I am submitting herewith a dissertation written by Dabney K. Johnson entitled "Reverse Genetics and Genes That Control Development: Initial Molecular Characterization of Two Deletion Complexes in Mouse Chromosome 7 by Random Clone Mapping and Genetic Linkage Analysis". I have examined the final copy of this dissertation for form and content and recommend that it be accepted for the degree of Ph. D., with a major in Biomedical Sciences.

Eugene M. Rinchik
Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

REVERSE GENETICS AND GENES THAT CONTROL DEVELOPMENT: INITIAL MOLECULAR CHARACTERIZATION OF TWO DELETION COMPLEXES IN MOUSE CHROMOSOME 7 BY RANDOM-CLONE MAPPING AND GENETIC LINKAGE ANALYSIS

A Dissertation

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ABSTRACT

The observation of a mutant phenotype gives insight into the normal functions associated with the gene (or genes) that has undergone mutation. The strategy of "reverse genetics," in which a gene is cloned by beginning with a phenotype that has been mapped to a known chromosomal subregion, is one way of analyzing genes that control development. Induced deletion mutations, selected by the visible mutant phenotype of a marker locus, often also cause embryonic lethality in homozygotes because other genes contiguous with the selected marker locus are deleted. Because the map position of the selected marker locus is known, the chromosomal subregion associated with the embryonic-lethality phenotype is automatically also known. If molecular access to that chromosomal subregion is achieved, then the genes responsible for the lethal phenotypes can be analyzed by reverse genetics.

Two large complexes of overlapping, radiation-induced mutations of the *p* (pink-eyed dilution) and *c* (albino) loci in mouse chromosome 7, both of which are known to include recessive-lethal genes, offer the opportunity to apply a reverse-genetics strategy to the cloning of genes that control development in the mouse. Molecular access has been achieved to both regions by the mapping of random clones from genomic libraries enriched for chromosome-7 sequences. These clones have: (1) provided a point

of molecular access to the distal end of the *c* region, near the neurological mutation, shaker-1, and near four embryonic-lethal functions; (2) provided molecular access to the *p* function and to closely-linked phenotypes; and, (3) provided proof that many of the radiation-induced *p* mutations are indeed deletions.

In addition, genetic linkage analyses have determined or refined the map locations of several genes thought to lie near *p*. Knowledge of the relationships among the many radiation-induced *p* mutations has been incomplete because of a dearth of flanking-marker loci and also because of very complicated results from complementation testing of the *p*-lethal mutations. The mapping of these genes near *p*, along with the molecular probes derived from random-clone mapping, should contribute greatly to the clarification of the linkage relationships, and eventually, the functional characteristics, of *p*-region loci.

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CHAPTER 1

OVERVIEW AND GENERAL INTRODUCTION

It is often only through the recognition of a phenotype that has resulted from a mutation in a gene that not only the existence but also the sphere of function of that gene is realized. Without mutations to draw attention to aberrations at specific stages of the developmental process, the process itself cannot be dissected into the genetic components that blend to yield a normal organism. The observation of a mutant phenotype gives insight into the normal functions associated with the gene (or genes) that has undergone mutation. If the mutation in question can be assigned to a known region of the genome, then access to that same region can lead to the molecular analysis of the gene that controls a vital step in the process of development even if the gene in question does not encode an easily recognized gene product.

It has been said, in recent conversations among molecular biologists, that all of the "easy genes" have been cloned. "Easy" mammalian genes have been amenable to isolation by molecular techniques because functional information is available about their specific expression in cells or tissues; "difficult" genes will be in the majority. Recent successes in cloning "difficult" genes responsible for human diseases (Monaco, et al., 1985;

Koenig, et al., 1987; Kunkel, et al., 1987; Dryja, et al., 1986; Friend, et al, 1986) have relied on knowledge of the map position of the locus responsible for the mutant phenotype associated with the disease in the absence of any knowledge of the molecular defect that results in the disease phenotype; this cloning strategy is known as "reverse genetics."

In the study of human genetic disease, reverse genetics has become a widely used strategy for bypassing the requirement for prior knowledge of the biochemical or functional properties of the mutant locus. The requisite knowledge of the map position has been acquired both from studies of linkage of the phenotype to known markers and from association of the phenotype with specific chromosomal subregions that are pinpointed by relevant chromosomal aberrations. To quote Rommens, et al., (1989), whose elegant but arduous strategy resulted in the cloning of the human gene responsible for the cystic fibrosis phenotype without prior knowledge of the basic defect, "A major difficulty in identifying the CF locus has been the lack of chromosomal rearrangements or deletions, which greatly facilitated all previous successes in the cloning of human disease genes by knowledge of map position." Thus, when the phenotype of interest can be pinpointed by useful cytogenetic landmarks, the search for the relevant gene becomes more straightforward.

Because research that involves human subjects involves so many ethical limitations, the mouse, as a model system, is a

nearly ideal substitute that can be bred to exact genetic specifications. This is especially true when the genes of interest include those that control the process of embryogenesis; these are the archetypical "difficult" genes because mutations are most often lethal, resulting in the unavailability of the biological material in which the mutant phenotype is expressed. With the mouse available for the study of mammalian embryogenesis, however, the knowledge of map position of the relevant genes, even in the absence of any functional information, becomes sufficient for the isolation of genes that control development.

When a genetically well-defined chromosomal region in a model system like the mouse is amenable to the use of reverse-genetics cloning strategies to identify expressed sequences that play a role in development, then those sequences can be employed to find and analyse homologous human sequences. The use of mouse-human homology to study a human disease in a model system is becoming more common, but chiefly in the human-to-mouse direction, as investigators search for a suitable experimental system in which to explore the function of the gene in question. For example, the cloning of the human genes involved in Duchenne's muscular dystrophy (DMD) (Monaco, et al., 1985; Koenig, et al., 1987; Kunkel, et al., 1987) and retinoblastoma (RB) (Dryja, et al., 1986; Friend, et al., 1986), which are both significant health problems, has led to the isolation of the cognate sequences in the mouse, a genetically

and technically malleable mammalian system. If the advantages of the mouse as a model system can be exploited initially in the isolation of genes that are centrally important in embryogenesis, then the cognate sequences in the human will become available for study without the ethical limitations that make the isolation of those genes from human material so difficult.

Knowledge of the map position of a subset of genes important to development has been acquired over the years as a part of the process of analysis of mutations induced during radiation mutagenesis experiments conducted in the Mammalian Genetics Section at ORNL since 1949. Using seven recessive marker loci [*a* (agouti), *b* (brown), *c* (albino, or tyrosinase), *d* (dilute), *se* (short-ear), *p* (pink-eyed dilution), and *s* (piebald spotting)] in the specific-locus test (W. L. Russell, 1951) to detect mutations, many mutagens have been tested and over a thousand germline mutations have been isolated and maintained. Both radiation and agents such as chlorambucil (L. B. Russell, et al., 1989; Rinchik, et al., in press)) are known to induce deletions, which often affect other genes that are, by chance, closely linked to one of the seven marker loci. Two of the seven loci, *c* (albino, the structural gene for tyrosinase,) and *p* (pink-eyed dilution), reside in mouse chromosome 7 and are an integral part of the work reported in this dissertation; however, the concepts and strategies involved are equally applicable to any

chromosomal region for which chromosomal aberrations, especially deletions, are available.

Once large numbers of mutations at *c*, *p* and *d-se* were generated by radiation mutagenesis, genetic studies were undertaken to characterize the phenotypes observed when each mutation was made homozygous as well as when each was combined with other independent mutations at the same locus in compound heterozygotes. One-hundred-twenty-two mutations at *d*, 43 mutations at *se*, and 39 mutations that involved both loci were examined by L. B. Russell (1971) in the first study in a mammalian system of a complex of overlapping deletions. This analysis revealed the presence of at least six prenatally lethal and at least two neonatally lethal "functions," possibly genes, in the 2.6 cM chromosome-9 region covered by the *d-se* deletion complex. L. B. Russell, et al. (1982), reported the results of complementation/deletion testing of 34 homozygous-lethal mutations involving the *c* locus, and hypothesized the presence of at least six developmentally lethal genes. Complementation analysis of the mutations surrounding the *p* locus is ongoing, and developmental lethals are found also in this region. (L. B. Russell, personal communication). The years of analysis by Russell and her colleagues were of crucial importance to this study because their analysis pinpoints the map location of genes essential to the progress of normal development. Their observation of various different lethal phenotypes that result from the coincident inclusion of genes important to development

within the radiation-induced deletions provided the starting point for the "reverse genetics" approach described in this work. There is no reason to believe, however, that the *c, p*, and *d-se* chromosomal subregions are unusual in the abundance of genes whose homozygous deletion causes developmental failure; it can only be assumed that any chromosomal subregion for which deletions become available will afford the developmental geneticist a means for studying a rich source of genes that participate in the processes of growth, differentiation, successful attainment of adulthood, and successful reproduction.

Developmental biology has progressed in recent years from a largely descriptive science to one that is now capable of analysis at the gene level by molecular techniques. Central to this progress has been the availability of developmental mutations, such as those described above, that interrupt normal development in characteristic ways, and thereby supply information about the function, spatial/temporal expression, and map locations of genes that control all stages of development from the fertilized egg to the adult. An important subclass of these mutations, as outlined above, are those that result in the complete failure of embryogenesis, with early lethality as the only observable mutant phenotype. Mutations in other later-acting genes may lethally disrupt the normal course of development at later embryonic, fetal, and postnatal stages, may be "lethal" to the gene (though not to the individual) only in that

they cause reproductive failure in an otherwise healthy organism, or may cause abnormalities that are not lethal.

The work presented in this thesis was designed to explore some of the ways in which the wealth of radiation-induced deletion mutations generated and maintained in the Mammalian Genetics Section at Oak Ridge National Laboratory could be exploited to gain molecular access to a subset of genes that participate in the process of development. The total approach proposed by the new research program initiated by E. M. Rinchik in 1985 is four-pronged: (a) the mapping of random, anonymous DNA clones, such as those generated by flow-sorting or microdissection of a specific chromosome, into the intervals covered by the largest members of a deletion complex, followed by the submapping of those clones into the intervals defined by overlap among other members of the deletion complex; (b) the disclosure of as many visible phenotypes as possible by saturation point-mutagenesis of the region covered by the largest members of the same deletion complex, again followed by submapping to specific ones of the other deletions; (c) the use of the molecular clones as access points in the development of long-range physical maps to be correlated with developmentally lethal phenotypes; and, (d) the use of molecular clones as access points to other kinds of genes (shaker-1, *sh-1* for example; see Chapters 2 and 4), that map within the deletion complex. Part (a) was undertaken in the thesis research to be reported below.

The initial biological reagents required for the initiation of part (a) were (1) genomic DNA from mice whose chromosomes carry the deletion mutations and (2) libraries of DNA clones enriched for sequences from the *c* and *p* deletion-complex intervals. Those reagents were provided by, first, the breeding of interspecfic (*Mus musculus/Mus spretus*)F_1 hybrid mice that carry one normal *M. spretus* chromosome 7 and a mutant *M. musculus* chromosome 7 that is deleted for a segment of DNA that includes either the *c* locus or the *p* locus. A clone mapping to a *c*- or *p*-region deletion is identified when restriction-fragment-length polymorphism (RFLP) analysis of these hybrids detects the absence of a *musculus*-specific hybridization band for any clone whose cognate genomic sequence is deleted in the mutant *musculus* chromosome 7. The second reagent, the enriched region-specific DNA libraries needed for mapping of relevant clones, was generated by either fluorescence-activated flow-sorting or chromosome microdissection of a large, morphologically distinct translocation chromosome that carries the wild-type *p*- and *c*-region sequences from chromosome 7. Together, these two reagents, along with region-specific probes provided by collaborating laboratories, have produced the necessary information for evaluation of the proposed strategy for achieving molecular access to the developmentally important genes that map into the deletions that overlap at the *p* locus or the *c* locus on mouse chromosome 7.

Table 1-1 gives of details of molecular probes used throughout this dissertation.

Table 1-1. Listing of Probes Used

Locus	Gene Name	Probe Name	Probe Description	Reference
Lhb	b-subunit, luteinizing hormone	pLHB-2	0.35-kb rat cDNA	Chin, et al., 1983
Igfr-1	Insulin-like growth factor receptor-1	pIGF-I-R.8	0.7-kb *Eco*RI fragment of human cDNA	Ullrich, et al., 1986
Saa-1	Alpha-subunit, serum amyloid protein-1	pSA30.1	1.5-kb *Pst* I mouse genomic fragment of pSA30	Lowell, et al., 1986
MyoD-1	Striated muscle-specific differentiation protein	pMyoD1	1.9-kb *Eco*RI fragment of human cDNA	Davis, et al., 1987
Ldh-1	Lactate dehydrogenase-A	pUCLD14	1.7-kb *Eco*RI-*Hin*dIII 5' mouse genomic fragment	Mendel, 1987
D7OR2	Anonymous sequence	TM15	0.2-kb *Eco*RI mouse anonymous genomic fragment	This thesis
*	Anonymous sequence	0.4	0.4-kb *Eco*RI-*Bam*HI mouse genomic fragment	This thesis
*	Anonymous sequence	0.6	0.6-kb *Bam*HI-*Pst*I mouse genomic fragment	This thesis
*	Anonymous sequence	0.8	0.8-kb *Bam*HI-*Hin*dIII mouse genomic fragment	This thesis
D7OR1	Anonymous sequence	11B2	0.09-kb mouse anonymous *Eco*RI genomic fragment	Johnson, et al., 1989
Hbb	Hemoglobin b-chain	pPK268	0.8-kb-*Eco*RI-*Hin*dIII mouse genomic fragment	Skow, et al., 1983

*Loci recognized by these probes are currently undefined

CHAPTER 2

GENERAL INTRODUCTION TO THE *c* REGION

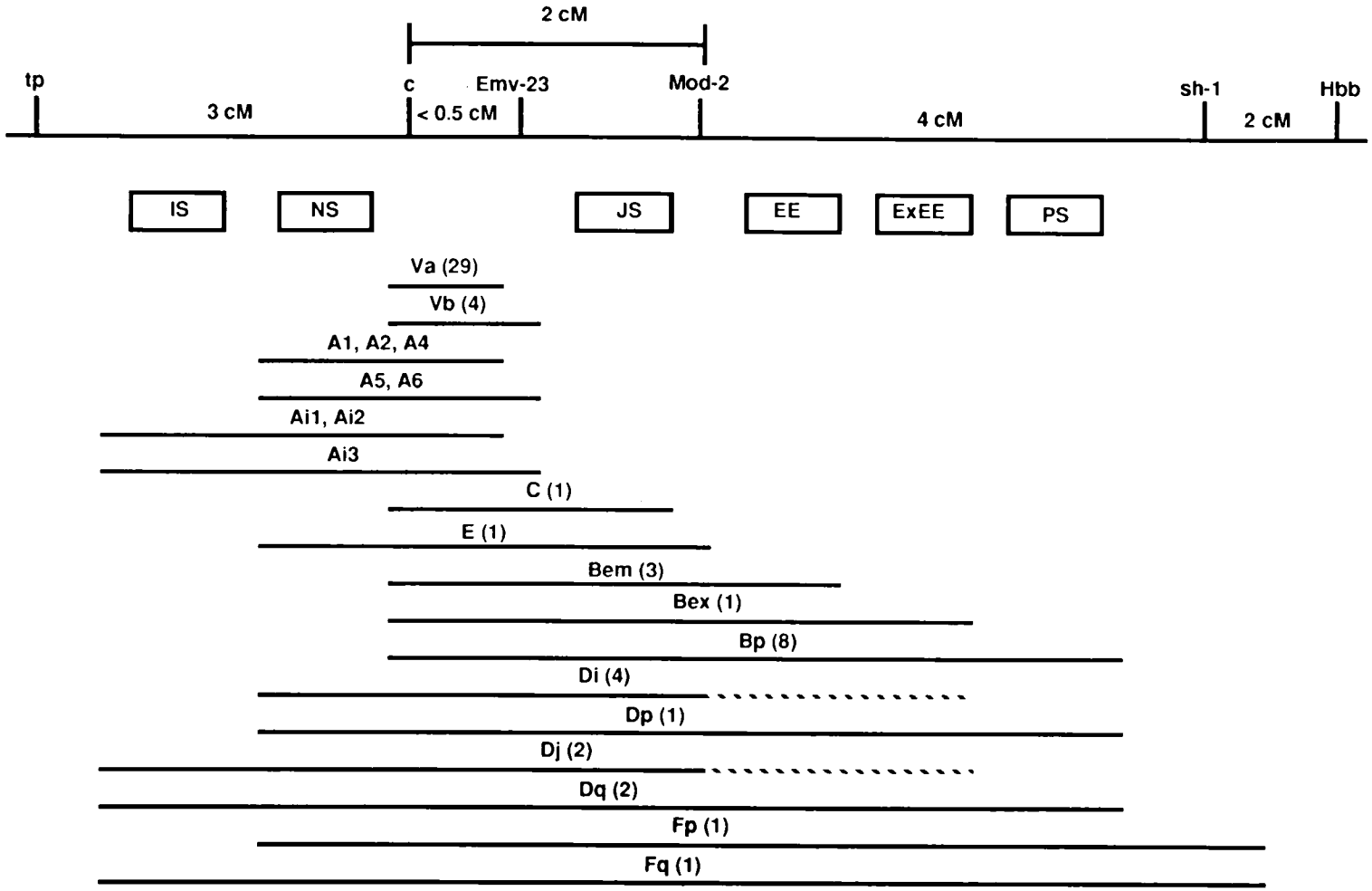
For the purposes of the research reported here, the *c* region has been defined as the region of mouse chromosome 7 covered by the presumed-largest of 34 recessive-lethal deficiency mutations that were recovered during experimental radiation mutagenesis using the seven-specific-loci system of detecting mutations (W. L. Russell, 1951; L. B. Russell, et al., 1979; L. B. Russell, et al., 1982). Among the spectrum of mutations recovered at the seven marker loci, 119 were detected that had lost normal function of the tyrosinase (*c*) gene carried by the mutagenized wild-type (*+/+*) chromosome 7. These mutations at *c* were recognized by the presence of a light-chinchilla mouse (genotype c^{ch}/c^{*}, where c^{*} represents any *c* mutation) in litters that would otherwise be all wild-type ($c^{ch}/+$). The cross used to identify the mutants (c^{*}) was c^{ch}/c^{ch} (from the tester "T" stock) x *+/+* , the mutatgenized hybrid stock [(C3H x 101)F1 or (101 x C3H)F1] (W. L. Russell, 1951; L. B. Russell, et al., 1982). These 119 mutations at *c* included 34 homozygous-lethal mutations, which were classified with respect to the developmental stage at which homozygotes die (Russell and Raymer, 1979; Russell, et al., 1982), and which were also tested for involvement of nearby marker loci (taupe, *tp*, 3cM proximal; mitochondrial malic enzyme, *Mod-2*, 2 cM

distal; shaker-1, *sh-1*, 6 cM distal; and hemoglobin beta chain, *Hbb*, 8 cM distal) (Russell, et al., 1982). The test for involvement of flanking markers was accomplished by crossing the mutant stock with a stock that carries a recessive marker, such as *sh-1*, that maps to a segment of DNA that flanks the specific-locus mutation. If the recessive phenotype associated with that marker (shaker-1 in this example) appears in the F_1, then the wild-type counterpart of the shaker-1 locus is assumed to be deleted; this phenomenon is called "pseudodominance", and simply means that the deletion of a wild-type allele allows the hemizygous expression of a normally recessive allele in an F_1 animal.

All 34 recessive-lethals were also crossed pairwise in a full-scale complementation grid in which 435 of the 561 possible crosses were made (Russell, et al., 1982). From the results of this series of genetic tests, the complementation/deletion map shown in Figure 2-1 was drawn, and its components will be discussed in detail below.

In the original complementation/deletion map published by Russell, et al., in 1982, the 34 lethal mutations fell into 12 complementation groups, A, Ai, C, E, Bi, Bp, Di, Dp, Dj, Dq, Fp, and Fq. A 13th group, V, consists of 52 viable albino mutations that were also found in the progeny of irradiated mice. All 34 lethal mutations were also crossed to stocks carrying 2 mutant loci , *tp*, and *sh-1*, and 2 loci with easily detected allelic variants (*Hbb* and *Mod-2*); all of these markers map within 8 cM of the *c*

Figure 2-1. Complementation/deletion map of a 6- to 11-centimorgan (cM) region of Chromosome 7 defined by the *Df(c Mod-2 sh-1)*^{12FR60Hb} (Fq1) deletion (Russell, et al., 1982). Marker loci are taupe, *tp*; albino (tyrosinase), *c*; mitochondrial malic enzyme, *Mod-2;* shaker-1, *sh-1*; and globin (beta chain), *Hbb*. *tp* is the most proximal locus. *Emv-23* is the integration site of an endogenous murine ecotropic provirus (Rinchik, et al., 1989). Distances between marker loci are given in cM. "Functional units" (shown as open boxes), defined by developmental phenotypes expressed by segments of unknown physical size and complexity, are defined by complementation analysis of 34 independent *c*-locus deficiency mutations (Russell, et al., 1982); they are abbreviated as IS, implantation survival; NS, neonatal survival; JS, juvenile survival, and PS, preimplantation survival. The EE and ExEE functional units (Niswander, et al., 1989) are associated with the development of the embryonic ectoderm and the extraembryonic ectoderm, respectively, and are subdivisions of Russell's (Russell, et al., 1982) second implantation-survival unit. Deletions of the IS, PS, EE, and ExEE functional units are associated with death of the embryo before (PS) or shortly after (IS, EE, ExEE) implantation (Lewis et al., 1976; Lewis, 1978; Russell and Raymer, 1979; Niswander, et al., 1989) The NS unit is needed for neonatal survival and corresponds to a locus encoding a *trans*-acting regulator for metabolic enzymes and other proteins (reviewed Gluecksohn-Waelsch, 1979), and the JS unit is associated with male sterility (Lewis, et al., 1978) and reduced fitness in both sexes (Russell, et al., 1982). The heavy lines below the functional map indicate the presumed extents of the Oak Ridge *c* deletions in each complementation group. The letter designation (Va, Vb, A, Ai, Bem, Bex, Bp, C, Di, Dj, Dp, Dq, E, Fp, Fq) indicates the complementation group, and the numeral in parenthesis represents the number of independent mutations assigned to each group. All deletions are homozygous lethal except for the V groups, Va and Vb; occasionally, however, some C/C homozygotes can survive to well past a year. Broken lines shown for complementation groups Di and Dj indicate uncertainties about the distal extents of these deletions with respect to the EE and ExEE regions.


2 cM
tp
c
Emv-23
Mod-2
sh-1
Hbb
3 cM
< 0.5 cM
4 cM
2 cM
IS
NS
JS
EE
ExEE
PS
Va (29)
Vb (4)
A1, A2, A4
A5, A6
Ai1, Ai2
Ai3
C (1)
E (1)
Bem (3)
Bex (1)
Bp (8)
Di (4)
Dp (1)
Dj (2)
Dq (2)
Fp (1)
Fq (1)

locus. The results of both the complementation and the deletion-mapping are summarized below.

The loci used for deletion-mapping were the following:

a) Taupe (*tp*) is a recessive coat-color mutation 3 cM proximal to *c*. No taupe progeny were recovered from crosses of any of the *c* radiation mutants (c^{ch}/c^{*}) to *tp/tp*, indicating that none of the deletions extends proximally as far as *tp*.

b). Mitochondrial malic enzyme (*Mod-2*) activity can be quantitated in heart mitochondrial fractions (Bernstine, et al., 1978). *Mod-2* activities were compared in c^{ch}/c^{*} mutants and c^{ch}/c^{ch} T-stock animals;.a ratio of 1.0 indicates a normal "two-dose" activity level, whereas a ratio of 0.5 indicates that one chromosome is deficient in *Mod-2* activity, suggesting deletion of the *Mod-2* structural locus 2 cM distal to *c*. Among the 34 mutants tested, 22 were found to be deficient for *Mod-2* activity. These were later assigned to the groups E, Bi, Bp, Di, Dp, Dj, Dq, Fp, or Fq both on the basis of their *Mod-2* genotype and on their other characteristics revealed in the full complementation/deletion analysis as described below.

c). Of the 22 mutations in the nine complementation groups in Figure 2-1 that are deficient for *Mod-2*, two mutations, Fp and Fq, are also deficient for shaker-1 (*sh-1*, 6 cM distal to *c*), a locus involved in inner-ear development. This determination was made by crossing the each of the mutant stocks (c^{ch} +/c^{*})to + *sh-1*/+ *sh-1* stocks, and looking for progeny with the shaker-1 phenotype. The $c^{ch}+/c^{Fp1}$ and $c^{ch}+/c^{Fq}$ mutant stocks produced

sh-1 progeny (+ *sh-1/c*^{Fp1} and + *sh-1/c*^{Fq1}), assumed to be of the genotype *sh-1*/0, indicating deletion (0) of the wild-type *sh-1+* locus normally found on the wild-type chromosome.

d). The *c*^{Fp1} and *c*^{Fq1} mutations were also tested for deletion of the hemoglobin (*Hbb*, 8 cM distal to *c*) allele carried by the wild-type C3H or 101 irradiated chromosome. Neither was deficient for that allele, indicating that the most distal extent of the deletion complex is a locus somewhere between *sh-l* and *Hbb.* The possible deletion of *Hbb* was tested for by examination of the pattern of electrophoretic variants of *Hbb* alleles.

When the 34 recessive-lethal mutations were crossed pairwise in most of the possible combinations, the following complementation groups could be established:

A group. This group includes 6 independent mutations that cause death in homozygotes at or shortly after birth. It should be noted here (this fact applies to all members of all the complementation groups to be described) that compound heterozygotes between members of the same complementation group are non-complementing. Thus, compound heterozygotes within a group exhibit the same phenotypes as homozygotes. Recent mapping (Rinchik, et al., 1989; Klebig and Rinchik, unpublished) of molecular probes has shown that the A2, A3, A4, and A6 deletions extend more proximally than the A1 and A5 deletions, and that the A3, A5 and A6 deletions extend the farthest distally, with A1, A2, and then A4 being progressively

shorter. The A-group deletions include a gene (or genes) necessary for survival in the neonatal period; this functional unit, neonatal survival (NS) lies proximal to *c*. Studies by Erickson, et al., (1968); DeHamer, (1975); Gluecksohn-Waelsch, (1979); and Gluecksohn-Waelsch, et al., (1974) have suggested the existence within this functional unit of a regulator of the inducible hepatocyte-specific expression of certain enzymes; this regulator has been termed "hepatocyte-specific developmental regulator" (*hsdr-1*) (Gluecksohn-Waelsch, 1989). The A-group mutations are deficient, by complementation-mapping, only for *hsdr-1*, and not for any of the other 3 flanking marker loci (*tp*, *Mod-2*, *sh-1*). A-group mutations are fully complemented by the V-, C- and B-group members, but not by any others.

Ai Group. Ai homozygotes (and Ai/Ai compound heterozygotes, as noted in the A group description above) die at or soon after implantation as evidenced by the increased presence, compared to normal controls, of resorption moles in the uterine contents of mated females (Russell and Raymer, 1979). Ai-group mutants are fully complemented by the C and B groups. They are partially complemented by the A, E, Di, Dp, and Fp groups, so that lethality occurs in the neonatal period instead of at or near implantation. Like the A-group mutations, the Ai mutations do not involve any of the 4 flanking marker loci, but their failure to complement A-group mutations suggests they are deficient for the *hsdr-1* function proximal to *c*. Recent data

from the molecular probes, including 23.3, a genomic sequence that flanks the integration site of an ecotropic provirus, *Emv-23*, has shown that Ai1, Ai2, and Ai3 are indeed deletions, that the Ai3 deletion extends more distally than does Ai1 or Ai2, (Rinchik, et al., 1989), and that Ai2 extends less distally than Ai1 (Klebig and Rinchik, unpublished data).

C Group. The single C-group mutation is often lethal at or before weaning and usually so by about four months of age; it defines the "juvenile-survival" (JS) function distal to *c*. This juvenile lethality is complemented only by the A and Ai mutations. The C deletion involves neither *hsdr-1*, proximal to *c*, nor *Mod-2*, the closest distal marker. However, data from probe 23.3, tightly-linked to the *c* locus, prove that the C mutation is a deletion (Rinchik, et al., 1989; Klebig and Rinchik, unpublished data).

E Group. Homozygotes for the E mutation die neonatally, as do the A-group members, but E must also extend distally because it is deficient for *Mod-2* activity. The mutant "juvenile survival" phenotype is masked since E homozygotes die at birth, although C x E compound heterozygotes survive into the juvenile period and exhibit reduced fitness. The E mutation complements the implantation lethality of Ai, Bem, Bex, Bp, and Di and the preimplantation lethality of Bp, Dp, Dq, Fp, and Fq. However, E/Ai heterozygotes, for example, die at birth since both mutations are deleted for the *hsdr-1* function. E/Bem, E/Bex, and E/Bp heterozygotes often die before weaning because of

deficiency for the "juvenile survival" function distal to *c*. E/Di heterozygotes die neonatally because both mutations are deleted for the NS function proximal to *c*.

Bi Group (Bem and Bex). The 4 Bi mutations, described by Russell, et al. (1982), die at or shortly after implantation as evidenced by the increased number, compared to normal controls, of uterine resorption moles; c^{ch}/c^{Bi} mutants are deficient for *Mod-2* activity, indicating that the members of the Bi (Bem + Bex) group delete *Mod-2* in addition to *c*. Bi lethality is fully complemented by A, and Ai; Bi implantation lethality is complemented by the C, and E mutations.

Recent embryological studies by Niswander, et al. (1988, 1989) have further separated the former Bi group into two new groups; Bem (Bi1, Bi3, and Bi4), where structures derived from embryonic ectoderm are aberrant (the EE function), and Bex (Bi2), with additional abnormalities in extraembryonic ectoderm development (the ExEE function).

Bp Group. Homozygotes (and, again, Bp/Bp compound heterozygotes) for any of the 8 Bp mutations die in the preimplantation period as evidenced by an increased excess compared to controls of corpora lutea (indicative of ovulations) over the total number of live and dead fetuses plus resorption moles. The Bp deletions are deficient for *Mod-2* activity (but not for *tp*, *sh-1*, or *Hbb*), are complemented to juvenile survival by the C, and E groups, and are fully complemented by the A and Ai mutations.

Di Group. The 4 Di mutations are deleted for *Mod-2* (but not for the other flanking markers) and die at or after implantation. The phenotypes of Di1 and Di2 homozygotes are identical to those of Bem homozygotes, suggesting that Di1 and Di2 include EE, but not ExEE (T. Magnuson, unpublished data). Di3 and Di4, however, have not yet been characterized to determine their extent into the EE and ExEE functions defined by the Bem and Bex complementation groups. Di mutations are presumed to delete at least part of the distal implantation-survival functions defined by Bem and Bex since Bi/Di compound heterozygotes are non-complementing. Di mutations do not complement A mutations for neonatal lethality, and are therefore presumed to delete the proximal *hsdr-1* function. The Di mutations do, however, complement the Ai mutations for implantation death, and so are presumed not to delete the proximal implantation (IS) function.

Dp Group. The single Dp mutation also deletes *Mod-2* and, presumably, *hsdr-1*, but dies in the preimplantation period and must therefore extend through a more distal "preimplantation survival" (PS) function than do the Di-group mutations. The A, Ai, and E group mutations partially complement Dp (to neonatal lethality); therefore, Dp must not delete IS but does delete *hsdr-1*. C complements Dp to juvenile survival. It is should be noted that none of the B (Bi, Bp), D (Di, Dp, Dj, Dq), or F (Fp, Fq) mutations complement each other since all members of these groups are deleted, at least, for the distal (to *c*) implantation-

survival function, EE.

Dj Group. The 2 Dj mutations are presumed to delete the same proximal "implantation survival" (IS) function as the Ai mutations since the 2 groups are non-complementing. Unlike the Ai mutations, however, these Dj mutations also delete *Mod-2*; like the Di3 and Di4 mutations, Dj have not yet been studied for their extent into the EE and ExEE functions. The Dj's cannot extend to the PS function since they complement Bp for preimplantation survival (Russell and Raymer, 1979).

Dq Group. The 2 Dq deletions, large enough to be detectable cytologically (Russell and Cacheiro, 1977), are also preimplantation lethals, and are deficient in *Mod-2* activity but do not include *sh-1* or *tp*. They do not complement the Ai group, and so are presumed to delete all the currently defined functional untis that must be present for survival to implantation (IS. EE, ExEE, PS).

Fp and Fq Groups. The Fp and Fq mutations, also cytologically detectable, do delete the *sh-1* locus 6 cM distal to *c* (Russell, et al., 1982). Fp complements Ai mutations to neonatal lethality, and therefore must be shorter proximally than Fq which does not complement for implantation lethality in combination with the Ai mutations. Fq therefore must delete the proximal "implantation survival" function.

Although all of the 34 *c*-region homozygous-lethal mutations have been proved, by deletion-mapping or molecular techniques, to be deletions, nothing is known about the number

of genes or the actual physical distance involved in any deletion. Dq, Fp, and Fq are all visible deletions in metaphase chromosomes (Russell and Cacheiro, 1977); Fp and Fq may be judged, vis-a-vis adding genetic distances (centimorgans) of flanking markers, to delete up to 11 cM. Dq, which could delete up to 9 cM, includes neither the extreme proximal marker, *tp*, nor the distal locus, *sh-1*, but deletes at least 6 functional units in addition to *c* and *Mod-2*. Any of the functional units might include one gene or many; a functional unit whose deletion causes very early lethality might, for example, contain many contiguous genes whose expression never occurs (so no phenotype is seen) because of the early death of the developing embryo. Only the molecular isolation of the DNA sequences in the segment covered by a "functional unit" will offer the opportunity to confirm the numbers and functions of the genes included.

The Fp and Fq deletions also cover 6 functional units in addition to *c, Mod-2*, and *sh-1*. Again, these deletions could be 11 cM in length if either extends distally nearly to *Hbb*, but there is as yet no information about how close to *Hbb* (or to *tp*, proximally) either deletion may end. Physical distances cannot accurately be related to cM distances; only accurate physical mapping of regions of DNA with well-studied genetic linkage relationships will provide dependable information about physical distances in DNA terms vs. genetic distances in terms of recombination frequencies. The mapping of molecular probes

into the intervals spanned by the collective proximal and distal limits of the deletion complex will provide the means for constructing a long-range physical map of the DNA, and its component genes, that constitute the *c* region.

In summary, the 6-11cM "*c* region" is defined by radiation-induced *c*-locus mutations that can be placed into 17 groups (including viables) that span at least 8 functional units. Doubtless many other "functions" may exist within this region, and no correlation is implied or can be drawn between any function and the number of genes that may be involved. Since early lethality is a common phenotype for *c*-region deficiencies, many other phenotypes may be masked by the early loss of homozygous embryos. The relevance of the *c*-region deletions is, then, that at least several genes essential for development clearly lie within a defined segment of DNA, and, with the overlapping deletions available as mapping tools, molecular probes can be localized to the various "functional units" that comprise that defined segment. Once molecular access has been achieved, then the isolation and characterization of these hitherto unknown genes, whose normal function is necessary for development, can follow.

