that 700H3 failed to detect any band in \( c^{14CoS}/c^{14CoS} \) DNA, whereas the 3.0 kb \textit{Bam HI} fragment (cloned from \( c^{6H}/c^{14CoS} \) DNA using 700H3 as a probe)
Figure 21. Mapping of the $c^6H$ breakpoint-fusion-fragment. (A) Complementation map of the relevant deletion chromosomes. $c^{ch}$ represents the non-deleted chromosome, $c^{14CoS}$ and $c^6H$ are the deletion chromosomes. Vertical lines are schematic representations of the regions of each chromosome to which the $c^6H$ breakpoint-fusion-fragment would hybridize. The hatched box below the $c^6H$ chromosome shows the location of the 3.0 kb breakpoint-fusion-fragment, p3Bc6j. (B) Southern blot analysis of Bam HI-digested DNA hybridized with random primer labeled p3Bc6j. Lanes 1-3 represent DNA from the following animals: (1) $c^{14CoS}/c^6H$; (2) $c^{ch}/c^{6H}$; (3) $c^{14CoS}/c^{14CoS}$. 
hybridizes to the same DNA \((c^{14}CoS/c^{14}CoS)\), suggests that the insert contains DNA from two different regions of the genome (Fig. 21A). These two regions are the proximal and the distal breakpoints of \(c^6H^\) deletion. Therefore, the 2.3 kb band seen in all lanes is from the distal end of the \(c^6H^\) deletion. In addition, the 3.0 kb band present in \(c^6H/c^{4h}\) and \(c^6H/c^{14}CoS\) DNA is from the \(c^6H\) DNA and marks the presence of the deletion junction fragment. Finally, the 1.5 kb band in \(c^6H/c^{4h}\) DNA is from the proximal end of the \(c^6H\) deletion. These results confirm that the 3.0 kb \(Bam\ HI\) fragment detected by 700H3 contains the breakpoint-fusion fragment of \(c^6H\) deletion. Thus, the distal end of this \(Bam\ HI\) fragment defines the genomic locus \(D7Cw6D\), the distal breakpoint of \(c^6H\) deletion, and also the distal limit of the \(exed\) region.

3. Isolation of YAC clones from the \(exed\) region:

The proximal limit of the \(exed\) region is defined by the \(D7Cw2D\) locus, the distal breakpoint of \(c^2YPSj\) deletion. PCR primers from a genomic clone, p1.4SacI, isolated from this region were used to screen a mouse YAC library. Five clones, 2DYACB3, 2DYACB7, 2DYACB8, 2DYACB14 and 2DYACB22 with insert sizes of 500 kb, >1.6 Mb, 700 kb, 785 kb and >1.2 Mb were isolated. All five inserts hybridized to P1.9H3, the probe that defines the distal limit of \(exed\) region, suggesting that the maximum size of the \(exed\) region is 500 kb, the size of the smallest YAC insert.
4. Estimation of size of the *exed* region:

Size of the *exed* region was estimated by determining the distance between the two loci, *D7Cw2D* and *D7Cw6D*, defining the proximal and distal limits, respectively. When hybridized to DNA isolated from the three smaller YAC clones, the p1.4SacI and p1.9H3 probes detected common restriction fragments. The smallest fragment common to both was a 40 kb *Sal* I fragment (Fig. 22 A and B).

