To the Graduate Council:

I am submitting herewith a dissertation written by Lorraine Moore Albritton entitled "The Albino Locus in the Laboratory Mouse Mus musculus: Analysis by Somatic Cell Genetics and Interspecies Meiotic Recombination." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Edward G. Bernstine, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

THE ALBINO LOCUS IN THE LABORATORY MOUSE MUS MUSCULUS: ANALYSIS BY SOMATIC CELL GENETICS AND INTERSPECIES MEIOTIC RECOMBINATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Lorraine Moore Albritton

June 1986

DEDICATION

I dedicate this thesis to Richard Albritton and Ryan Albritton in my deepest appreciation of their loving support throughout my graduate studies.

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ABSTRACT

Homozygous deletion of the c-locus region of mouse chromosome 7 results in embryonic lethality. A major focus of the thesis was to develop experimental approaches for the study of large c-locus deletions at the DNA level which deal with the difficulties posed by homozygous lethality. Two approaches were developed: (1) Construction of somatic cell hybrids carrying a large, lethal deletion of the c-locus and (2) Generation of F_1 animals from Mus musculus deletion stocks mated to the wild-type feral mouse Mus spretus. Five somatic cell hybrids were obtained that segregated the normal mouse chromosome 7, uncovering the sequences deleted in c^{26DVT} on the chromosome 7 retained. Mus musculus × Mus spretus F_1 animals carrying the c^{14CoS}, c^{146G}, c^{26DVT}, and $c^{12FR60Hb}$ deletions were obtained. DNA obtained from these somatic cell hybrids and interspecies hybrid animals was characterized by Southern blot hybridization to a known c-locus deletion DNA sequence, clone 12A. The results demonstrated the ability of this method to reveal sequences missing in the c-deletions. This method was applied to the mouse chromosome 7 sequences, Ct, Pth, Hras-1, Ins-2, H19, and CLX18, CLX23, and 11B6-9 (three anonymous sequences selected from chromosome 7-enriched recombinant DNA libraries using mouse-specific repeats). Results suggest that none of the sequences are missing in any of the c-locus deletions.

Another goal of the thesis was to develop a detailed map of the c-locus region. The segregation of alleles was followed by analysis of RFLPs in the backcross progeny of a Mus musculus to wild-type feral

mouse, Mus spretus, mating. 44 offspring of such matings were examined at six defined loci and with three anonymous DNA sequences. The segregation of alleles for these markers demonstrated linkage relationships from which a most probable gene order of:

Centromere---Ldh-1---Hras-1---c---12A---Hbb---(Ct,Pth)---CLX18---H19

was established. The linkage map of the mouse was compared with the human linkage map. The comparison suggests a minimum of four rearrangements has occurred in the divergence between mouse and man.

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CHAPTER I

INTRODUCTION

The albino-locus was the first coat color locus to be analyzed genetically (Cuenot, 1902; Castle and Allen, 1903). Besides the wild type, four alleles of the c-locus were found: c^{ch}, chinchilla (gray with black eyes); c^{e}, extreme dilution (light gray with black eyes); c^{h}, himalayan (light, almost white body with dark ears, nose and tail and red eyes); c, albino (white with pink eyes). All the alleles except the dominant wild-type, are epistatic producing recognizable intermediate coat color (e.g., c^{ch}/c, chinchilla-albino, is a medium gray intermediate between the dark gray of c^{ch}/c^{ch} and the white of c).

One of the first biochemical studies of the albino locus involved work with the enzyme tyrosinase. Since pigmentation in the mouse is derived from the synthesis of melanin catalyzed by the enzyme tyrosinase, it was suspected that the c-locus might encode the structure gene for that enzyme. Russell and Russell (1948) determined tyrosinase activity by measuring the oxidation of DOPA (dihydroxyphenylalanine), an intermediate in melanin synthesis, in frozen skin sections from mice carrying the wild-type, c^{ch}, c^{e}, or c alleles and found that activity correlated with coat color -- the lighter the coat color, the lower the enzyme activity, with albino homozygotes showing no activity. Coleman (1962) obtained similar results for tyrosinase activity in skins of mutant mice and in addition found that tyrosinase isolated from c^{h}/c^{h} mice was heat labile. These observations strongly support the theory that the c-locus is the structural gene for tyrosinase.

Progress toward a genetic analysis of the region around the albino locus was accelerated by the generation of a large number of independent mutations at the albino locus. In the course of studying the mutagenic effects of radiation on mice, Dr. W. L. Russell and Dr. L. B. Russell at the Oak Ridge National Laboratory developed the specific locus method (Russell, 1951). In this method radiation treated mice were mated to a tester stock which is homozygous recessive for seven loci, including the c-locus. At the albino-locus, the tester stock was homozygous for the chinchilla allele, which is co-dominant with the albino, c, allele. Radiation induced mutations of the c-locus (c*) in the treated mice were then identified by scoring progeny for diluted chinchilla coat color resulting from a genotype of c^*/c^{ch}. Thirty-seven independent mutations ranging in size from 2 cM to 6-11 cM, were identified in these studies (Russell and Montgomery, 1982). This wealth of genetic materials has provided the basis for extensive biochemical and genetic studies of several phenotypes associated with the various c-locus deletions.

One result of the biochemical and genetic analysis of the albino locus mutants was the identification of several liver-specific functions involved in some of the albino locus deletions. Twenty-four of the thirty-seven known c-locus deletions result in deficiencies of the liver enzymes glucose-6-phosphatase (G-6-Pase), tyrosine aminotransferase (TAT), serine dehydratase (SD), glutamine synthetase (GS), UDP glucuronosyltransferase and aldolase B (Erickson et al., 1968; Russell and DeHamer, 1972; Gluecksohn-Waelsch et al., 1974; Gluecksohn-Waelsch et al., 1975; Thaler et al., 1976; Sala-Trepat et al., 1985) and of the serum proteins' albumin (ALB), alphafetoprotein (AFP) and transferrin

(Erickson et al., 1976; Garland et al., 1976). The structural genes for some of the affected proteins have been mapped to their respective chromosomes: the TAT gene on chromosome 8 (Cori et al., 1978); ALB and AFP genes on chromosome 5 (Miller and Miller 1975; D'Eustachio et al., 1981); transferrin gene on chromosome 9 (Nichols et al., 1975); G-6-Pase gene on chromosome 4 (Chapman, 1975). This and other evidence has led to the conclusion that the structural genes for all of the above affected proteins reside elsewhere than the deleted region and that it is a regulatory gene or genes that is involved in the deletion (Russell et al., 1982; Gluecksohn-Waelsch, 1979).

Other biochemical evidence has shown many of the deletions result in a deficiency of the enzyme mitochondrial malic enzyme due to the absence of the structural gene, Mod-2 (Erickson et al., 1974; Bernstine et al., 1978). Studies of deletion animals also led to the discovery of a cis-acting regulatory gene of MOD2, Mdr-2 (Bernstine, 1979), and allowed the demonstration of that gene's control on the rate of MOD2 subunit synthesis (Bernstine and Koh, 1980).

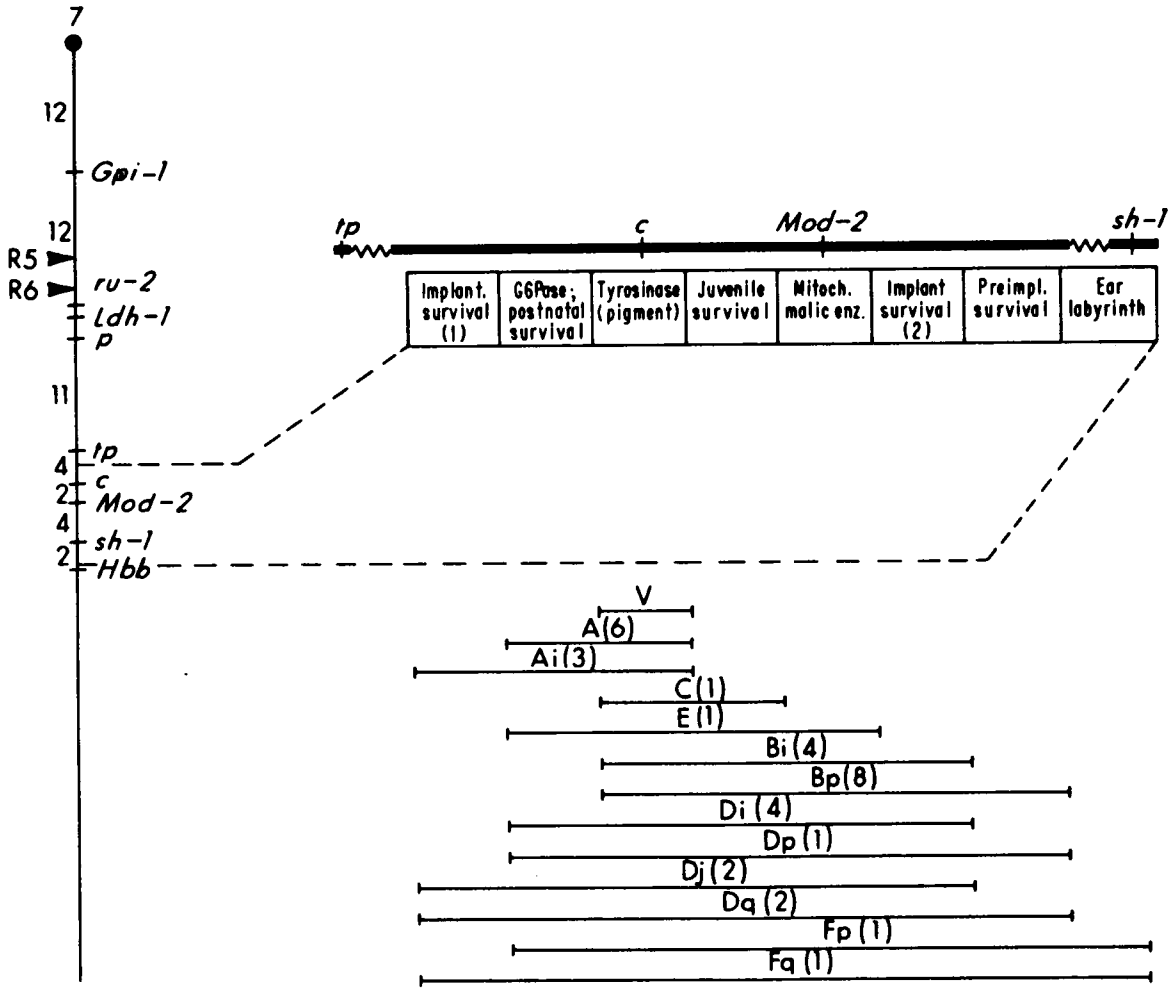
Genetic studies showed that many of the deletions were recessive lethals acting both before and after birth (Russell and Montgomery, 1970). The nature and stage of development at which the prenatal lethal deletions have effects has been studied. These studies revealed two independent functional units which effect survival prior to implantation and an additional unit which affects survival during or following implantation as well as a functional unit which affects juvenile survival (Russell and Raymer, 1979). One of the functional units affecting implantation survival, that which maps between Mod-2 and

shaker-1 (sh-1), is probably the same function as that described by Lewis et al. (1976), which shows abnormal development of the ectoplacental cone and extraembryonic ectoderm in deletion homozygotes. Subsequent complementation mapping based on the eight phenotypic markers identified by the biochemical and genetic studies, led to the establishment of a functional map of the albino region, as shown in Figure 1 (Russell et al., 1982).

Additional genetic materials involving the albino region came from mutagenesis experiments which produced several reciprocal translocations between chromosomes X and 7 in irradiated mice. Two of these translocations, T(X;7)5RL and T(X;7)6RL, were shown to have autosomal breakpoints at approximately 8 cM and 2 cM, respectively, proximal to the pink-eyed locus (p) (Russell and Montgomery, 1970). Thus the long translocation product in each of the translocations contains more than one-half of chromosome 7, including the c-locus. The breakpoints on the X-chromosome are distal to mottled (Mo) in both translocations (Russell and Montgomery, 1970). Studies by Dr. Peter Lalley (personal communication) have shown that the long translocation product of each translocation includes the hypoxanthine phosphoribosyl transferase (HPRT) gene, which proves important in the construction of somatic cell hybrids (Chapter II).