The normal functions of the some of the genes within the *c* region appear to form the very foundations of mammalian embryogenesis, and their cloning and characterization would contribute greatly to knowledge in the field of early development. For example, homozygous deletion of genes within

the functional units Bem and Bex (Niswander, et al., 1989) cause lethality around the time of gastrulation (Niswander, et al., 1988). Originally designated as the five members of the Bi group (L. B. Russell, et al., 1982) [four Oak Ridge deletions, plus a fifth deletion, c^{6H}, isolated at Harwell, that appeared to belong in the Bi group judging from published information (Glueckson-Waelsch, 1979)], these mutations include a distal functional unit called "implantation-survival-2" (IS) in the 1982 study. Histological examination of the c^{11DSD} (Bi1) and c^{6H} (Niswander, et al., 1988) and the $c^{4FR60Hd}$ (Bi2), $c^{5FR60Hg}$ (Bi3), and c^{2YPSj} (Bi4) (Niswander, et al., 1989) mutations led to the separation of the distal "IS" functional unit into two subunits, Bex, with breakpoints more distal, (c^{6H}, $c^{4FR60Hd}$) and Bem, with breakpoints more proximal (c^{11DSD}, $c^{5FR60Hg}$, and c^{2YPS}) (Figure 2-1). These results suggest the presence, within the original distal "IS" functional unit, of at least two genes of central importance to embryogenesis. The Bem-group embryos begin to die at day 8.5 (E8.5), and exhibit severe (and lethal) defects in development of the embryonic ectoderm. The Bex-group embryos, which begin to die a full day earlier (E7.5), manifest lethal abnormalities in both the embryonic and extraembryonic ectodermal structures (Niswander, et al, 1989). These investigations have shown that the previously defined functional unit "IS-2" contains at least one gene that is involved in development of the extraembryonic ectoderm and another gene (or genes) important for normal development of the embryonic

ectoderm.

These aspects of embryogenesis lie at the very core of mammalian development, yet nothing is known of the mechanisms of gene regulation that govern these processes. Because, however, we can locate phenotypes to a defined and well-characterized area of the mouse genome, we now have the tools at hand to clone and characterize these vital genes by application of the reverse genetics approach.

Each radiation-induced mutation generated in the specific-locus mutagenesis experiments is independent, so that within each complementation group, individual members have different breakpoints. Separation of these individual members, by molecular (Rinchik, et al., 1989; Klebig and Rinchik, unpublished data), or histological (Niswander, et al., 1988; 1989) examination leads not only to finer physical mapping but to more precise delimitation of functional regions associated with a phenotype. Eventually, these physical maps can be associated with the DNA sequences that encode gene products responsible for the phenotypes observed. Not only the *c* locus, but others of the seven specific loci are the focal points for genome regions that contain genes that are essential for normal development. These loci were chosen simply for easy recognition of mutant phenotypes; it seems reasonable to suppose that any other seven marker loci chosen at random should also have contiguous "lethal" genes amenable to isolation by strategies that employ the same reverse genetics approach.

CHAPTER 3

GENERAL INTRODUCTION TO T(X;7)5R*I*, THE LARGE TRANSLOCATION CHROMOSOME USED IN THE CONSTRUCTION OF THE CHROMOSOME-7 RANDOM-CLONE LIBRARIES

Mouse X-autosome translocations [T(X;A)s], first revealed by the mosaic phenotypes that result when recessive autosomal coat color markers were translocated to an X chromosome, provided the first evidence for the single-active-X hypothesis (L. B. Russell, 1961; M. F. Lyon, 1961). Since that important discovery, the T(X;A)s have proved useful in developmental (reviewed in Monk, 1978), cytogenetic (Nesbitt and Gartler, 1980), genetic (L. B. Russell, et al., 1962; L. B. Russell, 1964; Russell and Montgomery, 1965), and, now, molecular genetic studies. In the current study, an X;7 translocation chromosome has provided the means for constructing recombinant DNA libraries that are enriched for sequences from the *c*- (Chapter 2) and p- (Chapter 6) regions of chromosome 7.

Fifteen X-autosome rearrangements were known in the mouse as of 1983, (L. B. Russell, 1983), and, of these, two, T(X;7)5R*I* ("R5") and Is(In7;X)1Ct ("T1Ct") (Cattanach, 1961; 1966) have contributed heavily to this report. R5 was used to enrich for chromosome-7 sequences in genomic libraries generated by flow sorting (Chapter 4) or chromosome microdissection (Chapter 7), whereas T1Ct was employed in

schemes designed both to facilitate molecular mapping and genetic linkage studies (Chapter 10) and to locate embryonic-lethal genes within the p-region deletion complex (Chapter 10).

Two chromosome-7 segments (surrounding *c* and surrounding *p*), for which a large number of deletion chromosomes are available have been shown in genetic studies to include genes that control steps of normal embryogenesis (Russell, et al., 1982; L. B. Russell, personal communication). Since the functions of these developmental genes are as yet unknown, their molecular cloning and characterization may provide the means for ascertaining those functions. Molecular cloning will require recombinant DNA clones that map in or near the genome segments known to contain those genes. Once again, a radiation mutagenesis experiment has produced a useful aberrant chromosome for construction of recombinant genomic libraries highly enriched for sequences from the *c* and *p* regions. This aberrant chromosome is useful for library construction because its size distinquishes it from among all the other mouse chromosomes.

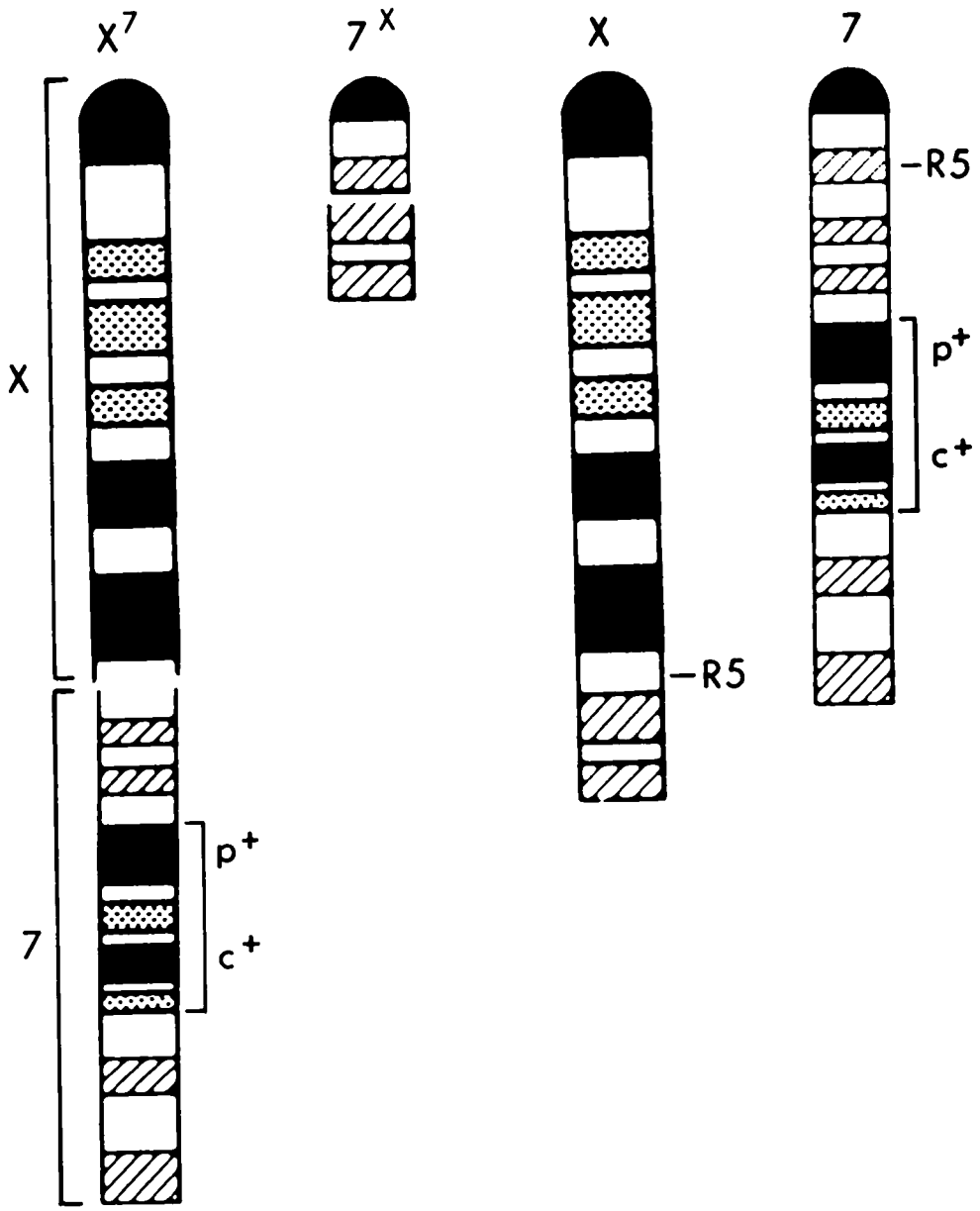
T(X;7)5R*l* ("R5") is a simple reciprocal translocation between a chromosome 7 carrying the wild-type p^+ and c^+ loci and an X chromosome. Generated during an X-ray-mutagenesis experiment, in which irradiated wild-type males were crossed to a tester stock (T) that carries a $p\ c^{ch}/p\ c^{ch}$ chromosome, R5 was recognized because female carriers are variegated for *c* and *p* mutant coat-color phenotypes. The chromosome-7 breakpoint

is proximal to ruby-eye-2 (*ru-2*) (gene order: centromere - R5 - *ru-2* - *p* - *tp* - *c*) and the X chromosome breakpoint is distal to *Ta* (Tabby) (L. B. Russell, 1983). The resulting balanced karyotype has a large derivative chromosome (X^7) consisting of the X centromere, about half the (genetic) length of the X, and the distal two-thirds of the 7 (Figure 3-1); and a small chromosome (7^X) with the 7 centromere, the proximal one-third of the 7, and the distal half of the X (L. B. Russell, 1983). The large X^7 is 1.3 to 1.4 times as long as chromosome 1 (the longest normal mouse chromosome) (Cacheiro and Russell, 1969) and carries the wild-type counterparts of the *ru-2*, *p*, and *c* loci from chromosome 7.

X-inactivation has been found to spread into autosomal segments that are physically joined to X-chromosome material, resulting in the inactivation of genes within the translocated autosomal segments (L. B. Russell, 1983). There is no *trans* effect; that is, the allele on the intact autosome is not inactivated. For R5, the resulting variegated phenotypes at both albino (*c*) and pink-eyed dilution (*p*) confirm the cytogenetic finding (Cacheiro and Russell, 1969) that the *c+* and *p+* loci reside in the large translocation chromosome.

Because the normal *Mus musculus* karyotype consists entirely of acrocentric chromosomes that range evenly along a size continuum, neither the human eye nor a computer-assisted fluorescence-activated cell sorter (FACS) can distinguish an individual chromosome in a uniformly-stained (non-banded)

Figure 3-1. Idiogram illustrating the two translocation chromosomes, X^7 and 7^X, and their normal counterparts in the X-autosome translocation T(X;7)5R*l*. On X^7, the brackets at left show the approximate proportions, based on Giemsa-banding, of X and 7; the brackets at right on X^7 and on 7 show the approximate target area for microdissection. The R5 breakpoint, based on Giemsa-banding, is shown on both normal chromosomes.


X7
7X
X
7
X
7
p+
c+
−R5
−R5
p+
c+

mouse chromosome complement. However, since the X^7 chromosome of R5 stands out as a single, large, chromosome in the diploid balanced karyotype, both the eye and the cell sorter can separate it easily. Therefore, the large translocation chromosome stained with fluorescent dye can be collected as a relatively pure fraction by the FACS (Figure 3-2 and Figure 3-3), and molecular cloning of enzyme-digested DNA from that fraction results in a recombinant genomic library highly enriched for sequences from the chromosome X and 7 components that comprise the R5 chromosome. Likewise, chromosome microdissection and microcloning can be performed on defined regions of the R5 chromosome (Figure 3-1) because the micromanipulator operator can distinguish the large chromosome from all others in the karyotype.

Efficient flow-sorting and microdissection require not only a morphologically distinct chromosome but also a large number of synchronous mitotic metaphases. Normal mouse tissues that are the usual sources for cytogenetic studies (spleen, kidney, bone marrow) provide too few cells and an insufficient percentage of cells in metaphase. Consequently, a tetraploid transformed cell line (Figure 3-4) was established (Chapter 4) from a male R5 carrier to supply not only the millions of live cells but the large percentage of synchronous metaphases needed for both FACS and for microdissection of the large T(X;7) chromosome.

Figure 3-2. Reproduction of the profile for the single-laser flow-sorting of the X^7 chromosome in a tetraploid, SV-40-transformed fibroblast cell line derived from a male carrier of T(X;7)5R*l*. The small peak between channels 450 and 500 was collected as the fraction containing the X^7 chromosome. The Y-axis is the relative number of chromosomes at each fluorecence level (the X axis). The chromosomes were stained with propidium iodide.

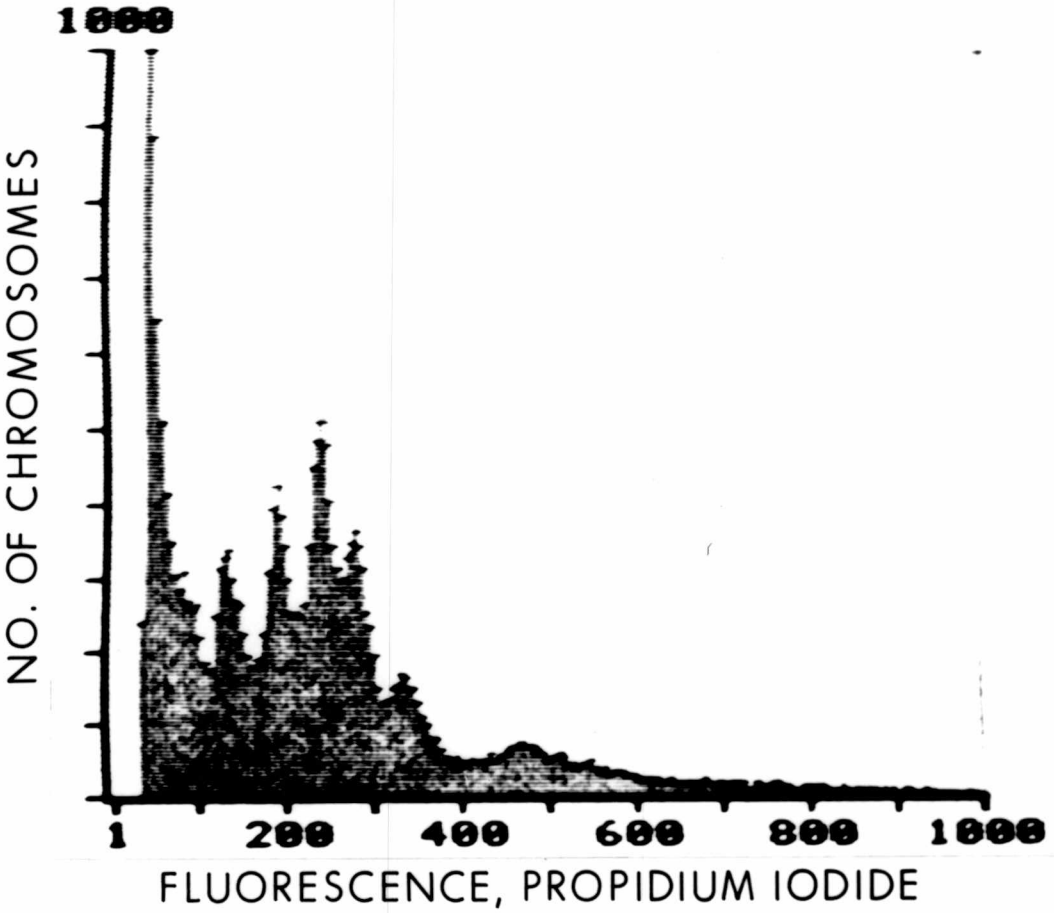
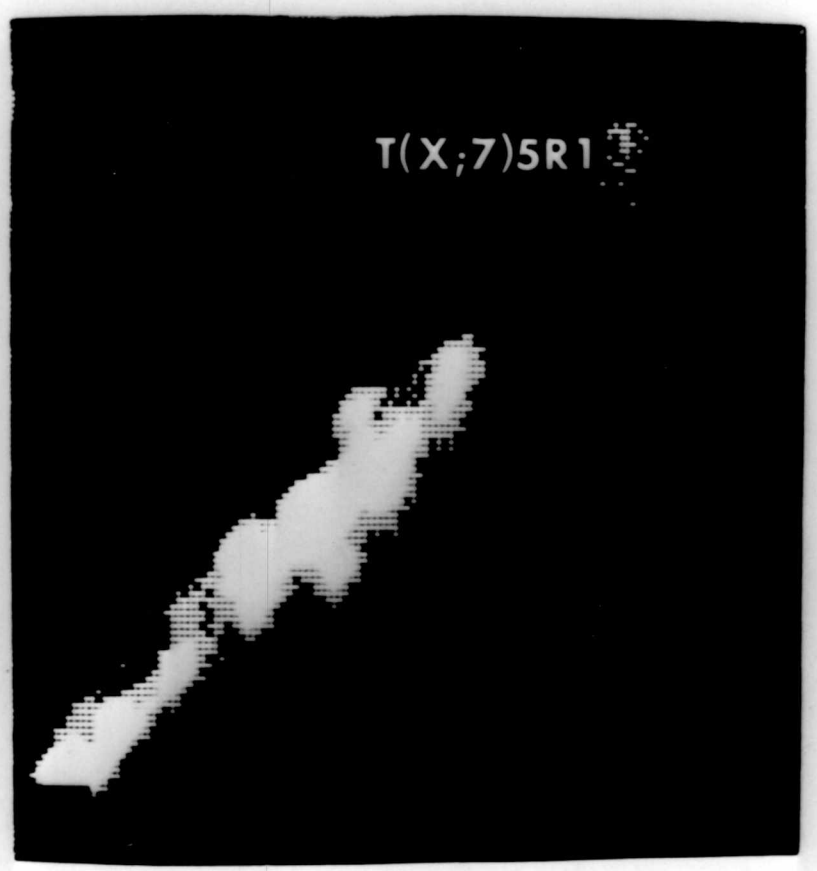

1000
NO. OF CHROMOSOMES
1
200
400
600
800
1000
FLUORESCENCE, PROPIDIUM IODIDE

Figure 3-3. Photograph, supplied by M. Bartholdi of Los Alamos National Laboratory, of the FACS profile of dual-laser-sorted chromosomes from the tetraploid, SV-40-transformed fibroblast cell line, SVR5-Af_3. The X-axis is the fluorescence signal generated by the stain Hoescht 33258, and the Y-axis is the fluorescence from the second stain, chromomycin A3. The fraction thought to contain the large T(X;7)5R*l* chromosome is designated. Isolation and sorting of chromosomes were performed at Los Alamos.

Mouse Fibroblasts SVR5-Af2

Hoechst 33258



Chromomycin A3

Figure 3-4. Giemsa-banded karyotype, courtesy of N. L. A. Cacheiro, of the SV-40-transformed tetraploid fibroblast cell line cultured from a male carrier of T(X;7)5R*l*. Two copies of the large X^7 chromosome are seen at the lower right.

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CHAPTER 4

MOLECULAR MAPPING WITHIN THE MOUSE ALBINO-DELETION COMPLEX

General Introduction

Chapter 4 consists of a journal article, entitled, "Molecular Mapping Within the Mouse Albino-Deletion Complex," that has been published in the Proceedings of the National Academy of Sciences (Johnson et al., 1989). This article describes the construction and screening of a genomic library, enriched for chromosome-7 sequences, from the large T(X;7)5R*l* chromosome described in Chapter 3. Screening of clones from this library, using DNAs from (*Mus musculus/Mus spretus*)F_1 hybrid carriers of the *c*-region deletions, has resulted in the isolation of an anonymous sequence, 11B2, which recognizes the locus *D7OR1,* which is deleted only in the two deletions, Fp1 and Fq1, that extend the farthest distally from *c. D7OR1,* therefore, maps in the distal end of the c region deletion complex (Chapter 2). This map position was confirmed by hybridization of 11B2 to a 130-segregant chromosome-7 *musculus/spretus* linkage backcross panel. Because deletions that include this area of the *c*-region include not only the neurological mutation shaker-1, but also at least four new, ethylnitrosourea-induced, presumed intragenic embryonic lethals (Rinchik et al., in press; Rinchik and Carpenter, unpublished data), the locus *D7OR1* may provide a point of molecular access to any or all of these interesting

developmental-control genes. This publication also offers important insights into the logistics of screening random-clone libraries with a panel of nested deletions carried by *M. musculus/M. spretus* interspecific hybrids.

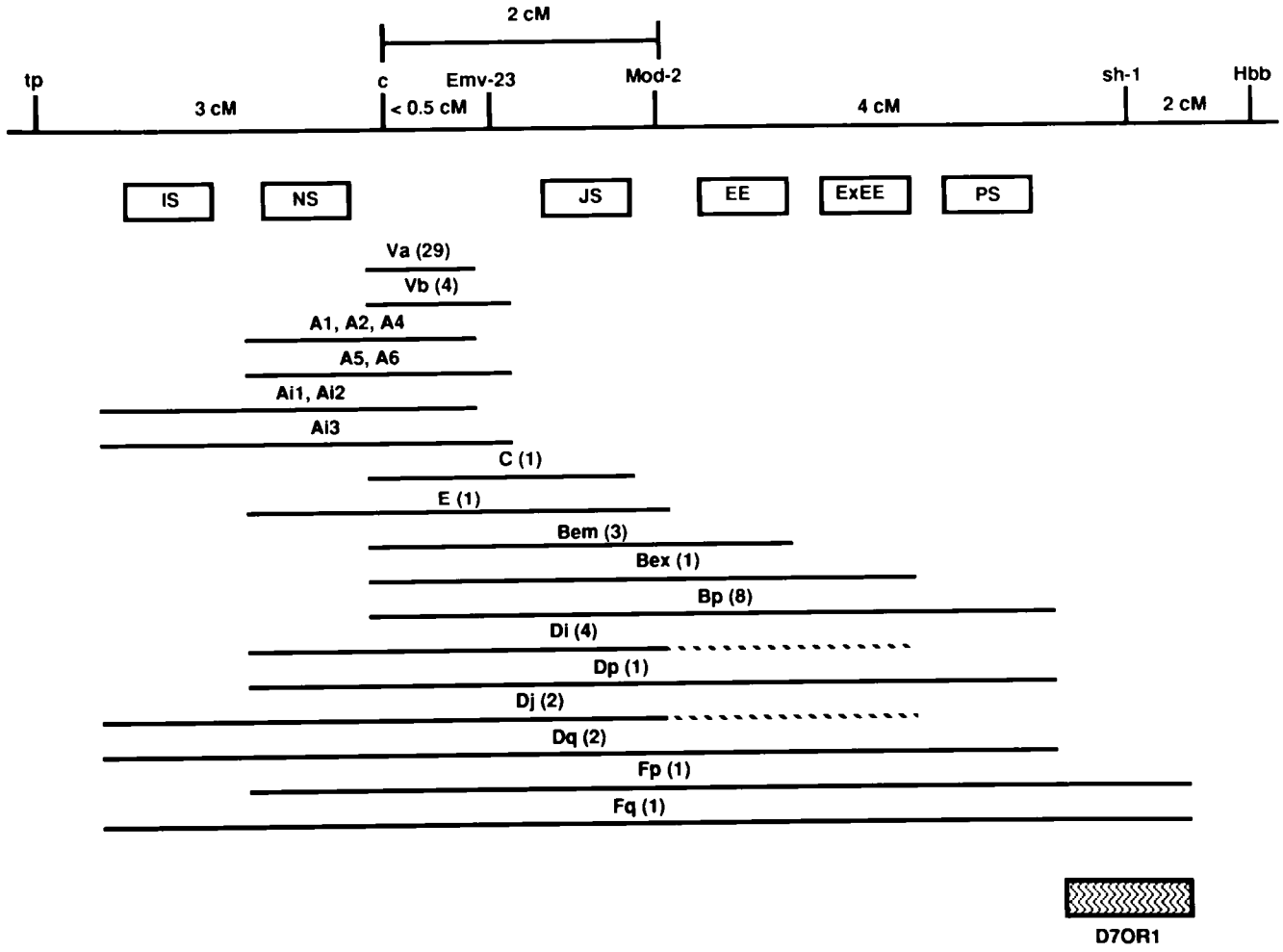
Abstract

Induced germline deletion mutations in the mouse provide a malleable experimental system for in-depth molecular and functional analysis of large segments of the mammalian genome. To obtain an initial bank of molecular probes for the region of mouse Chromosome 7 associated with the albino (*c*) deletion complex, random, anonymous DNA clones, derived from a library constructed from flow-sorted chromosomes, were screened on DNAs from *Mus musculus-M. spretus* F_1 hybrids carrying large, multi-locus, lethal *c* deletions. Clones falling within a given deletion interval can easily be recognized because hybridization bands that represent RFLPs specific for the mutant (deleted) chromosome inherited from the *musculus* parent will be absent. Among 72 informative clones used as probes, one, which defines the locus *D7OR1*, mapped within two deletions that are 6-11cM in length. Submapping of this anonymous clone across a panel of 27 smaller deletions localized *D7OR1* distal to a chromosomal subregion important for survival of the preimplantation embryo, proximal to globin [b-chain (*Hbb*)], and near the shaker-1 (*sh-1*) locus. The results of these deletion-mapping experiments were also confirmed by standard three-point linkage analysis. This

strategy for selection and rapid mapping of anonymous DNA probes to chromosomal segments corresponding to germline deletion mutations should contribute to the generation of more detailed physical and functional maps of genomic regions that are associated with mutant developmental phenotypes.

The generation and study of heritable mutations in the mouse provide the means for identifying and localizing genes essential for normal mammalian development by associating mutant developmental phenotypes with specific subregions of the genome. Radiation-induced, germline deletion mutations, such as those surrounding the albino (*c*) locus in Chromosome 7 of the mouse (1-3), have been useful for building detailed functional maps of genomic regions (2-7). Crosses among 34 independent, recessive-lethal *c* mutations recovered in radiation-mutagenesis experi-ments originally yielded a linear, 13-group complementation/deletion map comprised of at least 8 functional units (2). Figure 4-1 depicts the current proposed genetic map of this region. The *Df(c Mod-2 sh-1)*^{Fq1} mutation, which may be the longest of the deletions, is 6-11 cM in genetic length (2), and may be missing 6-20 megabase pairs of DNA. The segment of DNA deleted in this mutant includes the marker loci *c* (albino; tyrosinase), *Mod-2* (mitochondrial malic enzyme) and its closely linked regulator *Mdr-1* (8-11), and *sh-1* (shaker-1), a gene that participates in inner-ear development (12-14). Moreover, complementation analysis has provided evidence for the following: at least four subregions necessary for survival of

Figure 4-1. Complementation/deletion map of a 6- to 11-centimorgan (cM) region of Chromosome 7 defined by the *Df(c Mod-2 sh-1)^{12FR60Hb}* (Fq1) deletion (Russell, et al, 1982). Marker loci are taupe, *tp*; albino (tyrosinase), *c*; mitochondrial malic enzyme, *Mod-2;* shaker-1, *sh-1*; and globin (beta chain), *Hbb. tp* is the most proximal locus. *Emv-23* is the integration site of an endogenous murine ecotropic provirus (Rinchik, et al, 1989). Distances between marker loci are given in cM. "Functional units" (shown as open boxes), defined by developmental phenotypes expressed by segments of unknown physical size and complexity, are defined by complementation analysis of 34 independent *c*-locus deficiency mutations (Russell, et al., 1982); they are abbreviated as IS, implantation survival; NS, neonatal survival; JS, juvenile survival, and PS, preimplantation survival. The EE and ExEE functional units (Niswander, et al., 1989) are associated with the development of the embryonic ectoderm and the extraembryonic ectoderm, respectively, and are subdivisions of Russell's (Russell, et al., 1982) second implantation-survival unit. Deletions of the IS, PS, EE, and ExEE functional units are associated with death of the embryo before (PS) or shortly after (IS, EE, ExEE) implantation (Lewis et al., 1976; Lewis, 1978; Russell and Raymer, 1979; Niswander,et al., 1989) The NS unit is needed for neonatal survival and corresponds to a locus encoding a *trans*-acting regulator for metabolic enzymes and other proteins (reviewed Gluecksohn-Waelsch, 1979), and the JS unit is associated with male sterility (Lewis, et al., 1978) and reduced fitness in both sexes (Russell, et al., 1982). The heavy lines below the functional map indicate the presumed extents of the Oak Ridge *c* deletions in each complementation group. The letter designation (Va, Vb, A, Ai, Bem, Bex, Bp, C, Di, Dj, Dp, Dq, E, Fp, Fq) indicates the complementation group, and the numeral in parenthesis represents the number of independent mutations assigned to each group. All deletions are homozygous lethal except for the V groups, Va and Vb; occasionally, however, some C/C homozygotes can survive to well past a year. Broken lines shown for complementation groups Di and Dj indicate uncertainties about the distal extents of these deletions with respect to the EE and ExEE regions. The shaded box at lower right represents the proposed map position of *D7OR1*


2 cM
tp
c
Emv-23
Mod-2
sh-1
Hbb
3 cM
< 0.5 cM
4 cM
2 cM
IS
NS
JS
EE
ExEE
PS
Va (29)
Vb (4)
A1, A2, A4
A5, A6
Ai1, Ai2
Ai3
C (1)
E (1)
Bem (3)
Bex (1)
Bp (8)
Di (4)
Dp (1)
Dj (2)
Dq (2)
Fp (1)
Fq (1)
D7OR1

the preimplantation and implantation embryo (1-3, 15-19); one subregion, proximal to *c*, that is important for survival of the neonate (2,3) and is hypothesized to encode a *trans*-acting factor important for regulation of metabolic enzymes and serum proteins (3, 20-22); and one subregion, denoted in Fig. 4-1 as JS ("juvenile survival"), which, when deleted, gives rise to infertility in males, spontaneous,mid-gestation "abortion" in females, and decreased fitness in both sexes (23, 2).

Although it has been possible to associate general developmental phenotypes with this nested set of germline deletions, the actual number and nature(s) of genes present within the region associated with *Df(c Mod-2 sh-1)^{Fq1}* have yet to be determined. Indeed, the early-death phenotypes associated with some of the longer deletions may be masking the effects of additional early-acting, as well as later-acting, genes. We have, therefore, been developing strategies to refine both the physical and functional maps of the entire *Df (c Mod-2 sh-1)^{Fq1}* region. This report describes the use of genomic DNAs from *Mus spretus-M. musculus* interspecific hybrids carrying *c*-region deletion chromosomes for the selection and rapid subregion-mapping of random, anonymous clones derived from a DNA library enriched for Chromosome-7 sequences.

Materials and Methods

Mice The 129/Rl - *p c^{ch}/p c* segregating inbred strain was bred at ORNL, as were closed-colony, outbred stocks of *Mus spretus* and of *Mus musculus domesticus* carrying radiation-induced

deletions of the *c* locus or the balanced reciprocal translocation T(X;7)5RI. Breeding nuclei of outbred *Mus spretus* were generously provided by Dr. V. Chapman (Roswell Park Memorial Institute). Each lethal albino deletion [designated *Df(c)*] was maintained heterozygously opposite a chinchilla (c^{ch}) chromosome. This heterozygote [c^{ch} /*Df(c*)] manifests a light-chinchilla coat color, in contrast to full-chinchilla color evident in c^{ch}/c^{ch} segregants. A *spretus/musculus* backcross (24), segregating for the Chromosome-7 visible markers c^{ch} and *p* (pink-eyed dilution), was generated by crossing 129/RI-*p* c^{ch}/p c^{ch} females to a *Mus spretus* male, then crossing the resultant F_1 females to 129/RI-*p* c^{ch}/p c^{ch} males to provide the required N2 segregants.

Each lethal *c* deletion was made heterozygous with a *M. spretus* Chromosome 7 by a progeny-testing protocol. *Musculus* females carrying a specific deletion [c^{ch}/*Df(c)*] were crossed to *spretus* males ($+^{SPT}/+^{SPT}$). To determine which of the phenotypically wild-type F_1 females carried the deletion [*Df(c)*] chromosome (rather than the c^{ch} chromosome), each was crossed to a male (*c/c*) homozygous for the standard, non-lethal albino mutation. F_1 females yielding albino [*c* /*Df(c*)] and wild-type ($+^{SPT}/c$) offspring were of the desired $+^{SPT}$/*Df(c)* genotype. The $+^{SPT}$/*Df(c)* F_1 females, along with their c^{ch}/*Df(c)* dams and *spretus* sires, were sacrificed, and high-molecular-weight DNA was prepared from their spleen or liver as previously described (25).

Construction of a Library from Flow-Sorted Translocation Chromosomes.

An SV40-transformed cell line was produced, using methods described elsewhere (26), from muscle fibroblasts derived from a male carrying the T(X;7)5R*l* translocation (27). Nuclei in this cell line contain two copies of the T(X;7)5R*l* translocation chromosome, which carries the distal four-fifths of Chromosome 7 and is approximately 1.4 times the length of Chromosome 1, the longest normal mouse chromosome (27). Chromosomes were isolated from this line (which was tetraploid, XY) using the technique of Fuscoe, et al. (28) with modifications. Cells were blocked in mitosis using colchicine ($5x10^{-7}M$), and resuspended in 0.75*M* KCl for 30 min at room temperature. Chromosomes were released into the polyamine isolation buffer by passage through a 26-gauge needle. The chromosome suspension was stained with propidium iodide (10 ug/ml), and 2 X 10^6 T(X;7)5R*l* chromosomes were sorted in 0.5-ml aliquots on an Ortho 50H cytofluorograf cell sorter equipped with a 5W LEXEL argon-ion laser with an output of approximately 350 mW at 488 nm. Each aliquot was incubated overnight at 37∞ C in 0.5% SDS and 100 ug/ml proteinase K, extracted, and dialzyed. Following digestion to completion with *Sau*3A, the chromosomal DNA was ligated into *Bam*HI-digested pUC13. The ligation products were transformed into E. coli (DH5a) (Bethesda Research Laboratories) and plated onto LB agar + ampicillin.

White colonies were screened with radiolabelled total mouse DNA to identify clones bearing highly repetitive mouse sequences, and DNA minipreps were made from non-hybridizing clones. *Pvu*II restriction fragments from plasmids containing inserts were isolated by electrophoresis in low-melting agarose, radiolabelled by random-hexamer priming, and hybridized to appropriate Southern blots by methods described elsewhere (29).

Results

Use of musculus/spretus deletion heterozygotes for selection of c-region-specific clones.