5. Cloning the *exed* region:

To clone the region between the two breakpoints that define the *exed* region, a genomic library was screened using p1.3BII/PI (Fig. 23, probe 3). Several genomic clones spanning the proximal 25 kb of this region were isolated. The clones were oriented by comparing the restriction maps of these clones with that of λ14Rlc2, the lambda clone containing the c2*YPSj* breakpoint-fusion fragment (Chapter II). Clones with most distal ends were identified by mapping the T3 and T7 end probes. The T3 end of λ2D-1/4a was identified as the most proximal end and the T3 end of λ2D-1/2 as the most distal (Fig. 23). A single copy probe, p1.3H3 (Fig. 23, probe 6), was obtained from the T3 end of λ2D-1/2 and was used to rescreen the genomic library. No new genomic clone was obtained from the library. Later, the library was replated and screened using p1.3H3. None of the clones had
Figure 22. Estimation of size of the *exed* region. Southern blot analysis of *Sal* I (lanes 1, 2, and 3) and *Sma* I (lanes 4, 5, and 6) YAC clone DNAs. (A) The blot was hybridized with random primer labeled p1.4SacI probe. (B) The same blot was stripped and reprobed with random primer labeled p1.9H3. Both probes hybridize to the same 40 kb *Sal* I fragment. Lanes 1-6 represent DNA from the following YAC clones: lanes 1 and 4, *D7YACB3*; lanes 2 and 5, *D7YACB8*; lanes 3 and 6, *D7YACB14*. 
inserts from the region beyond p1.3H3. In addition, no clone could be isolated when the library was screened with p1.9H3, the probe defining the c^6H distal breakpoint (Fig. 23 probe 7). It was then concluded that the region distal to p1.3H3 was not present in the library. Another mouse genomic library in a cosmid vector (received from Dr. Evan Deneris, CWRU) was then screened with p1.3H3 and p1.9H3. No clones were isolated. When this library was screened with p2.2SacI, a probe that maps about 12-14 kb proximal to p1.3H3, two clones with insert size of 15-20 kb were isolated. However, none of these hybridized to p1.3H3 or p1.9H3.

Since no clone from the distal end of the exed region was obtained from two genomic libraries, a subgenomic library was made from the YAC DNA isolated from this region. It was found that p1.5H3 and p1.9H3, probes from the distal end of exed region, hybridize to the same 15 kb Sma I/Sal I fragment on a Southern blot containing restriction digested YAC DNA (Fig. 24A and B, lane 1). Therefore, the total YAC DNA was digested with Sal I and Sma I restriction enzymes, and the fragments in 15 kb size range were cloned into lambda Dash (Stratagene) vector. No positive clones were obtained from 10^5 recombinant phages screened with p1.3H3. The reason for the inability to clone this region is not known. Recombination between repetitive elements has been known to result in instability of recombinant clones (Sambrook et al. 1989). Such 'unclonable' sequences have also been found in Drosophila (Surdej et al. 1990).
6. Mapping the breakpoints within the 40 kb *Sal*I fragment:

To determine the precise location of the c^{2YPSj} breakpoint in the 40 kb *Sal*I fragment, the restriction maps of the genomic clones, λ2D-1/2 and λ2D-1/4a, were compared to the clone containing the breakpoint. The breakpoint was found to be within a 4 kb *Sac*I fragment that mapped 13-15 kb distal to the proximal *Sal*I site (Fig. 23). The exact location of the c^{6H} breakpoint in the 40 kb *Sal*I fragment could not be determined due to failure to obtain any genomic clone from the region. However, this breakpoint was found to be within 9 kb of the p1.3H3 probe. This was concluded from the observation that p1.3H3 and p1.9H3 hybridize to the same 9 kb *Sma*I/*Xho*I fragment (Fig. 23A and B, lane 2). Further, the distal breakpoint of c^{2YPSj} deletion is 10-12 kb proximal to p1.3H3 (Fig. 23) suggesting that the maximum distance between the two breakpoints that define the *exed* region is approximately 20 kb.