A basic goal of the experimental work described in this thesis is to develop a systematic approach for generating a detailed map of a large region of a mammalian chromosome. The chromosomal region chosen for study is centered around the c-locus on mouse chromosome 7. The

Figure 1. Genetic map of mouse chromosome 7 in the region of the c-locus. Only those loci utilized in analysis of the region are indicated. Complementation map of the region from taupe (tp) through shaker-1 (sh-1), shown blown up to the right, is adapted from Russell et al. (1982). Functional units which define the phenotypes assayed, are enclosed in blocks under the map and are not drawn proportional to actual distances, which are as yet unknown. The bars under the map indicate the 12 complementation groups into which the known c-locus deletions fall. Numbers in parenthesis after complementation group indicate the number of independent deletions which belong to that group. Loci are glucose phosphate isomerase (Gpi-1), ruby-eyed (ru-2), lactate dehydrogenase A (Ldh-1), pink-eyed (p), taupe (tp), albino (c), mitochondrial malic enzyme (Mod-2), shaker-1 (sh-1), and beta-hemoglobin (Hbb). Breakpoints of the T(X;7)5R7 and T(X;7)6R7 chromosomes are indicated as R5 and R6, respectively.



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approach taken applies the powerful combination of somatic cell genetics and molecular genetics to these mouse mutants.

The wealth of genetic material involving the c-locus and the well-laid foundations of biochemical analysis of the c-locus region, point out its potential. The focus of this work was to increase the utility of the genetic material already available and to develop new material for further genetic mapping studies.

Homozygous deletion of a significant chromosomal segment is likely to lead to deletion of a gene vital for the development of the organism and hence be lethal. This phenomena is in fact observed at the c locus. Only animals of classes A, V, and C survive to birth when deletions are homozygous. Homozygous A animals survive less than 2 days post partem. In order to circumvent the problem of the lethality large c-locus deletions two approaches were explored: (1) Construction of mouse-Chinese hamster somatic cell hybrids carrying a large lethal deletion of the c-locus and (2) Generation of F_1 animals from Mus musculus deletion stocks mated to the wild type mouse Mus spretus. The construction of the somatic cell hybrids carrying the c-locus deletion was made possible by the development of a mouse carrying the deletion on a T(X;7) chromosome. Both methods isolate the deleted chromosome from its normal homolog and place it in another species which provides a naturally polymorphic background. These DNA polymorphisms between the host Chinese hamster cell line, in the case of the somatic cell hybrids, or M. spretus in the case of the F_1 animals, and the M. musculus deletion stock expose deleted DNA sequences on the musculus chromosome.

A unique set of backcross animals from M. musculus × M. spretus F_1 animals to was generated as a source of recombination data for positioning genetic markers on mouse chromosome 7, and indeed, on any mouse chromosome. Then to utilize the new materials developed, known but unmapped, chromosome 7 DNA sequences were acquired and new DNA sequences on chromosome 7 were isolated.

Somatic cell hybrids and M. musculus × M. spretus F_1 mice were utilized to identify DNA sequences from chromosome 7 which are present in the c-locus deletions. Hybridization of a DNA probe to genomic DNA permits the identification of sequences present within a c-locus deletion. The presence or absence of a DNA sequence in the deletion can be detected using the somatic cell hybrid approach when RFLP is observed between hamster and mouse sequences. Similarly, F_1 animal can be used to determine the presence or absence of a DNA sequence in the deletion provided RFLP exists between M. spretus and M. musculus. Using the combination of these two approaches the DNA sequences are assigned to one of the eight complementation groups described by Russell et al. (1982) for the c-locus region.

To extend the capability of mapping sequences over the entire chromosome, interspecific hybrid mice were examined. Mus musculus females were mated to Mus spretus males. The male F_1 animals are sterile but the female F_1s are fertile. Meiotic recombination events occurring in the F_1 females between homologous musculus and spretus chromosomes were recovered by backcrossing to M. musculus males. The offspring of these backcrosses were analyzed for well-mapped genetic markers, and for the various unmapped chromosome 7 sequences. With

these recombination data, gene order and linkage relationships were assigned for the markers examined.

To utilize the materials developed, DNA sequences satisfying one or both of two criteria were examined: (1) DNA sequences that have been assigned to mouse chromosome 7, but whose position on the chromosome relative to established genetic markers is unknown; (2) Human DNA sequences that have been assigned to the short arm of human chromosome 11 (11p) and for which a mouse homolog exists. Chromosome 7 cDNA and genomic clones from other laboratories were examined and the number of sequences available from mouse chromosome 7 were expanded by isolating new clones from recombinant DNA genomic libraries. The use of human 11p sequences was promising by inference of known homologies between mouse 7 and human 11p, notably between the Hbb and HBBC loci and the Ldh-1 and LDHA loci. As genetic references genomic clones of the well-mapped B-hemoglobin (HBB), and lactate dehydrogenase-A (LDH-A) genes were used.

Additional cloned sequences from chromosome 7 were generated by screening three libraries enriched for mouse chromosome 7. Disteche et al. (1982) constructed one library from flow-sorted Is(X;7)Ct chromosomes. This chromosomal aberration consists of a segment of mouse 7 from sh-1 to ru-2 inverted into the X-chromosome. This library was screened with total mouse DNA and negative clones were picked as potentially containing completely unique inserts. Then these clones were assigned to their respective mouse chromosomes by Southern blot hybridization to a panel of well-characterized somatic cell hybrids obtained from Dr. Peter Lalley (Gazdar et al., 1977).

The other two libraries were constructed from a mouse-Chinese hamster somatic cell hybrid line retaining mouse chromosomes 7. Since total mouse DNA cross hybridizes with Chinese hamster DNA, it was necessary to identify clones containing mouse DNA inserts by hybridization to species-specific cloned repetitive sequences in the initial screening of these two libraries. Verification of the species of origin of inserts is based on hybridization of the sequence to mouse genomic DNA. The clones obtained were hybridized to the somatic cell hybrid cell lines and assigned to their respective mouse chromosomes.

DNA sequences mapping to mouse chromosome 7 were hybridized to the T(X;7def)5RL-containing somatic cell hybrids as a primary screen for involvement in an albino deletion. A search for musculus-spretus restriction fragment length polymorphisms (RFLPs) was performed for the sequences. Those sequences implicated by the somatic cell hybrid data as missing in an albino deletion, were hybridized to the appropriate restriction enzyme digestion of musculus deletion stocks × spretus F_1 animals and of overlapping doubly heterozygous deletion animals by Southern blot hybridization to verify absence and determine the complementation group or groups involved. Then all available chromosome 7 sequences were hybridized to Southern blots of backcross animals to obtain data on gene order and linkage relationships to known genetic markers. Thus each of the DNA sequences was placed on the genetic map of the mouse. The result is a more detailed map of mouse chromosome 7, based on DNA markers upon which future molecular analyses of this region of 7 may draw.

CHAPTER II

CONSTRUCTION AND CHARACTERIZATION OF SOMATIC CELL HYBRIDS

INTRODUCTION

Analysis of the albino region mutants requires special handling to circumvent the lethality of the large deletions in the homozygous state. Stocks of deletion animals must be maintained as heterozygotes, in whom the DNA sequence present on the normal homolog masks the absence of the deleted sequences. One of the ways this problem of heterozygosity was circumvented was by construction of somatic cell hybrids carrying a large, lethal deletion of the c-locus. In such hybrids, the deleted chromosome 7 was isolated from its normal homolog in a back ground of Chinese hamster chromosomes. This method takes advantage of the natural polymorphisms occurring between mouse and hamster to allow detection of missing DNA sequences.

An important consideration in generating useful somatic cell hybrids was how to achieve stable retention of the deleted mouse chromosome 7 in the hybrids, while obtaining segregation of the normal mouse homolog. There is no known selective marker on mouse chromosome 7. However, two of a series of T(X;7) chromosomes generated in radiation-treated mice, T(X;7)5RL and T(X;7)6RL include the mouse HPRT locus on the X-portion and the albino region on the 7-portion (Russell and Montgomery, 1970). Thus these translocation chromosomes can be selected for by utilizing the hypoxanthine-aminopterin-thymidine (HAT) selection system for HPRT.

With this selection scheme in mind, the c-locus deletion, 26DVT, was genetically transferred onto a T(X;7)5RL chromosome. Spleenocytes of the animal carrying the deleted translocation chromosome were then fused to HPRT-deficient Chinese hamster cells to produce somatic cell hybrid lines. A control hybrid line was constructed from a similar fusion of the spleen of a mouse carrying a non-deleted T(X;7)6RL chromosome, to HPRT deficient hamster cells. The objective was to obtain one or more hybrid cell lines in which the c-deficiency translocation chromosome was retained while the normal chromosome 7 was segregated, and one or more hybrid lines where the intact translocation chromosome was retained and the normal chromosome 7 was lost. Southern blot hybridization of DNA sequences from chromosome 7 to these cell lines would allow identification of sequences deleted in the 26DVT deficiency by a positive hybridization to hybrid lines retaining only the T(X;7)6RL chromosome and absence of hybridization to hybrid lines retaining only the deleted T(X;7)5RL chromosome.

MATERIALS AND METHODS

The translocation mice and the albino deletion mouse were provided by Dr. Liane B. Russell (Oak Ridge National Laboratory). All matings were done under the supervision of Dr. Liane B. Russell and Clyde Montgomery at the Oak Ridge National Laboratory. The translocation stocks 1 SaSd(5RL) and 30 FuFo(6RL) were maintained as (+++R/+ tp p) and +++R/$\underline{c^{ch}}$+p), respectively, at the c (albino), tp (taupe) and p (pink-eyed) loci, on chromosome 7. The following crosses were made to

introduce the albino deletion, 26DVT (indicated as c*), into the T(X;7)5RL chromosome (breakpoint indicated as R):

Cross 1 --- ♀ ♀ 1 SaSd (+++R/+ tp p) × ♂ ♂ 26DVT (c^{ch}++/c*++)

Expected female noncrossover progeny:

+++R/c*++: one-fourth of females are wild-type with white spots, the desired type

+++R/c^{ch}++: one-fourth of females are wild type with medium gray spots

+ tp p/c^{ch}++: one-half of females are wild-type

Cross 2 --- ♀ ♀ (+++R/c*++) × ♂ ♂ 22A (c^{ch}++/c^{ch}++)

Expected male progeny (nonrecombinant):

+++R/c^{ch}++: 38.5% of males are wild-type with small testes.

c*++/c^{ch}++: 38.5% of males are medium gray with normal testes.

Expected male progeny (recombinant, based on an R to c distance of 23 cM):

c*++R/c^{ch}++: 11.5% of males are light gray with small testes, the desired type.

+++/c^{ch}++: 11.5% of males are wild-type with normal testes.

In collaboration with Dr. Peter Lalley, spleen cells of 1 SaSd ♂ ($\underline{c}$*++R/$\underline{c^{ch}}$++) were fused to the HPRT-deficient Chinese hamster lung cells, clone E36, derived from V79, A-3 cells (Gillin et al., 1972), with inactivated Sendai virus as described in Minna et al. (1975). Fusion products were maintained in Dulbecco's modified Eagle's medium (DME) for 24 hr. Then they were switched to DME plus 100 μM hypoxanthine 0.4 μM aminopterin and 16 μM thymidine (HAT) for selection of hybrids retaining the mouse $\underline{hprt}$ gene. Colonies were selected from separate plates, except where two well-separated colonies were selected from the same plate and designated A and B. They were maintained in HAT for at least five weeks prior to analysis to allow for segregation and stabilization of the mouse chromosome complement. Spleen cells of 30FuFo ♂ (++R/$\underline{c^{ch}}$+p) were fused to E36 cells as described above with the exception that the fusion agent was 1 ml of 50% polyethylene glycol-6000 (PEG-6000) exposed to the cells at 37°C for two minutes, then washed off with DME minus fetal calf serum before plating the cells. The fusion products were selected and maintained in HAT as described above.

Characterization of hybrids was performed by isozyme analysis using starch gel electrophoresis and by Southern blot hybridization of genomic DNA to specific DNA probes. Cell extracts and nuclear DNA for electrophoresis were isolated from cells grown to confluence on three 150 mm plate in HAT plus 10% fetal calf serum. Cells were removed with trypsin (Gibco), washed twice with DME plus 10% fetal calf serum and then washed three times with ice cold DME minus fetal calf serum. Cells were then suspended in 1 ml of ice cold 50 mM Tris pH 7.6 and 0.05% Triton X-100

per 6×10^7 cells and homogenized with ten strokes of a Potter-Elbejheim homogenizer at 4°C. Homogenates were centrifuged in a Sorvall RT6000 at 2000 rpm for 10 min at 4°C, to pellet nuclei. Supernatant was removed and quickly frozen in liquid nitrogen then stored at -80°C until used for electrophoresis. Just prior to electrophoresis samples were thawed on ice then spun for 15 min in an Eppendorf microfuge at 4°C. The layer of lipids was aspirated off the top and the sample spun an additional 15 min at 4°C. Pelleted nuclei were resuspended in 1 ml of 10 mM Tris pH 7.8, 100 mM NaCl, 1 mM EDTA (TNE) then lyzed by addition of 1 ml of 10 mM Tris pH 7.8, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS) and 600 µg of proteinase K (Boehringer-Mannheim). Incubation of the released nucleic acids with proteinase K overnight at 37°C, was followed by extraction against equal volumes of phenol and then of chloroform. After adding an equal volume of isopropyl alcohol, DNA was wound on a glass hook, rinsed in 70% ethanol, air-dried, and resuspended in 0.5 ml of 10 mM Tris pH 7.8 and 1 mM EDTA (TE).