A plasmid library of approximately 20,000 recombinants was constructed from DNA isolated from flow-sorted T(X;7)5R/ chromosomes. DNA minipreps from 162 clones, which were judged to be free of repetitive sequences because they failed to hybridize to radiolabelled total mouse DNA, were digested with *Pvu*II to release the mouse insert plus 322 base pairs of flanking vector sequence. These *Pvu*II fragments were individually radiolabelled and hybridized to Southern blots bearing digests of the following DNAs: *Mus musculus* *c*-deletion heterozygotes (c^{ch}/c^{Ai1} and c^{ch}/c^{Fp1}); *musculus/spretus* F_1 deletion heterozygotes ($+^{SPT}/c^{Ai1}$ and $+^{SPT}/c^{Fp1}$); and wild-type *Mus spretus* ($+^{SPT}/+^{SPT}$). Each clone was hybridized to this same set of DNAs digested with at least two different enzymes.

Of the 162 clones screened, 72 could be unequivocally mapped relative to the two overlapping *c*-region deletions Ai1 and Fp1. Of these 72, one clone, 11B2, mapped within the limits of the Fp1 deletion and will be described in detail below. Of the 90 clones for which no result was obtained, 28 gave no *musculus/spretus* RFLP for any of 4 enzymes (*Eco*RI, *Bam*HI, *Taq*I, and *Msp*I), 21 apparently contained repetitive sequences not detected by the pre-screen (as indicated by hybridization patterns ranging from multiple bands to dark smears), and 41 gave no hybridization signal at all, probably due to very small insert size.

The hybridization pattern of one clone that did map to the *c* region, 11B2, is shown in Figure 4-2A. 11B2, a 90-base-pair unique-sequence probe, recognizes an 11-kb *Bam*HI fragment (designated $D7OR1^{SPT}$) in *spretus* DNA and an 18-kb fragment ($D7OR1^{M}$) in *musculus* DNA. The *musculus*-specific fragment is absent from the *musculus* (*c* deletion) */spretus* F_1 hybrid DNA ($c^{Fp1}/+^{SPT}$) in lane 5 (which displays only the 11-kb *spretus*-specific fragment), but is present both in *musculus* heterozygotes (c^{ch}/c^{Ai1} and c^{ch}/c^{Fp1}) in lanes 1 and 4 and in the *musculus/spretus* F_1 ($c^{Ai1}/+^{SPT}$) in lane 2. The *D7OR1* locus, defined by clone 11B2, therefore maps in the interval defined by the distal breakpoints of the Ai1 and Fp1 deletions (Fig. 4-1).

Figure 4-2. Selection and subregion mapping of an anonymous DNA probe to the *c*-region deletion complex. (A) A representative *c*-region screening blot; and (B) a representative subregion-mapping blot. *M. musculus* deletion heterozygotes (c^{ch}/c^{Fp1}, for example), *musculus/spretus* deletion heterozygotes (c^{SPT}/c^{Fp1}, for example), and *spretus* homozygotes (SPT) are indicated. The deletion abbreviations correspond to complementation groups depicted in Figure 2-1. Fragment sizes in this and all figures were determined by comparison to *Hind*III-restricted lambda DNA (not shown).

A
kb
18 –
11 –
1 cch/Ai1
2 SPT/Ai1
3 SPT
4 cch/Fp1
5 SPT/Fp1
B
kb
18 –
11 –
1 cch/Ai2
2 SPT/Ai2
3 SPT/Ai3
4 cch/Bem1
5 SPT/Bem1
6 cch/Bex1
7 SPT/Bex1
8 cch/Bem2
9 SPT/Bem2
10 cch/Bp7
11 SPT/Bp7
12 cch/Bp8
13 SPT/Bp8
14 cch/Di1
15 SPT/Di1
16 cch/Dj2
17 SPT/Dj2
18 SPT
19 SPT/Dq1
20 SPT/Fq1
21 cch/Fq1

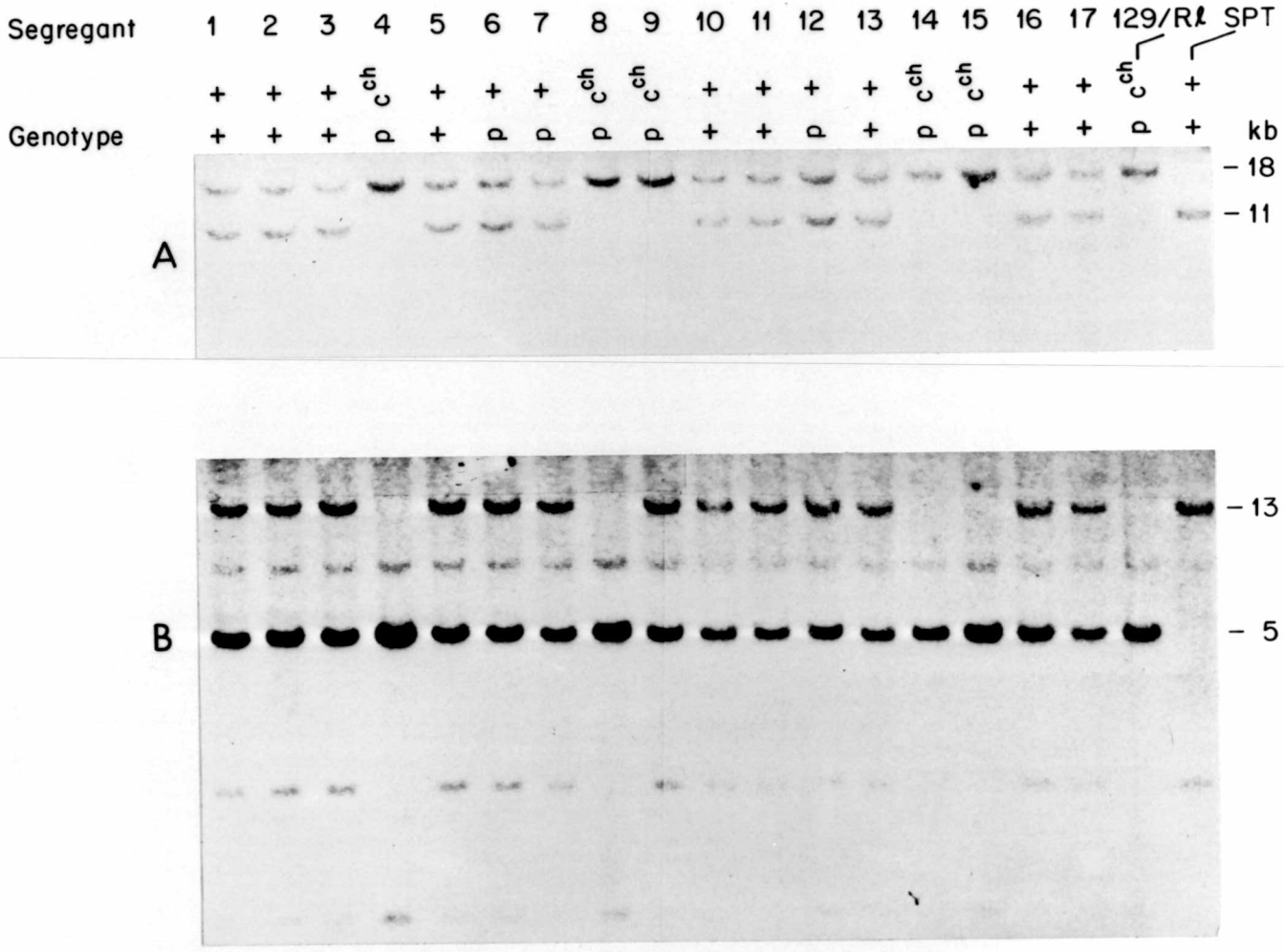
Deletion-Mapping of D7OR1 Within The *c*-Region Deletion Complex.

Figure 4-2B shows hybridization of clone11B2 to *Bam*HI-digested genomic DNAs from selected *musculus/spretus* deletion heterozygotes [$+^{SPT}/Df(c)$] and their *musculus* dams [$c^{ch}/Df(c)$]. Only *musculus/spretus* animals that carry the F-group deletions (Fp1, Fig. 2A; Fq1, Fig. 4-2B) are missing the *musculus*-specific fragment detected by 11B2. DNAs from all other *spretus*-balanced deletion chromosomes (i.e., all members of groups Ai, Bem, Bex, Bp, Di, Dp, Dj, and Dq) carry the fragment (Fig. 4-2B and data not shown). This indicates a map position for the locus *D7OR1* in the most distal end of the *c* -region deletion complex, near the *sh-1* locus (see Fig. 4-1).

Confirmation of the Map Position of *D7OR1* by Standard Backcross Analysis.

The *D7OR1* locus was also mapped in a standard linkage backcross with respect to Chromosome-7 anchor loci to provide a test of the accuracy and reliablility of selecting and submapping region-specific clones with the *musculus/spretus* deletion panel. *Bam*HI-digested spleen DNAs from segregants of the backcross (129/RI-*p* c^{ch}/p c^{ch} X *spretus*) F_1 X 129/RI-*p* c^{ch}/p c^{ch} were hybridized with clone 11B2 (Fig 4-3A). [In this particular backcross, progeny will segregate *p* (pink-eyed dilution) and c^{ch} (chinchilla) along with RFLPs detected by 11B2 and other DNA clones.] Following hybridization with 11B2 and

Figure 4-3. Interspecific backcross mapping of *D7OR1* with respect to Chromosome-7 anchor loci. Pictured is a Southern blot of representative segregants from the backcross (129/R*l-p* c^{ch}/*p* c^{ch} X *spretus*)F_1 X 129/R*l-p* c^{ch}/ *p* c^{ch}. The same segregants (1-17) are shown in both panels, and the abbreviated pink-eyed dilution (*p*) and albino genotypes (*c*) are given for each segregant. The full genotype for each segregant includes a *p* c^{ch} chromosome; for example, segregant 1 is $+^{SPT}$ $+^{SPT}$/ *p* c^{ch} whereas segregant 4 is *p* c^{ch}/ *p* c^{ch}. (A) *Bam*HI-digested spleen DNAs were electrophoresed, blotted to nylon, and probed with the 412-bp *Pvu*II fragment containing clone 11B2 (*D7OR1*). (B) The same Southern blot as in panel A was stripped and reprobed with the 1.1-kb *Hin*dIII-*Eco*RI fragment of pPK268 (*Hbb*). Note that segregant 9 is a *D7OR1* - *Hbb* recombinant.


Segregant
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 129/Rℓ SPT
+ + + p^{ch} + + + p^{ch} p^{ch} + + + + p^{ch} p^{ch} + + p^{ch} +
Genotype
+ + + p + p p p p + + p + p p + + p +
kb
A
18
11
B
13
5

autoradiography, the blots were stripped and rehybridized with a 0.8-kb *Eco*RI-*Hin*dIII fragment of pPK268 (30), which is derived from the ß-globin (*Hbb*) locus that maps 8 cM distal to *c* and 2 cM distal to *sh-1*. This *Hbb* probe recognizes a 5-kb fragment (designated Hbb^{M}) in 129/RI DNA and a 13-kb fragment (Hbb^{SPT}) in *spretus* DNA (Fig. 4-3B). Results from the genotyping of 134 backcross progeny, summarized in Table 4-1, indicate that *D7OR1* maps 3.7 ± 1.6 cM distal to *c* and 1.5 ± 1.0 cM proximal to *Hbb*. This map position is entirely consistent with the results of the deletion-mapping experiments with *musculus/spretus* F_1s, which also place *D7OR1* between *c* and *Hbb*, in the vicinity of *sh-1*.

DISCUSSION

The goal of the experiments reported here was to develop and test a strategy for taking the first step toward detailed molecular characterization of the genes and developmental functions associated with large segments of the mouse genome. An integral component of this strategy is the exploitation of extensive "complexes" of agent-induced, overlapping deletion mutations to map DNA clones to specific chromosomal subregions associated with specific developmental phenotypes. The panel of *c*-locus deletions in Chromosome 7 (2, 19) is particularly suited to this strategy because extensive genetic analysis of *c*-deletion complementation patterns has produced a functional map that localizes loci responsible for such phenotypes as early embryonic, perinatal, and juvenile lethality,

TABLE 4-1. Mapping of *D7OR1* with Respect to Mouse Chromosome-7 Markers Pink-Eyed Dilution (*p*), Chinchilla (c^{ch}), and Beta Globin (*Hbb*)

Cross: (129/RI X M. *spretus*)F_1 X 129/RI

I	II	III		
+	+	$D7OR1^{SPT}$	Hbb^{SPT}	
			X	
p	c^{ch}	$D7OR1^{M}$	Hbb^{M}	

p c^{ch} $D7OR1^{M}$ Hbb^{M}

Allele Configuration*	Genotypes†	Number of Progeny
P	+ + $D7OR1^{SPT}$ Hbb^{SPT}	61
	p c^{ch} $D7OR1^{M}$ Hbb^{M}	47
I	+ c^{ch} $D7OR1^{M}$ Hbb^{M}	7
	p + $D7OR1^{SPT}$ Hbb^{SPT}	12
II	+ + $D7OR1^{M}$ Hbb^{M}	3
	p c^{ch} $D7OR1^{SPT}$ Hbb^{SPT}	2
III	+ + $D7OR1^{SPT}$ Hbb^{M}	0
	p c^{ch} $D7OR1^{M}$ Hbb^{SPT}	2
	Total	134

*P = parental configuration; I = crossover between *p* and c^{ch}; II = crossover between c^{ch} and *D7OR1;* III = crossover between *D7OR1* and *Hbb.* No double crossovers were observed in any progeny. On the basis of these data, the order is as follows (with genetic distances): *p* -- (14.1 ± 3.0 cM) -- c^{ch} -- (3.7 ± 1.6 cM) --*D7OR1* -- (1.5 ± 1.0 cM) -- *Hbb.*

†All segregants carry a *p* c^{ch} $D7OR1^{M}$ Hbb^{M} chromosome.

male sterility, and inner-ear abnormalities.

A transformed cell line carrying a large and morphologically distinct translocation chromosome that includes the wild-type *c* region (27) made possible the construction of a flow-sorted chromosome library enriched for Chromosome-7 sequences. Random, anonymous DNA clones from chromosome-specific libraries are especially useful for gaining molecular access to relatively large chromosomal regions because all DNA sequences within the region should be equally represented.

Because long deletions are almost always homozygous lethal at or before implantation, DNA cannot readily be obtained from deletion homozygotes. Indeed, even embryonic stem-cell (ES) lines cannot be generated from embryos homozygous for one of the relatively shorter deletions of the Bem complementation group (Fig. 4-1). As a consequence, we have based our clone-selection and mapping strategy on a panel of DNAs derived from interspecific hybrids. Once large *Mus musculus*-derived deletions are balanced opposite a non-deleted chromosome from a *Mus spretus* sire, RFLP analysis can readily, and unequivocally, identify a clone that falls within the deletion interval because the maternal, *musculus*-specific hybridization band contributed by the deletion carrier will be absent from DNA of the F_1 animal. This method of mapping obviates the need for analysis of one- vs two-copy hybridization-intensity differences between deletion heterozygotes and wild-type controls, which can be an inexact and tedious procedure for screening large numbers of clones.

Indeed, this *musculus/spretus* strategy has recently been successful for mapping of DNA clones to a deletion involving the dominant spotting (*W*) locus in mouse Chromosome 5 (31, 32).

In a pilot experiment, 162 anonymous clones derived from flow-sorted T(X;7)5R*I* chromosomes were screened on DNAs from *musculus/spretus* F_1s carrying the Ai1 or Fp1 *c* deletions (See Fig. 4-2A). (We are using the Dq and Fq deletions in current experiments.) This pilot experiment provided valuable logistical information on how best to handle large numbers of clones and screening blots, and proved the efficacy of detecting a clone that falls within the limits of the largest deletions. Of the 72 clones that could be fully tested, one, 11B2, was found to map within the limits of the Fp1 deletion. If chromosome sorting could yield a pure fraction of the T(X;7)5R*I* chromosome, one would expect, based on the estimated sizes of the deletions compared to the overall size of the chromosomes, to find 1 in 20 clones mapping to the region. We expect that multivariate chromosome-sorting and chromosome microdissection will greatly increase the purity of subsequent DNA libraries and the efficiency of deriving clones that map to this region.

The clone, 11B2, which defines the locus *D7OR1* and which maps within the limits of the *c*-locus deletions Fp1 and Fq1, was also mapped against all the other lethal deletions of this complex. The locus *D7OR1* is not deleted in any of the other prenatally lethal deletions (Ai-, B-, or D- groups), suggesting a position for *D7OR1* between *c* and *Hbb* on the standard genetic

map, and distal to the preimplantation functional unit (PS) on the complementation/deletion map in Fig 4-1. This map position was confirmed by standard linkage analysis, demonstrating that, at least for this locus, deletion-mapping utilizing the panel of *musculus/spretus* DNAs enabled both accurate selection and accurate subregional mapping of a *c*-region specific clone from an enriched library.

It is noteworthy that *D7OR1* maps in the vicinity of shaker-1 (*sh-1*). Mutations of this locus result in anomalies of the inner-ear labyrinth (12- 14). Although no information yet exists about how close it is to *sh-1*, *D7OR1* could prove useful as a molecular access point to this interesting mutation affecting inner-ear development. Likewise, *D7OR1* maps in the area near the distal breakpoints of the Bp, Dp, and Dq deletions, and should be useful for expanding the molecular map of a subregion required for development of the preimplantation embryo. Moreover, clone 11B2 can now be used to initiate molecular walks to these deletion breakpoint-fusion fragments, resulting in chromosome "jumps" across the deleted regions into the proximal end of the albino-deletion complex.

Several DNA clones, most notably the anonymous sequence 12A, also isolated from a flow-sorted-chromosome library (33), tyrosinase clones of both human (34) and mouse (35, 36) origins, and 23.3, derived from the *Emv-23* locus (29), have been mapped near *c* by their inclusion in the smaller homozygous or overlapping deletions. Anonymous clones mapped into the region

defined by the largest deletions should result in a more extensive, ordered series of molecular access points that span the functional/complementation map, and should be useful for generating a long-range physical map of the entire region. Moreover, as additional *N*-ethyl-*N*-nitrosourea-induced single-gene mutations of loci encompassed by these deletions become available (E. M. Rinchik and D. A. Carpenter, manuscript submitted), it should become possible to correlate a genetic locus with a specific function, a precise physical-map location, and a specific DNA sequence.

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CHAPTER 5

CONSTRUCTION AND SCREENING OF A RANDOM-CLONE GENOMIC LIBRARY FROM T(X;7)5R*I* CHROMOSOMES ISOLATED BY DUAL-LASER FLOW SORTING

The enrichment for chromosome-7 sequences in the genomic library constructed from single-laser-sorted *T(X;7)5R*I chromosomes (Chapter 4) was lower than desired as evidenced by the isolation of only a single *c*-region (and no *p*-region) clone from 162 clones tested. Therefore, a second library was constructed from T(X;7)5R*I* chromosomes sorted by dual-laser technology (Fig 3-4) courtesy of M. Bartholdi at Los Alamos National Laboratory. By using two fluorescent dyes (Hoechst 322588 and chromomycin A_3), which preferentially bind to DNA sequences of different composition (A-T vs. C-G), more effective separation of the chromosome of interest from other chromosomes in the karyotype could be achieved. Chromosome suspensions were prepared, stained, and sorted at Los Alamos by the standard methods in use there (Fuscoe et al, 1986).

In an initial cloning attempt, DNA was extracted as described in Chapter 4 from 1.2 x 10^6 chromosomes from a fraction expected to be highly enriched for the X^7 chromosomes from T(X;7)5R*I*. The DNA was digested with *Hin*dIII and cloned into *Hin*dIII-digested pUC13 (Pharmacia). Transformation into

competent cells resulted in the recovery of only 88 recombinants.

A second cloning experiment was then undertaken. Approximately 9.5 x 10^5 chromosomes were extracted as above, digested with *Eco*RI, and ligated into lambda gt10, yielding 7000 recombinant phage. The entire library was grown *en masse,* phage DNA was prepared by the liquid-lysis method (*Current Protocols in Molecular Biology,* Unit 1.31.1), and then *Eco*RI-digested fractions were size-selected on a sucrose gradient. The fractions centered around 0.5-kb and 1.0-kb were ligated into EcoRI-digested pBSM13+ (Stratagene). Seven-hundred-sixty-two recombinants were prescreened for the presence of highly-repeated sequences as described in Chapter 4. Of those 762, 359 (53%) were eliminated outright from use as probes by hybridization to radiolabelled total mouse DNA. DNA minipreps were made from 148 of those recombinant clones not eliminated in the prescreening process. *Eco*RI inserts from those preparations were radiolabelled by random-hexamer priming (Chapter 4) and were hybridized to Southern blots containing the following DNAs: a *Mus spretus* male ($+^{SPT}/+^{SPT}$); a *M. musculus* female carrier of the *c*-region deletion c^{Fp1} (c^{ch}/c^{Fp1}); (*musculus/spretus*)F_1 hybrid carriers of two *c*-region deletions ($+^{SPT}/c^{Dq1}$ and $+^{SPT}/c^{Fq1}$); and (*musculus/spretus*)F_1 hybrid carriers of three lethal *p*-region mutations ($+^{SPT}/p^{46DFioD}$, $+^{SPT}/p^{2HATh}$, and $+^{SPT}/p^{12R30Lb}$). The *M. musculus* female provided a generic *M. musculus*-specific control hybridization

for all lanes containing F_1 hybrid carriers. Had it been necessary, any F_1 hybrid DNA could have been compared on a second Southern blot directly with the actual parental DNAs. The DNAs on one series of screening blots were digested with *Eco*RI and *Bam*HI, and on a second series, with either *Msp*I and *Taq*I.

Of the 148 inserts hybridized to the Southern blots just described, 71 proved, by smeared hybridizations, to contain repetitive sequences, not detected in the prescreen, which made those clones unsuitable for use as hybridization probes. Another 17 failed to detect an RFLP between the *M. musculus* and *M. spretus* genomes with either of two enzymes used to digest the DNAs on the screening blot. Forty-four other clones (a) gave no signal, (b) had no insert, (c) did not grow in (ampicillin) liquid culture, or (d) did not digest with *Eco*RI. Of the remaining 19, which gave clear results, none mapped to any of the *c*- or *p*-region mutations tested on the screening blots.

There is no immediately apparent explanation for the poor quality of this second flow-sorted library. Since results were clear for only 17 recombinant clones, no assessment can be made of the level of enrichment for chromosome-7 sequences in the library. Nearly half (71 of 148) probes contained repetitive sequences not detected in the prescreen but which rendered those probes useless. Perhaps the extra step in the second cloning experiment, in which inserts were first cloned into lambda gt10 and only then into pBSM13+ introduced some bias

toward repetitive sequences. In any case, the construction and screening of a second library from flow-sorted chromosome unfortunately proved to be a fruitless endeavor.

CHAPTER 6

GENERAL INTRODUCTION TO THE *p* REGION

Pink-eyed dilution (*p*) is a very old mutation carried by many varieties of the mouse fancy. Mutations at the pink-eyed dilution (*p*) locus in chromosome 7 drastically reduce the pigmentation of both the eyes and coat. The mutation is thought to affect chiefly the black and brown pigments (eumelanin) with little if any reduction of the yellow pigments (phaeomelanin) (Silvers, 1979). The original *p* mutation can be shown to retain some activity since, in females carrying X-autosome translocations (X^7/X; $7^X/7$, where X^7 carries *p* and the normal 7 also carries *p*) functionally hemizygous (*p*/0) pigment cells (those with the *p* mutation on the X^7 in the inactive state) produce a lighter color than homozygous cells (*p/p*, where the *p*-bearing X^7 is active). These females are mottled for *p*/0 on a slightly darker *p/p* background (L. B. Russell, 1983); this observation also proved that the original *p* mutation is not cell lethal. Russell (1964) also showed that females who carry a wild-type p^+ locus on a T(X;7) chromosome and a lethal *p* mutation on the normal autosome 7 were variegated for *p* coat color. The variegation indicated that the cells in which the p^+ locus on the X^7 translocation chromosome was inactivated, that is, those cells in which the *p* phenotype was expressed, were viable despite the organismic lethality of the *p* mutation (p^{48PB})

used in the experiment.

The color disturbance is seen histologically as a change in the size, shape, and distribution of melanin granules in the hair (E.S. Russell, 1949), but nothing is yet known about the molecular nature of the mutation. In *p/p* animals, the structural fibers in melanosomes are not arranged in parallel fashion as is seen in wild-type animals, and much less cross-linking of fibers is observed (Moyer, 1961; 1963; 1966; Wolfe and Coleman, 1966). As a result, the mature melanin granules are smaller in size than normal and are very irregular in shape. These "shred-like" granules clump in the melanocyte, and are not distributed distally in the hair bulb (E. S. Russell, 1949). It may be that *p* alters the protein matrix of the melanosome; the action of the product of the *p* gene is known to be localized in the melanosome itself since a *p/p* host supports the normal development and function of transplanted normal melanocytes (Silvers, 1956; 1958).

Recent studies by Halaban, et al. (1988) on the activity and expression of tyrosinase, the enzyme product of the *c* locus, have shed further light on the possible role of the *p* locus in melanocytes. Although no experiments were done using cultured melanocytes mutant only at *p*, c^{ch} *p/c p* melanocytes had much lower tyrosinase activity than c^{ch} *+/*c^{ch} *+* melanocytes; c^{ch} *p/c p* melanocytes produce very little melanin, whereas c^{ch} *+/*c^{ch} *+* melanocytes show only slightly reduced melanin compared to wild-type. c^{ch} *p/c p* melanocytes yield very little

immunoprecipitatable tyrosinase, and what little is present is in lower-molecular weight forms indicative of protease degradation. How much of this difference is attributable to the mutation at *p* is uncertain since melanocytes included in the study also differed at *c* (c^{ch}/c vs. c^{ch}/c^{ch}). c^{ch} *p*/*c p* melanocytes, which have a well-developed Golgi apparatus, can be induced by the addition of DOPA to produce tyrosinase; the tyrosinase mRNA is the same size and appears to be of the same abundance as in wild-type melanocytes. These findings have led Halaban, et al. (1988) to suggest that the mutation of the *p* gene "affects the ability of tyrosinase to be transported to melanosomes or to be active in melanosomes."

Common alleles of *p* are p^d (*p* "dark"), p^x (*p* "extra-dark"), p^m (*p*-"mottled", autosomal somatic mottling of *p* and wild-type coat color), and p^{un} (*p*-"unstable", a *p* mutation exhibiting spontaneous somatic and germline reversion to wild-type at an increased frequency). The alleles that have contributed to the current report will be discussed in more detail later.

The *p* locus, which lies about 15 cM proximal to the *c* locus (Green, 1966), is another of the seven specific loci used as markers for experimental mutagenesis over the years at ORNL and elsewhere. Many radiation-induced *p* mutations have been isolated from irradiated animals; of those, 40 (36 *p*, 3 p^x, and 1 p^m) that are homozygous-lethal are presently being propagated in breeding stocks. Those stocks are maintained opposite a balancer chromosome as $+/p^*$ heterozygotes (where p^*

represents the mutant chromosome), except for p^m, which is maintained as p/p^m so that the mottled phenotype can be selected. Also being propagated in breeding stocks are seven *p*-locus mutations that survive to the perinatal or weaning period, and hence are classified "sublethal" (3 *p*, 2 p^x, 1 p^m).

The utility of the *c*-region deletion complex (described in Chapters 2 and 4) is inherent in the unambiguous, linear deletion/complementation map that reveals the outline of the constellation of genes and gene functions that lie within the region. Deletion-mapping for the *c*-locus deficiencies was simplified by the availability of four close flanking marker loci [1 proximal (*tp*) and 3 distal (*Mod-2*, *sh-1*, and *Hbb*)]. These loci aided in determining (a) that many of the lethal mutations were indeed deletions and, (b) the approximate extent in cM of some of the deletions. For the *p* region, similar deletion mapping could be done only for *ruby-2* (*ru-2*), a coat-color mutation that maps 3 cM proximal to *p*; there have been neither known distal markers closer than about 12 cM (*tp*) nor other close proximal markers. Of the 40 lethal *p*-locus radiation mutations, only one, Df(*ru-2 p*)^{46DFioD} encompasses both *p* and *ru-2*. Of the 39 remaining lethal mutations, many have been shown to be deletions by the molecular analyses to be reported below, but nothing is known of the proximal or distal extent of any *p*-region deletion. Twister (*twt*), a neurological mutation, was reported to map within 4 cM of *p* (Lane, 1981); crosses of thirteen lethal *p* deletions to *twt/twt* stocks, however, in no case elicited

pseudodominance of the twister phenotype (Chapter 10), so that no *p* mutation deletes as much as 4 cM distal to *p*.

Complementation analysis, involving pairwise crosses of most of the radiation-induced *p* mutations, has to date provided only a very rudimentary map of the proposed regions of overlap of the deletion mutations (L. B. Russell, personal communication). This rudimentary map suggests the presence of at least 5 embryonic lethals flanking the *p* locus, along with other phenotypes such as male sterility, neonatal lethality, and retardation of normal development evident from data gathered from both homozygotes (where possible) and non- or incompletely-complementing compound heterozygotes. There are also many examples of fully complementing compound heterozygotes whose only mutant phenotype is pink-eyed dilution. However, unlike the complementation data from the *c*-region deletion complex, the data from the *p*-region complementation testing do not suggest a simple, linear map of clearly defined functional units. Instead, the *p*-region complex appears to include deletion mutations that must consist of locally rearranged or "skipped" DNA segments, so that functions that appear contiguous in one series of complementation crosses appear to be non-adjacent in another. As a result, it has thus far proved impossible to postulate phenotype-based "functional units" whose limits are drawn by the relative breakpoints of overlapping deletions as has been done in the *c* region.

It is hoped that as molecular probes become available throughout the *p* region, those probes will act as "flanking markers" which can be used to define the relative distance encompassed by each member of the deletion complex. Those probes might also assist in determining if the hypothesized "skipping," or other large-scale, rearrangements have indeed occurred during the radiation induction of the *p*-locus mutations. When that information is available, together with more complete data from complementation crosses to identify those compound heterozygotes that do or do not include any of the same recessive-lethal loci, then the task of determining the patterns of overlap of the complex of deletion mutations can be completed.

Of the 47 lethal and sublethal *p*-locus mutations outlined above, 39 have been examined for complementation of lethal phenotypes by other *p* mutations, with most of the possible crosses in the complementation grid having been made (the cross that has been employed is $+/p^{la} \times +/p^{lb}$, where p^{la} and p^{lb} are independent, lethal *p* mutations; p^{la}/p^{lb} progeny would be of the pink-eyed-dilute phenotype) (L.B. Russell and C.S. Montgomery, unpublished information). To date, two-hundred thirty-eight combinations produce pink-eyed dilute compound heterozygotes, indicating full complemention for all lethal phenotypes. One-hundred ninety-five combinations of embryonic-lethal mutations complement at least for embryonic lethality, so that neonates can be obtained, whereas 531 combinations are non-

complementing and exhibit embryonic lethality as evidenced by a complete absence of pink-eyed dilute progeny. The stage of development at which each *p*-region embryonic lethal acts has not yet been investigated.

At least seven of the lethal mutations seem to complement other mutations in combinations that should be prenatally lethal according to the rudimentary map. These have been designated as "skippers"; that is, these deletions appear to "skip" over (fail to delete) proposed intermediate recessive-lethal loci while deleting other recessive-lethal loci that are apparently further from *p*. The availability of molecular probes will be an important asset in determining if and where the local DNA arrangements that seem to abound in the *p* region have occurred.

The seven available homozygous sublethal radiation mutations present a very interesting variety of phenotypes (Table 6-1) Three of these ($p^{7FR60Lb}$, p^{9DTW}, and $p^{4THO-II}$) produce not only the *p* phenotype in homozygotes, but are neonatal or juvenile lethals characterized by extreme runting and ataxia. Two others, (p^{103G} and $p^{12R250M}$) are p^{x} mutations, with $p^{12R250M}$ homozygotes exhibiting the runting and "nervous" phenotypes, while p^{103G} homozygotes frequently survive to adulthood (but males appear to be sterile; E. M. Rinchik, unpublished observations). p^{6H}, a radiation mutant isolated at Harwell but used in this study, is another juvenile-lethal *p* mutation that exhibits male sterility in the occasional animals who survive to post-pubertal stages. One sublethal mutation,

Table 6-1. Sublethal Radiation Mutations at *p*.

Mutation	Phenotype
p^{18CoS}	mottled males and females; juvenile lethal; nervousness and runting.
p^{9DTW}	*p*; juvenile lethal; nervousness and runting.
p^{6H}	*p*; juvenile lethal; male sterile; nervousness and runting.
$p^{7FR60Lb}$	*p*; neonatal lethal.
p^{4ThoII}	*p;* neonatal lethal.
p^{103G}	p^{x}; male sterile?
$p^{12R250M}$	p^{x}; juvenile lethal; nervousness and runting.

p^{18CoS}, has proved to particularly interesting and useful for the *p*-region mapping and cloning experiments reported in Chapter 8. p^{18CoS} is a p^{m} mutation; that is, both homozygotes (p^{18CoS}/p^{18CoS}) and heterozygotes (p/p^{18CoS}) have coats that are mottled for the *p* coat color on a wild-type background. The heterozygotes exhibit much more obvious mottling than the homozygotes, and both males and females exhibit the mottled phenotype. Since all melanocytes are presumed to be genotypically identical in a single animal, no immediately apparent mechanism explains why some patches of hair express *p* coat color while others of identical genotype express wild-type color.

Using the same breeding scheme described in Chapter 4 for the production of *c*-locus deletion carriers, 37 of the 40 homozygous-lethal *p* mutations have been bred into F_1 interspecific hybrid animals. These animals, progeny-tested to confirm that they carry the lethal *p* mutation on the maternal *Mus musculus* chromosome 7, carry a wild-type *Mus spretus* balancer chromosome 7. DNAs from these animals have been used to map random, anonymous probes from libraries generated by chromosome-sorting or microdissection of the large T(X;7)5R*l* translocation chromosome described in Chapter 3 .

CHAPTER 7

MATERIALS AND METHODS FOR *p*-REGION RANDOM CLONE MAPPING

The probe TM15, which defines the mouse locus *D7OR2*, is a 200-base pair microclone derived from a chromosome-microdissection library constructed by Niswander at Case Western Reserve University (see Chapter 8). Microdissection was performed on the X^7 chromosome, described in Chapter 3, in metaphases from the SV-40-transformed *T(X;7)5RI* cell line described in Chapter 4 and illustrated in the karyotype and idiogram in Figures 3-1 and 3-4. The microdissected chromosome segments from the region of X^7 that is thought to include both the *c* and *p* loci from chromosome 7 were digested with *Eco*RI, cloned into lambda gt10 and subsequently subcloned into the plasmid vector pUC13 by Niswander and Magnuson at Case Western Reserve University (Niswander, et al., 1990, in press). Inserts were excised from plasmids by digestion with *Pvu*II, which results in probes that include the mouse insert plus 323 base pairs of flanking plasmid sequences. Probes were isolated by electrophoresis through low-melt agarose, and hybridized to the *Mus musculus/Mus spretus* screening blots described in Chapter 4. To reiterate: the screening blots carry, in addition to the *c*-region DNAs detailed in Chapter 4, DNAs from a *Mus musculus* female carrier ($+^{MSC/p46DFioD}$) of the presumed-largest *p*-region deletion, from a *Mus spretus* male

($+^{SPT}/+^{SPT}$), and from the (*Mus musculus/Mus spretus*)F_1 hybrid carrier ($+^{SPT}/p^{46DFioD}$). All methods used for Southern blot preparation, prehybridization, radiolabelling, hybridization, washing, and blot stripping are outlined in the Materials and Methods section of Chapter 4.