7. Identification of a transcription unit in the *exed* region:

The genomic sequences isolated from the *exed* region were analysed for the presence of a transcription unit. DNA fragments (2.5 kb, 4.0 kb and 6.0 kb *Sac*I fragments) were used as probes to hybridize against Northern blots made from PolyA⁺ RNA extracted from various adult tissues. A 5 kb transcript was seen in brain when hybridized to the 6.0 kb *Sac*I fragment. Subsequently, a 1.8 kb *Pst*I
Figure 23. Restriction map of the *exed* region. Restriction sites are shown on the wild-type chromosome at the top (S represents the *Sac I* restriction site). Limits of the *exed* region are shown by the distal breakpoints of the *c²YP5j* and *c⁶H* deletions. Stippled boxes below the wild-type chromosome show the location of the following genomic clones described in the present work: (1) p1.4Sacl; (2) p2.2Sacl; (3) p1.3BII/PI; (4) p1.8PstI; (5) region from the T7 end of p7.5(X/RI) from which riboprobes were transcribed as described in chapter II; (6) p1.3H3; and (7) p1.9H3. λ2D-1/4a and λ2D-1/2 represent the overlapping lambda clones isolated from the *exed* region.
Figure 24. Estimation of the distance between p1.3H3 and p1.9H3.
Southern blot analysis of Sal I + Sma I (lanes 1) and Xho I + Sma I (lane 2) digested D7YACB3 YAC clone DNA. (A) The blot was hybridized with random primer labeled p1.5H3 probe. (B) The same blot was stripped and reprobed with random primer labeled p1.9H3. Both probes hybridize to the same fragments.
fragment, p1.8PstI (Fig. 23, probe 4) was isolated from the 6.0 kb Sac I fragment which detected the same 5.0 kb transcript in the brain tissue on the Northern blot.

8. Mapping of the cloned portion of exed region relative to \( c^{4FR60Hd} \):

The region around p1.8PstI was mapped relative to the \( c^{4FR60Hd} \) deletion which shows the exed phenotype, like the \( c^{6H} \) deletion (Niswander et al. 1989). p2.2SacI, which is one of the most proximal probes available (Fig. 23, probe 2) was mapped relative to this deletion. It was hybridized to Pst I-digested DNA from an F1 hybrid that carried a Mus spretus non-deleted chromosome 7 and a M. musculus \( c^{4FR60Hd} \) deletion chromosome (Fig. 26). A Pst I polymorphism was detected between the two species. A 5.0 kb fragment is detected in M. spretus (Fig. 26, lane 1) while in M. musculus a 2.7 kb fragment is present (Fig. 26, lane 3). Presence of a M. musculus specific band in F1 hybrid (Fig. 26 lane 2) shows that p2.2SacI is present in \( c^{4FR60Hd} \) deletion chromosome. The region distal to p1.8PstI was shown in chapter II (Fig. 11) to be present in this deletion chromosome. Therefore, it can be concluded from these results that the region surrounding probe p1.8PstI is present in \( c^{4FR60Hd} \) deletion.
Figure 25. Detection of a 5 kb transcript in adult brain. (A)
Hybridization of a multiple tissue Northern blot with p1.8PstI. Each lane contains 2 µg of polyA⁺ RNA. Lanes 1-8 represent the following adult tissues: (1) testis; (2) kidney; (3) skeletal muscle; (4) liver; (5) lung; (6) spleen; (7) brain; and (8) heart.
(B) Rehybridization of the same blot with β-actin cDNA probe to show the presence of RNA in each lane.
Figure 26. Mapping of p2.2SacI relative to $c^{4FR60Hd}$ deletion.

Southern blot analysis of Pst I digested DNA. Lanes 1-3 represent DNA from the following animals: (1) Mus spretus/M. spretus, (2) M. spretus/M. musculus ($c^{4FR60Hd}$) and (3) M. musculus ($c^{ch}$)/M. musculus ($c^{ch}$). The blot was hybridized with random primer labeled insert of p2.2 SacI.
D. DISCUSSION

One of the regions in the albino deletion complex that is required around the time of gastrulation is the extraembryonic ectoderm development (*exed*) region. The molecular basis of the phenotype resulting from lack of this region is not known. A molecular characterization of this region, which is defined by the distal breakpoints of two of the albino deletions, *c2YPSj* and *c6H*, is described here. Cloning of the distal breakpoint of *c2YPSj* deletion (described in Chapter II) which resulted in the isolation of the probe that defined the proximal limit of *exed* region. Cloning of the *c6H* breakpoint fusion fragment, as reported here, has provided a molecular marker for the distal end of the *exed* region. The distance between the two breakpoints has been estimated to be approximately 20 kb.