Starch gel electrophoresis was performed in collaboration with Dr. Peter Lalley as described in Nichols and Ruddle (1973) for peptidase-C (PEP-C EC 3.4.11), peptidase-S (PEP-S, EC 3.4.11), peptidase-B (PEP-B, EC 3.4.11), cytoplasmic malic enzyme (MOD-1, EC 1.1.1.40), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucomutase (PGM-2, EC 2.7.5.1), adenine phosphoribosyltransferase (APRT, EC 2.4.2.7), esterase D (ES-D, EC 3.1.1.1), mannose phosphate isomerase (MPI, EC 5.3.1.8), glucose phosphate isomerase (GPI, EC 5.3.1.9), and lactate dehydrogenase A (LDH-A, EC 1.1.1.27); Swallow et al. (1973) for acid phosphatase (ACP-1 and ACP-2, EC 3.1.3.2); Minna and Coon (1974)

for glutathione Reductase (GR, EC 1.6.4.2) and galactokinase (GLK, EC 2.7.1.6); Lalley et al. (1977) for peptidase-D (PEP-D, EC 3.4.11); Minna et al., 1975) for adenylate kinase (AK-1, EC 2.7.4.3), glyoxylase (GLO, EC 4.4.1.5), and sorbital dehydrogenase (SODH, EC 1.1.1.14).

Hybridization of selected probes to restriction enzyme digests of hybrid cell DNAs was as described Sy Southern (1975), except that DNA was transferred to a nylon membrane (zetabind from American Lab Supplies) and after baking 2 hr at 80°C, was washed one hour in 0.5% sodium dodecyl sulfate (SDS) and 0.1 × standard saline citrate (SSC) at 65°C, then prehybridized and hybridized in 50% formamide, 5 × SSC, 100 μg/ml of denatured salmon sperm DNA, 1× Denhardt's solution, 20 mM sodium phosphate pH 6.5 at 42°C for 44-48 hr. DNA probes were labelled either by nick-translation as described by Rigby et al. (1977) or by random-primer labelling modified from Feinberg and Vogelstein (1983) as follows: DNAs were digested with the appropriate restriction enzyme to release inserts, then electrophoresed in 1% low gelling temperature agarose (Seakem) in Tris-acetate buffer. Insert band were cut from the gel and carefully trimmed of all excess agarose. One microliter of water was added per microliter of gel slice, then the slice was boiled for 10 min. An appropriate amount of boiled gel slice that contained 10-30 ng of DNA was brought up to 32 μl volume then 50 μCi of α-P^{32}dCTP (2000-3000 Ci/mmole from New England Nuclear), 20 μg of bovine serum albumin (nuclease-free from Bethesda Research Laboratory), 10 μl of OLB (0.1 mM dATP, 0.1 mM dTTP, 0.1 mMdGTP, 1 M Hepes pH 6.6, and 15 μg/ml random undecanucleotides from P. L. Laboratory), and 2 units of the large fragment of E. coli DNA polymerase I (New England Biolabs) were

added. The reaction was incubated at room temperature for 18-36 hr. Three cloned probes from mouse chromosome 7 were used to characterize the somatic cell hybrids: (i) beta-hemoglobin - from plasmic clone pMSeHbb2, containing a 1.9 kbp EcoRI/Bam HI fragment in pUC19 (Tom Glaser, unpublished), subcloned from the mouse beta major genomic clone M beta G2 (Tilghman et al., 1977); lactate dehydrogenase A - from the plasmid clone pUCLD 14, containing a 2 kbp BglII fragment of the mouse Ldh-A genomic sequence (Jane Mendel, unpublished); clone 12A - pBR322 containing a 5 kbp fragment of mouse genomic DNA isolated by Christine Disteche and shown to be deleted in $\underline{c}^{3H}$ (complementation group B) × $\underline{c}^{6H}$ (complementation group E) animals (Disteche and Adler, 1984).

RESULTS AND DISCUSSION

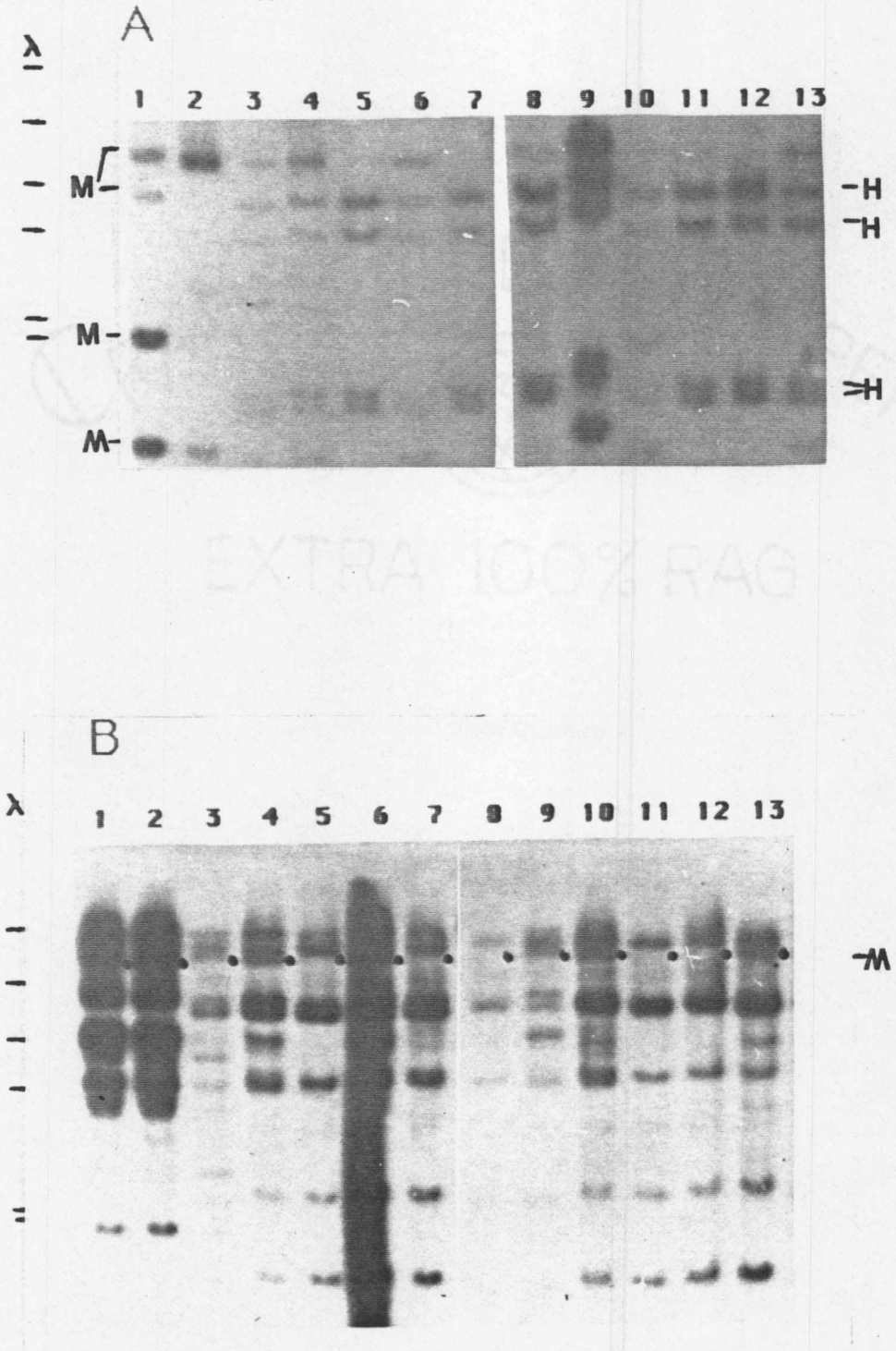
A female doubly heterozygous for the c-deficiency, 26 DVT, and for the T(X;7)5RL chromosome, was obtained from a cross of the translocation to deletion stocks. She was identified by the white spots on a wild-type coat which result from inactivation of the wild-type allele on the translocation chromosome (Russell and Montgomery, 1970). During meiotic recombination in this double heterozygote, the deleted c-region should be involved in a crossover event with the translocation breakpoint in one out of 4.5 meiosis (based on a R to c-locus distance of 23 cM). In a mating of the female double heterozygote to a male 22A ($\underline{c}^{ch}$++/$\underline{c}^{ch}$++), 11.5% of the offspring would be expected to be of the genotype c*++R/$\underline{c}^{ch}$++. From such a cross, a male offspring of this genotype was identified uniquely from other possible male offspring by

coat color, chinchilla-albino, and by small testes, which occur in male translocation carriers. The use of a male of the genotype $\underline{c}$*++R/$\underline{c}^{ch}$++ as a mouse donor for making somatic cell hybrid lines allowed for a tighter HAT selection of hybrids retaining the T(X;7df)5R126DVT chromosome, since the only possible contribution of HPRT is from that chromosome.

The spleenocytes from 1 SaSd ♂ ($\underline{c}$*++R/$\underline{c}^{ch}$++) were fused to E36 cells yielding nine somatic cell hybrid lines upon selection in HAT, which were designated as the R5D cell lines. Fusion of spleenocytes from 30FuFo ♂ (+++R/$\underline{c}^{ch}$++) to E36 cells yielded one hybrid line, designated R6. Southern blot hybridization of genomic DNAs from the hybrid lines to the beta-hemoglobin probe is shown in Figure 2A. Five of the R5D lines show 8.2 kilobase pair (kbp) and 1.15 kbp mouse HindIII fragments characteristic of the $\underline{Hbb}^{d}$ (diffuse) allele and the 5.5 kbp, 4.2 kbp, 1.6 kbp and 1.45 kbp hamster fragments. The hybrid line R6 shows 8.4 kbp, 5.5 kbp, 2.1 kbp, and 1.15 kbp mouse HindIII fragments characteristic of $\underline{Hbb}^{s}$ (single) allele, as well as the hamster fragments. Hybrid lines R5D-2 and R5D-7 had only the hamster fragments.

Southern blot hybridization of the hybrid lines to the lactate dehydrogenase-A probe is shown in Figure 2B. The hybridization pattern of this probe is complex and suggests the presence in the mouse genome of multiple pseudogenes and/or of cross-hybridizing sequences with other genetic loci encoding perhaps structural genes for different dehydrogenases utilizing the same coenzyme (Jane Mendel, personal communication). However the 14 kbp fragment, indicated on the figure by a black dot, has been identified as the fragment containing the structural gene

Figure 2. Hybridization of somatic cell hybrids to DNA probes for the (A) beta-hemoglobin and (B) lactate dehydrogenase genes. Lane 1. C57BL/6. Lane 2. Tester stock 22A. Lanes 3-9. R5D-1A, R5D-1B, R5D-2, R5D-5, R5D-7, R5D-12, R5D-14, respectively, somatic cell hybrids from the fusion of spleen cells of a male mouse carrying a T(X;7def)5RL chromosome to E36 Chinese hamster cells. Lane 10. R6, a somatic cell hybrid from a fusion of spleen cells of a male mouse carrying a T(X;7)6RL chromosome to E36 Chinese hamster cells. Lane 11. E36, an HPRT deficient Chinese hamster cell line used as the parental cell line for the R5D hybrids and the R6 hybrid. Lane 12. EBS-4, a somatic cell hybrid from the fusion of BALB/c spleen cells to E36, retaining mouse chromosomes 13, 15, 17, and X. Lane 13. EBS-11B6, derived from EBS-11, a BALB/c × E36 somatic cell hybrid, by back-selection in 6-thioquanine. A. 10 μg DNA was digested with HindIII and loaded in single lane. Probe is 1.9 kbp EcoRI/BamHI fragment of the beta-hemoglobin clone, pMSeHbb2. B. 10 μg DNA was digested with EcoRI and loaded in a single lane. Probe is the 500 bp PstI fragment of the mouse LDH-A cDNA clone, pML2. Position of the mouse genomic Ldh-1 fragment is indicated by a dot for each lane. In both panels, M indicates mouse fragments; H indicates hamster fragments. Size standard generated by a lambda cI857 HindIII digestion in kilobase pairs (kbp).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for LDH-A (Jane Mendel, personal communication). As seen in the figure, all the hybrids but R5D-2 and -7 retain Ldh-1. These results suggest that hybrid lines R5D-2 and -7 have undergone rearrangements in which some portion of both the normal mouse 7 and the T(X;7def)5RL chromosome, including Hbb and Ldh-1, and probably the albino locus, were lost. Based on this evidence, no further analysis of those two hybrids was done. The remaining eight hybrid lines were characterized by isozyme analysis with starch gel electrophoresis.