The *p*-region *Mus musculus/Mus spretus* mapping (as opposed to screening) blots contain DNAs from a *Mus spretus* male, from *Mus musculus* female carriers of each *p*-region mutation, and from (*Mus musculus/Mus spretus*)F_1 hybrid carrier of each *p* mutation.

Compound heterozygotes for *p*-region mutations that complement for lethality were provided by C. S. Montgomery and L. B. Russell. Stocks carrying a homozygous-lethal *p* mutation are maintained as $+/p^{la}$ heterozygotes, where p^{la} represents any homozygous-lethal mutation. Such $+/p^{la}$ heterozygotes were crossed to animals heterozygous for different lethal *p* mutations (p^{lb}). In any such cross from which pink-eyed-dilute progeny are produced, the two *p* mutations are considered to be complementing, with the pink-eyed progeny being compound heterozygotes for the two distinct *p* mutations.

Sublethal (p^{sl}) *p* mutations (Table 6-1) are also maintained as heterozygous $+/p^{sl}$ stocks, except for those that result in a mottling phenotype (p^{m}). In the latter case, the stocks are maintained as p/p^{m} to allow easy selection for the mottled animals.

The 3.0-kb p-specific *Eco*RI fragment (Chapter 8) was cloned into *Eco*RI-digested lambda-ZAP (Stratagene) from a complete *Eco*RI digest of p^{18CoS}/p^{18CoS} spleen DNA, utilizing TM15 as a hybridization probe. The fragment was then subcloned into *Eco*RI-digested pUC13 (Pharmacia) after gel-isolation and elution from NA45 paper (Schleicher and Schuell) following manufacturer's directions. The end clones, 0.4 and 0.6, were isolated after digestion with the appropriate enzymes (*Eco*RI-*Bam*HI for 0.4, *Eco*RI-*Pst*I for 0.6) by electrophoresis in low-melt agarose, and radiolabelled in the melted agarose by random-hexamer priming (Chapter 4) The internal fragment, 0.8, was similarly prepared for use as a probe by digestion with *Bam*HI and *Hin*dIII.

Lambda libraries were made from both 129/R*I* (*p/p*) and C3Hf (p^+/p^+) DNAs by partial digestion with *Sau*3A, followed by sizing on a sucrose gradient. Ten- to twenty-five-kb fragments were cloned into *Bam*HI-digested lambda 2001. Positive clones from the 129/R*I* library were isolated by screening with the 0.8 probe, whereas positive clones from the C3Hf library were isolated after screening with TM15. Both libraries were plated on 23-cm^2 disposable plates (Nunc), and lifted (Benton and Davis, 1977) onto Nytran (Schleicher and Schuell). Secondary and tertiary screening were done on nitrocellulose filters (MSI).

The yeast artificial chromosome (YAC) clone containing the *D7OR2* locus recognized by TM15 was a kind gift from Lisa Stubbs (ICRF, London) This YAC clone is a 90-kb *Not*I fragment

from C3Hf carried in the yeast vector pYAC55. Total genomic yeast DNA was prepared in agarose blocks according to the protocol given in the manual supplied with the BioRad CHEF gel-electrophoresis system. A stationary-phase (ura^- and trp^-) yeast culture, grown for 48-72 hours in 0.67% yeast nitrogen base without amino acids, 2.0% dextrose, and 0.5% acid-hydrolyzed casamino acids, was digested in lyticase (2 mg/ml in 50 mM EDTA, pH 8.0). The resulting spheroplasts were embedded in 0.75% low-melting-point agarose (Incert, FMC) in 125 mM EDTA, (pH 7.5), and then incubated in 50 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 7.5; 7.5% 2-mercaptoethanol. After thorough rinsing in 50 mM EDTA, pH 8.0, the agarose blocks were incubated in a 1 mg/ml proteinase K solution (50 mM EDTA, pH 8.0; 1% laurylsarcosine), and then were incubated in two changes of 1 mM PMSF (phenylmethylsulfonylfluoride) in 10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0. After rinsing in 50 mM EDTA, pH 8.0, the blocks were either digested immediately or stored in 0.5M EDTA, pH 8.0. It was found that digestion of the total yeast DNA was more efficient when the low-melt agarose blocks were melted at 68 C, diluted to 5 mM EDTA and the appropriate salt content for the restriction enzyme of choice, and incubated overnight with agarase (Calbiochem, 10 units/100 microliters of digestion volume) and the restriction enzyme of choice.

CHAPTER 8

RESULTS AND DISCUSSION OF *p*-REGION RANDOM CLONE MAPPING

A Chromosome-Dissection Microclone Maps to the *p*-Region Deletion Complex.

The large T(X;7)5R/ chromosome (Chapter 3) used in the construction of the flow-sorted random-clone library described in Chapter 4 was also used by Niswander, (Niswander, et al., 1990, manuscript submitted) for construction of a random microclone genomic library by microdissection. The region of the R5 X7 chromosome (Figure 3-1) that was the target of the microdissection was the *c*-region. However, the *p*-region, which lies about 15 cM proximal to *c,* could easily have been included in the library of microclones. For this reason, the screening blots described in Chapter 4 contained DNA from a (*M. musculus/M spretus*)F_1 carrier of the presumed-largest *p*-region deletion, $p^{46DFioD}$, as well as F_1 carriers of the two *c*-region mutations, in order to take advantage of a single genomic library that may be enriched for sequences from two chromosome-7 deletion complexes.

Chromosome microdissection of the large T(X;7)5R/ chromosome resulted in a library of 108 microclones. Of eight subclones from that library sent to us by Magnuson, one, TM15, a 200-bp *Eco*RI insert in pUC13, mapped into the largest *p*-region

deletion, $p^{46DFioD}$. In lane 4 of Figure 8-1, TM15 recognizes a 2.9-kb allele in DNA from *Mus spretus* and, in lane 3, a 4.2-kb *Mus musculus*-specific allele in a *Mus musculus* carrier of a *c*-region deletion. Lane 5 has DNA from the (*musculus/spretus*)F_1 hybrid that carries a *spretus* chromosome and the $p^{46DFioD}$ mutant chromosome. It is apparent that only the 2.9-kb *spretus* allele is present in lane 5, indicating the deletion from the *musculus* chromosome of the DNA sequence recognized by TM15. Subsequent mapping of TM15 using other lethal *p* mutations balanced in (*Mus musculus/Mus spretus*)F_1 hybrids proved that 17 of 29 lethal mutations tested are deleted for the DNA sequence recognized by TM15. Table 8-1 summarizes the results for each of the 29 lethal mutations tested with TM15 (and with three additional probes to be described below). Seventeen of those radiation-induced mutations, suspected prior to this study of being deletions because of the associated lethal phenotypes, are proved to delete at least the sequence recognized by the molecular probe TM15. This sequence has been assigned the locus name *D7OR2*.

In an experiment to determine how close to *p* the locus *D7OR2* might map, homozygous DNAs from five of the sublethal *p* mutations (Chapter 6) were digested with *Eco*R1 and probed with TM15 (Figure 8-2). The wild-type control (C3H) in lane 7 has a hybridizing fragment of 0.5 kb; p^{9DTW}/p^{9DTW} (lane 2), and p^{103G}/p^{103G} (lane 3) show the same 0.5-kb band; therefore, they are not deleted for TM15. However, p^{6H}/p^{6H} (lane 4) and

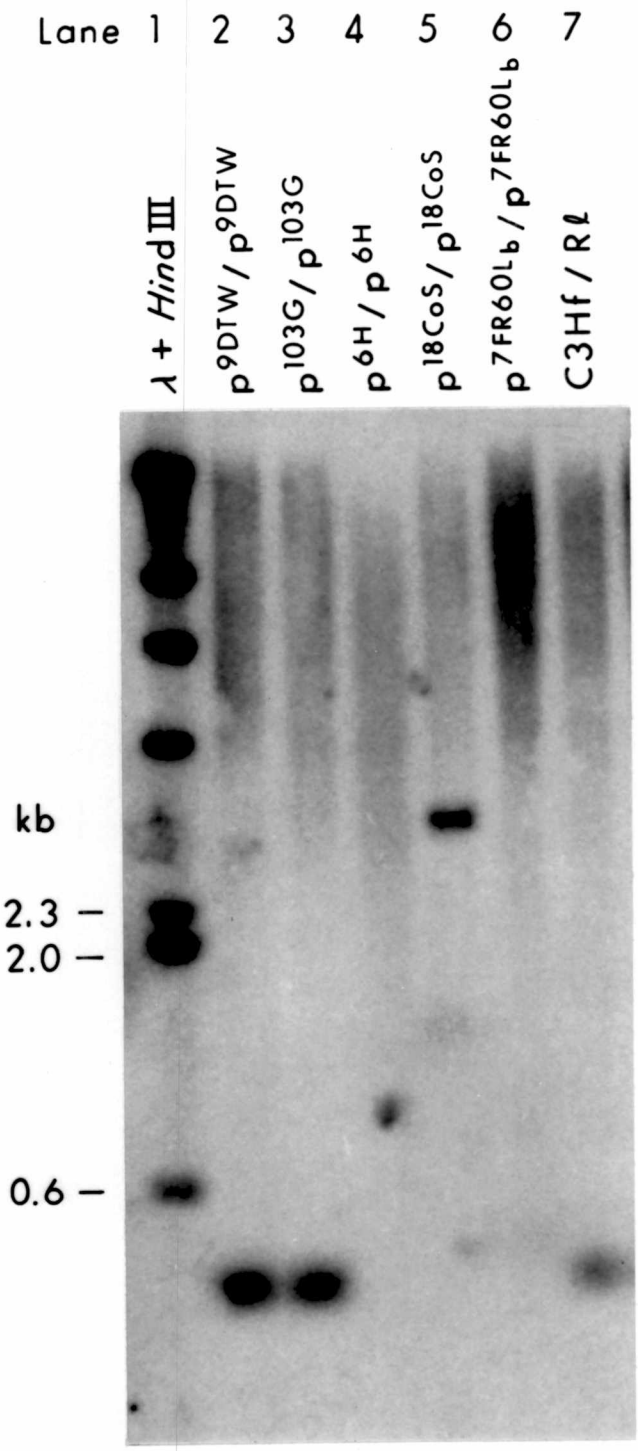
Figure 8-1. Autoradiogram showing hybridization of the probe TM15 to *Taq*I-digested spleen DNAs. DNAs are from: (*M. musculus/M. spretus*)F_1 hybrid carriers of the lethal *c* mutations c^{Ai1} and c^{Fp1}($+^{SPT}/c^{Ai1}$, lane 1; $+^{SPT}/c^{Fp1}$, lane 2); a *M. musculus* carrier of c^{Fp1} (c^{ch}/c^{Fp1}, lane 3); a *M. spretus* male ($+^{SPT}/+^{SPT}$, lane 4), a (*musculus/spretus*)F_1 hybrid carrier of the *p*-region deletion, $p^{46DFioD}$ ($+^{SPT}/p^{46DFioD}$, lane 5).

Lane 1
2
3
4
5
SPT /c Ai2
SPT /c Fp1
c ch /c Fpl
SPT
SPT / p 46DFioD

Table 8-1. Summary of Results of Each of 29 *p*-Lethal Mutations Hybridized With TM15.

Deleted	Not Deleted
p24Zb	*p41DTD*
p30PUb	*p4R250H*
p132G	*p3DTR*
p25DVT	*p12R30Lb*
p80K	*p12R250M*
p23DFioD	*p3FR60Lg*
p2HATh	*p2DFioD*
p45DTD	*p3R30M*
p116G	*p58HATh*
p8FDFoD	*p2MNURf* (p^x)
p15DThWb	*p1MNURf* (p^x)
p46DFioD	*p17MNURf* (p^x)
p4THO-II	
p83FBFo	
p47DTD	
p8R250M	
p1DDTMb (p^x)	

Figure 8-2. Autoradiogram showing hybridization of the probe TM15 to *Eco*RI-digested spleen DNAs from homozygous *p*-sublethal mutant animals. Lanes 2 (p^{9DTW}/p^{9DTW}), 3 (p^{103G}/p^{103G}), 4 (p^{6H}/p^{6H}), 5 (p^{18CoS}/p^{18CoS}), and 6 ($p^{7FR60Lb}/p^{7FR60Lb}$) are the *p* mutations; lane 7 is wild type (C3Hf/R*f*).


Lane 1 2 3 4 5 6 7
λ + HindIII
p9DTW / p9DTW
p103G / p103G
p6H / p6H
p18CoS / p18CoS
p7FR60Lb / p7FR60Lb
C3Hf / Rℓ
kb
2.3
2.0
0.6

$p^{7FR60Lb}/p^{7FR60Lb}$ (lane 6) are missing the 0.5-kb fragment that hybridizes with TM15. $p^{4THO-II}$, another sublethal *p* not shown in Fig. 8-2, is known to be deleted for *D7OR2* from analysis of *musculus/spretus* hybrids.

Definition and Characterization of a DNA Polymorphism Detected by TM15.

In addition to finding that some of the sublethal *p*'s and p^x's are deleted for the *D7OR2* locus, the hybridization pattern of p^{18CoS}/p^{18CoS} (lane 5 in Fig. 8-2) appeared to be of particular interest because it shows an aberrantly-migrating 3.0-kb band. This unusual fragment could represent a simple RFLP or, significantly, a genomic rearrangement due to a deletion (or other mutational) event. A hybridization fragment of novel size may be a breakpoint-fusion fragment, the site where the broken ends of DNA generated in the deletion (or rearrangement) event have rejoined. In the process of breakage and rejoining, rearrangement of restriction enzyme sites that existed in the pre-deletion DNA occurs, resulting in a novel-sized fragment that contains the sequence recognized by the original probe. Breakpoint-fusion fragments are very useful mapping tools because they juxtapose segments of DNA that had not originally been contiguous. This means that a probe that recognizes a breakpoint-fusion fragment gives automatic access to the "other end" of the deletion, thus effectively "jumping" across whatever length the deletion has involved.

The aberrantly migrating 3.0-kb *Eco*RI fragment recognized by TM15 in p^{18CoS} DNA was cloned (Chapter 7), and a partial restriction map was constructed (Figure 8-3). The end clones, "0.4" (a 0.4-kb *Eco*RI-*Bam*HI fragment) and "0.6" (a 0.6-kb *Eco*RI-*Pst*I fragment), of the 3.0-kb fragment were isolated from agarose and used as probes on the Southern blots shown in Figure 8-4. This Southern blot includes DNAs from the sublethal mutations, two of which (p^{6H} and $p^{7FR60Lb}$) were now known to be deleted for *D7OR2*, as well as from complementing (at least to perinatal survival) compound heterozygotes. Figure 8-4 shows the hybridization pattern with 0.6; the pattern is identical for 0.4 (data not shown). Both of the end clones, which hybridize to a single 3.0-kb *Eco*RI band in p^{18CoS}/p^{18CoS} DNA, recognize, in this experiment, four *Eco*RI fragments (15 kb, 8 kb, 1.5 kb, and 0.5 kb) in wild-type DNA of some F_1 hybrids of inbred strains 101/RI and C3Hf/RI (lane 16). Two homozygous sublethals (p^{6H}, lane 13; $p^{7FR60Lb}$, lane 11) are deleted for all bands recognized by 0.6. The same is true for the compound heterozygotes in lanes 5, and 6; these three mutations, p^{116G}, $p^{46DFioD}$, and $p^{7FR60Lb}$, are all known to be deleted for *D7OR2* (TM15). The remaining compound heterozygotes in lanes 1, 3, and 4, have at least one copy of the wild-type sequences recognized by 0.6. Lanes 7, 8, 9, 10, 12, 14, and 15 are homozygous DNAs not deleted for any of the wild-type sequences; p^{34UThb} (lane 7), p^{4PU} (lane 10), $p^{17DThWb}$ (lane 14),

Figure 8-3. Diagram of a partial restriction map of the 3.0-kb *p*-specific *Eco*RI fragment recognized by probe TM15. This fragment was cloned from the genomic DNA of a p^{18CoS}/p^{18CoS} (*p*-sublethal) homozygote. Enzyme sites are: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; and P, *Pst*I. The thin, hatched bars below indicate the end clones, "0.4" and "0.6", of the 3.0-kb fragment. The thicker, hatched bar below 0.6 indicates that the TM15 probe, a 200-bp *Eco*RI insert in a microclone, falls somewhere within the 0.6-kb *Eco*RI-PstI fragment.

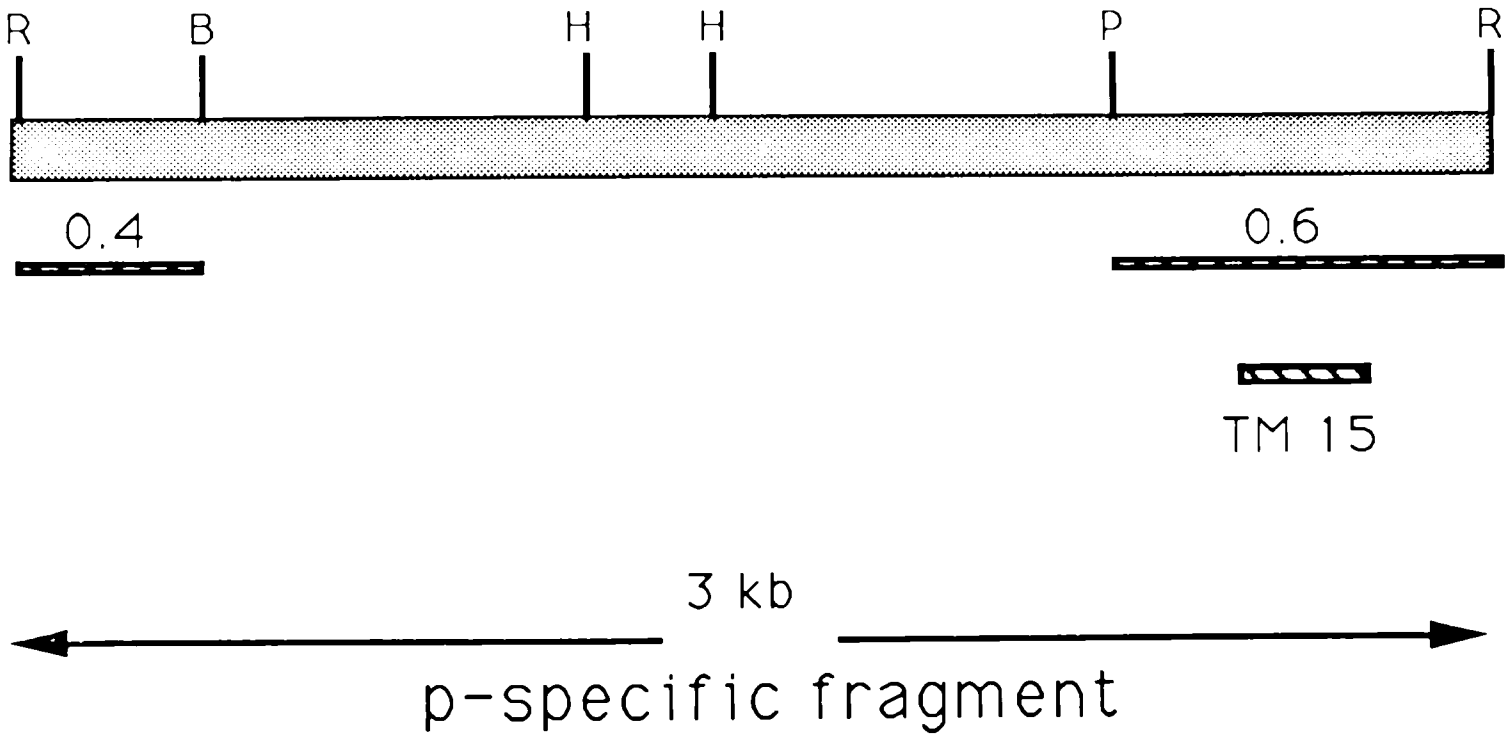
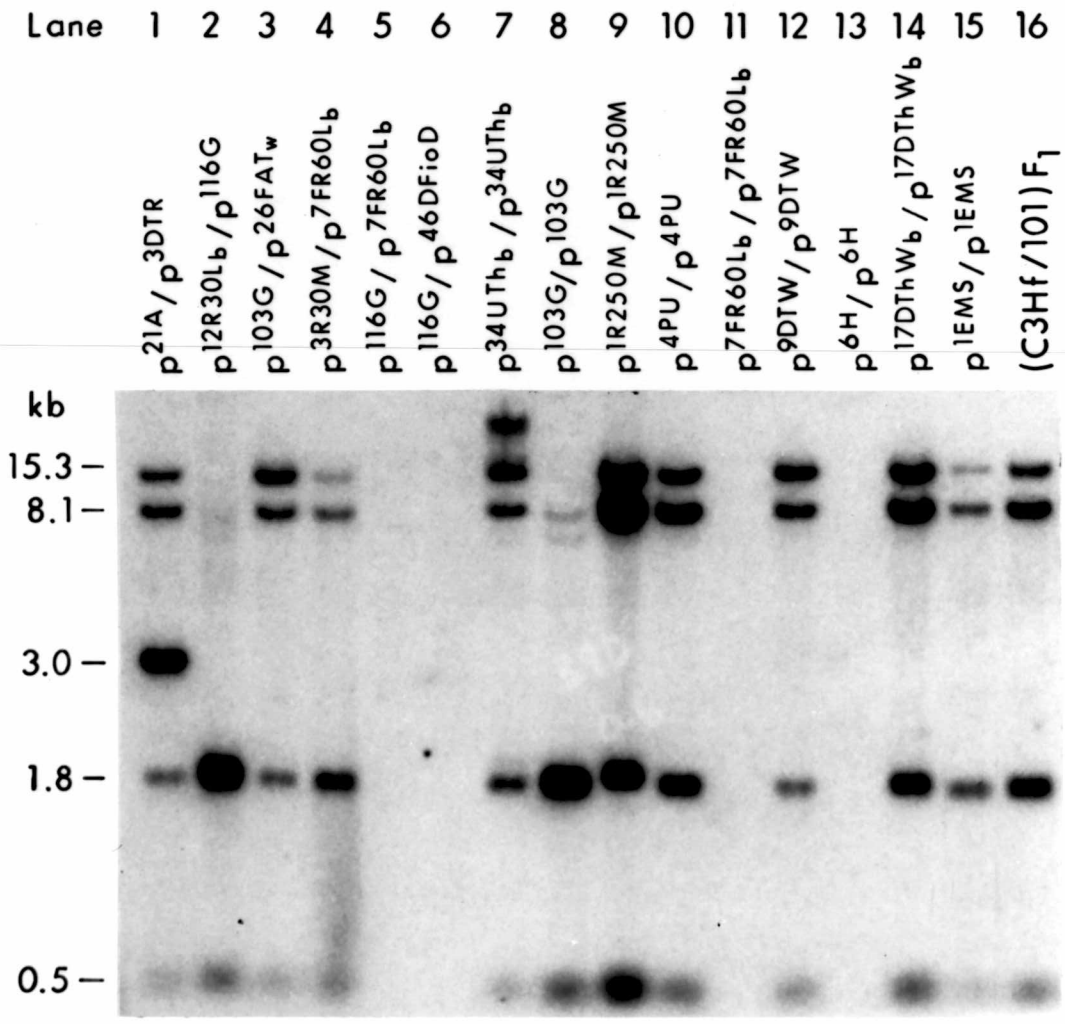

R
B
H
H
P
R
0.4
0.6
TM 15
3 kb
p-specific fragment

Figure 8-4. Autoradiogram showing hybridization of the probe 0.6 to *Eco*RI-digested DNAs from *p*-sublethal homozygotes and complementing *p*-lethal heterozygotes. *p*-sublethal homozygotes: ($p^{7FR60Lb}/p^{7FR60Lb}$, lane 11; p^{6H}/p^{6H}, lane 13); p^{x}-sublethal homozygotes (p^{34UThb}/p^{34UThb}, lane 7; p^{103G}/p^{103G}, lane 8; p^{1R250M}/p^{1R250M}, and 9; p^{4PU}/p^{4PU}, lane 10; $p^{17DThWb}/p^{17DThWb}$, lane 14; and p^{1EMS}/p^{1EMS}, lane 16); complementing heterozygotes for lethal *p* mutations ($p^{12R30Lb}/p116G$, lane 2; p^{103G}/p^{26FATw}, lane 3; $p^{3R30M}/p^{7FR60Lb}$, lane 4; $p^{116G}/p^{7FR60Lb}$, lane 5, and $p^{116G}/p^{46DFioD}$, lane 6); a *p*-lethal, p^{3DTR}, balanced by a *p/p* (Stock 21A) chromosome (lane 1); and a wild-type hybrid DNA, C3Hf/101 (lane 16).


Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
p21A / p3DTR
p12R30Lb / p116G
p103G / p26FATw
p3R30M / p7FR60Lb
p116G / p7FR60Lb
p116G / p46DFioD
p34UThb / p34UThb
p103G / p103G
p1R250M / p1R250M
p4PU / p4PU
p7FR60Lb / p7FR60Lb
p9DTW / p9DTW
p6H / p6H
p17DThWb / p17DThWb
p1EMS / p1EMS
(C3Hf/101) F1
kb
15.3
8.1
3.0
1.8
0.5

and p^{1EMS} (lane 15) are viable p^{X} mutations tested on the chance they might be deletions.

TM15 and 0.6 Recognize a Polymorphism Specific to Chromosomes Carrying the Original *p* Mutation.

Two lanes, 1 and 2, in Fig. 8-4 illustrate some interesting results. Lane 1 contains DNA from a p^{21A}/p^{3DTR} heterozygote. The p^{21A} chromosome carries the original *p* mutation, not a new radiation-induced *p* mutation. However, the 3.0-kb TM15 fragment, originally thought to be a breakpoint-fusion fragment generated in the p^{18CoS} mutational event, is also seen in lane 1. Therefore, the 3.0-kb *Eco*RI fragment must represent either the p^{21A} chromosome or the p^{3DTR} chromosome. Further testing showed that the 3.0-kb fragment recognized by both TM15 and 0.6, which was originally assumed to have been generated in the p^{18CoS} mutational event, is actually a consistent fragment that appears in *Eco*RI-digested genomic DNA from every animal tested that carries a *p* chromosome (data not shown). Therefore, it can be assumed that the 3-kb *Eco*RI fragment recognized by 0.6 in Lane 1 of Fig. 8-4 can be attributed to the p^{21A} chromosome in the compound heterozygote p^{21A}/p^{3DTR}. This result suggested that wild-type and *p* chromosomes differ at *D7OR2*, and that the difference might be due either to a simple *Eco*RI RFLP or to a larger-scale rearrangement. Fig. 8-5 shows that 0.6 likewise detects a *Bam*HI polymorphism between wild-type chromosomes (lanes 1 and 2) and *p* chromosomes [lanes 3,

Figure 8-5. Autoradiogram showing hybridization of probe 0.6 to *Bam*HI-digested spleens from animals carrying wild-type and p chromosomes. Wild type chromosomes (*+/+*); lane 1, 101/R*l*; lane 2, C3Hf/R*l*; lane 6, *M. spretus*); *p/p* chromosomes (lane 3, 129/R*l*; lane 4, 21A; lane 5, T); radiation-induced *p*-mutant chromosomes (lane 7, p^{18CoS}/p^{18CoS}; lane 8, $p^{116}/p^{7FR60Lb}$). p^{116} is a prenatal lethal that is partially complemented by the *p*-sublethal, $p^{7FR60Lb}$.

Lane 1 2 3 4 5 6 7 8
101/Rℓ
C3Hf/Rℓ
129/Rℓ
21A
T
SPT
p18CoS/p18CoS
p116G/p7FR60Lb
kb
9.6
9.3

4, and 5, which contain *Bam*HI-digested spleen DNAs from *p/p* stocks 129/RI (inbred), 21A, and T]. In fact, probing of additional blots with both 0.6 and TM15 (not shown) demonstrated that these probes recognize RFLPs between wild-type and *p* chromosomes with five other enzymes (*Kpn*I, *BstE*II, *Taq*I, *Msp*I, and *Pvu*II). The *p* chromosome present in all the stocks used in these experiments is the original *p* mutation. Therefore, it is likely that a rearrangement of the original wild-type genome in which *p* arose resulted in the *p*-specific novel fragments carried by these stocks. It is not yet known if such a rearrangement caused the *p* mutation.

Since the original p^{18CoS} mutation was induced in a wild-type chromosome (C3H/R*f* or 101/R*f*), one would expect p^{18CoS}/p^{18CoS} DNA digested with *Bam*HI to have only the wild-type 9.3-kb fragment recognized by 0.6, and p^{18CoS}/p DNA to have one wild-type and one *p* (9.6-kb) hybridizing fragment. However, in Figure 8-6, both p^{18CoS}/p^{18CoS} and p^{18CoS}/p DNAs (lanes 2 and 3) have only the *p*-specific 9.5-kb *Bam*HI fragment. DNAs from p^{9DTW}/p^{9DTW} and p^{103G}/p^{103G} homozygotes, known not to be deleted for TM15 or 0.6, (Figure 8-2) carry the 9.3-kb wild-type fragment. The DNAs in lanes 5 and 8 do not carry either fragment, confirming that they are deleted for these sequences. p^{18CoS}/p^{4THOII} (p^{18CoS} is abbreviated to p^{m} in lane 7 of Fig. 8-6) heterozygotes carry only the *p*-specific fragment contributed by p^{18CoS}, since p^{4THOII} is deleted for these sequences.

Figure 8-6. Autoradiogram showing hybridization of the probe TM15 to *Bam*HI-digested spleen DNAs from *p*-sublethal mutations. DNAs in lanes 3 (p^{18CoS}/p^{18CoS}), 4 (p^{9DTW}/p^{9DTW}), 5 (p^{6H}/p^{6H}), 6 (p^{103G}/p^{103G}), and 8 ($p^{7FR60Lb}/p^{7FR60Lb}$) are from homozygotes. DNAs in lane 2 (p^{18CoS}/p) and 7 [(p^{18CoS} ("p^{m}")/p^{4THOlh}] are heterozygous. Lane 1 contains wild-type C3Hf/R/ DNA.

1
2
3
4
5
6
7
8
C3H/Rℓ
p18CoS/p
p18Cos/p18CoS
p9DTW/p9DTW
p6H/p6H
p103G/p103G
pm/p4ThoII
p7FR60Lb/p7FR60Lb
kb
9.6
9.3

To summarize the findings discussed in the paragraph above, all radiation-induced *p* mutations tested so far, except p^{18CoS}, are either deleted for the sequences recognized by TM15 and 0.6, or they carry the wild-type fragment. Only p^{18CoS} displays the *p*-specific allele. It may be concluded from these data that the p^{18CoS} mutation must have recombined with the TM15-recognition sequences in the *p* chromosome during stock maintenance. Because p^{18CoS}/p^{18CoS} animals die soon after birth, the stock is maintained as p^{18CoS}/p x *p/p* to preserve viability and allow easy selection for the mottled phenotype. The fact that the recombination did occur proves that the *D7OR2* locus and the p^{18CoS} mutation are separate; however, through three decades of stock maintainence, even a very closely linked p^{18CoS} mutant locus would have had an opportunity to recombine with *D7OR2.*

The 0.8-kb internal *Bam*HI-*Hin*dIII fragment (termed "0.8") derived from the 3.0-kb *p*-specific *Eco*RI fragment (Fig. 8-3) was also isolated and used to probe *p*-region DNAs (data not shown). 0.8 is a unique-sequence probe that recognizes the 3.0-kb *Eco*RI *p*-specific fragment and a 6.5-kb *Eco*RI fragment in wild-type DNAs (101/RI, C3Hf, Stock 2A). The 0.8-kb *Hin*dIII-*Pst*I and 0.4-kb *Hin*dIII internal fragments (Fig 8-3) contain highly repetitive sequences and were, therefore, not suitable for use as probes on Southern blots.

Additional Characteristics of the Subclones Defining the *p*-specific Rearrangement.

Comparison of the hybridization patterns when *Eco*RI-digested wild-type DNAs are probed with TM15, 0.6, 0.4, and 0.8 has revealed some unusual features. First, as can be seen in several lanes on the Southern blot in Fig 8-4, the probe 0.6 recognizes four fragments in non-p DNA, and, in experiments not shown, O.4 recognizes these same 4 fragments. However, in *Eco*RI digests of spleen DNAs from other C3Hf animals, for example, which would be presumed to be genetically identical to all other C3Hf animals, only two of the four fragments (1.8-kb and 0.5-kb) are recognized by 0.6 and 0.4. One consistent observation, however, is that both 0.6 and 0.4 always recognize the 1.8-kb fragment in addition to the 0.5-kb fragment recognized by TM15. Interestingly, in *Eco*RI-digested *p* chromosomes, neither 0.4 nor 0.6 has ever recognized any fragment other than the expected 3.0-kb fragment, so the phenomenon of variable recognition of the two largest of the 4 *Eco*RI fragments is peculiar to wild-type DNA.

Secondly, no matter what the hybridization pattern seen, in *p* or in wild-type chromosomes, 0.6 and 0.4 always recognize identical fragments. This result indicates that the two end clones, which are about 2 kb apart in *p* chromosomes (Fig. 8-3) and (currently) an unknown distance apart in wild-type chromosomes, contain homologous (duplicated?) sequences.

Thirdly, it is interesting to note (in Fig. 8-3) that, in *p* DNA, the "repeated" sequences found within the 0.6 and 0.4 subclones lie on either side of the unique sequence 0.8. As described above, the 0.8 clone recognizes a 6.5-kb *Eco*RI fragment in wild-type DNA, a completely different fragment from those fragments recognized by 0.6 and 0.4. The formation of any hypothesis to account for these unusual features of genomic organization must await the isolation of larger genomic clones from both wild-type and *p* DNA (see below).

Mapping of 0.6, 0.4, and 0.8 Within the *p*-Deletion Complex.

Because 0.6, 0.4, and 0.8 are subclones of a fragment thought to be the result of a rearrangement carried by *p* chromosomes, it was of interest to map these clones across the panel of DNAs derived from animals carrying lethal *p* deletions balanced with *M. spretus* chromosomes 7. Therefore, the 0.6, 0.4, and 0.8 probes were hybridized to Southern blots containing DNAs from the p-region *musculus/spretus* lethal mutation panel. A representative blot is shown in Fig. 8-7, and results are summarized in Table 8-1. In each case in Fig. 8-7, which shows *Bam*HI-digested DNAs hybridized with the 0.6 probe, the *musculus/spretus* carrier of each lethal mutation and the appropriate *musculus* parent are side by side, with a representative *spretus* male in lane 1. In lane 2, the *musculus/spretus* hybrid carrying the lethal p^x mutation p^{1DTTMb}

Figure 8-7. Autoradiogram showing hybridization of the probe 0.6 to *Bam*HI-digested spleen DNAs from carriers of homozygous-lethal p mutations. The DNAs are as follows: *M. spretus* male (lane 1); *M. musculus* female carriers (*+/p^{l}*) of homozygous-lethal *p^{l}*mutations (lanes 3, 5, 7, 9, 11, and 13); and (*musculus/spretus)*F_1 hybrid carriers of each of the same *p^{l}* mutations (lanes 2, 4, 6, 8, 10, and 12). *p^{l}* signifies any of the representative sample of *p*-lethal mutations listed at the top of the figure.