A Northern blot containing polyA⁺ RNA from various adult tissues was used to identify transcription units in the cloned portion of the *exed* region. A 5.0 kb transcription unit was detected in adult brain. The fragment used as a probe maps to the region which is deleted in the *c6H* deletion, indicating that this 5.0 kb transcript must be physically disrupted in the mutant chromosome and could be responsible for the *exed* phenotype. Expression of the gene in adult brain does not diminish its possible role in embryonic development. Several genes are known to be expressed at various developmental stages as well as in certain adult tissues. For example, *Pax8*, a murine
paired box gene is expressed in the developing excretory system and thyroid gland as well as in adult kidney (Plachov et al. 1990).

$c^{4FR60Hd}$ deletion and the exed gene

Based on complementation analysis and embryological studies, the $c^{4FR60Hd}$ deletion, like the $c^{6H}$ deletion, has also been proposed to remove the exed region (Niswander et al. 1989). Molecular mapping studies described in Chapter II have shown that the distal breakpoint of $c^{4FR60Hd}$ deletion was proximal to the distal breakpoint of $c^{2YPSj}$ deletion, which defines the proximal limit of exed region. This suggests that if $c^{4FR60Hd}$ deletion is a simple deletion with two breakpoints, then the region containing the exed gene(s) should be present in this chromosome. This prediction is contradicted by the phenotype of embryos homozygous for the $c^{4FR60Hd}$ deletion. To explain the results obtained from genetic as well as molecular studies, the $c^{4FR60Hd}$ deletion was proposed to be a complex deletion. In the work described here, the probe that detects the 5.0 kb transcript is from the region that has been shown to be present in the $c^{4FR60Hd}$ deletion. However, this result does not rule out the possibility that the exed gene is mutated in the $c^{4FR60Hd}$ deletion. This deletion could be associated with a second abnormality that disrupts the exed gene. Further molecular characterization of this region and the $c^{4FR60Hd}$ deletion would provide answers to these problems.
Future experiments

Future experiments will be focused on characterizing the 5 kb transcript expressed in adult brain and also on determining if it is the gene responsible for the exed phenotype. The gene will be characterized by isolating cDNA clones from a mouse brain cDNA library. Determining the sequence of the cDNA and comparing it with sequences stored in the database will be the next step. This will show whether the cloned gene codes for a novel mouse protein. It will also identify any homologous gene known in some other organism or determine the presence of any functionally important domain known in some other protein(s).

There are essentially two requirements for any gene to be a candidate gene responsible for the exed phenotype. First, the gene must map to the exed region. This can be shown by mapping the cDNA inserts using the deletions that define the region. The gene should be completely or partially deleted from the c6H chromosome which shows the exed phenotype. The second requirement is that the gene should be expressed in wild-type embryos prior to day 7.5 of development, the time of manifestation of the mutant phenotype. Sensitive techniques that can detect rare messages can be employed for expression studies in embryos. Some of these techniques include RNase protection assay (Kinloch et al. 1993) or amplification of mRNAs using Reverse Transcription-PCR (Arcellana-Panlilio and
Schultz 1993). If feasible, the precise domain of expression within the embryos will be identified by in situ hybridization techniques (Wilkinson and Neito 1993).