Table 1 shows a summary of the results of the isozyme analysis of the somatic cell hybrids. Analysis of markers on mouse chromosomes 3, 6, 13, 15, 16, 18, 19 and X was not done. Chromosome retention is correlated to the characteristic enzyme activity. Of primary concern to these studies is the profile of mouse chromosome 7 markers for each hybrid. Figure 3A shows analysis of LDH-A, Figure 3B shows analysis of GPI, and Figure 3C shows a schematic of analysis of PEP-D, all established chromosome 7 markers. The gene for LDH-A is distal to the breakpoints, R, of the long X^7 chromosomes of T(X;7def)5RL and T(X;7)6RL, while in both cases the genes for GPI and PEP-D reside on the reciprocal translocation chromosomes, the short 7^X. LDH-A was present in all hybrids tested, confirming the results of Southern blot hybridization. GPI and PEP-D are present in all the hybrids except R5D-1B and R6. In these two hybrids it is readily apparent from the absence of GPI and PEP-D, that both the normal chromosome 7, and the short 7^X translocation chromosomes have segregated, leaving the long 7^X chromosome of T(X;7def)5RL and the T(X;7)6RL chromosome isolated in the hamster background of the respective hybrids. These two hybrids appear

Table 1: Isozyme Analysis of Somatic Cell Hybrids

Mouse Chromosome	Mouse Isozyme	Somatic Cell Hybrid Line								
		R5D-1A	R5D-1B	R5D-9A	R5D-9B	R5D-14	R5D-12	R5D-5	R6	EBS-11B6
1	PEP-C	-	-	-	-	-	(-)	+	+	-
2	ACP-2	+	+	+	+	+	ND	ND	-	-
	SODH	+	ND	+	+	+	ND	ND	-	-
	AK-1	+	+	+	+	+ (red)	ND	ND	-	-
4	PGD	+	-	+	+	+	ND	ND	-	-
	PGM-2	+	-	+	+	+	ND	ND	-	-
5	PEP-S	+	-	-	ND	-	-	+	-	-
7	GPI	+	-	+	+	+	+ (red)	+	-	+
	PEP-D	+	-	+	+	+	ND	ND	-	+
	LDH-A	+	+	+	+	+	+	+	+	+
8	GR	-	-	-	+	-	ND	ND	+	-
	APRT	-	-	-	+	-	ND	ND	+	ND
9	MOD-1	-	-	+	-	-	ND	ND	-	-
	MPI	-	-	+	-	-	ND	ND	-	-
10	PEP-B	-	-	-	ND	-	+ (red)	-	+	+
11	GLK	-	-	-	-	-	ND	ND	(-)	-

(Table 1 continued)

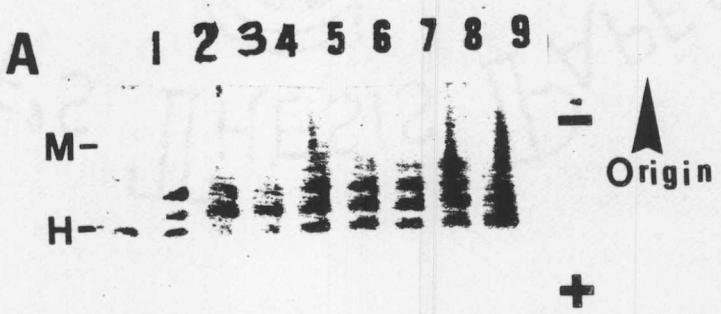
Table 1 (continued)

Mouse Chromosome	Mouse Isozyme	Somatic Cell Hybrid Line								
		R5D-1A	R5D-1B	R5D-9A	R5D-9B	R5D-14	R5D-12	R5D-5	R6	EBS-11B6
12	ACP-1	+	+	+	+	+	ND	ND	+	+
14	ES-D	+	+	+	+ (red)	+ (red)	ND	ND	-	-
17	GLO	+	+	+	+	+	ND	ND	+	-

+, mouse isozyme activity present; -, mouse isozyme activity absent;

+ (red), reduced mouse isozyme activity present; ND, not done; (-), mouse isozyme activity appears negative, but determination uncertain.

Figure 3. Isozyme analysis of mouse chromosome 7 markers LDH-A, GPI and PEP-D in somatic cell hybrids. Starch gel electrophoresis of 30 µl of cell extract was performed in Tris-borate buffer at pH 8.6 at 300 v for 18 hr. Gels were sliced in half, one-half being stained for PEP-D and the other half stained for LDH-A and GPI. M, indicates mouse isozyme; H, indicates Chinese hamster isozyme; intermediate bands are heteropolymers of mouse and hamster subunits. Lanes 1. E36, Chinese hamster. 2. R5D-1A. 3. R5D-1B. 4. R5D-9A. 5. R5D-9B. 6. R5D-14. 7. R6. 8. EBS-11B6, BALB/c mouse × Chinese hamster hybrid. 9. EBS-12, BALB/c mouse × Chinese hamster hybrid lacking mouse chromosome 7. A. LDH-A, a tetramer, exhibits formation of the three possible heteropolymers in lanes 2-8, indicating the presence of mouse LDH-A. Pure mouse tetramer is not visible because the majority of mouse subunits are tied up in the heteropolymers, therefore scoring is based on heteropolymer formation. B. GPI, a dimer, exists in two forms in the mouse, indicated as M^a and M^b. Mouse isozyme forms and heteropolymer formation are seem in lanes 2, 4, 5, 6, and 8. Lanes 3, 7, and 9 do not exhibit mouse isozyme. The high degree of sensitivity of this assay is shown in lane 1, where a small amount of contaminating mouse isozyme is seen without heteropolymer formation, therefore scoring is on the basis of mouse isozyme and heteropoly. C. PEP-D, a dimer, schematic shows presence of mouse isozyme and heteropolymer formation in lanes 2, 4, 5, 6, and 8. Lanes 3, 7, and 9 lack mouse isozyme. Because of overlap of the heteropolymer with a minor hamster band, scoring is based on mouse isozyme only.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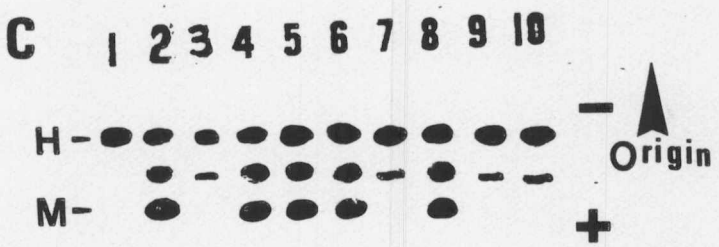
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to be suitable for use in detecting DNA sequences deleted in c-locus region by Southern blot hybridization.

The six hybrids which were positive for mouse GPI and PEP-D could be expressing the enzymes from the reciprocal translocation and/or the normal chromosome 7. In order to determine whether these hybrids had the reciprocal translocation or a normal chromosome 7, we performed Southern blot hybridization of the hybrids to the EcoRI insert of clone 12A, a sequence included in the overlap region of $\underline{c^{3H}} \times \underline{c^{6H}}$ animals and based on the present complementation map, expected to be deleted in $\underline{c^{26DVT}}$. The results of this hybridization are shown in Figure 4. The 5 kbp EcoRI band hybridizing to clone 12A is present in the hybrid R5D-5. This suggests that R5D-5 retains the normal mouse chromosome 7, which would provide the fragment seen hybridizing with clone 12A. Hybrids R5D-1A, -1B, -9A, and -14, and -9B (data not shown) do not contain sequences hybridizing with clone 12A. This suggests that no normal mouse chromosome 7 is retained in these hybrids and indicates that the activity of enzymes GPI and PEP-D found in all of the hybrids except R5D-1B and R6, stems from the presence of the reciprocal T(7;X) chromosome and not a normal mouse 7.

With the construction of the somatic cell hybrid lines as described above, a primary screening system for detection of DNA sequences included in functional units 2 through 8 of the c-locus deletions has been established. The use of the somatic cell hybrids in Southern blot hybridization source DNA allows detailed analysis of DNA sequences removed in c locus deletion. The segregation of the normal mouse chromosome 7 from five of the deletion carrying somatic cell hybrids

Figure 4. Hybridization of DNA of somatic cell hybrids to a cloned sequence missing in c-locus deletions. 10 μg of DNA digested with either EcoRI (A) or BamHI (B) was loaded per lane. Probe is random-primer labelled clone 12A insert. A. EcoRI digestion, Lanes 1. C57BL/6. 2. Tester stock 22A. 3. R5D-1A. 4. R5D-1B. 5. R5D-5. 6. R5D-12. 7. R5D-14. 8. R6. 9. E36, Chinese hamster. 10. EBS-4, BALB/c mouse × Chinese hamster hybrid segregating mouse chromosome 7. 11. EBS-11B6, BALB/c mouse × Chinese hamster hybrid. B. BamHI digestion, Lanes 1. C57BL/6. 2. R5D-1A. 3. R5D-9A. 4. R6. 5. E36, Chinese hamster. 6. EBS-11B6, BALB/c mouse × Chinese hamster hybrid.

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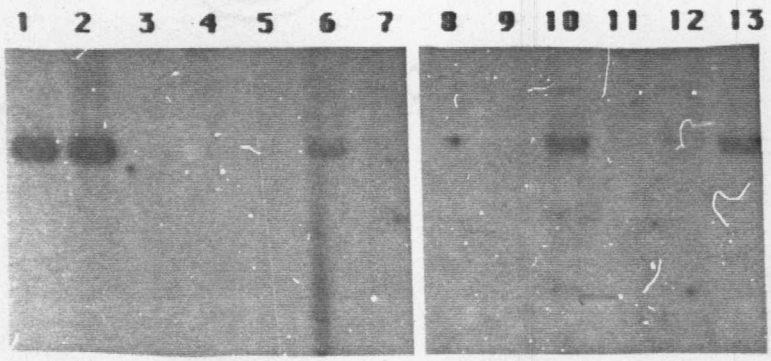

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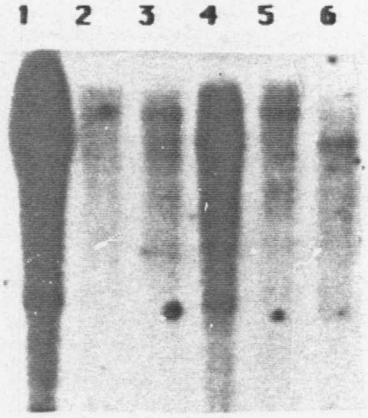


unmasks the sequences present on and, more importantly, absent from the T(X;7def)5RL chromosome. Southern blot hybridization of a given DNA sequence from mouse chromosome 7, to the hybrids allows identification of sequences included in the $\underline{c^{26DVT}}$ deletion by exhibiting the same pattern of hybridization as clone 12A - positive hybridization to the mouse fragment(s) in hybrids R5D-5, R6 and EBS-11B6 and absence of hybridization to the mouse fragment(s) in hybrids R5D-1A, -1B, -9A, -9B, and -14. An additional asset to this screening system is its ability to improve the genetic map of regions other than the c-locus region of mouse chromosome 7.

The same Southern blot hybridization will identify sequences from the region of mouse chromosome 7 proximal to the breakpoint of the T(X;7)5RL chromosome by absence of hybridization to the R5D hybrids and to the R6 hybrid, and from the region of chromosome 7 between the breakpoints of the T(X;7)5RL and T(X;7)6RL chromosomes by positive hybridization to the R5D hybrids and absence of hybridization to the R6 hybrid. Southern hybridization of a mouse chromosome X sequence to the somatic cell hybrids will allow assignment of that sequence to the regions proximal, distal and between the respective breakpoints of the two translocation chromosomes. Thus the somatic cell hybrid lines developed in the course of this work provide a source of genetic mapping information for both mouse chromosomes 7 and X.