Lane
1
2
3
4
5
6
7
8
9
10
11
kb
9.4
8.0
SPT
SPT/p DTTMb
+/p DTTMb
SPT/p 58HATh
+/p 58HATh
SPT/p 2MNURf
+/p 2MNURf
SPT/p 1MNURf
+/p 1MNURf
SPT/p 17MNURf
+/p 17MNURf

Table 8-2. Summary of Results of Each of 29 *p*-Lethal Mutations Hybridized With 0.4, 0.6, and 0.8

Deleted	Not Deleted
p24Zb	*p41DTD*
p30PUb	*p4R250H*
p132G	*p3DTR*
p25DVT	*p12R30Lb*
p80K	*p12R250M*
p23DFioD	*p3FR60Lg*
p2HATh	*p2DFioD*
p45DTD	*p3R30M*
p116G	*p58HATh*
p8FDFoD	*p2MNURf* (p^x)
p15DThWb	*p1MNURf* (p^x)
p46DFioD	*p17MNURf* (p^x)
p4THO-II	
p83FBFo	
p47DTD	
p8R250M	
p1DDTMb (p^x)	

is seen to be missing the 9.4-kb *musculus*-specific allele, and carries only the 8.0-kb *spretus* allele. Of the remaining four lethal *p* or p^x mutations in Fig. 8-7, none is deleted for the 9.4-kb *Bam*HI *musculus* fragment recognized by the 0.6 probe [F_1 hybrids in lanes 4 (p^{58HATh}), 6 (p^{2MNURf}), 8 (p^{1MNURf}), and 10 ($p^{17MNURf}$)] All of the 17 lethal deletions (of the 29 total mutations tested) that are deleted for TM15 are also deleted for the 0.6, 0.4, and 0.8 (see Table 8-1), so that the results are concordant for the four probes. In every examined case, the mapping profile for each probe was identical, indicating that TM15 and 0.8, for example, cannot be in different intervals as defined by the *p* deletions.

It is noteworthy that the deletion of these molecular probes is the first proof that any of the radiation-induced *p* mutations, with the exception of $Df(ru\text{-}2\ p)^{46DFioD}$, are actually deletions; perhaps new probes will increase the number of these mutations known to be deletions or other rearrangments. As was discussed in Chapter 6, several *p* mutations appear to be "skippers", and may represent more complex mutational events than simple deletions. The nature of these rearrangements may be deciphered by the mapping of additional molecular probes.

D7OR2 Maps Within the "*p*" Functional Unit.

Hybridization of the 0.6 clone with homozygous *p*-sublethal DNAs demonstrated that the two *p* sublethals that are

deleted for TM15 (Fig. 8-2) are also deleted for 0.6.(Fig. 8-4). As reported earlier, the 0.5-kb wild-type *Eco*RI fragment recognized by TM15 is deleted in p^{6H} and $p^{7FR60Lb}$; the 1.8-kb and 0.5-kb band *Eco*RI bands recognized by 0.6 are also deleted in these two sublethal *p*'s. In p^{9DTW} and p^{103G}, the 0.5-kb wild-type TM15 band is not deleted, and neither of the two *Eco*RI bands recognized by 0.6 is deleted.

DNAs from complementing compound heterozygotes that carry two different, overlapping deletions that are both developmental lethals in homozygotes (Chapter 6) were also tested for the sequences that hybridize to TM15 and 0.6. Lethal *p* mutations that are known to be deletions (Table 8-1), and which exhibit either full or partial complementation for lethality in compound heterozygotes, are valuable for mapping probes close to *p*. These heterozygotes, as well as homozygotes for the *p* sublethals, could potentially be animals carrying small regions of homozygous deletion. Therefore, a probe that is deleted in these DNAs may be located close to the *p* locus itself or to gene(s) responsible for one of the phenotypes associated with p sublethals (Table 6-1).

The hybridization of a subset of complementing compound heterozygotes to 0.6 is shown in Figs 8-4 and 8-8. In two representative cases (Fig 8-8, lanes 6 and 4) where complementation is complete ($p^{4THO\text{-}II}/p^{15DThWb}$ and $p^{4THO\text{-}II}/p^{46DFiOD}$), that is, where the mating of heterozygotes carrying two different lethal deletions produces normal, healthy pink-

Lane 1 2 3 4 5 6 7 8
(C3Hf/101)F1
p4THOII/p4R250M
p4THOII/p17FATw
p4THOII/p46DFioD
p58HATh/p2HATh
p4THOII/p15DTHWb
p4THOII/p25DVT
p48PB/p2DFioD

Figure 8-8. Autoradiogram showing hybridization of the probe 0.6 to *Eco*RI-digested spleen DnAs from complementing compound heterozygotes. The prenatal- and neonatal-lethal p^{l} mutations are: lane 2, $p^{4THOII/p4R250M;}$ lane 3, $p^{4THOII/p17FATw}$; lane 4, $p^{4THOII/p46DFioD}$; lane 5, $p^{58HATh/p2HATh}$; lane 6, $p^{4THOII/p15DThWb}$; lane 7 $p^{48PB/p2DFioD}$. Lane 1 has wild type (C3Hf/101)F_1 DNA.

eyed offspring, 0.6 is deleted in the DNA from the compound heterozygote. Likewise, in a representative case of partial complementation ($p^{116G}/p^{46DFiOD}$), that is, where the mating of heterozygotes carrying two different *pre*natally lethal mutations produces pink-eyed progeny that die as neonates, 0.6 is also deleted (Fig. 8-4, lane 6).

In discussing the importance and utility of fully and partially complementing and sublethal classes of *p* mutations, the compound heterozygotes $p^{4THO\text{-}II}/p^{46DFiOD}$ and $p^{4THO\text{-}II}/p^{15DThWb}$ are good cases in point. The mutation $p^{46DFioD}$ is thought to be the longest of the Oak Ridge *p* deletions and, along with $p^{15DThWb}$, is a prenatal lethal; $p^{4THO\text{-}II}$ is a late fetal or neonatal lethal, and all three are proved to be deletions by the molecular studies presented here. When carriers of these deletions are mated, normal pink-eyed offspring are produced. The expectation is that some or all of the *p* locus only is absent in the area of overlap between the respective deletions, so that the compound heterozygotes are complemented for all other phenotypes known (from the sublethal class of mutations) to be closely apposed to *p*. Since TM15 and its associated subclones are totally absent in these fully complementing overlaps, as well as in three of seven of the sublethal mutations, a reasonable hypothesis is that the probes map very close to the *p* locus itself, and probably close to, but not contained within, the chromosomal subregions responsible for the associated

phenotypes (male sterility, runting and nervousness, perinatal death).

The compound heterozygote $p^{116G}/p^{46DFioD}$ is another case in point. Both deletions are prenatal lethals, yet in combination complement partially so that embryos survive to the perinatal period. All *Eco*RI fragments recognized by TM15 and 0.6 are deleted in this compound heterozygote. Since the region of overlap for these two deletions includes, in addition to the *p* gene, a gene (or genes) that is necessary for postnatal survival, the probes TM15 and 0.6 must recognize sequences that fall within the DNA segment that carries both the *p* gene and the gene(s) necessary for postnatal survival, but not for prenatal survival (since the prenatal lethality is complemented). This deleted postnatal survival region may be the same region that is deleted in the sublethal mutations p^{6H}, $p^{7FR60Lb}$, and $p^{4THO-II}$, since these sublethals exhibit the same (gross) perinatal mortality as $p^{116G}/p^{46DFiOD}$ compound heterozygotes and are also deleted for both probes. However, as described above, the probes cannot map to the perinatal mortality function itself, since they are also deleted in some of the fully complementing compound heterozygotes. This illustrates the fact that DNAs from different *p* mutations, or combinations of *p* mutations, that exhibit different phenotypes can be useful in differentiating the probes that map to each separate phenotype.

An additional example of the use (as new probes become available) of phenotypic differences to map clones to functions

within the p region, is the difference seen in the $p^{7FR60Lb}$ and p^{103G} sublethal mutations. $p^{7FR60Lb}$ homozygotes usually die just at birth, whereas p^{103G} homozygotes often survive to weaning age or longer. Taking the logic, above, one step further, if a different probe were deleted in $p^{7FR60Lb}$, but not in p^{103G} or any available fully complementing compound heterozygote, the chance exists that the probe maps to the DNA region associated with the earlier mortalilty of the $p^{7FR60Lb}$ homozygotes. These sorts of comparison between differing phenotypes associated with different deletions of DNA sequences can be made among the large number of *p* mutations that are available. When more molecular probes, such as those already isolated in this study, can be mapped into each region that is associated with a particular phenotype, then molecular access to that phenotype is achieved and can lead to the cloning and characterization of the relevant gene or genes.

Expansion of the Molecular Map Around *D7OR2* By Preliminary Analysis of TM15$^+$ YAC and Lambda Clones.

In an effort to expand this initial molecular access provided by probe TM15 to the wild-type *p* region, both yeast artificial chromosome (YAC) and lambda clones were isolated from wild-type (C3Hf) genomic libraries by hybridization with TM15. The YAC clone (YAC-TM15), containing a 90-kb *Not*I mouse fragment, was kindly provided by Dr. Lisa Stubbs of the

Cancer Research Fund in London. The Southern blot pictured in Fig. 8-9 contains the total genomic DNA from YAC-TM15 digested with *Not*I and electrophoresed on a BioRad CHEF gel for visualization of large DNA fragments. The probe TM15 hybridized uniquely to a 90-kb *Not*I fragment, as measured by comparison to lambda multimers. YAC-TM15 total genomic DNA was also digested with *Eco*RI to create the Southern blot pictured in Fig. 8-10. When this blot was sequentially hybridized with TM15, 0.6, and 0.8, interesting results were obtained. In the C3H spleen DNA in lane 1, TM15 recognized the expected 0.5-kb wild-type *Eco*RI fragment (not shown), and 0.6 recognized its expected 1.8-kb and 0.5-kb wild-type C3Hf *Eco*RI fragments (Fig. 8-10). However, in lanes 2 and 3 containing YAC-TM15 DNA (lane 2 contains about five times more DNA than lane 3), 0.6 recognizes only the 0.5-kb wild-type *Eco*RI fragment. This result indicates that 0.6 recognizes an additional sequence in spleen DNA not contained on this 90-kb *Not*I fragment. There could be three explanations for this result: (1) YAC-TM15 contains a fragment artifactually rearranged during the cloning process; (2) TM15 and the portion of 0.6 not homologous to TM15 (defined for this discussion as W) are juxtaposed in wild-type DNA, and the 90-kb mouse insert in YAC-TM15 begins at a *Not*I site in TM15 and extends, in relation to Figure 8-3, to the right and away from W; and (3) W and TM15, not more than 400 base pairs apart in *p* DNA (see Fig. 8-3), are more widely separated in wild-type DNA. Explanation (1) is made less likely by Dr. Stubbs'

Figure 8-9. Autoradiogram showing hybridization of the probe TM15 to total genomic DNA from YAC-TM15 digested with *Not*I. Sizes given at the left are estimated by comparison to lambda multimers.

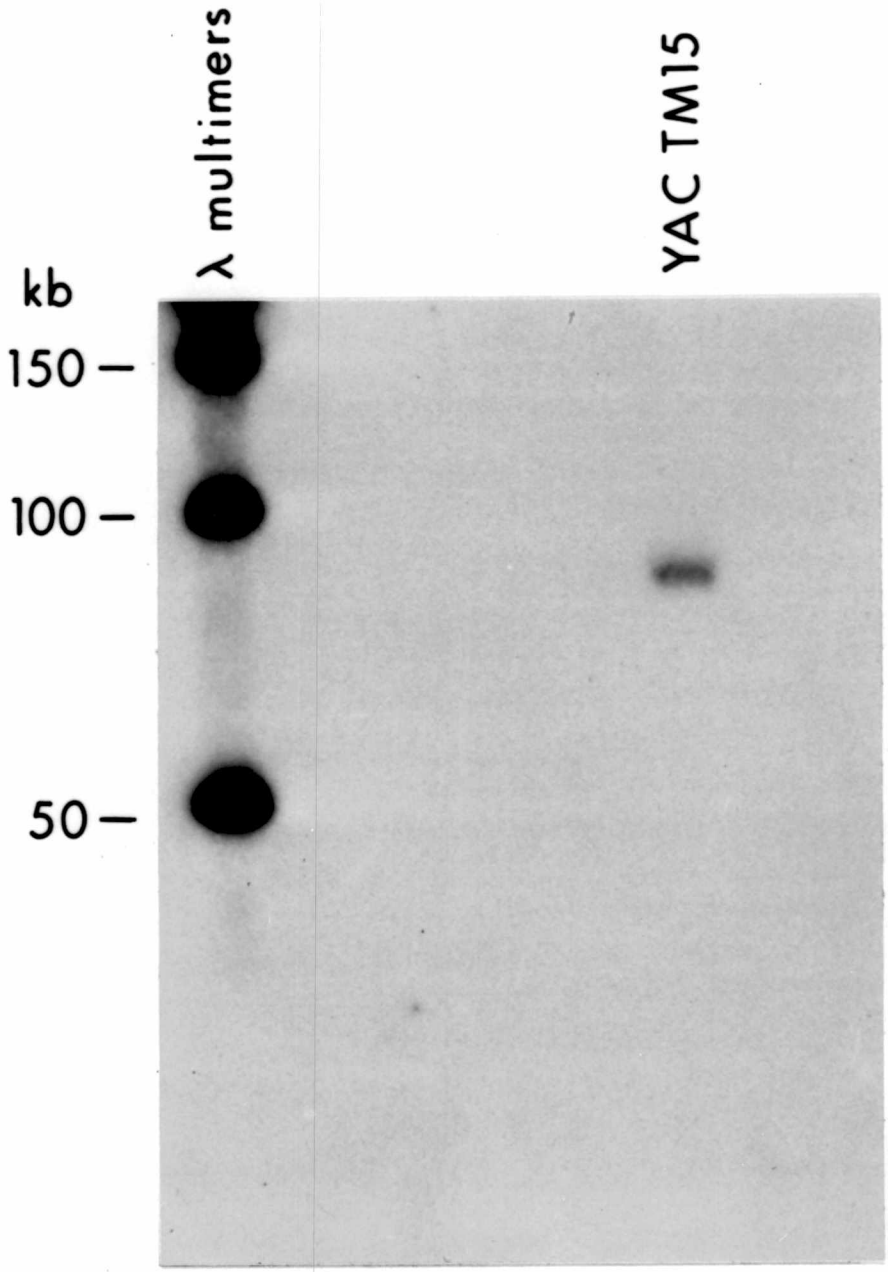
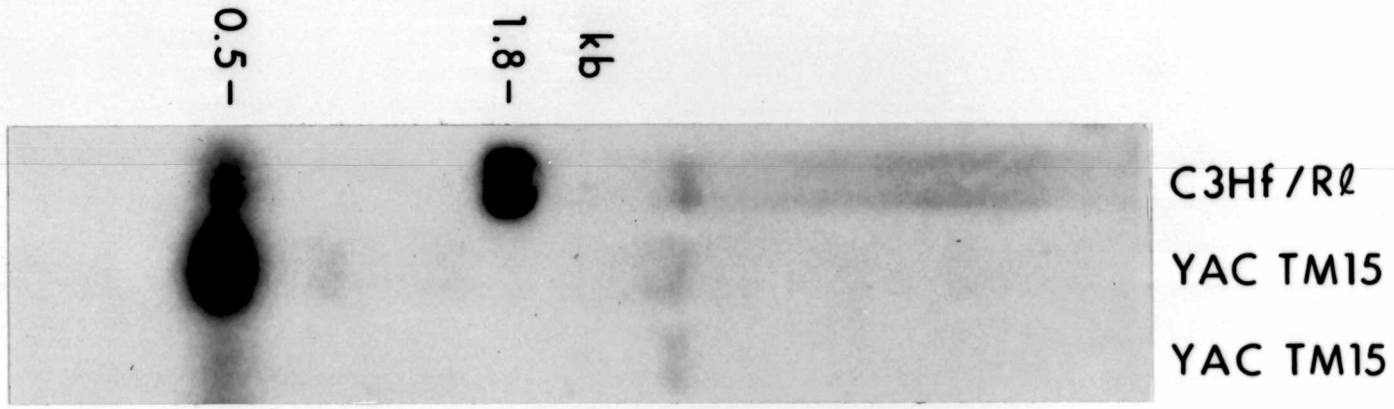

λ multimers
YAC TM15
kb
150
100
50

Figure 8-10. Autoradiogram showing hybridization of the probe 0.6 to total DNA from YAC-TM15 (two lanes), and to spleen DNA from a C3Hf/R*l* animal, both digested with *Eco*RI. Sizes at left are estimated from comparison to lambda DNA digested with *Hin*dIII. The middle lane contains about 5 times more YAC-TM15 DNA than the lane on the right.


0.5–
1.8–
kb
C3Hf/Rℓ
YAC TM15
YAC TM15

finding (personal communication) that TM15 recognizes a 90-kb *Not*I fragment in a pulsed-field-gel Southern blot of C3Hf ultra-high-molecular-weight DNA. The Southern blot shown in Figure 8-10 was also hybridized to probe 0.8, which lies on the side of 0.6 away from TM15 (Fig. 8-3). Again, no hybridization to YAC-TM15 was seen with 0.8, indicating that TM15 and 0.8 are not both contained in this YAC clone. The question of whether TM15 and 0.8 are on the same *Not*I fragment in wild-type DNA must await the hybridization of both probes to *Not*I digests of wild-type spleen DNA in PFG analysis. Another means of distinguishing among the above (or other) possible explanations for the results seen from hybridization of TM15, 0.6, and 0.8 with YAC-TM15 may come from the analysis of other wild-type (C3Hf/RI) genomic clones (both lambda and cosmid) isolated by hybridization to TM15.

What is the Null Phenotype at the *p* Locus?

The existence of fully-complementing compound heterozygotes among *p* mutations now known to be deletions raises the interesting question of what might constitute a null mutation for the *p* locus. The existence of compound heterozygotes carrying fully complementing lethal deletions at the albino (*c*), brown (*b*), or short-ear (*se*) loci demonstrates that these genes are nonvital, and that the null phenotype is simply the change in coat color or cartilage structure (Russell,

et al., 1990, in press; Kingsley et al., 1990). However, an interesting contrast to these genes is observed with overlapping lethal deletions at the dilute (*d*) locus. In no cases have there been dilute compound heterozygotes that did not express an associated neurological abnormality known as *d*-opisthotonus (d^{op}); in fact, the homozygous deletion of only four kb within the *d* transcription unit causes the d^{op} phenotype (N. Jenkins, personal communication). This result suggests that the null phenotype at *d* is not simply a change in coat color, but includes a neuological problem as well.

As explained in Chapter 6, studies by Russell and Montgomery (1965) studied coat-color-variegation patterns of females carrying X-autosome translocations and concluded that the original *p* mutation is not a null (0) mutation. Variegation resulting from the presence of cells either homozygous for *p* (*p/p*) or functionally hemizygous for *p* (*p*/0) in the same animal shows that *p/p* is not an absence of color since *p*/0 is an even lighter color, albeit only sightly lighter. Therefore, the original *p* cannot be a null mutation unless the coat color is modified by the effects of a different locus.

The homozygous deletion of the *p* locus by overlapping, fully-complementing deletions (e.g., $p^{4THO\text{-}II}/p^{46DFiOD}$ and $p^{4THO\text{-}II}/p^{15DThWb}$) appears to result only in the pink-eyed phenotype. Ideally, the hypothesis that the original *p* mutation is not a null should be testable by comparing the coat colors of *p/p* animals (darkest from two doses of *p)* to $p/p^{46DFioD}$ animals [hemizygous

(*p*/0)} and therefore of a lighter color that *p*/*p*, and finally to $p^{4THO\text{-}II}/p^{46DFioD}$ compound heterozygotes [both deleted for *p* (0/0), and therefore lighter still]. Russell and Montgomery (1965) were only able to make their observations about color differences produced by the *p*/*p* and *p*/0 cellular genotypes only because those colors were present in the same animal; the *p*/*p*, *p*/0, and 0/0 genotypes all appear to give the same color when whole animals of those genotypes are compared. Nonetheless, the phenotype of $p^{4THO\text{-}II}/p^{46DFiOD}$ must be the null phenotype, and may be different from *p*/*p*. The elucidation of the question of the null phenotype at *p* will have to await the analysis of transcription and translation products from the locus. The combination of the molecular probes isolated in the research reported here, along with the many *p* mutations at hand, should aid in that analysis.

CHAPTER 9

INTERSPECIFIC BACKCROSS ANALYSIS OF *p*-REGION LOCI

Probes have become available for several loci thought, either by linkage to mapped mouse loci or by conservation of linkage relationships with other species, to map to chromosome 7. For example, the human locus *MYOD1*, which encodes a protein thought to be involved in the regulation of striated muscle differentiation (Scrable, et al., 1986; Tapscott, et al., 1988), is found in the human genome on chromosome 11 p (Davis, et al., 1987; Scrable, et al., 1986), in a region which also carries other genes (e. g., *CALC*, calcitonin; *PTH*, parathyroid hormone; *HBBC*, hemoglobin, beta cluster) that have cognate sequences that map to mouse chromosome 7. Human *MYOD1* is tightly linked to *LDHA*, which encodes a subunit of a muscle-specific lactate dehydrogenase; *LDHA* is known to map to human chromosome 11p by linkage to the human locus, *RD*, thought to control the development of the embryonal tumor, rhabdomyosarcoma (Scrable, et al., 1987). *MYOD1* had not been mapped directly to human 11p because no RFLP could be found in human DNAs, but *MYOD1*, because of its myogenic action, was considered a candidate gene for the *RD* locus. The mouse locus, *Ldh-1*, was mapped to mouse chromosome 7, within the limits of the $p^{46DFioD}$ deletion by Mendel (1987). It became of interest to test

(1) the possible linkage of (mouse) *Ldh-1* with the mouse homolog (*MyoD-1*) of the human *MYOD1* locus and (2) the map positions of both mouse loci on chromosome 7. Both loci were found to be deleted in the $p^{46DFioD}$ deletion, but information about their linkage to each other and their map positions relative to *p* was unknown. Since deletions, and especially those near p (Chapter 6) are often not clean, linear breakages, standard genetic linkage testing is necessary to insure that wild-type gene orders have been maintained in the course of a deletion event.

Another human gene, insulin-like growth factor receptor (*IGFR*), was also mapped to mouse chromosome 7, and was found to be linked tightly to *p*, by interspecific linkage backcross analysis (N. Jenkins, personal communication). Testing of the human probe for *IGFR* (Table 1-1) on DNAs from the *p*-region panel of 29 (*M. musculus/M. spretus*)F_1 hybrids (Chapter 7) proved that the mouse locus, *Igfr-1*, is not deleted in $p^{46DFioD}$. It became necessary, then, to determine the linkage relationship of *Igfr-1* to the chromosome-7 anchor loci *p* and *c*.

Interspecific (*Mus musculus/Mus spretus*)F_1 hybrids, in which RFLPs are common, are very useful in standard backcross linkage analysis, since, in addition to segregation of visible markers, segregation of genomic DNA loci can be followed by RFLP analysis. In the case of probes that are thought to map near *p,* the data obtained by linkage backcross analysis and by

deletion-mapping can be pooled to yield valuable mapping information.

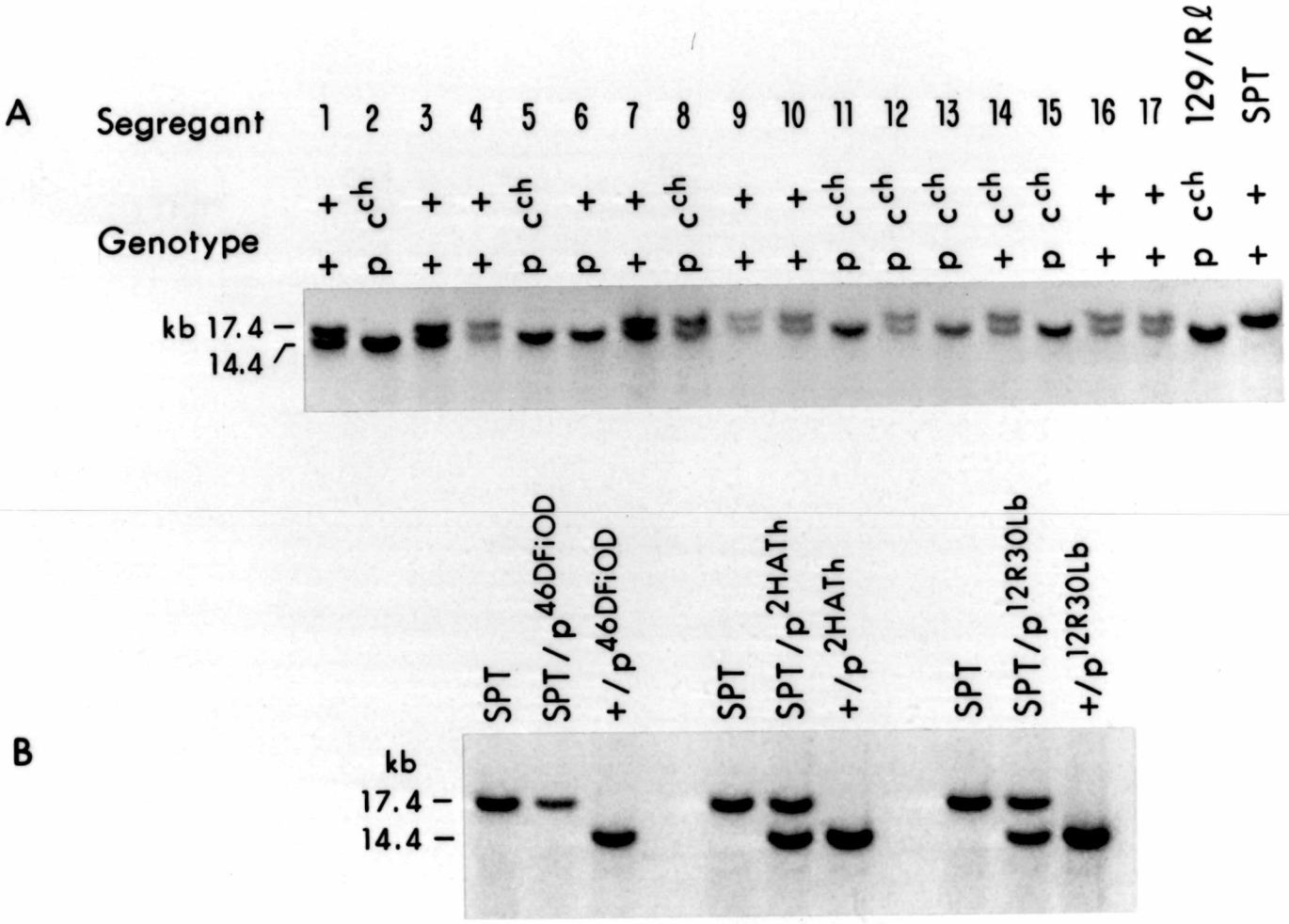
Probes for the two *p*-region loci, *MyoD-1* and *Ldh-1*, and for the chromosome-7 locus *Igfr-1*, obtained from other investigators, were mapped in relation to chromosome-7 anchor loci *p* and *c,* using linkage backcross data and to *p* by deletion-mapping. One hundred thirty segregants from the backcross (129/R*l* - *p* c^{ch}/*p* c^{ch} x *M. spretus*)F_1 x 129/R*l* *p* c^{ch}/*p* c^{ch} were typed for *p* and c^{ch}, and then spleen DNAs were tested by Southern blot analysis to determine linkage relationships for *Ldh-1*, *MyoD-1*, and *Igfr-1* (Table 1-1), three loci thought to map near *p:*

1) *Ldh-1*; lactate dehydrogenase-A. The probe, a 1.7-kb *Eco*RI-*Hin*dIII fragment of pUCLD-14, recognizes a 17.3-kb *Bam*H1 band in *M. musculus* and a 14.1-kb band in *M. spretus* DNA. Figure 9-1A is a representative autoradiogram of the hybridization of the probe for *Ldh-1* to seventeen segregants (lanes 1-17) from the backcross. Lane 1 has DNA from a *M. spretus* male, and lane 2 from a 129/R*l* *M. musculus* animal; these two lanes display the RFLPs that are followed for each locus in the backcross progeny (segregants). All segregants carry the *p* c^{ch} chromosome with the *musculus* allele for the locus being followed. Recombinants are recognized by a new (non-parental) association of alleles at those three loci. Lanes 18 and 19 contain DNAs from *M. musculus* (129/R*l*) and *M.spretus* ("SPT") animals to show the RFLPs for *Ldh-1* that are being

Figure 9-1. Autoradiograms showing the hybridization of the probe for *Ldh-1* to *Eco*RI-digested DNAs from interspecific linkage backcross progeny and from carriers of lethal *p* mutations.

A. Seventeen segregants from the backcross (129/R*l* - *p* c^{ch}/*p* *c* x *M. spretus*)F_1 x 129/R*l* - *p* c^{ch}/*p* *c*; abbreviated genotypes are shown, with each segregant also carrying a *p* c^{ch} chromsome. Phenotypes at *p* and *c* are shown. Lanes 18 (129/R*l*) and 19 [*M. spretus* ("SPT")] show the *Ldh-1* alleles that are followed.

B. Each set of three lanes contains DNAs from the *M. spretus* sire, the F_1 interspecific hybrid carrier of one of the three lethal *p* mutations shown, and the *M. musculus* heterozygous carrier dam of the F_1 for each *p* mutation.


A
Segregant
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 129/Rℓ SPT
Genotype
+ c^ch + + c^ch + + c^ch + + c^ch c^ch c^ch c^ch c^ch + + c^ch +
+ p + + p p + p + + p p p + p + + p +
kb 17.4 –
14.4
B
SPT
SPT/p^46DFiOD
+/p^46DFiOD
SPT
SPT/p^2HATh
+/p^2HATh
SPT
SPT/p^12R30Lb
+/p^12R30Lb
kb
17.4 –
14.4 –

followed. Abbreviated genotypes are given at the top; each segregant also has a *M. musculus p* c^{ch} chromosome. The segregant in lane 6 is recombinant between the *p* and c^{ch} loci, but no segregants shown are recombinant between *Ldh-1* and p. Of the 130 backcross progeny tested, 6 were recombinants between *Ldh-1* and *p*, indicating a centimorgan distance of 4.6 ± 1.8. Recombination patterns are best explained by the gene order *Ldh-1 - p - c*. Figure 9-1B shows the results when a Southern blot containing DNAs from a *M. spretus* male, from three *M. musculus* carriers of lethal *p* mutations ($p^{46DFioD}$, $p^{12R30Lb}$, and p^{2HATh}) and three *musculus/spretus* hybrid carriers of those three deletions were hybridized with the probe for *Ldh-1*. The musculus-specific 17.3-kb fragment is absent only in the hybrid carrier ($SPT/p^{46DFioD}$) of the $Df(ru\text{-}2\ p)^{46DFioD}$ deletion. Both the *Ldh-1* and *MyoD-1* loci (data not shown) are deleted in $p^{46DFioD}$, and in no others of the twenty-nine lethal *p* mutations tested.

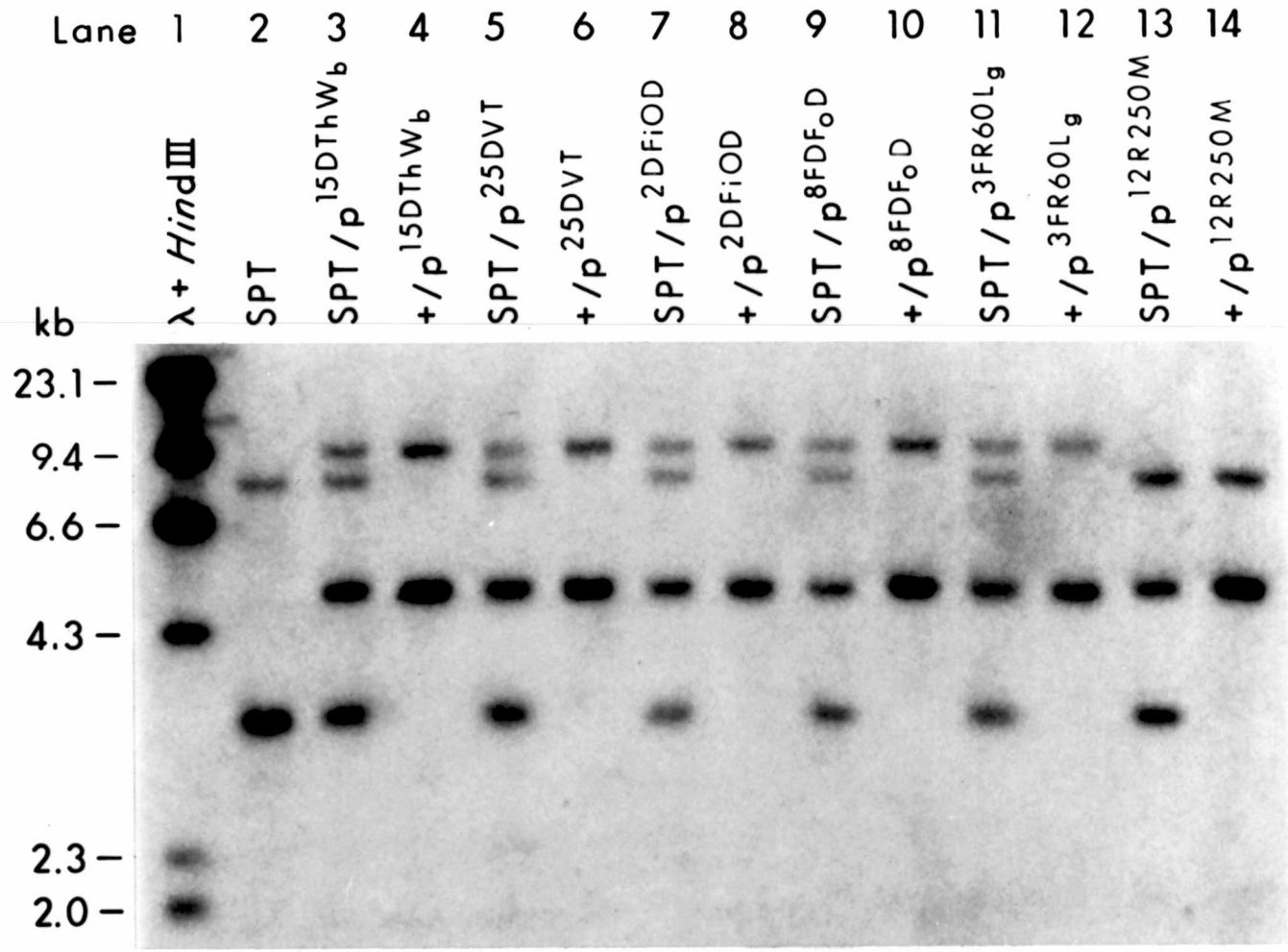
A third locus, *Saa*, was found (Chapter 13) to map to the same region of chromosome 7, but was not used on the linkage backcross blots because of band complexity due to homologous sequences.