If the cloned gene is a suitable candidate, then two approaches can be taken to determine whether it is the exed gene. One of the approaches is to test the ability of the candidate gene to rescue the mutant phenotype in transgenic c<sup>6H</sup> homozygous embryos. The transgenic mice, however, would not be viable and the embryos are expected to exhibit the eed phenotype at day 8.5 of development. This is due to lack of the other functionally important regions including the embryonic ectoderm development region in c<sup>6H</sup> deletion chromosome. To generate transgenic animals, DNA constructs containing the candidate gene will be injected into fertilized eggs (Hogan et al. 1986). To mimic the endogenous expression pattern, the gene should be under the control of its own promoter and other cis-regulatory sequences. Identification of such sequences may be difficult; however, a new technique is now available to overcome this problem. It involves introduction of large genomic fragments, about 150-600 kb in size cloned into Yeast artificial chromosomes directly into either ES cells or the pronucleus of fertilized oocytes (Strauss and Jaenisch 1992; Jakobovits et al. 1993; Schedl et al. 1993). Since several YAC clones are available from the exed region, the technique can be conveniently employed to generate transgenic mice. By setting up appropriate crosses, c<sup>6H</sup>/c<sup>ch</sup> animals homozygous for the transgene
will be obtained. When these animals are mated, then at day 7.5 of gestation, all the embryos should show the wild-type phenotype if the candidate gene is the \textit{exed} gene.

Disrupting the candidate gene in wild-type embryonic stem cells by gene targeting is the second approach that can be taken to analyze the candidate gene (Capecchi 1989). Mutant stem cell lines can then be used to generate chimeric mice, which can be bred to obtain heterozygous animals. Homozygous mutant embryos obtained from these animals can be examined at day 7.5 of gestation. Phenotypic similarity between embryos homozygous for the mutant candidate gene and the $e^{5H}$ deletion would provide evidence to support that the candidate gene is \textit{exed}.

Failure to rescue the mutant phenotype in transgenics, or absence of \textit{exed} phenotype in embryos lacking the candidate gene would suggest that the candidate gene is not \textit{exed}. Since the distal 9 kb of the \textit{exed} region has not been searched for transcription units, the presence of a second gene in the region cannot be eliminated. In addition, the phenotype seen in deletion homozygotes could be the result of the combinatorial effect of the absence of more than one gene instead of a single gene defect. Putative point mutants are ideal to analyze this. As described in chapter I, such mutants are being generated by using \textit{N-ethyl-N-nitrosourea} (ENU) in a saturation mutagenesis experiment (Rinchik \textit{et al.} 1990). Isolation of putative
point mutants from the exed region, their complementation and phenotypic analysis will help to determine the number of genes that are responsible for the mutant phenotype.

If the exed gene is not found in region defined by the deletions, then the possibility of the mutant phenotype resulting due to positional effects will have to be considered. In this case the phenotypic effect of the deletions would be due to inactivation of gene(s) near the breakpoints and not due to their absence. Such an inactivation is influenced by a new region juxtaposed to the gene(s) as a result of the chromosomal rearrangement. Such positional effects are frequently seen in transgenic mice, where the expression of the transgene is affected by the site of integration. ‘Silencing’ or inactivation of such transgenes has been shown to be associated with de novo methylation (Lettmann et al. 1991; Dandolo et al. 1993). The positional effect on the exed region can be explored by comparing the methylation pattern of the region surrounding the breakpoints in deletion DNA with the wildtype DNA.

*Clustering of deletion breakpoints?*

The distance between the distal breakpoints of the $c^{2YPSj}$ and $c^{6H}$ deletions has been found to be 20 kb. Similar results were obtained when the distal breakpoints of other radiation-induced deletions around the eed region were mapped (Holdener and Magnuson
unpublished data). Two of these deletions, \( c^{DENB} \) and \( c^{AL} \), map within 30 kb. Similarly, the distal breakpoints of \( c^{3H} \) and \( c^{112K} \) were found to be within 80 kb. Though these results indicate a possible clustering of the breakpoints, the number of deletions analysed so far is insufficient to predict the presence of hotspots for radiation-induced damage. However, recent studies of human fibroblasts have indicated that the breakpoints of four deletions involving a 45 kb region were associated with A+T rich sequences (Morris et al. 1988). Thus, it may be speculated that such regions of the genome result in a chromatin structure that is susceptible or prone to radiation-induced breakage. Mapping of other radiation-induced breakpoints and sequencing of the region around the clustered breakpoints may provide more conclusive results.
LITERATURE CITED


Rappolee, A., et al. (1988). Developmental expression of PDGF TGF-α,


approach. IRL press. 41-60.