CHAPTER III

MOLECULAR CLONING OF MOUSE REPETITIVE SEQUENCES

INTRODUCTION

To expand the number of sequences available from mouse chromosome 7, new clones were selected from recombinant DNA genomic libraries enriched for mouse chromosome 7. Two of the libraries screened were constructed from a mouse-Chinese hamster somatic cell hybrid line retaining mouse chromosomes 7. Since total mouse DNA cross hybridizes with Chinese hamster DNA (Nelson, 1984), these libraries could not be screened efficiently for mouse inserts by hybridization to total mouse DNA. It was necessary to obtain species-specific cloned repetitive sequences. One such cloned repeat, TU96, was obtained from Dr. Hans Lehrach of the European Molecular Biology Laboratory, Heidelberg (Roehme et al., 1984).

To obtain additional mouse-specific interspersed repetitive sequences a mouse genomic library was constructed in the plasmid vector pBR322, from a size-selected complete Sau3A restriction digest. The library was the screened with labelled total mouse DNA. Cloned sequences identified in this screening were checked for cross-hybridization to Chinese hamster DNA by colony hybridization to total Chinese hamster DNA. Repetitive clones showing no hybridization to total Chinese hamster DNA were checked further for species-specificity by colony hybridization to random mouse cosmid clones and random Chinese hamster cosmid clones. From these data the usefulness of the repetitive

sequences for screening mouse colonies from libraries constructed from mouse-hamster hybrids using colony hybridization was evaluated.

MATERIAL AND METHODS

Mouse genomic DNAs were prepared by homogenization of liver tissue in 0.25 M sucrose, 50 mM Tris pH 7.5, 10 mM EGTA, and 10 mM KCl using a Potter-Elbejheim homogenizer. Nuclei were pelleted by adding two times the volume of 2.3M sucrose, 50 mM Tris pH 7.5, 10 mM EGTA and 10 mM KCl, then spin over a 2.3M sucrose cushion in a SW27 rotor at 24 rpm for 45 min at 4°C. Nuclei were resuspended in cold TNE, and 300 μg/ml of proteinase K (Sigma) and SDS to 0.5% were added to lyse the nuclei. After overnight incubation at 37°C, DNA was phenol and chloroform extracted, then brought to 50% isopropanol at room temperature and wound on a glass rod. DNA was resuspended in one ml of TE. Twenty micrograms of genomic DNA was digested to completion with the restriction enzyme Sau3A and electrophoresed overnight on a 1% agarose gel in Tris-acetate buffer. Fragments of 400 bp and 700 bp length were cut from the gel, crushed and soaked in TNE overnight at 4°C. Supernatant was passed through a siliconized wool column, phenol-chloroform extracted twice and then ethanol-precipitated. These Sau3A fragments were then ligated in a 20 μl reaction at 12°C, into the BamHI site of the tetracycline resistance gene of the plasmid vector pBR322, with T4 DNA ligase (New England Biolabs).

Competent HB101 cells, a *recA* deficient strain of *E. coli*, were prepared by resuspending a 30 ml culture a OD_{600} = 0.19, in 15 ml

ice-cold CMT (0.1 M $CaCl_2$, 0.005 M $MgCl_2$, 0.01 M Tris-HCl pH 7.8) for 20 min, then resuspending cells in 0.75 ml CMT. Competent HB101 cells were transformed by adding 6.7 µl of the ligation reaction to 200 µl of HB101, storing on ice for 15 min, followed by a 5 min heat shock at 37°C. Transformed bacteria were plated on LB plates containing 50 µg/ml ampicillin (Sigma) and incubated overnight at 37°C. Colonies were placed in grids on duplicate plates, one containing 50 µg/ml ampicillin and other containing 170 µg/ml tetracycline (Sigma). Colonies negative on tetracycline and positive on ampicillin were selected as containing inserts and regrided on ampicillin plates for screening by colony hybridization.

Colony hybridization was performed as described by Grunstein and Hogness (1975). Nick-translated total mouse DNA was put through a BioRad A-0.5M column, eluted in TE, then brought to 0.18 M NaCl and boiled for 10 min to denature. The probe was allowed to reanneal at 65°C for one hour, then hybridized to the nitrocellulose filters containing colonies from the library. Positive colonies were rescreened by colony hybridization to total mouse probe prepared as before.

Plasmid DNAs from repetitive clones, for use in restriction enzyme digestions to analyze insert size and for use in random primer labelling reactions were isolated by alkaline lysis as described by Maniatis et al. (1982). Random primer labelling of plasmid inserts was carried out as described in Chapter II (page 16). Nick-translation of total mouse and total Chinese hamster DNA was done as described by Rigby et al. (1977).

A genomic C57BL/6 library in the cosmid vector pTCF and E. coli 803 host cells was obtained from Ginny Folsom, and a Chinese hamster ovary (CHO) genomic library in the cosmid vector pJB8 and E. coli 1046 host cells was obtained from Phillipe Gros. One hundred randomly plated cosmid colonies from the mouse library were grown on 100 mm petri dishes, then 82 mm nitrocellulose filters were laid on top of the colonies to replicate them. These master filters were placed, colony side up, on fresh agar plates, incubated 2 hr at 37°C and then used to make two more replicas on nitrocellulose filters. The same procedure was followed for 100 random colonies from the Chinese hamster cosmid library. One replica filter of both the mouse and Chinese hamster colonies was prepared for colony hybridization. The other replica of the mouse library was pressed onto the remaining replica of the hamster library to produce filters with a random mixture of colonies from both species. These replicas were incubated 2 hr at 37°C, then prepared for colony hybridization. Just prior to denaturation, 100 ng of purified DNA from plasmid pBR322 and from eleven randomly selected repetitive clones were spotted as controls on the perimeter of at least one filter of the set of replicas.

RESULTS AND DISCUSSION

Construction of the Sau3A digested mouse library resulted in 483 colonies, from which 333 colonies were randomly picked and placed in duplicate grids on one plate each of tetracycline and of ampicillin. Of these colonies, 236 contained inserts (as determined by susceptibility

to tetracycline). To enhance recovery of mouse-specific interspersed repetitive sequences, which make up approximately 20% of the mouse genome (Britten and Kohne, 1968), two strategies were utilized. One relied on the fact that the restriction enzyme Sau3A recognizes a four base pair DNA sequence which should occur every 4^4 base pairs, to give an average size fragment of about 256 bp. This enzyme though, has no recognition site within mouse satellite DNA, long stretches of tandem repetitive sequences localized mainly in the centromeric regions rather than being interspersed throughout the genome. Such repetitive sequences make up approximately 9% of the mouse genome (Britten and Kohne, 1968), and therefore could be expected to be highly represented in a genomic library. However, because they are not interspersed, they are not suitable for the purposes of this work. Thus a complete Sau3A digestion allowed agarose gel fractionation of the bulk of the genome containing interspersed repetitive sequences away from the undesirable satellite DNA.

The second enhancement step in the selection of the interspersed repetitive sequences over non-interspersed repetitive sequences such as satellite DNA, took advantage of the difference in hybridization kinetics between the two types of mouse repeats. For the satellite sequences of the mouse, $Cot_{1/2}$, the product of DNA concentration and time at which one-half the complementary base pairs are reassociated, is 10^{-3} mole-sec/liter. Thus for denatured complementary strands of satellite DNA at an initial concentration of 2 μg/ml or 6.4×10^{-6} moles of nucleotides/liter in 0.18 M NaCl at 60°C, complementary base pairs would be half-reassociated in 156 sec [(10^{-3} mole-sec/liter)/(6.4×10^{-6}

mole/liter)]. At Cot = 10^{-2} mole-sec/liter, virtually all satellite sequences have reassocciated, which for conditions as stated before, occurs at t = $10^{-2}/6.4 \times 10^{-6}$ mole/liter = 26 min. Under the same conditions, the $Cot_{1/2}$ of middle repetitive intersperse sequences is 0.1 mole-sec/liter, which means that at Co = 6.4×10^{-6} mole/liter half-reassociation will occur in 4.34 hr. This difference in the time for half-reassociation allowed the preparation of labelled mouse DNA in which satellite sequences were double stranded and unavailable for hybridization, while the majority of middle repetitive interspersed sequences and unique sequences were still single stranded and available for hybridization to complementary sequences in recombinant plasmids. We denatured labelled, EcoRI digested total mouse DNA by boiling in 0.18 M NaCl, then placed the denatured DNA at 60°C for one hour, after which it was hybridized to recombinant plasmids on nitrocellulose filters. Under these conditions virtually all of the satellite sequences should be reassociated but far less than half of other sequences would be.

Hybridization of total mouse DNA, prepared as stated, to colonies of the recombinant Sau3A library yielded 14% or 35 repeat-positive colonies, designated the pMR series (mouse repeat), out of 236 colonies. This recovery is in line with the known 20% representation of middle repetitive sequence in the mouse genome. Inserts of 12 randomly chosen repeat-positive plasmids were hybridized to EcoRI digested C57BL/6 and BALB/c DNA on Southern blots. Eleven of the sequences showed a smear of hybridization down the entire length of each mouse lane, which is characteristic of a repeated interspersed sequence (data not shown). One of the sequences hybridized only to a 1.3 kbp fragment in both mouse

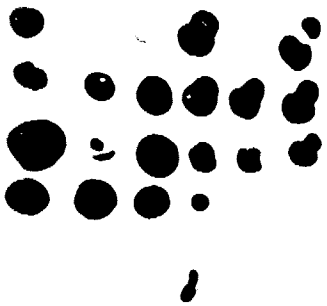
strains. This sequence is probably a member of a large family of 4.5 kbp BamHI interspersed sequences that are characterized not only by the parental BamHI fragment but also by a 1.3 kbp EcoRI fragment (Martin et al., 1984). These data indicate that the sequences in those plasmids are interspersed repetitive sequences.

To further characterize the repetitive clones and guide selection of mouse-specific sequences among them, total mouse DNA, total Chinese hamster DNA and the cloned mouse repetitive sequence in plasmid TU96 (Rohme et al., 1984) were hybridized to a representative sample of 22 randomly selected clones, which included the 12 clones tested above, from the 35 repeat-positive colonies. Hybridization results are shown in Figure 5. This primary screening for species specificity suggested cross-homology of pMR 81, pMR 120 and pMR 139 with Chinese hamster (Figure 5B) thus these clones are not useful for screening the interspecies recombinant libraries. The screening also suggested weak cross-homology between pMR 115 and TU96. Of the remaining 18 clones, four were chosen at random for further analysis.

To evaluate the species specificity of each of the four cloned sequences sets of nitrocellulose filters containing approximately 100 colonies from a mouse cosmid library and 100 colonies from a Chinese hamster cosmid library were prepared for colony hybridization to the cloned repeats. It should be noted that the vector-host combination of the hamster library showed slower growth than that of the mouse library. To compensate, hamster colonies were plated and grown one hour longer than mouse colonies, however the resultant hamster colonies were still smaller in size than mouse colonies. Thus, for the hamster-only

Figure 5. Colony hybridization of cloned repetitive sequences. 22 selected repetitive sequence colonies were placed in duplicate grids on nitrocellulose and hybridized to A. Nick-translated total C57BL/6 DNA. B. Nick-translated total E36, Chinese hamster DNA. C. Random primer labelled EcoRI insert from plasmid TU96 from Hans Lehrach. D. Schematic of colony positions on the grids. Numbers under the dot representing the colony, refer to the pMR plasmid growing in that colony.