(2) *MyoD-1*. The second probe tested was pMyoD1, a 1.9-kb human cDNA from human chromosome 11p. pMyoD1 recognizes a 16-kb *Eco*RI fragment in *M. musculus* DNA and a 13-kb fragment in *M. spretus* DNA. Of one-hundred-thirty segregants tested, six were recombinants between *MyoD-1* and *p*,suggesting a map distance of 4.6 ±1.8 cM, with recombination patterns dictating

distance of 4.6 ±1.8 cM, with recombination patterns dictating the gene order as *MyoD-1 - p - c.* No recombinations were seen between *Ldh-1* and *MyoD-1* in the 130 progeny tested, giving a centimorgan distance of less than 1 cM between the two loci. The lack of recombinant progeny made it impossible to order the loci with respect to *p*; it can only be concluded that the two loci are very tightly linked in the mouse as they are in the human. In addition, results reported in Chapter 13 also confirm the *MyoD-1/Ldh-1* linkage by proving that both loci are proximal to *ru-2* on chromosome 7. These are the first two loci to be reliably mapped to that region of mouse chromosome 7.

3) *Igfr-1.* A fourth probe thought to map near *p* and obtained from Nancy Jenkins was pIGF-I-R.8, which recognizes the human insulin-like growth factor receptor. In the mouse, pIGF-I-R.8 recognizes 8.4- and 5.5-kb *Bam*H1 fragments in *M. musculus* and and 8.4- and 3.5-kb fragments in *M. spretus* DNA. Eight of 130 segregants tested were recombinant between *Igfr-1* and *p*, while 12 of 130 were recombinant between *Igfr-1* and *c*, consistent with a gene order and distance of *p* - (6.1 ± 2.1 cM) - *Igfr-1* - (10.4 ± 2.6 cM) - *c.* Hybridization of the probe for *Igfr-1* to a representative *M. musculus/M. spretus p*-region deletion-mapping blot, containing six of the 28 *p* mutations tested, is shown in Figure 9-2. In each of the six mutations ($p^{15DThWb}$, p^{25DVT}, p^{2DFioD}, p^{8FDFoD}, $p^{3FR60Lb}$, and $p^{12R250M}$) shown, a *M. musculus* hybridizing fragment is present (lanes3, 5, 7, 9, 11, and 13) in the (*musculus/spretus*)F_1 hybrid carrier of the *p*

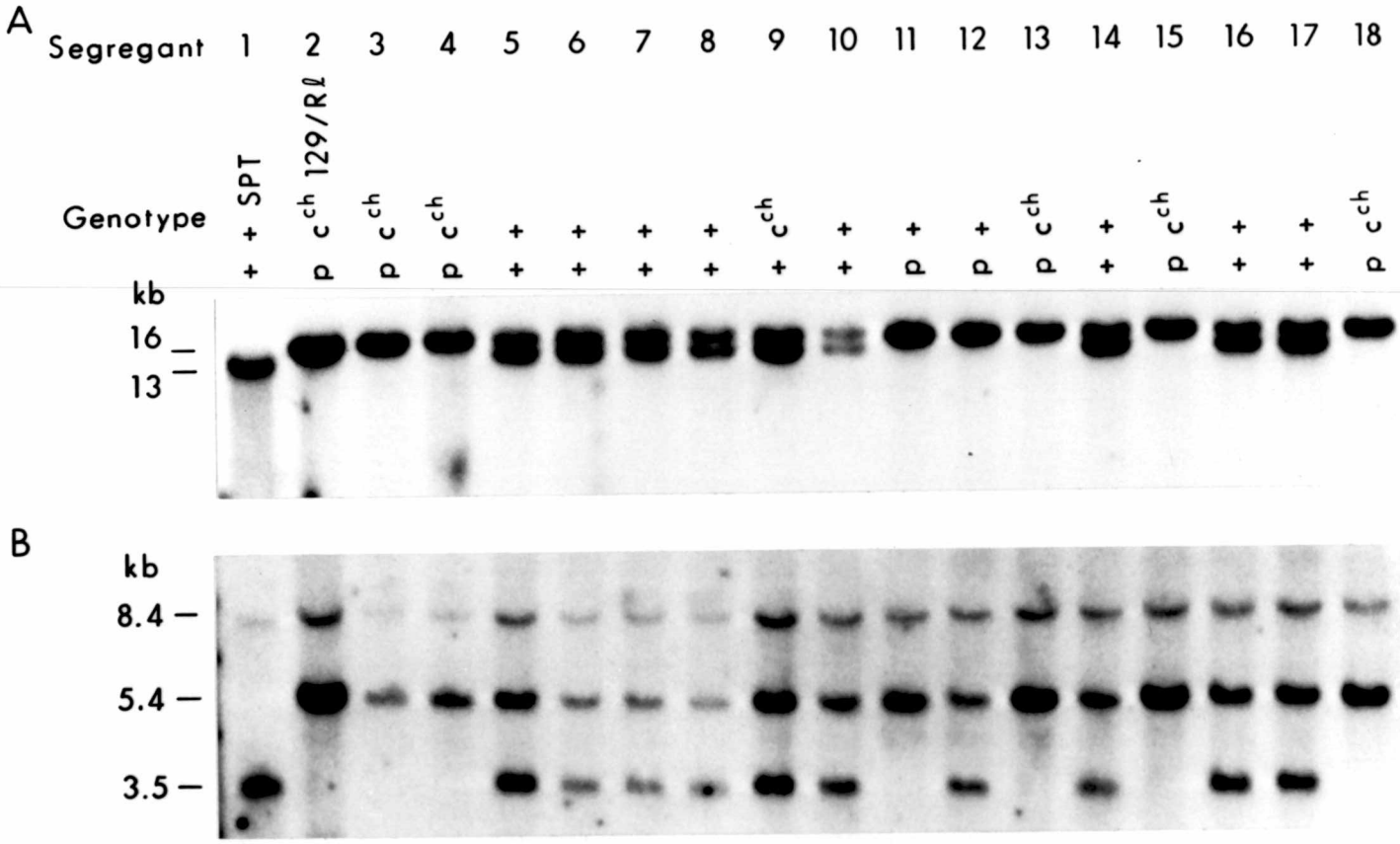
Figure 9-2. Autoradiogram showing the hubridization of the probe for *Igfr-1* to *Bam*Hi-digested spleen DNAs from carriers of six lethal *p* mutations. Lane 2 has DNA from a *M. spretus* male, lanes 4, 6, 8, 10, 12, and 14 contain DNAs from female *M. musculus* heterozygous carriers of the *p*-lethals, and lane 3, 5, 7, 9, 11, and 13 contain DNAs from (*M. musculus/M. spretus*)F_1 hybrid carriers of the *p*-lethal mutations.


Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
λ + HindIII
SPT
SPT / p15DThWb
+ / p15DThWb
SPT / p25DVT
+ / p25DVT
SPT / p2DFiOD
+ / p2DFiOD
SPT / p8FDFoD
+ / p8FDFoD
SPT / p3FR60Lg
+ / p3FR60Lg
SPT / p12R250M
+ / p12R250M
kb
23.1
9.4
6.6
4.3
2.3
2.0

mutation. It should be noted that in the case of the probe for *Igfr-1*, the *musculus*-specific fragment in lane 14 (and in the *SPT/p1*^{2R250M} hybrid carrier in lane 13) is larger than the musculus-specific fragments in all other lanes. This probably represents a simple RFLP between *M. musculus* chromosomes 7. None of the 28 *p* mutations tested was deleted for *Igfr-1*.

Figure 9-3 shows two more examples of the method of RFLP analysis that is used with the interspecific backcross linkage test. DNAs from eighteen progeny from the backcross (129/R*l* - *p c*^{ch}/*p c*^{ch} x *M. spretus*)F_1 x 129/R*l* - *p c*^{ch}/*p c*^{ch}, already scored for their coat color phenotypes at *p* and *c*, are hybridized with pMyoD (Fig. 9-3A; DNAs digested with *Eco*RI) and pIGF-I-R.8 (Fig. 9-3B; DNAs digested with *Bam*HI).Lane 1 (*M. spretus*), and lane 2 (129/R*l M. musculus*) display the RFLPs that are followed for each locus in the backcross progeny (segregants). All segregants carry, in addition to the abbreviated genotype listed at each lane, the *p c*^{ch} chromosome with the *musculus* allele for the locus being followed. For example, the segregant in lane 9 (*+ c*^{ch}) shows a crossover between *p* and *c*, so that the *spretus* parental phenotype (*+ +*) and the *musculus* parental phenotype (*p c*^{ch}) have recombined. No segregant in Fig. 9-3A is a recombinant between *MyoD-1* and *p*; however, the segregant in lane 12 in Fig 9-3B (*p +*, with the *spretus* allele for *Igfr-1*), is recombinant, between *p* and *Igfr-1*.

Figure 9-3. Interspecific backcross mapping of (A) *MyoD-1* and (B) *Igfr-1* with respect to chromosome-7 anchor loci *p* (pink-eyed dilution) and *c* (albino). This is a representative Southern blot showing eighteen segregants from the backcross (129/R*l*-*p* c^{ch}/*p* c^{ch} x *M. spretus*)F1 x 129/R*l*-*p* c^{ch}/*p* c^{ch}; the same segregants are shown in both panels. Abbreviated genotypes are given for each segregant; the full genotype for each includes a *p* c^{ch} *M. musculus* chromosome. DNAs in A were digested with *Eco*RI; the DNAs in B were digested with *Bam*HI.

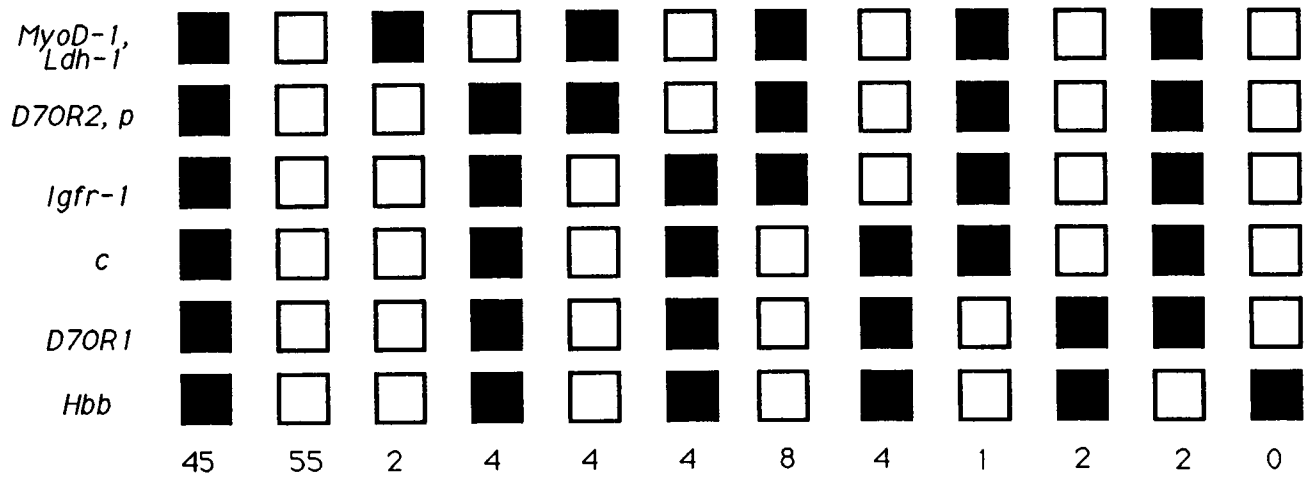

A
Segregant 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Genotype
+ + SPT
p c^ch 129/Rℓ
p c^ch
p c^ch
+ +
p c^ch
+ +
p c^ch
+ +
kb
16
13
B
kb
8.4
5.4
3.5

These data, along with the linkage backcross data from Chapter 4, are summarized in Figure 9-4. The results of linkage backcross analysis establish the gene order and distance as follows: [*MyoD-1*, *Ldh-1*] - 4.6 (± 1.8 cM) - *p* - 6.1 (± 2.1 cM) - *Igfr-1* -10.4 (± 2.6) - *c* -2.3 (±1.3) - *D7OR1* - 1.8 (± 1.1) - *Hbb.*

Deletion-mapping using the *p*-region (*M. musculus/M. spretus*)F_1 *p*-mutation carriers was used to refine the genetic map provided by the backcross data. *MyoD-1* and *Ldh-1* are both deleted in $p^{46DFioD}$, which is thought to be the largest Oak Ridge *p*-region deletion (Chapter 5) and the only one that extends proximally as far as *ru-2* (L. B. Russell, personal communication). None of the other 36 lethal *p*-region mutations include *Ldh-1* or *MyoD1*, a finding consistent with the experiments reported in Chapter 13 that proved both genes to be located proximal to *ru-2* and, therefore, outside the limits of any p-deletion except $p^{46DFioD}$. Since *ru-2* maps 3 cM proximal to *p* , and *MyoD-1* and *Ldh-1* are closely-linked at 4.6 ± 1.8 cM proximal to *p*, the mutation $p^{46DFioD}$, which deletes all three loci, must be at least 4.6 ± 1.8 cM in length. Thus, the ability to map *Ldh-1* and *MyoD1* using data from linkage backcross analysis, *p*-region deletion-mapping, and segregation of markers with the derivative chromosomes from the T1Ct rearrangement (Chapter 10) has provided a significant refinement of the genetic map proximal to *p*.

The genetic map distal to *p* has been sadly lacking in closely linked markers that could contribute information about

Figure 9-4. Summary of results from interspecific backcross analysis. Each of the loci mapped is listed on the left. A total of 130 N_2 segregants from the cross (129/R*l* - *p* c^{ch}/*p* c^{ch} x M. spretus)F_1 x 129/R*l* - *p* c^{ch}/*p* c^{ch} were typed for each locus. Each column represents the chromosome, parental or recombinant, that was inherited by each segregant, and the numeral at the bottom of each column is the number of segregants that inherited that particular chromosome. Black boxes represent *M. musculus* (129/R*l*) alleles; white boxes represent *M. spretus* alleles.

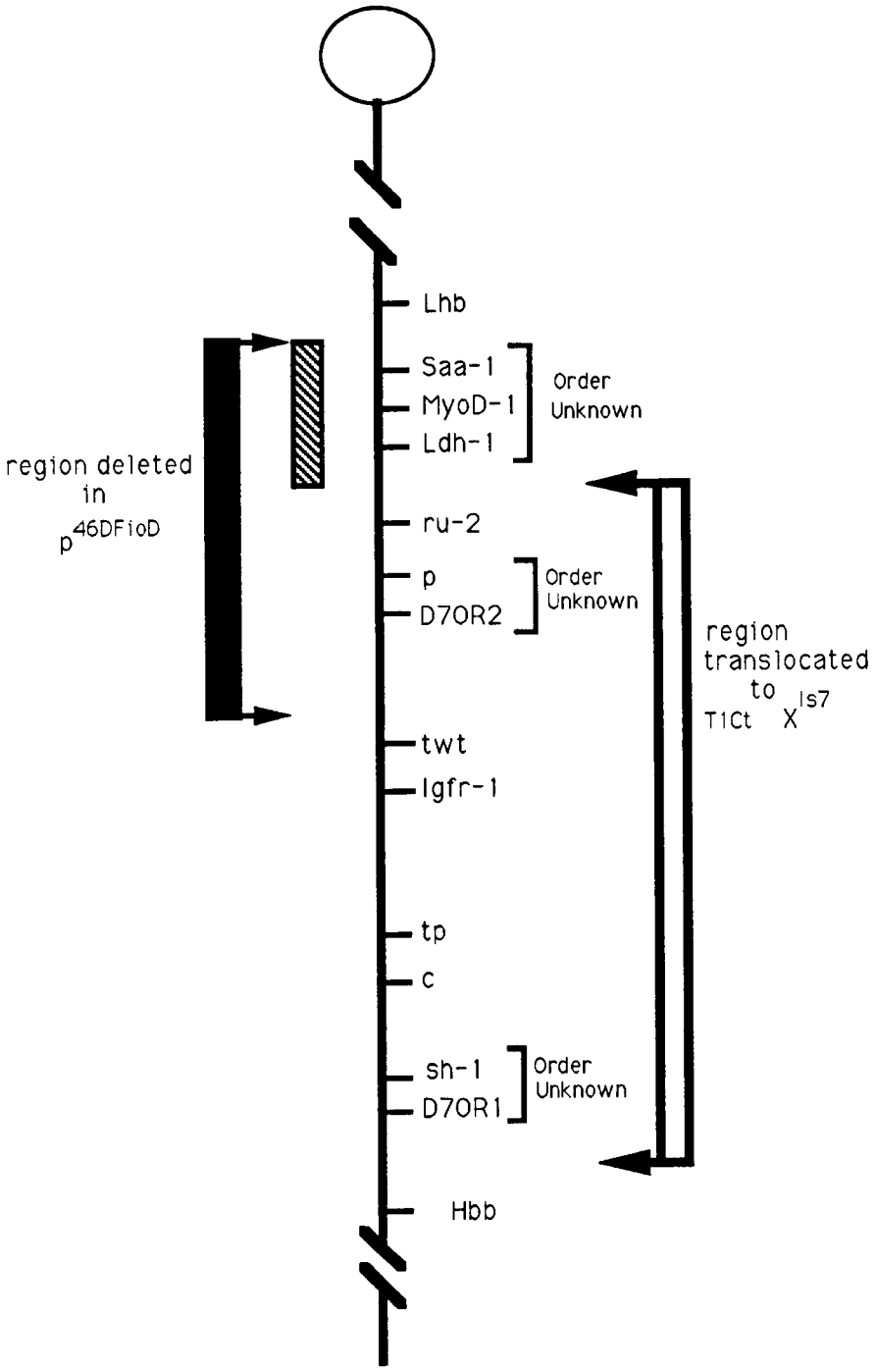

MyoD-1, Ldh-1
D7OR2, p
Igfr-1
c
D7OR1
Hbb
45
55
2
4
4
4
8
4
1
2
2
0

the distal extent of the *p*-region deletion complex. Consequently, the mapping of the *Igfr-1* locus has provided some new, useful information about the distal extent of $p^{46DFioD}$ and other *p* deletions. *Igfr-1* is not deleted in $p^{46DFioD,}$ and by the standard linkage backcross data reported above, maps 6.2 ± 2.1. cM distal to *p*. Because the map position of the *MyoD-1/Ldh-1* linkage is informative about the proximal extent of the deletion, $p^{46DFioD}$ could, by ending proximally past *MyoD-1/Ldh-1* and distally just short of *Igfr-1*, be more than 14.6 cM (4.6 + 1.8 + 6.2 + 2.1) in genetic length. No other *p* deletion can be greater than 7 cM in genetic length, because none goes as far proximally as *ru-2* (3 cM) and none deletes *twt* (Chapter 10). Figure 9-5 illustrates schematically the linkage relationships of the loci mapped in the chromosome-7 linkage backcross reported in this Chapter and in Chapter 4.

These analyses again emphasize the value of a series of independent mutations that involve a common locus. Without the $p^{46DFioD}$ and T1Ct mutations (Chapters 6 and 11), *MyoD-1* and *Ldh-1* (and *Saa*) could not readily have been mapped relative to either *ru-2* or the $p^{46DFioD}$ and T1Ct proximal breakpoints. As a result, valuable data about the proximal end of the *p*-region deletion complex would have been missed. The distal end of the complex is just beginning to be characterized, and the mapping of *Igfr-1* is a useful first step.

These data, gathered from both deletion-mapping and linkage backcross analysis in the *p* region, have contributed to

Figure 9-5. Cartoon showing the relationships of chromosome-7 loci and the breakpoints of T1Ct and *Df(ru-2 p)*^{46DFioD}. The open box to the right shows the region of chromosome 7 that is inserted into the X; the black box at left shows the region deleted in $p^{46DFioD}$. The hatched box is the region of non-overlap between T1Ct and $p^{46DFioD}$. Loci are: *Lhb* = beta subunit of luteinizing hormone; *Saa-1* = alpha subunit of serum amyloid protein; *MyoD-1* = striated muscle-specific myogenic protein; *ru-2* = ruby-eye-2; *p* = pink-eyed dilution; *D7OR1* and *D7OR2* are loci recognized by anonymous DNA probes; *twt* = twister; *Igfr-1* = insulin-like growth factor receptor; *tp* = taupe; *c* = albino; *sh-1* = shaker-1; *Hbb* = beta globin.


Lhb
Saa-1
MyoD-1
Ldh-1
Order Unknown
region deleted in p46DFioD
ru-2
p
D7OR2
Order Unknown
region translocated to T1Ct X Is7
twt
Igfr-1
tp
c
sh-1
D7OR1
Order Unknown
Hbb

the pool of the knowledge that is being gathered about a devastating human disease, the embryonal tumor rhabdomyosarcoma. *MYOD1*, a striated-muscle-specific gene on human chromosome 11p (Scrable, et al., 1986; Davis, et al., 1987;), had been postulated to be involved in the development of rhabdomyosarcoma, which is a tumor of striated muscle. Both the *MYOD1* and the rhabdomyosarcoma (*RD*) loci were shown (Scrable, et al., 1987; Scrable. et al., 1990, manuscript submitted) to map to human chromosome 11p, making *MYOD1* a candidate locus responsible for the tumor phenotype. Further mapping data, however, reported in a manuscript submitted to Proceedings of the National Academy of Sciences (Scrable, H. J., D. .K. Johnson, E..M. Rinchik, and W. K. Cavenee, 1990) and entitled, "*RD* and *MYOD* Are Syntenic But Separate Loci on Human Chromosome 11p", the authors provide proof that the human *MYOD1* locus maps near, but is not identical to, the locus thought to be involved in rhabdomyosarcoma. In the human, the *LDHA* locus also maps near *MYOD1*. Data from the mouse, provided by the experiments described in this chapter, including the deletion of the mouse *MyoD-1* locus in $p^{46DFioD}$, are included in the manuscript as further proof that *MyoD-1* and *Ldh-1* are tightly-linked genes in both mouse and human and could represent a cluster of coordinately-regulated muscle-specific genes in both species. Although there is no evidence that mice develop rhabdomyosarcomas, the fact that a mouse exists which carries a deletion of the *MyoD-1* locus might provide a means for

studying the regulation of the differentiation of striated muscle during embryogenesis. The mouse locus that is homologous to the human locus associated with the formation of rhabdomyocarcoma may or may not be syntenic with *MyoD-1* in the mouse; if it is, then mapping of that locus when an *RD* probe becomes available might provide a model mammalian system for analysing the regulation of the *RD* locus.

CHAPTER 10

p-REGION DELETION MAPPING USING THE *twt* MUTATION

As was pointed out in Chapter 5, the characterization of the radiation-induced deletion complex surrounding *p* has suffered from the lack of nearby markers useful for deletion mapping. In the *c* region (Chapter 2), taupe (*tp*) lies 3 cM proximal to *c*, and mitochondrial malic enzyme (*Mod-2*) and shaker-1 (*sh-1*) lie 2 and 4 cM distal to *c*, respectively. These flanking markers, then, are within a cM distance that might reasonably be expected, from the sizes of other known deletions (L. B. Russell, 1971; L. B. Russell, et al., 1982; Russell and Cacheiro, 1977), to be included in a large deletion event. The *p* region, however, is notable for the lack of closely-linked distal marker loci. Ruby-eye-2 (*ru-2*) lies 3 cM proximal to *p*, but is deleted in only one ($p^{46DFioD}$) of the 40 *p*-region lethal mutations tested (L. B. Russell and C. S. Montgomery, unpublished data). To date, *lgfr-1*, is the closest reliably mapped distal marker (Chapter 9) but the data reported in Chapter 9 have ruled out the inclusion of *lgfr-1* in any of the tested *p*-region mutations. This dearth of distal markers has made further *p*-region deletion mapping very diffcult.

Twister (*twt*) is a recessive neurological mutation that arose spontaneously in a (C57Bl/6 x C3H)F_1 mouse at the Jackson Laboratory. Linkage-mapping experiments, using

recombinant inbred strains (700 chromosomes tested) and linkage testing with *p* (70 segregants tested), placed *twt* within 4 cM of *p* (Lane, 1981). The mutation is characterized by tucking of the head "as if to somersault"(Lane, 1981) when the animal is picked up by the tail; homozygotes also display circling behavior, and inability to swim. It was reported in Mouse News Letter that *twt* mice can hear; however, it has also been reported (Paul Neumann, personal communication) that they are deaf. This mutation, besides being intrinsically interesting because of the apparent neurological defects, was a possible marker locus near enough to *p* to have been fortuitiously included in some of the p-region radiation mutations. To test this possibility, thirteen *p*-region mutations, judged from preliminary complementation mapping to be among those that might be deletions extending the farthest from *p*, were crossed to *twt* homozygotes or heterozygotes (*twt/twt* or *+/twt*). If any *p* mutation were indeed a deletion that also included the wild-type *twt* locus carried on the *p*-mutant chromosome, the crosses should produce progeny with the twister phenotype (50% *twt* : 50% *+* in the cross to *twt/twt;* 25% *twt* : 75% *+* in the cross to *+/twt*.) The results of these crosses are summarized in Table 10-1. None of the thirteen mutations tested, seven of which are now known to be deletions from data reported in Chapter 8, includes the wild-type *twt+* locus as evidenced by the finding of no *twt* progeny in any of the F_1 litters. These data suggest that (a) *twt* lies distal to *p*, since the deletion $p^{46DFioD}$, known to

Table 10-1. Summary of Results of Deletion-Mapping Crosses Between Radiation-Induced *p* Mutations and Stocks Carrying *twt*.

Cross	F_1s*	F_1s with *twt* phenotype Obs/Exp†	p of f(0)††
+/p2DFioD x +/twt	8/9	0/2.5	.1001
+/p2DFioD x twt/twt	40/42	0/20	9.1×10^{-13}
+/p46DFioD x +/twt	15/15	0/3.75	.0133
+/p46DFioD x twt/twt	20/20	0/10	9.5×10^{-7}
+/p17MNURf x +/twt	36/37	0/9	3.1×10^{-5}
+/p7FR60Lb x +/twt	28/28	0/7	3.2×10^{-4}
+/p19DVT x +/twt	36/40	0/9	3.1×10^{-5}
+/p19DVT x twt/twt	40/46	0/20	9.1×10^{-13}
+/p116G x twt/twt	10/11	0/5	.0009
+/p3R30M x twt/twt	8/10	0/4	.004
+/p41DTD x twt/twt	26/29	0/13	1.5×10^{-8}
+/p4/THOII x twt/twt	26/27	0/13	1.5×10^{-8}
+/p8FDFoD x twt/twt	21/25	0/10.5	4.8×10^{-7}
+/p15DThWb x twt/twt	10/10	0/5	.0009
+/p12R30Lb x twt/twt	48/48	0/24	3.5×10^{-15}
+/p2HATh x twt/twt	11/12	0/5.5	.0004

* F_1s weaned/F_1s born

† F_1s observed with *twt* phenotype/F_1s expected with *twt* phenotype if the *p* mutation in the cross deletes twt^+

†† Probability that no *twt* progeny [f(0)] will be observed in n weanlings when 0.50 (cross of $+/p^l$ x *twt/twt*) or 0.25 (cross of $+/p^l$ x *+/twt*) of the weanlings should exhibit the phenotype if the p^l mutation includes twt^+.

extend proximally at least 4cM, and probably more, (Chapter 12) from *p*, does not delete twt; and (b) no tested *p*-region mutation extends as far distally from *p* as 4 cM since none elicit pseudodominance of the twister phenotype. These two new pieces of information, although negative in a sense, are quite useful in helping set an outside limit for the distal extent of any of the known *p*-region deletions.

CHAPTER 11

IINTRODUCTION TO THE EXPERIMENTS THAT EXPLOIT Is(In7;X)1Ct FOR MOLECULAR AND GENETIC LINKAGE MAPPING IN THE *p* REGION

The results of experiments published by Eicher (1970) and by Taylor and Rowe (1984) suggested a way of taking advantage of two independently generated chromosomal aberrations, Df(*ru-2 p*)^{46DFioD} and Is(In7;X)1Ct, to map probes that lie near *p* and *ru-2*, a flanking coat-color marker 3 cM proximal to *p*. As described in Chapter 9, the Oak Ridge radiation-induced *p* mutation, $p^{46DFioD}$, extends proximally 4.6 ± 1.8 cM, and includes not only *ru-2*, but *MyoD-1*, *Ldh-1*, and *Saa-1*. The distal extent of $p^{46DFioD}$ is unknown, but it does not includes *Igfr-1*, which interspecific backcross analysis suggests is 6.2 ± 2.1 cM distal to *p* (Chapter 9) and it does not include *twt* (Chapter 10). The region of $p^{46DFioD}$ proximal to and including *ru-2* is not overlapped by any other Oak Ridge deletions, and no other markers have been available for exploring the length of the area of non-overlap, either proximal to or distal to *ru-2*. Complementation testing so far has failed to contribute consistent data on functional units covered by $p^{46DFioD}$ as has been done for the *c*-region deletion mutations. The experiments reported here used the breakpoints of the $p^{46DFioD}$ mutation and of Is(In7;X)1Ct ["Cattanach's translocation"], abbreviated T1Ct, as additional markers to provide information about the proximal end of the *p* region.

T1Ct is a non-reciprocal rearrangement in which an inverted central segment of chromosome 7 was inserted interstitially into an X chromosome (Cattanach, 1961; 1969). The resulting, balanced karyotype ("Type I") includes a normal X chromosome, a derivative X chromosome that consists of the full complement of X chromosome-DNA plus the inverted segment from the middle of chromosome 7 (X^{Is7}), and a small, deleted autosome 7 composed of the 7 centromere and only the most proximal and distal segments of chromosome 7 (7^{Df}). Additionally, it was found that the X^{Is7} can exist in an unbalanced genome ("Type II") that includes a normal X, the X^{Is7}, and two normal chromosomes 7; the deleted 7^{Df} is not present in this Type-II form. Because of the availability of both balanced and unbalanced (partially trisomic) animals, the segregation of the two rearranged chromosomes, marked by RFLPs, can be followed separately in different animals. This unusual feature makes it possible to assign genetic loci to one or the other derivative chromosome; this fact, combined with the knowledge that both Is(In7;X)1Ct and *p*^{46DFioD} involve breakpoints near the same region of chromosome 7, led to the design of genetic crosses that have permitted the mapping of several *p*-region probes relative to both *p* and *ru-2*.

Eicher's experiments (1970) were undertaken to map the proximal breakpoint of T1Ct in chromosome 7 relative to *ru-2*. Because T1Ct includes an inversion of the chromosome-7 segment inserted into the X, crossing over leads to unbalanced

gametes and makes standard linkage mapping impossible. Eicher's results strongly suggested that the proximal breakpoint in chromosome 7 in Cattanach's rearrangement occurred centromeric to *ru-2*, so that the wild-type *ru-2*$^{+}$ locus was translocated to the X^{ls7} chromosome along with the wild-type *p* $^{+}$and *c* $^{+}$ loci.

Taylor and Rowe's experiments (1984) were designed to determine if another chromosome-7 gene, *Saa-1* (Table 1-1) was also translocated to the X^{ls7}. Since the inheritance of *Saa-1*, the locus encoding the alpha subunit of a serum amyloid protein, can be followed with a molecular probe, rather than a visible phenotype as in Eicher's experiments, Taylor and Rowe bred T1Ct pedigrees in which they could follow the segregation of copies of the *Saa* hybridization band in the balanced (Type I; two copies, one on the normal 7 and one on either the X^{ls7} or $7^{Df)}$ and unbalanced forms (Type II, which carries, in addition to the X^{ls7}, two normal 7's; two copies if *Saa* is on the 7^{Df} chromosome which is absent, or three copies if *Saa* is on X^{ls7}) Taylor and Rowe's work demonstrated, by extrapolating gene copy number of *Saa* from comparing hybridization band-intensity, that the *Saa* locus resides in the portion of proximal 7 that was not translocated to the X^{ls7}, and therefore remained on the small derivative 7^{Df}.

Because, then, T1Ct can exist in two viable forms such that the two derivative chromosomes, X^{ls7} and 7^{Df}, can segregate serparately, molecular markers can be followed by RFLP analysis

and definitively mapped to one of the two derivative chromosomes. The technique is made difficult and unwieldy, however, by the necessity, in Taylor and Rowe's scheme, of judging two- versus three-gene copies by hybridization-band density comparison. If, on the other hand, the breeding scheme takes advantage of the availability of strains congenic for the segment of chromosome 7 surrounding *p*, a simple "plus-minus" screen for segregating RFLPs can be used to map the fate of any chromosome-7 gene in T1Ct, obviating the need for tedious and less-exact comparisons between hybridization intensities. The approaches used by both Eicher and Taylor and Rowe have been adapted in this study to confirm and expand their findings about the gene order and map positions in the region of chromosome 7 near *p*, *ru-2*, and the proximal T1Ct breakpoint.

The homozygous-lethal mutation $p^{46DFioD}$ deletes both *p* and *ru-2*, and is therefore at least 3 cM in genetic length; however; since no other markers have been available near *p*, nothing else has been known about the extent, proximal or distal, of $p^{46DFioD}$ or any other *p* mutation. It is known, however, from the *musculus/spretus* F_1 hybrid deletion mapping reported in Chapter 8 that not only *Saa* but also *Ldh-1* and *MyoD1* map near *p* because they all are deleted in $p^{46DFioD}$, and because of the results of the linkage backcross described in Chapter 9. In order to examine the segregation of each of these three genes and the anonymous DNA sequence, TM15, also deleted in $p^{46DFioD}$ (Chapter 8), a three-generation pedigree that combines the T1Ct

chromosomes and two strains congenic for the region near *p* [129/R*l* and B10.129 (21M)] was bred, and the DNAs from all family members were hybridized with probes for four loci deleted in $p^{46DFioD}$ (*Saa*, *Ldh-1*, *MyoD-1*, and *D7OR2*). The same DNAs were also hybridized with probes recognizing *Lhb* and *Igfr-1* (Table 1-1), two chromosome-7 loci that are not deleted in $p^{46DFioD}$.

T1Ct has proved indispensable in mapping proximal chromosome-7 genes, some of which lie within and some outside the largest *p*-region deletion, because its breakpoints and that for the independently generated aberration $p^{46DFioD}$ lie proximal to *ru-2* and close together. Because T1Ct can exist, in addition to the balanced form, in an unbalanced form that results in partial trisomy, the genes that segregate with each of the aberrant chromosomes can be followed in separate animals to determine if they lie proximal or distal to the T1Ct breakpoint. This same approach will work for distal-7 genes that lie either proximal or distal to the distal T1Ct breakpoint since the breakpoints themselves can be used as DNA markers to order loci. The idea is important because the genes within the inversion cannot be mapped by standard techniques that rely on recombination frequencies to order and map autosomal loci. Since the autosomal loci within this segment of chromosome 7 are now subject to X-inactivation, however, variegated phenotypes can be used, as Eicher did, to map some markers. RFLP analysis, using congenic strains to simplify patterns, can

be used to map others. In some cases, as with the genes *Saa*, *Ldh-1*, *MyoD-1*, and *Igfr-1*, these results can be combined with the knowledge of which probes are deleted in $p^{46DFioD}$ (or in other deletions) to permit the ordering of the relative breakpoints of the different aberrations.

The fact that the T1Ct pedigrees bred in the experiments reported in this Chapter include animals that carry both the X^{ls7} chromosome and *Df(ru-2 p)*^{46DFioD} raised an interesting question about the map locations of lethal genes in the proximal end of the *p* region. Experiment 2, below, provided the information that the proximal breakpoint on chromosome 7 of T1Ct lies within the segment of DNA that is deleted in $p^{46DFioD}$ (Figure 9-5) and that this T1Ct breakpoint is more distal than is the proximal $p^{46DFioD}$ breakpoint. Therefore, there is a region of DNA, proximal to *ru-2,* that lies between the two breakpoints (see the hatched area in Figure 9-5) where the two segments of DNA involved in the two rearrangements do not overlap. Because unbalanced carriers of the T1Ct chromosomes are viable, it is possible to breed animals that could be homozygously-deleted for the region of non-overlap; this chromosome constitution is illustrated schematically in Figure 11-1 and the breeding scheme is shown in pedigree form in Figure 11-2. If the animals that carry two $p^{46DFiOD}$ chromosomes (Figure 11-2) are viable, then the region of non-overlap between the T1Ct and $p^{46DFioD}$ breakpoints cannot include any lethals.

Figure 11-1. Idiograms illustrating the possible chromosome constitutions of the variegated G_3 females from the pedigree in Figure 11-2.

A. Genotype *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +.* These females are expected to be fully viable, and to carry at least one copy of all chromosome 7 loci.

B. Genotype *X^{ls7}/X; Df(ru-2 p) +/Df(ru-2 p) +.* These females are homozygously deleted for loci that lie between the proximal breakpoints of T1Ct and *Df(ru-2 p)^{46DFioD}* (see hatched area in Figure 9-5); they may or may not be viable.

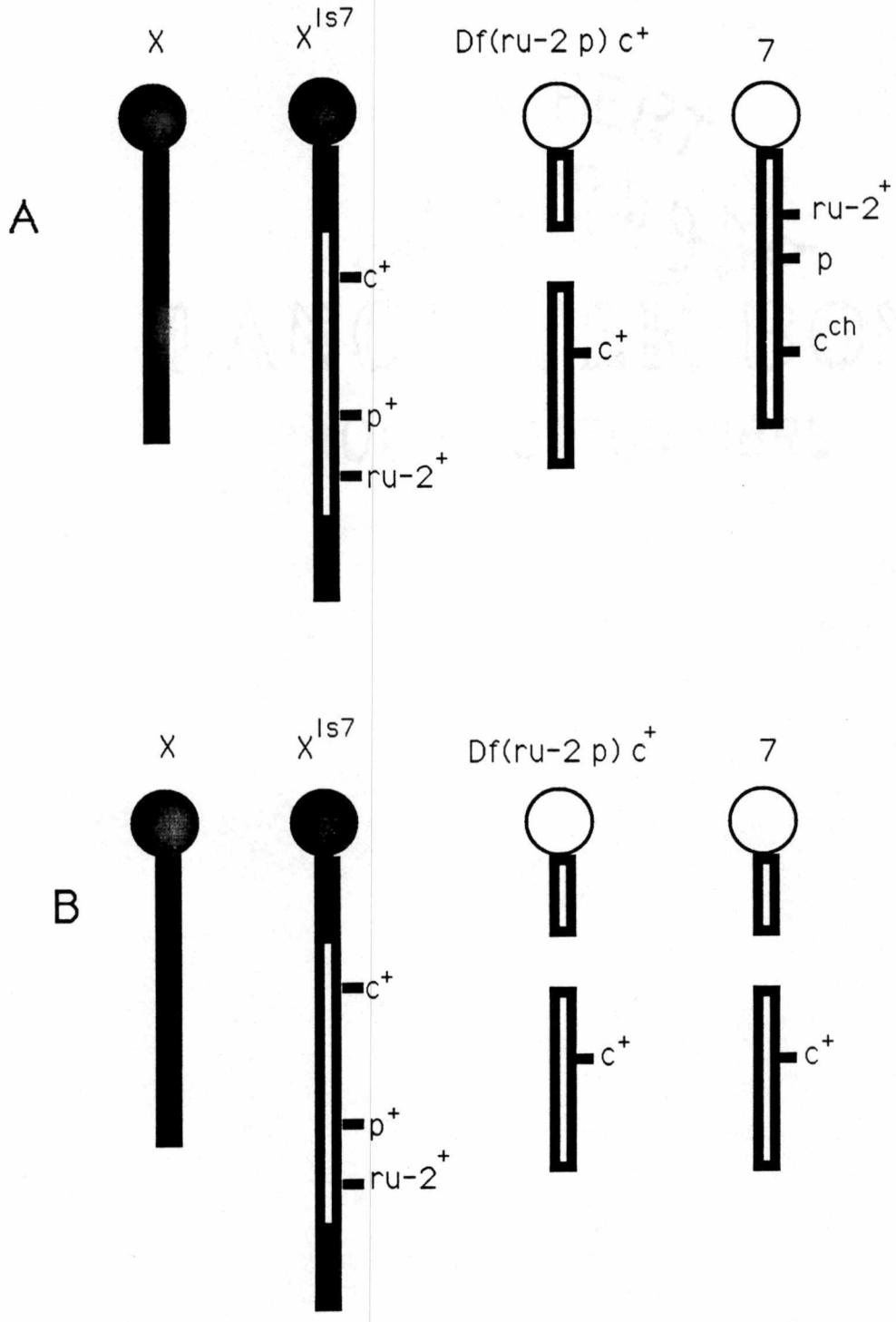
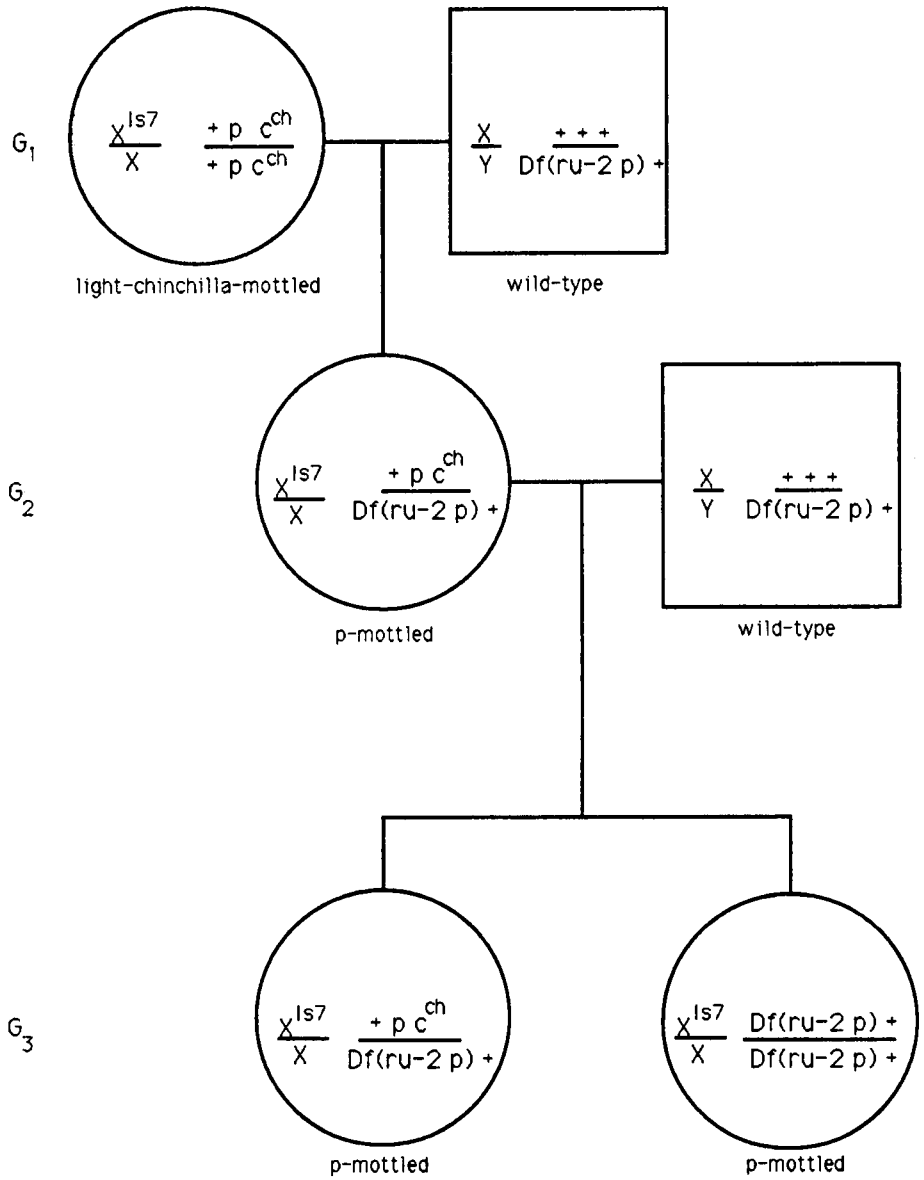

A
X
X^ls7
Df(ru-2 p) c+
7
c+
p+
ru-2+
c+
ru-2+
p
cch
B
X
X^ls7
Df(ru-2 p) c+
7
c+
p+
ru-2+
c+
c+

Figure 11-2. Three-generation pedigree (G_1, G_2, and G_3) illustrating the derivation of the two genotypes, *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +* and *X^{ls7}/X; Df(ru-2 p) +/Dfru-2 p) +*. Both would produce variegated (*p*-mottled) females in the third generation if females of both genotypes were viable. The pedigree is designed to test the possible viability of the G_3 females which carry two *Df(ru-2 p)^{46DFioD}* chromosomes.


G1
X ls7 / X
+ p c ch / + p c ch
light-chinchilla-mottled
X / Y
+ + + / Df(ru-2 p) +
wild-type
G2
X ls7 / X
+ p c ch / Df(ru-2 p) +
p-mottled
X / Y
+ + + / Df(ru-2 p) +
wild-type
G3
X ls7 / X
+ p c ch / Df(ru-2 p) +
p-mottled
X ls7 / X
Df(ru-2 p) + / Df(ru-2 p) +
p-mottled

p^{46DFioD}, and many other radiation-induced *p* mutations [17 of 29 now tested proved to be deletions (Chapter 8)] include at least one, and perhaps many, gene(s) whose homozygous absence is lethal to normal development. Since *p*^{46DFioD} is the only Oak Ridge *p*-deletion that also deletes both *ru-2*, and the *Saa*, *MyoD-1*, and *Ldh-1* loci, this proximal end of the *p* region is not overlapped by other Oak Ridge *p* mutations. It has therefore been impossible, as has been done by complementation testing for the *c* region (Chapter 2), to determine if "functional units" for embryonic lethality lie proximal to *ru-2*. Because, however, breeding schemes can be employed to obtain mice that carry both X^{ls7} and *p*^{46DFioD}, which have a region of non-overlap, and because available molecular probes can be used to prove, by the absence of hybridization bands, homozygous deletion of loci in the non-overlap region, the absence of lethal genes in this region can be proved. The converse, that lethal genes are present, can be suggested but not proved since an alternate explanation, the X-inactivation of autosomal loci in the proximal end of the chromosome-7 insertion in the T1Ct X^{ls7}, cannot be ruled out. This point will be discussed in Chapter 13.

Nothing is known of the physical size of this region of non-overlap; however, Experiment 2 proved that this region includes the genes *Saa-1*, *MyoD-1*, and *Ldh-1*, and perhaps many others. By simply comparing centimorgan distances, from standard linkage analysis of the *ru-2* - *p* interval (3 cM) and the *Ldh-1/MyoD-1* - *p* interval (4.8 ± 1.8 cM), the region of non–overlap

is, at most, 3.6 cM and could be less than 1 cM. No accurate relationship between physical and recombination distances has been established, so that useful information would be gained by the eventual molecular cloning of the T1Ct proximal breakpoint and the *p46DFioD* breakpoint; a long-range physical map derived from pulsed-field gel electrophoresis might physically link clones from the two breakpoint-fusion fragments. It is hoped that further genetic and molecular analysis of this interesting region will provide the necessary molecular access points that will make that long-range physical map a reality.

CHAPTER 12

MATERIALS AND METHODS USED IN THE T1Ct MOLECULAR MAPPING AND GENETIC LINKAGE EXPERIMENTS

Experiment 1

To confirm Eicher's conclusion that the *ru-2* locus was translocated to X^{ls7}, two mating schemes were designed:

(A) T1Ct Type I females (X^{ls7}/X; $7^{Df}/ru\text{-}2^{+}$ p^{+} c^{ch}) were mated to *RUT* males (*X/Y; ru-2 + +/ru-2 + +*). If any F_1 females were mottled for *ru-2*, they must be of genotype X^{ls7}/X; $7^{Df}/ru\text{-}2$ *+ +* and display a variegated phenotype because the wild-type $ru\text{-}2^{+}$ gene is on X^{ls7} and would be expected to be inactivated about half the time. A corollary would be that the 7^{Df} chromosome does not carry the $ru\text{-}2^{+}$ gene; otherwise, mottling would never be observed in any female. Similarly, the other class of translocation-carrier F_1 females (Type II, X^{ls7}/X; $ru\text{-}2^{+}$ p^{+} $c^{ch}/ru\text{-}2$ + +) would be phenotypically wild-type because the $ru\text{-}2^{+}$ gene on the maternally derived chromosome 7 is always expressed. In summary, then, the presence of ruby-mottled females in the F_1 would prove that *ru-2* was moved to the X chromosome by the T1Ct rearrangement as Eicher (1970) had concluded.

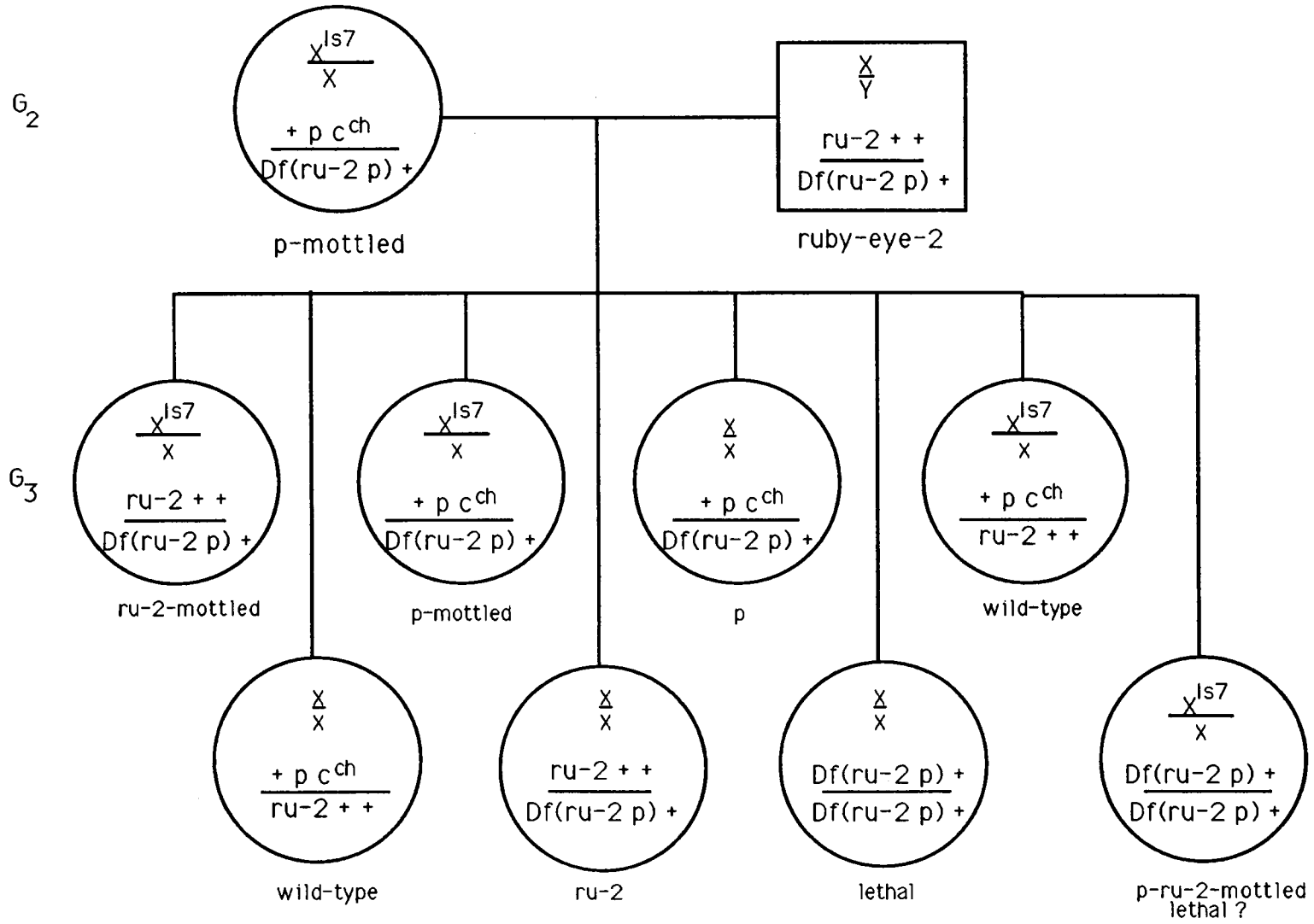
(B) T1Ct Type II females (G_1)[X^{ls7}/X; $ru\text{-}2^{+}$ *p* c^{ch} /+ *p* c^{ch}] were mated to males carrying $Df(ru\text{-}2\ p)^{46DFioD}$ (*X/Y; ru-2*$^{+}$ p^{+} $c^{+}/Df(ru\text{-}2\ p)$ +]. From the G_2 progeny, females mottled for *p* [X^{ls7}/X; + *p* $c^{ch}/Df(ru\text{-}2\ p)$ +] were selected and were mated to

ru-2 males who also carried the *Df(ru-2 p)^{46DFioD}* chromosome [*X/Y; ru-2 + +/Df(ru-2 p) +*] (Fig. 12-1). If *ru-2^{+}* is on the X^{ls7} chromosome, then G_3 female progeny who are *X^{ls7}/X; ru-2 + +/Df(ru-2 p) +* would show mottling for *ru-2* coat color because the X^{ls7} chromosome, which carries the translocated wild-type *ru-2^{+}* locus, would be inactivated in about 50% of cells.

Third-generation (G_3) females that appeared to be mottled for *ru-2* were progeny-tested using the following mating: *X^{ls7}/X; ru-2 + +/Df(ru-2 p) +* (assumed genotype) x *X/Y; + p^{un} +/+ p^{un} +*. The expected ratios of phenotypes if the assumed genotype is correct would be 1 female mottled with *p* : 5 wild-type (females and males): 2 *p* (females and males). The third generation could also include females mottled for *p* [*X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +*]; in the mating of this *p*-mottled female with a *p* male (*X/Y; + p^{un}+/+ p^{un} +*), expected phenotypic ratios would be 2 females mottled for *p* : 2 wild-type males : 0 wild-type females : 4 *p* males and females. The important difference is that a *ru-2*-mottled female of genotype *X^{ls7}/X; ru-2 + +/Df(ru-2 p) +* could give wild-type female progeny in the test cross to *+ p^{un} +/+ p^{un} +*, whereas a *p*-mottled female [*X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +*] could bear no female wild-type progeny.

A second test, using segregation of RFLPs for a chromosome-7 locus, *Saa*-1(serum amyloid, alpha subunit), was done to identify the genotypes of females who appeared to be mottled for *ru-2*. DNA extracted from tail segments of the grandparents (G_1),

Figure 12-1. Pedigree illustrating the female progeny expected from the cross, *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +* x *X/Y; ru-2 + +/Df(ru-2 p) +*. Note that both *ru-2*-mottled and *p*-mottled females can result if *ru-2* has translocated to X^{ls7}; *p* is known to reside in X^{ls7}, so that mottling for p is expected.

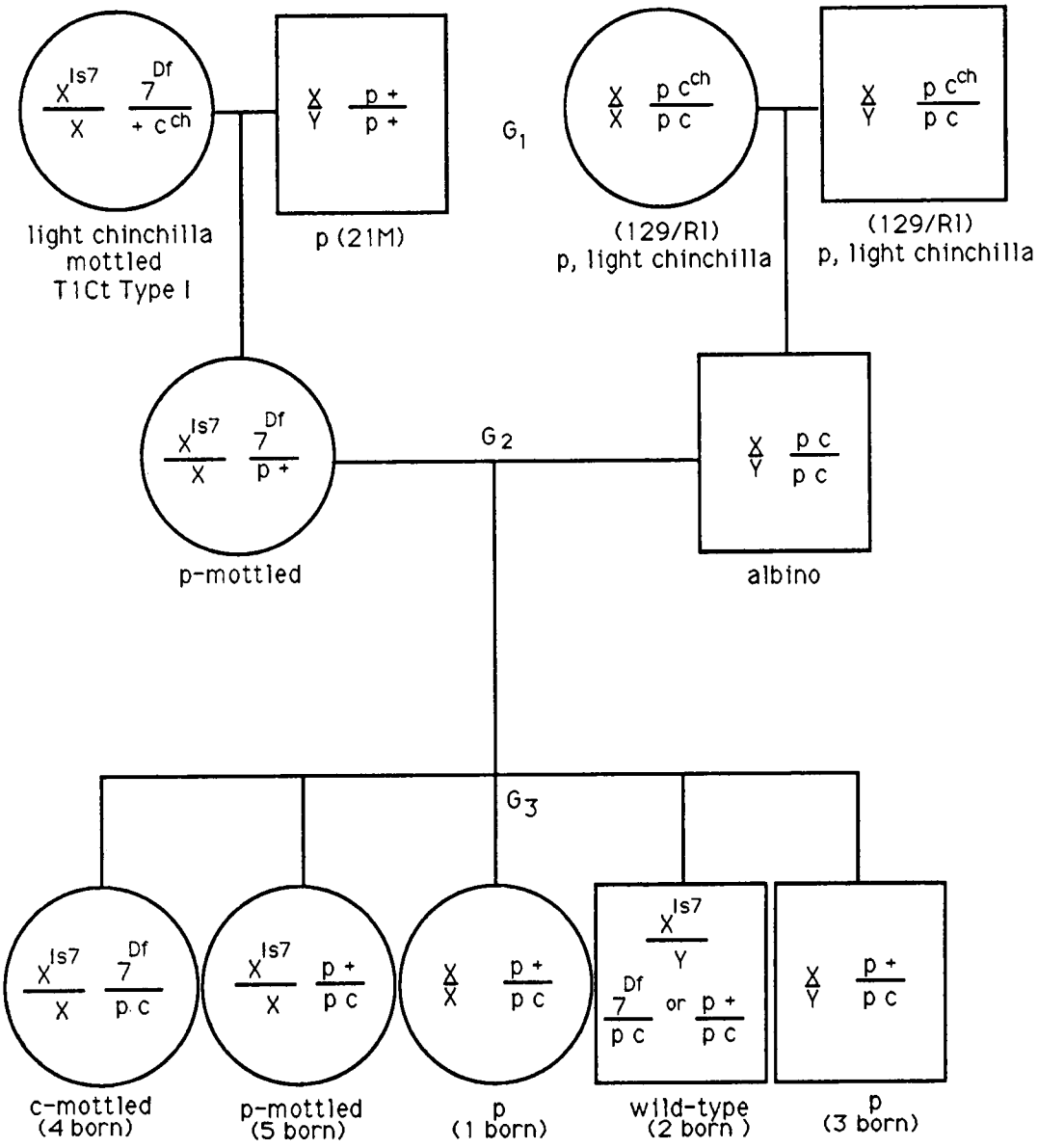

G2
X ls7 / X
+ p c ch
Df(ru-2 p) +
p-mottled
X / Y
ru-2 + +
Df(ru-2 p) +
ruby-eye-2
G3
X ls7 / X
ru-2 + +
Df(ru-2 p) +
ru-2-mottled
X ls7 / X
+ p c ch
Df(ru-2 p) +
p-mottled
X / X
+ p c ch
Df(ru-2 p) +
p
X ls7 / X
+ p c ch
ru-2 + +
wild-type
X / X
+ p c ch
ru-2 + +
wild-type
X / X
ru-2 + +
Df(ru-2 p) +
ru-2
X / X
Df(ru-2 p) +
Df(ru-2 p) +
lethal
X ls7 / X
Df(ru-2 p) +
Df(ru-2 p) +
p-ru-2-mottled
lethal ?

parents (G_2), G_3 siblings, and from the *ru-2*-mottled females themselves (also G_3). The DNAs were digested with *Eco*RI, electrophoresed, and Southern-blotted to Nytran. The probe for *Saa*-1 (Table 1-1), was radiolabelled using random-hexamer-priming and hybridized as described in Chapter 4. Tail DNAs were prepared by digesting a one-half-inch piece from the tip of the tail with proteinase K [(10 mg/ml) in a buffer of 50 mM Tris-HCl, pH 7.5; 100 mM EDTA, pH 8.0; 100 mM NaCl; 1% SDS], extraction with phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and then chloroform:isoamyl alcohol (24:1) and ethanol precipitation.

Experiment 2

In order to generate progeny in whom the X^{ls7} and 7^{Df} chromosomes are separated, the following crosses were done by E. M. Rinchik (note that *ru-2* is not involved in this mating scheme, shown in pedigree form in Figure 12-2): In G_1, T1Ct Type I females, mottled with light-chinchilla (X^{ls7}/X; $7^{Df}/+$ c^{ch}) were mated to males (X/Y; p +/p +) from B10.129(21M), a C57BL/10 congenic stock that derives the segment of DNA surrounding *p* from the 129 inbred strain. G_2 females mottled for *p* (X^{ls7}/X; $7^{Df}/p$ +) were then mated to male p c/p c segregants of the 129/RI- p c^{ch}/p c segregating inbred strain to generate G_3 segregants. DNA was extracted from the spleens of individuals from all three generations as described in Chapter 4. Initially, DNAs were cut with 9 different enzymes (*Eco*RI, *Bam*HI, *Pvu*II, *Pst*I, *Taq*I, *Msp*I, *Eco*RV, *Kpn*I, and *Xba*I) and

Figure 12-2. Three-generation pedigree illustrating the mating between carriers of the T1Ct chromosomes and two strains, B10.129 and 129/R*I*. B10.129 (21M) (*p +/p +*) and 129/R*I* (*p* c^{ch}*/p c*) are congenic for the region of chromosome 7 surrounding *p*. Note that G_3 progeny are of both balanced and unbalanced forms, so that the segregation of the X^{ls7} and 7^{Df} chromosomes can be followed separately. Genotypes are given within the circles (females) or squares (males), while phenotypes are listed below each circle or square.


light chinchilla
mottled
T1Ct Type I
p (21M)
G1
(129/R1)
p, light chinchilla
(129/R1)
p, light chinchilla
p-mottled
G2
albino
G3
c-mottled
(4 born)
p-mottled
(5 born)
p
(1 born)
wild-type
(2 born)
p
(3 born)

hybridized with probes for *Ldh-1*, *Saa-1*, *MyoD-1*, *Lhb*, and *D7OR2* loci (see Table 1-1 for probe details) to determine which enzyme gave an appropriate RFLP for each probe for all chromosomes segregating in the pedigree.

Experiment 3

In order to obtain Type II animals that were potentially carriers of both the X^{ls7} chromosome and two copies of the $p^{46DFioD}$ chromosome, and would thus be homozygously deleted for the region that lies between the proximal T1Ct breakpoint and the $p^{46DFioD}$ breakpoint, the crosses illustrated in pedigree form in Figure 11-2 were made. Type II females were mated to carriers of $p^{46DFioD}$, and the *p*-mottled G_2 progeny were again mated to carriers of $p^{46DFioD}$. Southern blots were made using DNA from the tails of all G_3 *p*-mottled progeny from the second cross of G_2 females to $p^{46DFio,D}$ and from appropriate controls; that blot was hybridized with probes from the region of non-overlap illustrated in Figures 9-5 and 11-1.

CHAPTER 13

RESULTS AND DISCUSSION OF THE MOLECULAR MAPPING AND GENETIC LINKAGE ANALYSIS OF LOCI NEAR THE PROXIMAL BREAKPOINT OF T1Ct

Experiment 1

Results from the (A) mating in Experiment 1 are shown in Table 13-1. Thirteen females were born; none was obviously mottled, although one-fourth of the females should have exhibited variegation if *ru-2*$^{+}$ were on X^{Is7} and were being inactivated. The number of females born is too small for statistical significance in this experiment, which will continue when more Type I T1Ct females become available. Eicher (1970) had reported difficulty in detecting *ru-2* mottling on a wild-type coat color in her experiments in which the mating *X^{Is7}/X; 7^{Df}/ru-2 p x X/Y; ru-2 +/ru-2 +* would potentially give *ru-2*-mottled females who would carry two autosomal copies of *ru-2* in addition to *ru-2*$^{+}$ on X^{Is7}. It is possible that two doses of *ru-2*, as in Eicher's experiment, might result in darker ruby patches that would be difficult to detect on a wild-type background. More animals are needed to conclude that the *X^{Is7}/X; 7^{Df}/ru-2 + +* genotype (only one autosomal copy of *ru-2*) also produces animals in which the *ru-2*-mottled phenotype is, as Eicher found, difficult to detect.

Table 13-1. Female Progeny In Six Litters from the Mating *X^{ls7}/X; 7^{Df}/+ + c^{ch}* by *X/Y; ru-2 + +/ru-2 + +* To Locate the *ru-2* locus in T1Ct.

Possible Female F_1s		Female F_1s Observed
Genotype	Phenotype	
X^{ls7}/X; 7^{Df}/ru-2 + +	ruby–mottled (X^{ls7}) or wild-type (7^{Df})*	0
X^{ls7}/X; + + c^{ch}/ru-2 + + *X/X; + + c^{ch}/ru-2 + +*	wild-type	13
X/X; 7^{Df}/ru-2 + +	lethal	- -

*If *ru-2* is on X^{ls7}, coat color will mottle due to random X-inactivation of the translocated autosomal *ru-2^{+}* locus; if *ru-2* is on 7^{Df}, mottling will not occur since *the ru-2^{+}* locus will be expressed in all cells.

The results of the mating in experiment 1 (B), *X^Is7/X; + p c^ch/Df(ru-2 p) +* x *X/Y; ru-2 + +/Df(ru-2 p) +*, however, gave a clear answer to the question about *ru-2* mottling. Each F_1 litter, shown in Table 13-2 included a female who was clearly mottled for *ru-2*, and the mottling was easily seen on both agouti (*A/a*) and non-agouti (*a/a*) coats. Eicher's conclusion that *ru-2* was translocated to the X^{Is7} chromosome is therefore confirmed, since only X inactivation, with concomitant inactivation of the wild-type locus on X^{Is7}, can reasonably explain the mottled phenotype. There is still no explanation of why mottling is easily seen in the mating (B) progeny (*X^Is7/X; ru-2 + +/Df(ru-2 p) +*) females but not in *X^Is7/X; 7^Df/ru-2 + +* females that should result from mating (A) or in the *X^Is7/X; ru-2 + +/ru-2 + +* females from Eicher's mating.

Both *ru-2*-mottled females from mating (B) (assumed to be *X^Is7/X; ru-2 + +/Df(ru-2 p) +*) were progeny-tested to confirm their genotypes by mating to a pink-eyed male (*X/Y; + p^un +/+ p^un +*). This mating is shown in pedigree form in Figure 13-1; the results are shown in Table 13-3. If the mottled females had been mottled with *p* (the other possibility) rather than with *ru-2*, then the progeny-test mating would have been *X^Is7/X; + p c^ch /Df(ru-2 p) +* (*p-* mottled) x *X/Y; + p^un +/+ p^un +* and could yield no wild-type females. This mating is shown in pedigree form in Figure 13-2 and the (theoretical) results are summarized in Table 13-4. Since ten wild-type females were among the progeny, the mottled females must indeed have been *ru-2*-

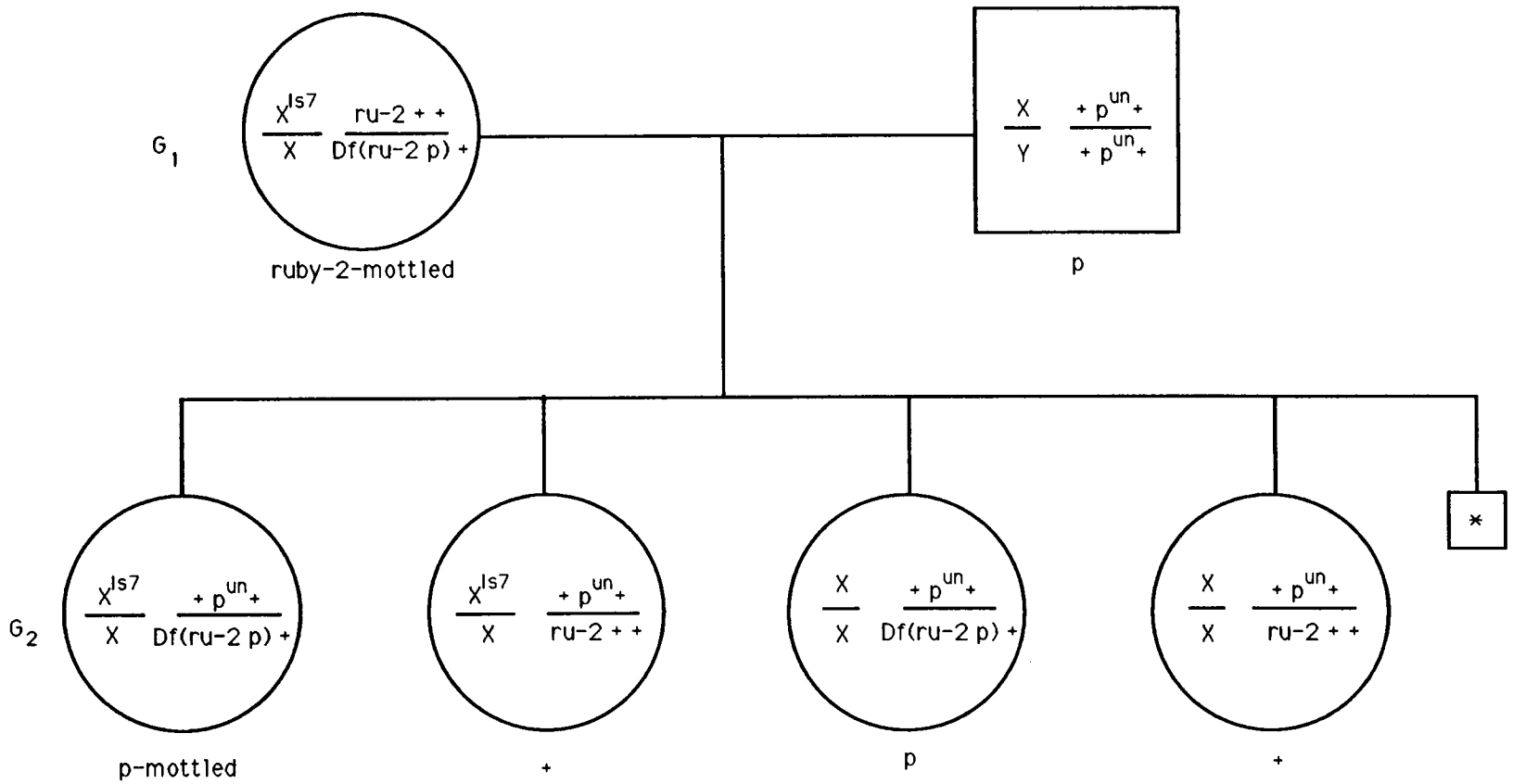
Table 13-2. Female Progeny* in Two Litters From the Cross *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +* by *X/Y; ru-2 + +/Df(ru-2 p) +* To Test for Mottling at *ru-2*.