A



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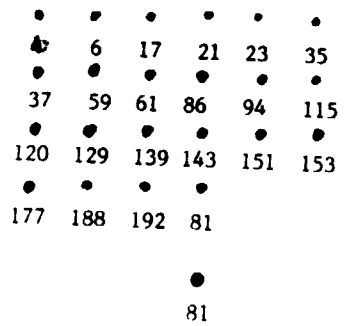
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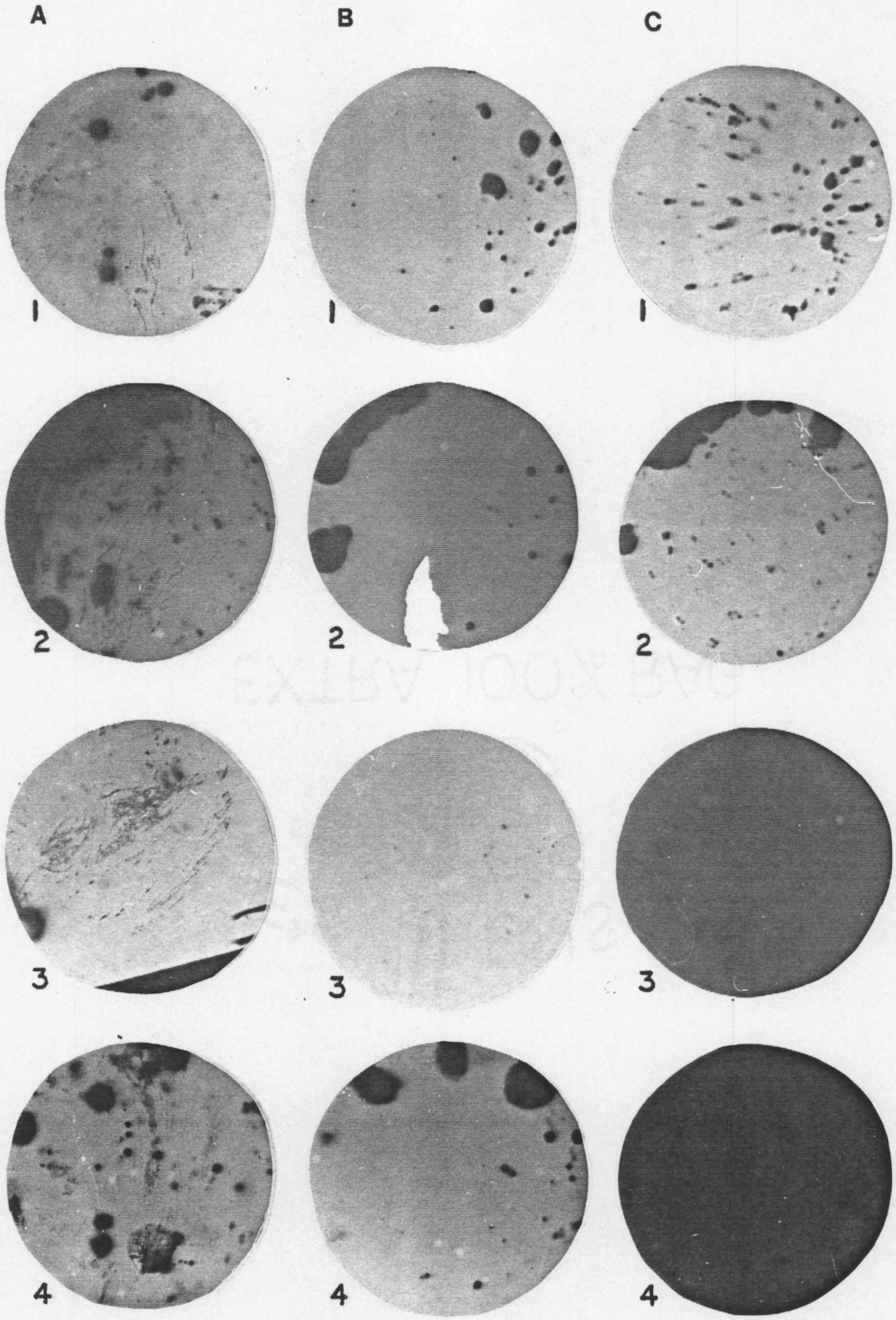
filters, longer exposures of autoradiographs were examined to improve scoring of positive colonies. DNAs from the four pMR clones examined were spotted on one filter of each set as positive controls. DNAs from seven additional pMR clones were spotted on the same filter so that possible homology between repetitive sequences could be detected to avoid redundancy in future choices of clones for analysis. Plasmid DNA from pBR322 was also spotted on the filter to control for background due to hybridization of contaminating vector DNA in inserts used as probes to cosmid vector DNA.

Hybridization of random primer labelled insert DNA from the four pMR plasmids and from TU96 to sets of replica filters of the mouse Chinese hamster libraries is shown in Figure 6. A summary of the hybridization results also appears in Table 2. Ideally, a cloned repetitive sequence for screening mouse-Chinese hamster hybrid libraries should be a high copy mouse repeat to maximize the percentage of mouse colonies recognizable, and yet have no homology to Chinese hamster to minimize the background of hamster colonies obtained in a screening of the library.

Plasmids pMR 153 and pMR 129 show the largest number of positive mouse colonies, 120 and 61, respectively. Species specificity of these sequences is reflected in the 18% (28/148) of colonies pMR 153 and 34% (32/93) of colonies pMR 129 hybridizes to, which are Chinese hamster colonies. The background of hamster colonies for these two sequences is probably not as high as these results indicate since part of the hybridization may be due to vector sequences contaminating the insert probes, as indicate by some hybridization to the pBR322 DNA on filter 2

Figure 6. Hybridization of repetitive sequences to mouse and to Chinese hamster cosmid colonies. Colonies were plated at random on ampicillin plates then transferred to nitrocellulose filters for replication and colony hybridization. Filters are arranged in sets of four: 1) replica of mouse colonies only; 2) and 4) replicas of mouse colonies and Chinese hamster colonies; 3) replica of the Chinese hamster colonies. Filters are shown in the same orientation throughout the set of four. Control DNA was spotted on the perimeter of filter 2 and where indicated, on filter 4. Control DNA spots are indicated by arrows. Numbers refer to the plasmid pMR DNA spotted. Hybridization probes are random primer labelled A. pMR 35 insert. B. pMR 86 insert. C. pMR 129 insert. D. pMR 153 insert. E. TU96 insert.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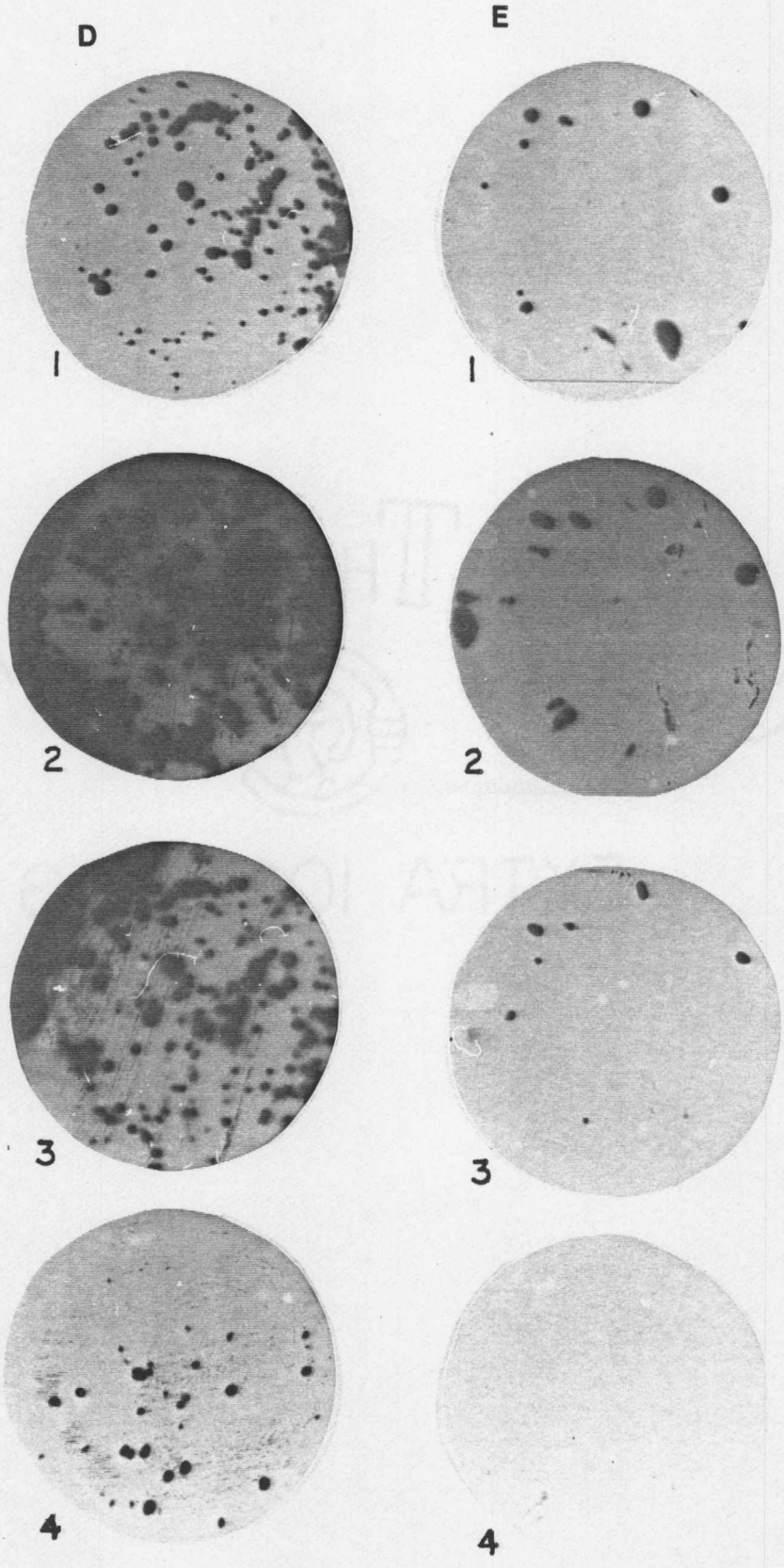
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Table 2: Species Specificity of Repetitive Sequences

Repetitive Sequence	No. of Positive Mouse Colonies[a]	No. of Positive Hamster Colonies[b]	Percent Hybridization to Chinese Hamster	Cross-Hybridization to other pMR plasmids
pMR35	8	2	20% (2/10)	151,188 115,129
pMR86	28	5	15% (5/33)	94,151,188 115, 129
pMR129	61	32	34% (32/93)	35,151 86,94,115,188
pMR153	120	28	18% (28/148)	151,188
TU96	9	0	0	115

[a]Colonies counted on mouse-only filters.

[b]Colonies counted on Chinese hamster-only filters.

in panels C and D of Figure 6. Plasmid pMR 86 picked up 28 mouse colonies on a background of 15% (5/33) Chinese hamster colonies and plasmid pMR 35 picked up eight mouse colonies on a 20% (2/10) background. The cloned repeat TU96 picked up nine mouse colonies with no Chinese hamster background.

Thus none of the five sequences are ideally suited for screening mouse-Chinese hamster hybrid libraries. However, pMR 153 could be a very useful probe in a situation where the most important criterion is the identification of as many mouse colonies as possible, with a tolerance for the 18% hamster background. In screening interspecies libraries with a goal of obtaining a relatively small number of mouse colonies to examine, then TU96 would be the most useful sequence. The repetitive sequence in pMR 35 hybridizes to a similar number of mouse colonies compared to TU96 approximately 8% of the colonies tested, plasmid pMR 86 hybridizes to 28% of the colonies tested. Both a significant background pMR 35 and pMR 86 exhibit hamster colonies. For this reason, these sequences are less useful for interspecies library screening in comparison with TU96. Plasmid pMR 129 is not suitable for screening mouse-hamster libraries. The level of background hybridization to hamster colonies prevents its use entirely for this purpose.

The organization of repetitive sequences within the mouse genome is still poorly understood but it is apparent that families of related repetitive sequences exist (Davidson et al., 1975; Ginelli et al., 1979). An analysis of the hybridization data in Figure 6 shows some of the interrelationships between the repeats. Plasmids pMR 35, 86, 129 and 153 show homology to both pMR 151 and 188, but appear themselves to

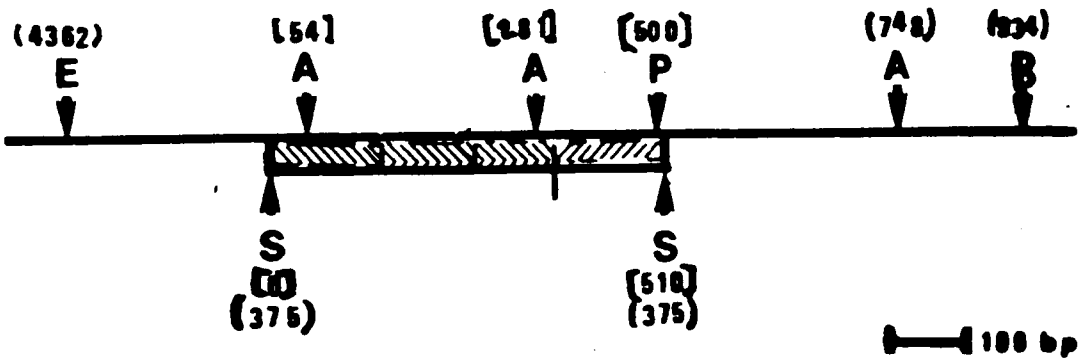
be nonhomologous, as judged by lack of cross-hybridization. Plasmid pMR 153 appears to represent a different portion of the repetitive sequences present in pMR 151 and 188, as it does not hybridize with any of the other eight repetitive clones on filter 2 of panel D. Plasmids pMR 86, 94 and 129 all cross-hybridize with each other but are still probably independent, as suggested not only by the greater number of mouse colonies to which pMR 129 hybridizes than pMR 86, but also by the evident homology of pMR 35 only to pMR 129 and not to pMR 86. The cloned repeat TU 96 shows hybridization only to pMR 115, which is in agreement with hybridization data in Figure 5C. TU 96 is probably a member of the 4.5 kbp BamHI repeat family discussed earlier, as upon Southern blot hybridization to EcoRI restriction digests of genomic mouse and mouse-hamster hybrid DNA, there is prominent and very intense hybridization to 1.3 kbp fragments (data not shown).