Possible† female F_1s		Observed female F_1s	
Genotype	Phenotype	Litter 1	Litter 2
X^{ls7}/X; + p c^{ch}/ru-2 + +	+	0	1
X/X; + p c^{ch}/ru-2 + +			
X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +	*p-* mottled	0	0
X^{ls7}/X; ru-2 + +/Df(ru-2 p) +	*ru-2*-mottled	1	1
X/X; + p c^{ch}/Df(ru-2 p) +	*p*	2	3
X/X; Df(ru-2 p) +/Df(ru-2 p) +	lethal	- -	- -
X^{ls7}/X; Df(ru-2 p) +/Df(ru-2 p) +	lethal (?)	- -	- -
X/X; ru-2 + +/Df(ru-2 p) +	*ru-2*	0	0

*Male F_1s not considered

†Only non-recombinant female F_1s considered

Figure 13-1. Pedigree illustrating the progeny-test cross to confirm the genotypes of the variegated females arising from the cross described in Table 13-2. This pedigree assumes that these females were *ru-2*-mottled, [X^{ls7}/X; *ru-2 + +/Df(ru-2 p) +*] rather than *p*-mottled [X^{ls7}/X; *+ p* c^{ch}*/Df(ru-2 p) +*]. Note that wild-type females are expected from this cross, whereas the cross in Figure 13-2 cannot produce wild-type females.



*Males not considered in this pedigree

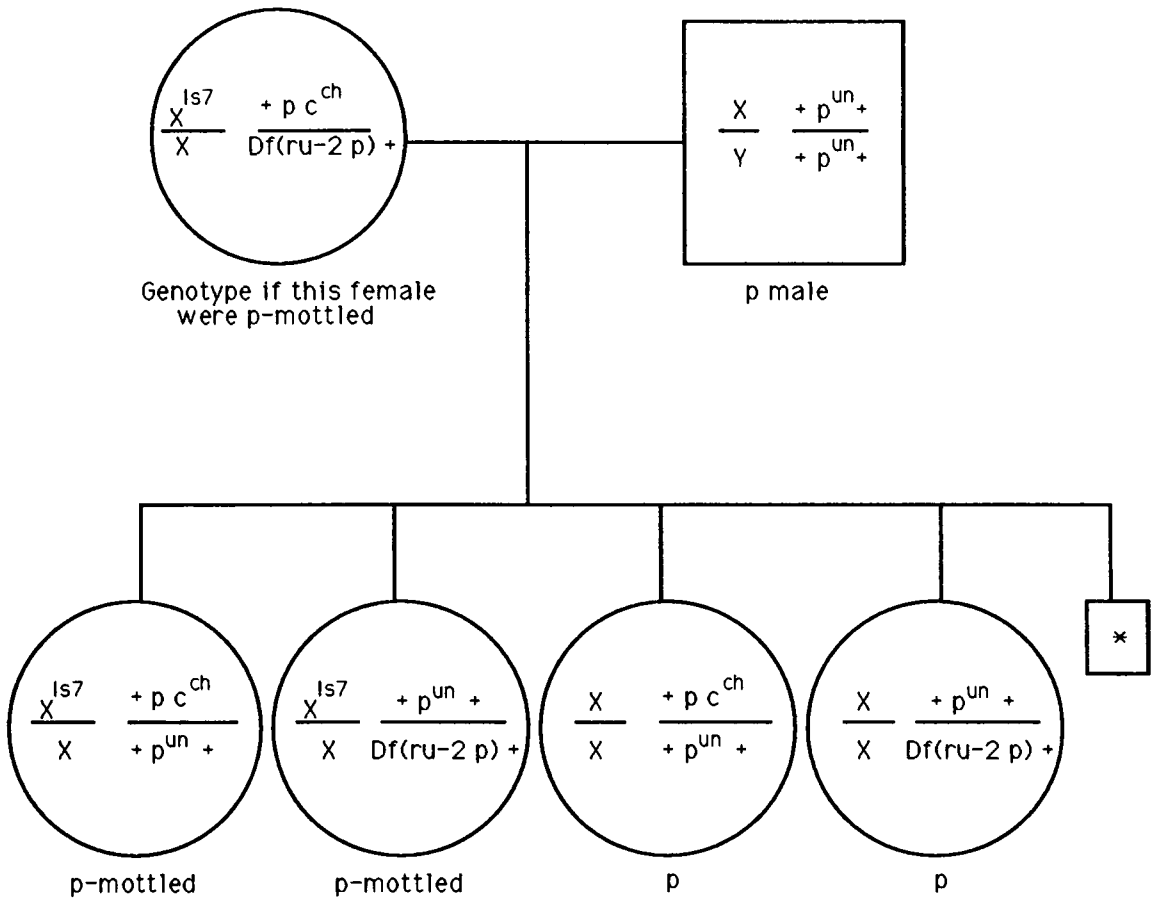
TABLE 13-3. Progeny Testing of Presumed Ruby-Mottled Females: Possible Progeny if the Genotype of the Two Mottled Females from the Cross: X^{ls7}/X; + *p* c^{ch}/*Df(ru-2 p)* + by *X/Y; ru-2* + +/*Df(ru2 p)* + (Table 13-2) are X^{ls7}/*X; ru-2* + +/*Df(ru-2 p)* + (*ru-2*-mottled).

Test Mating: X^{ls7}/*X; ru-2* + +/*Df(ru-2 p)* + x *X/Y;* + p^{un} +/+ p^{un} +

Possible Nonrecombinant Progeny

Genotype	Phenotype	Observed/Expected
X^{ls7}/*X; Df(ru-2 p)* +/+ p^{un} +	*p*-mottled	3/4.25
X^{ls7}/*X; ru-2* + +/+ p^{un} +	wild-type	10/8.5
X/X; ru-2 + +/+ p^{un} +	female	
X^{ls7}/*Y; ru-2* + +/+ p^{un} +		
X^{ls7}/*Y; Df(ru-2 p)* /+ p^{un}	wild-type	6/6.7
X/Y; ru-2 + +/+ p^{un} +	male	
X/X; + p^{un} +/*Df(ru-2 p)* +	*p*	4/4.25
X/Y; + p^{un} +/*Df(ru-2 p)* +	*p*	4/3.3

Figure 13-2. Pedigree illustrating the theoretical progeny-test mating had the variegated females described in Table 13-4 been *p*-mottled [X^{ls7}/X; + *p* $c^{ch}/Df(ru\text{-}2\ p)$ +] instead of being *ru-2* mottled [X^{ls7}/X; *ru-2* + +/$Df(ru\text{-}2\ p)$ +]. Note that this cross cannot produce wild-type females.



*Males not considered in this pedigree

TABLE 13-4. Progeny Test Cross: Possible Progeny if the Genotype of the Two Mottled Females from the Cross: *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +* by *X/Y; ru-2 + +/Df(ru-2 p) +* is *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +* (*p*-mottled).

Theoretical Test Mating: *X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +* x *X/Y; + p^{un} +/+ p^{un} +*

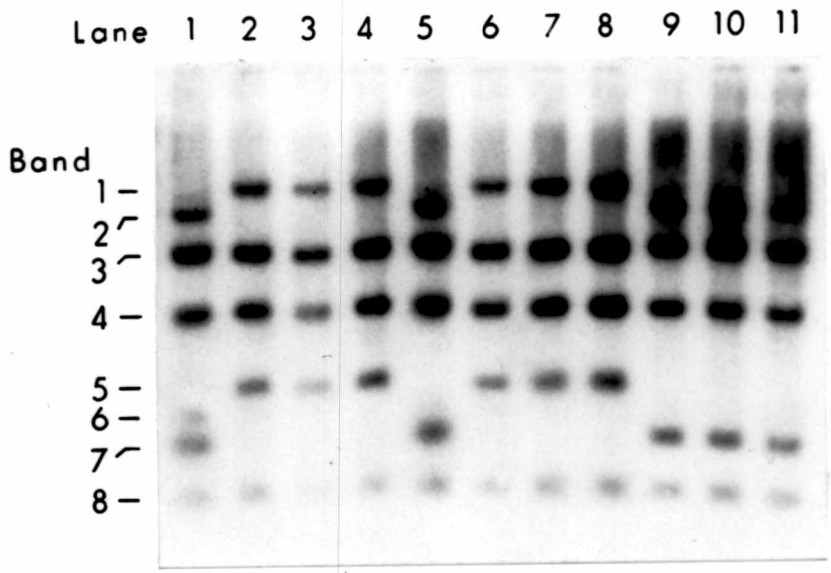
Possible Nonrecombinant Progeny Genotypes	Phenotype	Expected
X^{ls7}/X; + p c^{ch}/+ p^{un} + *X^{ls7}/X; + p^{un} +/Df(ru-2 p) +*	*p*-mottled	25%
X/X; + p c^{ch}/+ p^{un} + *X/X; + p^{un} +/Df(ru-2 p) +*	*p* female	25%
X/Y; + p c^{ch}/ + p^{un} + *X/Y; + p^{un} +/Df (ru-2 p) +*	*p* male	25%
X^{ls7}/Y; + p^{un} +/Df(ru-2 p) + *X^{ls7}/Y; + p c^{ch}/+ p^{un} +*	wild-type males (no females)	25%

mottled [X^{Is7}/X; *ru-2* + +/*Df(ru-2 p)* +] (pedigree in Figure 13-1) because the other potential variegating genotype (pedigree in Figure 13-2) could not produce wild-type females.

In a second experiment to confirm the genotypes of the *ru-2*-mottled females, tail DNAs from the pedigree shown in Figure 12-1 were digested with *Eco*RI and probed with the *Saa-1* cDNA (probe pSA30.1). The autoradiogram is shown in Figure 13-3. Bands 1 and 5 are present in lanes 2 [*p*-mottled G_2 female, genotype X^{Is7}/X; + *p* c^{ch}/*Df(ru-2 p)* +], 3 and 4 [pink-eyed dilute G_3 females, genotype *X/X*; + *p* c^{ch}/*Df(ru-2 p)* +], and in lanes 6, 7 (Type II females, genotype X^{Is7}/X; + *p* c^{ch}/+ *p* c^{ch}) and 8 (Type I females, genotype X^{Is7}/X; 7^{Df}/+ *p* c^{ch}). Bands 1 and 5, then segregate with the + *p* c^{ch} chromosome, because *Saa* is deleted in the *Df(ru-2 p)* chromosome and is not on X^{Is7}. Since the G_3 *ru-2*-mottled female (lane 1) has to be of genotype X^{Is7}/X; *ru-2* + +/*Df(ru-2 p)* +, she could not, and does not, carry the band 1 and band 5 alleles that mark the + *p* c^{ch} chromosome, more evidence that *Saa* is not on the X^{Is7}. Moreover, she cannot be a *p*-mottled female because the *p*-mottled genotype must be X^{Is7}/X; + *p* c^{ch}/*Df(ru-2 p)* +; the lane 1 female does not carry the + *p* c^{ch} alleles (bands 1 and 5).

Bands 2 and 7 are present in lane 1 [*ru-2*-mottled female, X^{Is7}/X, *ru-2* + +/*Df(ru-2 p)* +], lane 5 [G_2 ruby-2 male, *X/Y*, *ru-2* + + /*Df(ru-2 p)* +)] lane 9, [an additional ruby-2 male of genotype *X/Y*; *ru-2* + + /*Df(ru-2 p)* +], and in lanes 10 and 11 [wild-type males of genotype *X/Y*; + + +/*Df(ru-2 p)* +]. All these animals

Figure 13-3. Autoradiogram of a Southern blot of tail DNAs hybridized with pSA30.1. This probe for *Saa-1* was used to follow the segregation of the *Saa-1* locus in pedigrees that have produced females mottled for *ru-2* The genotypes for each DNA are listed below by lane number for simplicity.



Lane	Genotype	Phenotype
1	X^7/X; ru-2 + +/Df(ru-2 p) +	ru-2-mottled
2	X/X; + p c^{ch}/Df(ru-2 p) +	p
3	X/X; + p c^{ch}/Df(ru-2 p) +	p
4	X^7/X; + p c^{ch}/Df(ru-2 p) +	p-mottled
5	X/Y; ru-2 + +/Df(ru-2 p) +	ruby-eye-2
6	X^7/X; + p c^{ch}/+ p c^{ch}	p-c^{ch}-mottled
7	X^7/X; + p c^{ch}/+ p c^{ch}	p-c^{ch}-mottled
8	X^7/X; 7^{Df}/+ p c^{ch}	p-c^{ch}-mottled
9	X/Y; ru-2 + +/Df(ru-2 p) +	ruby-eye-2
10	X/Y; + + +/Df(ru-2 p) +	wild-type
11	X/Y; + + +/Df(ru-2 p) +	wild-type

carry either the + + + or the *ru-2* + + chromosome in addition to the *Df(ru-2 p)* + chromosome which is deleted for *Saa*. A *ru-2*-mottled female cannot carry a + + + chromosome (or she would not be mottled), and must therefore carry the *ru-2* + + chromosome, confirming again the assumed genotype of X^{Is7}/X; *ru-2* + +/*Df(ru-2 p)* + for these mottled females.

Bands 3 and 4 are constant in all lanes, and represent *Saa* homology units not segregating in these pedigrees. Band 6 is assumed to be a product of partial digestion since the DNA in lane 1 appears to be only partially digested on the ethidium bromide-stained gel before blotting.

To summarize the results seen on the autoradiogram in Figure 28, the banding patterns confirm that the females that appear to be mottled for *ru-2* coat color are genotypically X^{Is7}/X; *ru-2* + +/*Df(ru-2 p)* +.

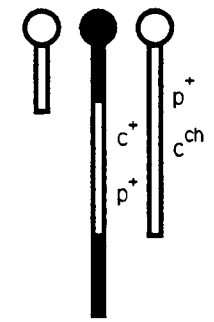
Experiment 2

This experiment was designed to use the segregation patterns of chromosome-7 loci to discover which of the loci linked to *p* and currently defined by molecular probes had been translocated to the X^{Is7} T1Ct chromosome and which had remained on the $7^{Df.}$ This knowledge could, and did, prove useful in the mapping of the proximal end of the large *p*-region deletion, *Df (ru-2 p)*^{46DFioD}. Three separate matings of X^{Is7}/X; $7^{Df}/p$ + (*ru-2* is not followed in this experiment) females were

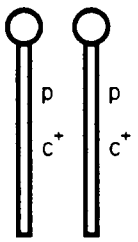
made to *X/Y; p c/p c* males. Figure 12-2 shows, in pedigree form, the results for one of the three matings.

There are 6 separate chromosomes 7 (or parts thereof) segregating in these pedigrees: X^{Is7}; 7^{Df}; + c^{ch}; *p* c^{ch}; *p c; and p* +, as illustrated in Figure 13-4; + c^{ch} and *p* c^{ch} are not followed after G_1. The autoradiogram in Figure 13-5 is from a Southern blot comprised of spleen DNAs from the pedigree in Figure 12-2 hybridized with the pSA30.1 probe for *Saa-1*. Bands 1, 3, 4, 5, and 7 are constant in all 18 lanes, and must represent, collectively, homology units segregating with the *p* + and *p c* chromosomes, at least one of which is present in each lane. The *p* + and *p c* chromosomes were expected to be identical for RFLPs in the p region since the strains of origin are congenic for that region. Bands 2 and 6 are present in lanes 1, 5, 9, 10, 12, 13, and 17; the DNAs in each of these lanes carry both the X^{Is7} (abbreviated to X^7 for simplicity in this Figure) and 7^{Df} chromosomes in these balanced animals, so that bands 2 and 6 might segregate with either of these two chromosomes. However, the DNAs in lanes 2, 3, 4, 8, and 11 carry only X^{Is7}, in the unbalanced form, and bands 2 and 6 are <u>not</u> present in lanes 2, 3, 4, 8, and 11. Therefore, bands 2 and 6 must segregate with the 7^{Df} chromosome, and not with the X^{Is7} (X^7 in this figure). These results allow the conclusion that the chromosome-7 locus *Saa-1* is proximal to the (proximal) T1Ct breakpoint, and remains on the small 7^{Df} chromosome instead of translocating to the X^{Is7}. Analyses of this kind, which led to the mapping of a

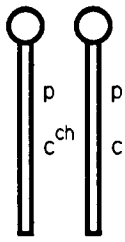
Figure 13-4. Idiograms of chromosomes arranged as if they were in the pedigree shown in Figure 12-2. Black centromeres and black boxes represent the X chromosome; white centromeres and white boxes represent chromosome 7. The autosomes 7 throughout the illustration are congenic for the region surrounding *p*. The small white chromosome is the T1Ct 7^{Df}, while the large translocation chromosome is the T1Ct X^{ls7}. Alleles at the *ru-2*, *p* and *c* loci are given on each chromosome; the phenotypes observed with each chromosome constitution are given below.



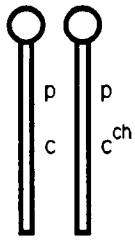
light-chinchilla
mottled



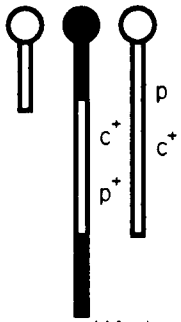
p



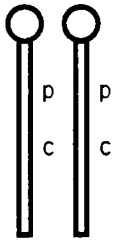
p, light-chinchilla



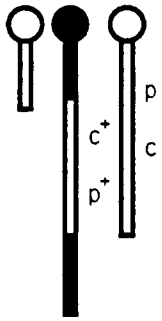
p, light-chinchilla



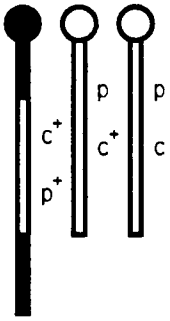
p-mottled



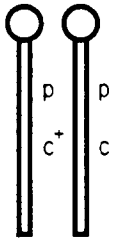
albino



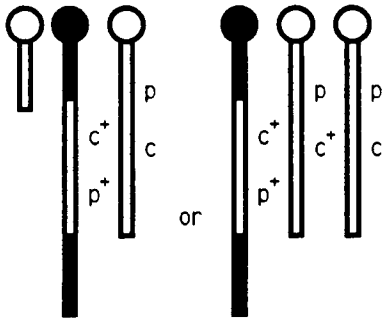
c-mottled
females



p-mottled
females

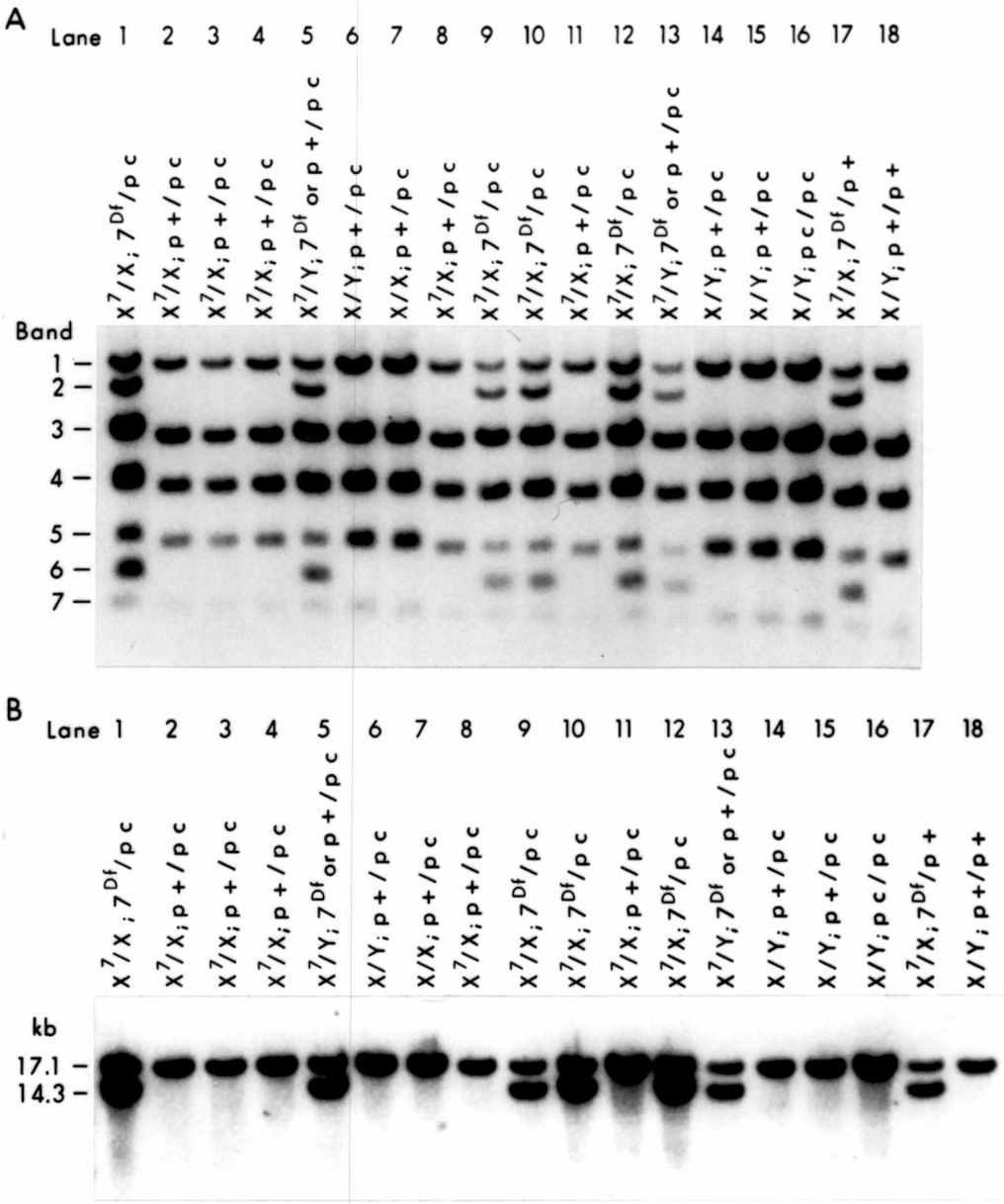


p
Male or female



Wild-type
Males

Figure. 13-5. Autoradiograms of Southern blots containing *Eco*RI-digested DNAs from members of the pedigree in Figure 12-2. Genotypes are listed for each lane. A. The Southern blot hybridized with pSA30.1, a probe for *Saa-1*. Because of band complexity, the bands were numbered from the largest to the smallest B. The same Southern blot hybridized with a probe for the *Ldh-1* locus. Band sizes at the left are derived from comparison of lambda DNA digested with *Hin*dIII.


A
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
X7/X; 7Df/p c
X7/X; p +/p c
X7/X; p +/p c
X7/X; p +/p c
X7/Y; 7Df or p +/p c
X/Y; p +/p c
X/X; p +/p c
X7/X; p +/p c
X7/X; 7Df/p c
X7/X; 7Df/p c
X7/X; p +/p c
X7/X; 7Df/p c
X7/Y; 7Df or p +/p c
X/Y; p +/p c
X/Y; p +/p c
X/Y; p c/p c
X7/X; 7Df/p +
X/Y; p +/p +
Band
1
2
3
4
5
6
7
B
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
X7/X; 7Df/p c
X7/X; p +/p c
X7/X; p +/p c
X7/X; p +/p c
X7/Y; 7Df or p +/p c
X/Y; p +/p c
X/X; p +/p c
X7/X; p +/p c
X7/X; 7Df/p c
X7/X; 7Df/p c
X7/X; p +/p c
X7/X; 7Df/p c
X7/Y; 7Df or p +/p c
X/Y; p +/p c
X/Y; p +/p c
X/Y; p c/p c
X7/X; 7Df/p +
X/Y; p +/p +
kb
17.1
14.3

chromosome-7 locus with respect to the T1Ct rearrangement, was made possible by the mating of T1Ct animals, viable in both balanced and unbalanced forms to animals expected to carry identical RFLPs for *p*-region probes.

The same line of reasoning has been applied to Figure 13-5B. The same Southern blot has been stripped and rehybridized with pUCLD14, a probe (Table 1-1) that recognizes the chromosome-7 *Ldh-1* locus. Only the H ("high", 17.1-kb) allele is present in lanes 6, 8, 14, 15, 16, and 18; these animals carry the p + and/or p c chromosomes, which have the identical RFLPs expected from the use of strains congenic for the p region. Lanes 1, 5, 9, 10, 12, 13, and 17 display the L ("low", 14.3-kb) allele; the DNAs in these lanes carry both the X^{Is7}(X^7 in this Figure) and 7^{Df} chromosomes. However, lanes 2, 3, 4, 8, and 11 are unbalanced Type II animals which carry only the X^{Is7} chromosome, and not the 7^{Df}. These lanes have only the H allele that segregates with the two autosomes (*p* + and *p c*; note that the + c^{ch} chromosome is not present after G_1 and is not considered further). Therefore, the L allele segregates invariably with the 7^{Df} chromosome, and not with the X^{Is7} (X^7). Thus, the genotypes of individuals in this pedigree (shown in Figure 12-2) allow the conclusion that the *Ldh-1* locus segregates with the 7^{Df} chromosome, and therefore has not been translocated to the X^{Is7}.

To reiteriate, the reasoning is as follows: the *p*-mottled females (X^{Is7}/X; *p* +/*p c*) in lanes 2, 3, 4, 8, and 11 must carry only the X^{Is7} chromosome and not the 7^{Df}. They carry only the H

allele, however, which is known to segregate with the *p* + and *p c* chromosomes; the L allele, which marks either the X^{Is7} or 7^{Df}, is not present. They must, however, carry the X^{Is7} chromosome (they are mottled) and since both the L allele and the 7^{Df} are absent, *Ldh-1* must be on the 7^{Df} chromosome. This finding is confirmed by lanes 9, 12, and 17, in which the L allele is present in genotype X^{Is7}/X; $7^{Df}/p\ c$ (mottled albino phenotype). The same method and reasoning have been applied to DNAs from the two other families (pedigrees not shown) bred using the same matings shown in the pedigree in Figure 12-2. From results from Southern blots hybridized with probes for *MyoD-1*, *Lhb*, *D7OR2*, and *Igfr-1* (data not shown), conclusions can be drawn that *Ldh-1*, *Saa*, *MyoD-1*, and *Lhb* segregate with the 7^{Df} chromosome, while *D7OR2* and *Igfr-1* are on the X^{Is7} chromosome. Since *Lhb* is not deleted in $p^{46DFioD}$ (data not shown) but is proximal to *Ldh-1* (Tom Glaser, personal communication), it can be concluded that *Lhb* must be more proximal than the other 7^{Df} loci (*Ldh-1*, *MyoD-1*, and *Saa*) tested here. *D7OR2* is known to map very near *p* (see Chapter 8), while *Igfr-1* is 6.2 $\pm$ 2.1 cM distal to *p*; the map positions of these two loci are, again, consistent with their location on the X^{Is7} chromosome.

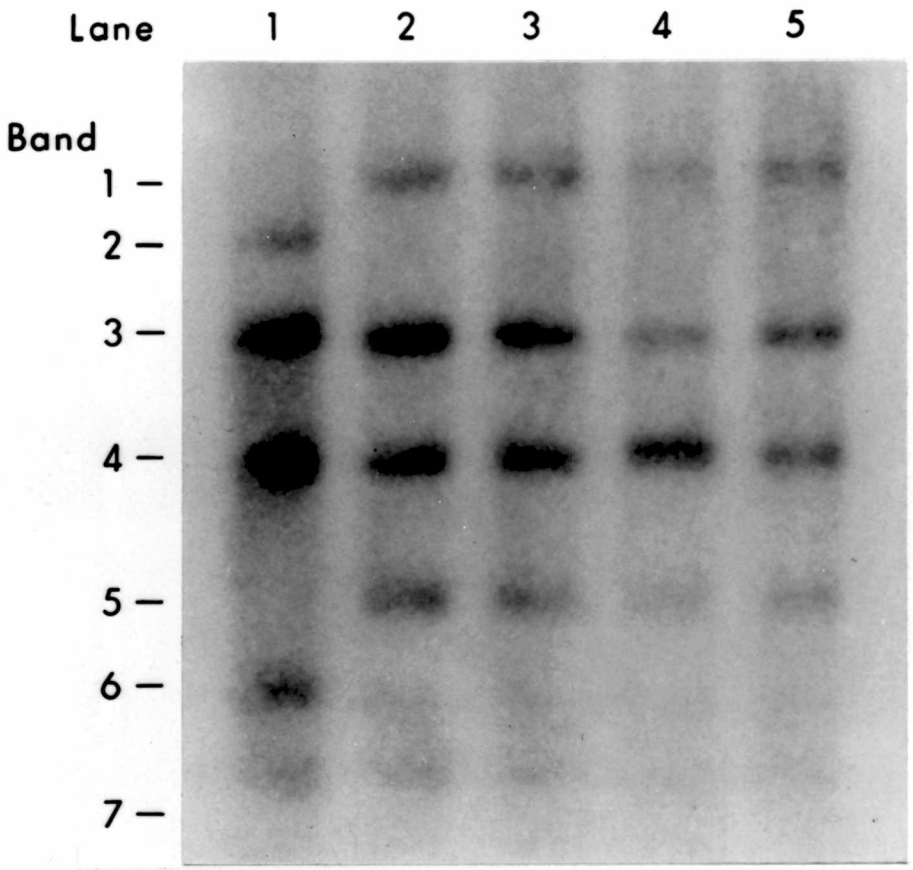
Combining the data reported here and the data from the *spretus/musculus* backcross described in Chapter 9, the map shown in Figure 9-5 has been drawn for this region of chromosome 7. The relationship of *p* and *ru-2* have been known (L. B. Russell, 1983), and $p^{46DFioD}$ was known to be at least 3cM

long because it deletes both *p* and *ru-2* (L. B. Russell, personal communication). All other relationships shown in Figure 9-5 have been determined by the experiments reported here. Knowledge of these relationships in a genome region that has until now been devoid of marker loci will greatly facilitate the use of the great wealth of mutations that have been generated at *p* in the specific-locus test.

Experiment 3

The results from Experiment 3 (Figure 11-2), designed to test for the presence of recessive lethal genes in the region of chromosome 7 that lies between the proximal breakpoint of T1Ct and the proximal $p^{46DFioD}$ breakpoint (the hatched area in Figure 9-5), are shown in Figure 13-6. Seven *p*-mottled females were born in the third generation (G_3) (Figure 11-2); one died just after birth before DNA was obtained. The remaining six appeared to be healthy, normal animals. The Southern blot in Figure 13-6, hybridized with the pSA30.1 probe for *Saa-1* contains, in lanes 3, 4, and 5, tail DNAs from three of the six *p*-mottled G_3 females tested. Lanes 1 and 2 contain tail DNAs from the G_2 parents [*X/Y; + + +/Df(ru-2 p) +* and X^{Is7}*/X; + p* c^{ch}*/Df(ru-2 p) +*]. All three G_3 females display the same banding pattern as the G_2 female parent in lane 2. They must, therefore, carry her + *p* c^{ch} chromosome, and share her genotype [X^{Is7}*/X; + p* c^{ch}*/Df(ru-2 p) +*]; they cannot carry the + + + paternal chromosome or they would not mottle. The results for the other three p-mottled females are not shown, but were identical.

Figure 13-6. Autoradiogram showing hybridization of pSA30.1, the probe for *Saa-1*, to *EcoRI*-digested tail DNAs from members of the pedigree shown in Fig. 11-2. Genotypes and phenotypes are listed for each lane. Because of band complexity, the hybridizing fragments were numbered from the largest to the smallest. Lanes 1 and 2 are the (G_2) parents; lanes 3, 4, and 5 are G_3 p-mottled females.



Lane	Genotype	Phenotype
1	X/Y; + + +/Df(ru-2 p) +	wild type
2	X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +	p-mottled
3*	X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +	p-mottled
4*	X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +	p-mottled
5*	X^{ls7}/X; + p c^{ch}/Df(ru-2 p) +	p-mottled

*These three p-mottled G_3 females (Figure __ pedigree) could potentially have been of genotype X^{ls7}/Df (ru-2 p) +/Df(ru-2 p) +

Therefore, all six third-generation p-mottled females have sequences that hybridize to the *Saa-1* locus. These results prove that none of these six females was of the genotype X^{Is7}/X; *Df(ru-2 p) +/Df(ru-2 p) +*

Third-generation females who were *p*-mottled could be one of the two genotypes, X^{Is7}/X; *+ p* c^{ch}*/Df(ru-2 p) +* or X^{Is7}/X; *Df(ru-2 p) +/Df(ru-2 p) +* (Figure 11-2). Any of those females that carried sequences from the *Ldh-1*, *MyoD-1*, or *Saa-1* loci would have to carry the *+ p* c^{ch} chromosome since neither the X^{Is7} chromosome nor the *Df(ru-2 p) +* chromosome carries those sequences. A *p*-mottled female that showed no hybridization bands with probes to any of those three loci would be presumed to carry two copies of the *Df(ru-2 p) +* chromosome; this female would then carry no copies of the sequences recognized by those probes, and would be homozygously deleted for that region of DNA. If such a female were viable, it would mean that the homozygous deletion of *MyoD-1*, *Ldh-1*, *Saa-1*, and all other genes in the same region is compatible with normal development.

It is perhaps not surprising that all six females tested were of the genotype X^{Is7}/X; *+ p* c^{ch}*/Df(ru-2 p) +*. *MyoD-1*, for example, might be expected to be a gene necessary for survival since the human MYOD protein is known to play a role in the differentiation of the striated muscle (Davis, et al., 1987; Scrable, et al., 1989). The region, which might be up to 3 cM in (genetic) size, might also reasonably be expected to carry other essential

genes. If a homozygously-deleted female had been born, it would prove the absence of lethal genes; since none was born among those tested, and since the numbers are too small yet for significance, the presence of lethal genes, perhaps *MyoD-1*, for example, can only be suggested.

An alternate explanation for the (presumed) lethality of the X^{Is7}/X; *Df(ru-2 p) +/Df(ru-2 p)* + genotype exists. Inspection of Figures 9-5 and 11-1 will show that $p^{46DFioD}$ deletes a region of chromosome 7 that is the same part (the *ru-2 - p* interval, and some DNA proximal and distal to that interval) that is inserted into the X^{Is7}. The autosomal genes in this inserted segment are subject to X-inactivation in all cells in which the X^{Is7} is the inactive chromosome (L. B. Russell, 1965; 1979). If a female carried two $p^{46DFioD}$ chromosomes, she would be expressing, in approximately 50% of her cells, none of those genes that are common to $p^{46DFioD}$ deletion and the autosomal segment in X^{Is7}. If those cells in which X^{Is7} were the inactive X were inviable, it is possible that the death of that percentage of cells would be lethal to the embryo after the time of X-inactivation.

In either case, whether (a) the homozygous deletion of the region between the proximal T1Ct and $p^{46DFioD}$ breakpoints, or (b) random inactivation of autosomal genes in and near the *ru-2 - p* interval, is a lethal condition, the creative genetic manipulation of available mutations in combination with molecular probes has contributed to the analysis of a relatively uncharacterized region of chromosome 7.

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