Although the primary reason for developing cloned mouse repetitive sequences was for use in screening interspecies libraries, the sequences have other uses. David Nelson and Shawnee Weider utilized pMRs 120 and 129 to evaluate the amount of mouse DNA stably integrated into African green monkey CV-1 cells following metaphase chromosome transfer from mouse donors (Housman et al., 1983). Primary and secondary transferrants retaining resistance to the drug ouabain were selected. Hybridization of the two repetitive sequences to Southern blots of DNA from primary and secondary transferrants allowed identification of band patterns common to all the transferrants and therefore, likely to include the sequences conferring ouabain resistance. In collaboration with Robert Levenson, the presence of sequences homologous to one of the

repetitive clones, pMR 81, on the same EcoRI restriction fragment as a mouse sequence conferring ouabain resistance in a secondary transferrant of mouse into human cells, allowed for the molecular cloning of that ouabain resistance sequence (Levenson et al., 1984). In developing the cloned sequence for ouabain resistance for further study, it was desirable to remove the repetitive sequences from the unique ones. To facilitate this, the restriction map of pMR 81 shown in Figure 7 was constructed. Restriction enzymes analyzed were limited to those known not to interfere with expression of ouabain resistance upon digestion with that enzyme. This study demonstrated the utility of cloned repetitive sequences as hybridization probes to isolate nearby sequences selected for in transfection experiments.

Five cloned mouse repetitive sequences were examined for their utility as mouse-specific probes to screen mouse-Chinese hamster libraries made from the mouse chromosome 7-enriched somatic cell hybrid, EBS-11B6. Of the five sequences, plasmid TU96 was deemed the most useful sequence to obtain high levels of specificity for mouse sequences from the EBS-11B6 interspecies libraries. Since a limited number of mouse chromosome 7 sequences were to be examined for involvement in the c-locus deletions, the desired number (approximately 20) of mouse positive cloned sequences should be obtained by screening 6,000 cosmid colonies or 12,000 phage plaques from the EBS-11B6 libraries with plasmid TU96 (Chapter IV, page 66). In future screenings of mouse-Chinese hamster interspecific libraries it may be desirable to obtain high yields of mouse sequences, in which case plasmid pMRs 153, 35 and 86 would be useful probes.

Figure 7. Restriction map mouse repetitive clone pMR 81. Plasmid DNA was digested with restriction enzymes and analyzed by electrophoresis on 1.2% agarose gels and 5% acrylamide gels. Restriction enzymes AvaII (A), BglI (B), EcoRI (E), PvuII (P), and Sau3A (S) were shown to leave ouabian resistance intact by recovery of ouabain resistant colonies from transvection of DNA from a primary ouabain^r transferrant digested with these enzymes. The insert of pMR 81 did not contain restriction sites for BglI, EcoRI or PstI. Numbers in parenthesis refer to the original position of restriction enzyme sites in the vector, pBR322, while numbers in brackets refer to positions within the insert DNA.


(4362)
E
[54]
A
[500]
P
(748)
A
S
S
100 bp

CHAPTER IV

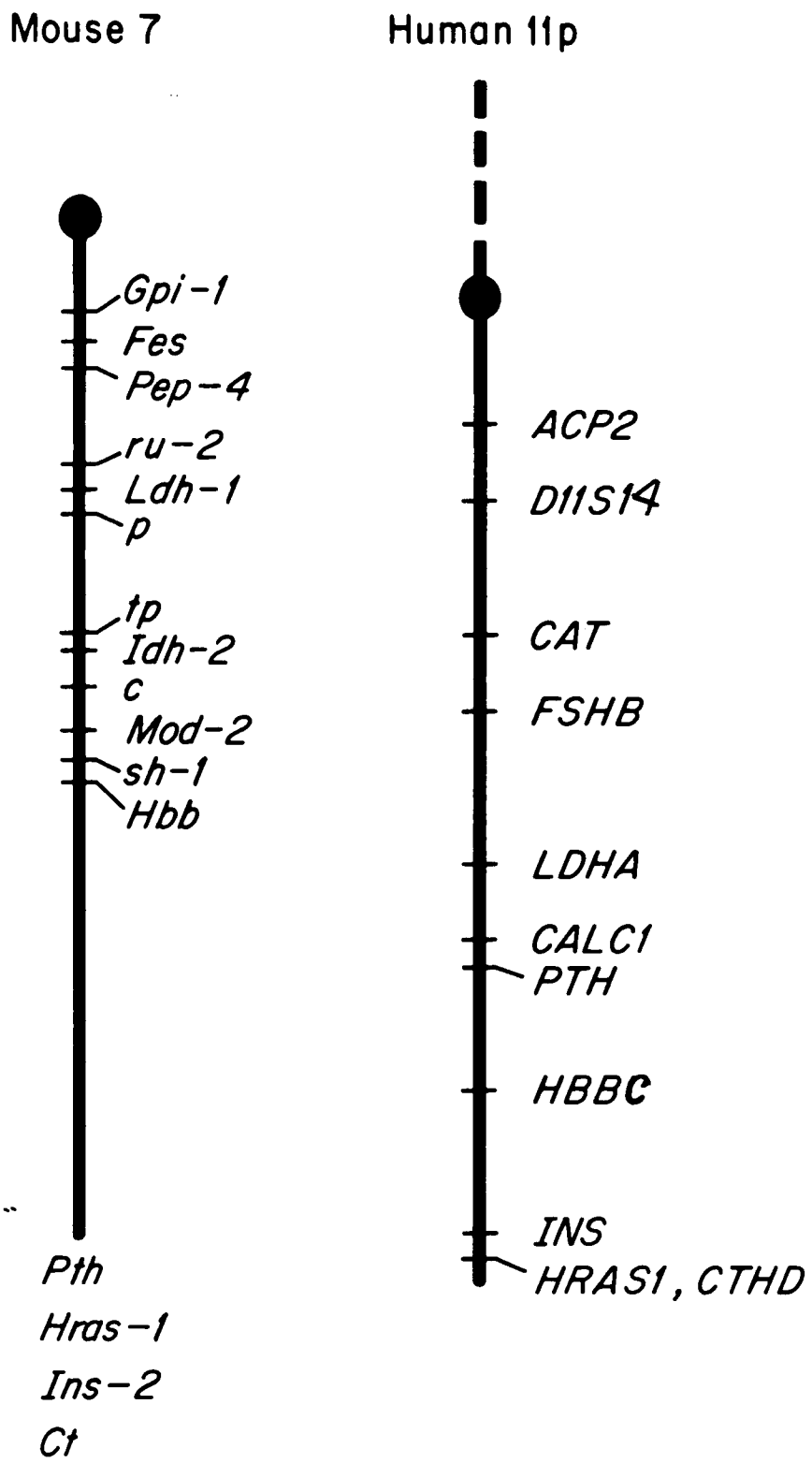
SELECTION OF MOUSE CHROMOSOME 7 SEQUENCES

INTRODUCTION

The extensive biochemical, genetic, and morphological studies of the albino-locus region have established the beginning of a detailed map of this region of mouse chromosome 7. They have demonstrated the region's potential as a source of information about early development of the organism and about gene function and regulation. Every new marker added to the genetic map increases the potential of the region for future studies, and so one objective of this work was to assemble a set of cloned DNA sequences with which to expand the genetic map of not only the c-locus region but of all of mouse chromosome 7.

The criteria used to select sequences for examination were prior assignment of the sequence to mouse chromosome 7, but with map position with respect to established genetic markers unknown and/or prior assignment of the sequence or its homolog, to human 11p. Figure 8 shows the current status of the genetic maps of mouse chromosome 7 and human chromosome 11p, for the relevant markers. Homology between the two chromosomes is known for the Ldh-1 and LDHA, and the Hbb and HBBC loci. The recent assignment of parathyroid hormone (Pth), calcitonin (Ct), insulin (Ins-2), and Harvey murine sarcoma virus (Hras-1) loci to mouse chromosome 7 extends the known homology significantly (Davisson and Roderick, 1985). The availability of cloned sequences for these markers, and the prospect of gaining more insight into the comparative

Figure 8. Genetic maps of mouse chromosome 7 and human chromosome 11p. Mouse map is adapted from published linkage data (Davisson and Roderick, 1985). Human map is adapted from Housman et al. (1985). Distances are not drawn exactly to scale.


Mouse 7
Human 11p
Gpi-1
Fes
Pep-4
ru-2
Ldh-1
p
tp
Idh-2
c
Mod-2
sh-1
Hbb
Pth
Hras-1
Ins-2
Ct
ACP2
D11S14
CAT
FSHB
LDHA
CALC1
PTH
HBBC
INS
HRAS1, CTHD

map between mouse 7 and human 11p, led to an examination of these sequences with the materials developed in this work. Plasmid H19, a mouse cDNA clone coding for an unknown protein, which had been mapped to mouse 7, clone 12A, a cloned DNA sequence which maps to mouse 7 between c and sh-1, and Ds-1, an anonymous human sequence from 11p between CAT (catalase) and APC-2 (acid phosphatase-2) were also examined.

To supplement the available unmapped sequences on mouse chromosome 7, unique sequences from recombinant DNA libraries were isolated. Screening of a total mouse genomic library for random clones should give one out of fifteen clones on chromosome 7 (based on 85 cM in chromosome 7 from the total 1300 cM mouse genome) and one out of 150 clones in the approximately 8 cM region involved in the largest c-deletion. To avoid having to isolate so many clones it is desirable to work with libraries enriched for mouse chromosome 7. Three such libraries were screened in this work. The first library, the lambda CLX library, was constructed by Dr. Christine Disteche from flow-sorted Cattanach's translocation chromosomes. The second library and the third library (constructed by Jane Mendel) were constructed from a mouse-Chinese hamster somatic cell hybrid retaining mouse chromosome 7.

The Cattanach's translocation consists of an inverted insertion of part chromosome 7 (from ru-2 to sh-1) into the X-chromosome. The lambda CLX library was estimated by metaphase chromosome banding of the sorted fraction of Cattanach's translocation chromosomes used as insert, to be 40% pure (Disteche et al., 1982). Since approximately 25 cM of chromosome 7 is inserted in the X-chromosome (which contains a minimum of 85 cM), chromosome 7 sequences should comprise less than one-fourth of

the sequences of the entire Cattanach's translocation chromosome. Thus one out of nine cloned sequences in the library should be from chromosome 7. Approximately 8 cM or one-third the length of the chromosome-7 segment inserted in the Cattanach translocation, are missing in the largest c-deletion; thus one out of approximately 30 clones in the library should be missing in a c-locus deletion.

As a source of DNA for constructing recombinant libraries enriched for chromosome 7 sequences, the potential of mouse-Chinese hamster somatic cell hybrids was explored. In the stabilization of such hybrids, segregation of mouse chromosomes occurs, serving as an enrichment of the retained mouse chromosomes over their original composition in the mouse genome. For this purpose the mouse-hamster hybrid EBS-11 was obtained from Dr. Peter Lalley. Its mouse complement consists of chromosomes 2, 7, 10, 12, 15 and X. In a library from this hybrid, one out of 6 mouse sequences should be from mouse 7, and one out of 60 mouse sequences should be in the largest c-deletion. To improve enrichment for mouse 7, segregation of additional mouse chromosomes was selected for. Segregation of the mouse X chromosome was selected for by growing EBS-11 in 6-thioguanine, which results in the death of any cells retaining the hprt gene on mouse X. During the stress of selection, segregation of some of the mouse autosomes as well as of X might be obtained. Clonal lines were developed from surviving colonies and tested for retention of mouse chromosome 7 by isozyme analysis of LDH-A and GPI, and by Southern blot hybridization to a beta-hemoglobin sequence. The resultant hybrid subclone was then used as a source of DNA for constructing two recombinant DNA libraries.

One library was constructed in the BamHI site of the cosmid vector pCVN from a partial MboI digestion of hybrid cell DNA. As previously described (Chapter III), mouse-specific cloned repetitive sequences for screening this library were developed. Because of its high specificity, the repeat sequence utilized primarily in the screening of this library to identify mouse sequences was TU96. The other library was constructed by Jane Mendel in the phage vector MBL4 from the hybrid cell DNA. It was also screened with TU96.

Cloned sequences from all the libraries were analyzed to identify repeat-free restriction fragments. These fragments were rechecked again for repetitive sequence content. To verify the species of origin of a cloned DNA sequence the fragment was used as a hybridization probe on a Southern blot of genomic mouse and geomic hamster DNA digested with the same restriction enzyme which produced the fragment. Unique sequence mouse fragments should show a single band of the same size as the probe fragment itself upon hybridization to mouse DNA, but a different size fragment (or even no hybridization) upon hybridization to hamster DNA. Fragments containing unique mouse sequences were hybridized to Southern blots of thirteen well-characterized mouse-Chinese hamster somatic cell hybrids obtained from Dr. Peter Lalley. Each clone was assigned to a mouse chromosome by evaluating discordancies shown by these hybridization data.

MATERIALS AND METHODS

The following cloned sequences were obtained to be used as hybridization probes: (i) parathyroid hormone from the plasmid clone rPTHs-1, containing a 4.5 kbp BamHI rat genomic fragment in pBR322 (Heinreich et al., 1984); (ii) Harvey murine sarcoma virus from the plasmid clone BS9, containing a 460 bp fragment with EcoRI linkers (Ellis et al., 1980); (iii) an anonymous cDNA coding for a protein subject to the same regulation as alpha-fetoprotein for which no locus has been assigned (Shirley Tilghman, personal communication) containing a 300 bp insert in the PstI site of pBR322; (iv) calcitonin from the plasmid clone C89, containing a rat cDNA fragment in pBR322 (Jacobs et al., 1981) (v) insulin from the plasmid clone pI476, containing a 281 bp PstI rat cDNA fragment in pBR322 (Villa-Komaroff et al., 1978) (vi) lactate dehydrogenase as described in Chapter II (page 17) (vii) beta-hemoglobin as described in Chapter II (page 17) (viii) clone 12A as described in Chapter II (page 17) (ix) D11S14 from the plasmid clone pDs-1, containing a 3.2 kbp anonymous human sequence from the short arm of 11p (Glaser et al., 1985).

Three recombinant DNA libraries were selected for mouse chromosome 7 sequences: (1) The lambda CLX phage library constructed from flow-sorted Cattanach translocation chromosomes by Dr. Christine Disteche; (2) The EBS-11B6 cosmid library constructed from the somatic cell hybrid, EBS-11B6; and (3) The EMBL11B6 phage library constructed from a size-fractionated EcoRI digestion of the EBS-11B6 somatic cell

hybrid by Jane Mendel. Each of the three libraries was enriched for mouse chromosome 7.

The lambda CLX library was constructed by from a complete EcoRI digestion of flow-sorted mouse Cattanach's Is(X;7)1Ct chromosomes isolated from carrier embryos and inserted into the EcoRI sites of the phage vector lambda gtWesB (Disteche et al., 1982). The chromosome sorting yielded 40% purity for the translocation chromosome as assayed by metaphase banding of the sorted fraction from which the library was constructed. The original library contained 10% nonrecombinant phage and 2% recombinant phage with inserts of unknown origin. An amplified fraction of the library, stored in CsCl, was obtained and E. coli LE392 cells were infected then plated on NZYM agar (10g NZ amines, 5 g yeast extract, 5 g NaCl, 15 g agar and 1 ml of 1 M Mg SO per liter). 1000 individual plaques were placed in grids on lawns of LE392 cells with sterile toothpicks. After an 8 h incubation at 37°C, nitrocellulose filters were placed on each plate to absorb phage and then prepared for phage plaque hybridization, in triplicate, as described by Benton and Davis (1977). Filters were hybridized to nick-translated EcoRI digested total genomic mouse DNA in 6 × SSC, 1 × Denhardt's solution, 100 μg/ml single-stranded salmon-sperm DNA, and 0.1% SDS at 60°C for 18 hr. Filters were washed once at room temperature, three times at 65°C in 2 × SSC, 0.1% SDS, and once in 1 × SSC, 0.1% SDS at 65°C, then autoradiographed. Selected phage were maintained in LE392 cells on NZYM agar plated.

DNA from phage was isolated from 30 ml overnight cultures of infected LE392 cells by spinning out cell debris at 8000 rpm in a

Sorvall SS34 rotor for 10 min, then layering the supernatant over a double-step gradient of 1.6 g/ml CsCl and 1.4 g/ml CsCl. Gradients were spun at 45,000 rpm for 45 min in a 50.1 rotor. Phage bands were collected from the interface between the 1.4 g/ml and 1.6 g/ml CsCl layers, dialyzed against TE at 4°C to get rid of the CsCl, then phenol extracted twice and the DNA precipitated with 70% ethanol. Phage were examined for size of inserts, and inserts were prepared for random primer labelling by a complete EcoRI digestion of DNA run on 1% low gelling temperature agarose gel electrophoresis in Tris-acetate buffer. Hybridization of labelled insert DNA to Southern blots of mouse and Chinese hamster DNAs was carried out for 44-48 hr in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 100 μg/ml single-stranded salmon-sperm DNA, and 200 mM sodium phosphate pH 6.5. Repeat-free inserts hybridizing to mouse DNA were then hybridized to a panel of somatic cell hybrids containing known complements of BALB/c mouse chromosomes in a Chinese hamster background (Minna et al., 1975; Tukey et al., 1984) for assignment to the correct mouse chromosome.

Somatic cell hybrid EBS-11, a hybrid resulting from the fusion of a BALB/c mouse spleen to HPRT-deficient E36 Chinese hamster cells, was obtained from Dr. Peter Lalley. Cells were grown in DME for 10 days to allow non-selective segregation of mouse chromosomes, then switched to DME plus 5 μg/ml 6-thioquanine (Sigma). Colonies were picked when 2 mm in diameter and transferred and grown to confluence in DME in 24-well Linbro plates. At confluence, cells were trypsinized in a 2 ml volume, 100 μl of which was transferred to fresh DME in a new Linbro plate. The remaining 1.9 ml was used to prepare cell extracts as described in

Chapter II (page 14), except that cells were resuspended in 100 µl of lysis buffer and that DNA was not extracted from the nuclei. Starch gel electrophoresis of 30 µl samples from each subclone was performed as described in Nichols and Ruddle (1973).

Genomic DNA from the hybrid EBS-11B6, after back-selection in 6-thioguanine, was isolated from cells grown to confluence in DME. Cells were washed twice with PBS (phosphate buffer solution), then 10 ml/plate of 10 mM Tris pH 8.0, 10 mM EDTA, 10 mM NaCl, 0.5% SDS and 50 µg/ml of proteinase K were added on one side of the plate without agitation. Plates were incubated at 37°C overnight. Phenol and chloroform extraction was done by gently pouring the cell lysis material into a 250 ml conical polyproylene centrifuge tube (Nalgene), adding an equal volume phenol or chloroform, and laying the tube on its side for three to four hours. After extraction, the DNA was transferred to dialysis tubing, dialyzed against 10% ethanol and then against several changes of TE a 4°C. 300 µg DNA was digested with 24 units MboI for one hour at 37°C, phenol-chloroform extracted and precipitated in 70% ethanol. Digested DNA was resuspended in TE and phosphatased, using 1.4 units calf intestinal phosphatase (Boehringer-Mannheim) at 37°C for one hour. EGTA to 50 mM and DEPC (diethylpyrocarbonate) to 0.1% were added to the reaction which was then heated to 68°C for 10 min. Following phenol-chloroform extraction, DNA was precipitated in 70% ethanol, resuspended in 400 µl TE and loaded on top of a 1.25 M to 5 M NaCl gradient, spun at 36,000 rpm in an SW41 for four hours. Fractions of 1.0 ml each were collected from the gradients. 50 µl of each fraction was diluted to 500 µl with TE, precipitated in 70% ethanol thee times to

get rid of salt, then run on a 0.4% agarose gel in tris-acetate buffer overnight. Fractions showing the highest concentration of fragments in the 35 to 50 kbp size range were pooled and dialyzed to get rid of salt then ethanol precipitated for use as insert DNA. Vector DNA fragments were prepared from the cosmid vector pCVN by the method described by Ish-Horowitz and Burke (1981) for pJB8. Cosmid pCVN is a derivative of pJB8 which contains the bacterial neomycin resistance gene and a single HindIII restriction site, in addition to parental cosmid sequences. Ligation of 1.2 μg of the size-selected genomic MboI partially digested DNA to vector fragments in a 1:1 molar ratio with T4 DNA ligase at 15°C overnight was followed by packaging of the recombinant molecules using Packagene packaging extracts (Promega Biotech). E. coli 1046, a rec A^- strain, were infected with the packaged molecules and plated on agar plates containing 50 μg/ml ampicillin (Sigma). When colonies reached about 0.5 mm in diameter, they were transferred to nitrocellulose filters and incubated for 2 hr at 37°C. Two filters were replicated from each of these master filters and prepared for colony hybridization as described in Grunstein and Hogness (1975). Storage of the recombinant colonies of the library during screening was as described by Hanahan (1979). Master filters were incubated at 37°C for 2 hr on agar plates containing 50 μg/ml ampicillin and 10% glycerol. Then a fresh sterile nitrocellulose filter was pressed on top of each master filter to form a sandwich which was then placed between damp sterile 3MM paper, sealed in a plastic bag and stored at -80°C. Replica filters were hybridized to random primer labelled inserts from cloned repeats. Inserts were isolated by complete Sau3A digestion of pMR and complete

EcoRI digestion of TU96 DNAs run on a polyacrylamide gel. Insert bands were extracted from gel slices by crushing and soaking the slice overnight in TE. Following precipitation in 70% ethanol, insert DNA was run on a 1% low gelling temperature agarose gel in Tris-borate buffer. Insert bands were cut from the gels and labelled as described for random primer labelling in Chapter II (page 16). Positive colonies were recovered by thawing stored master filters and picking colonies. After each use, master filters were grown one hour on 10% glycerol plates and stored as before. DNA isolated from positive colonies after the second screening, was digested with EcoRI, run on 1% agarose gels in Tris-acetate buffer and transferred to nitrocellulose by the method of Southern (1975). Nick-translated EcoRI digesed total mouse DNA was hybridized to the Southern blots to identify fragments free of repetitive sequences. EcoRI digestions of colony DNA were then run on 1% low gelling temperature agarose gels. Bands not hybridizing to total mouse DNA were excised, random primer labelled, and used as hybridization probes on Southern blots of EcoRI digested mouse and Chinese hamster DNA. Repeat-free clones were hybridized to Southern blots of the thirteen BALB/c-Chinese hamster somatic cell hybrids from Dr. Peter Lalley, for assignment to their respective chromosomes.

Recombinant phage from a library constructed from a complete EcoRI digestion of DNA from the back-selected hybrid, EBS-11B6, size-fractionated by agarose gel electrophoresis to 13 to 14.5 kbp fragments and inserted into the EcoRI site of lambda EMBL4 vector by Jane Mendel, were hybridized to TU96 by the method described by Benton and Davis (1977). Phage DNAs were prepared by the plate lysis method as described

by Maniatis et al. (1982). Inserted sequences were examined, and fragments for random primer labelling obtained, by agarose gel electrophoresis of HindIII digested and EcoRI/BamHI double-digested phage DNA. Transfer of digested and agarose gel fractionated restriction fragments to nitrocellulose, identification of repeat-free fragments, and hybridization to somatic cell hybrids for chromosomal assignment was as described above for cosmid DNAs.

RESULTS AND DISCUSSION

1. The Flow-sorted Lambda CLX Library

Of the one thousand lambda CLX phage screened, 644 were judged to contain repetitive sequences in the inserts, because of positive hybridization with total mouse DNA probe. 356 phage did not hybridize to total mouse DNA and potentially contained only unique sequence inserts. 29 of the negative phage were examined for insert size- and inserts were hybridized to Southern blots of mouse DNA to check for any repetitive sequences present and to verify identity of the insert as mouse DNA by virtue of hybridization to the same size EcoRI fragment in total mouse DNA. Inserts were also hybridized to Chinese hamster DNA to identify a restriction fragment length polymorphism to be used in hybridization of the inserts to the somatic cell hybrid mapping panel for chromosomal assignment. Six phage (21%) contained repetitive sequences in their inserts and were not suitable without further development for hybridization probes on Southern blots of genomic mouse DNAs. Four phage (14%) were nonrecombinant, four (14%) contained only

the lambda gtWesB cloning arm fragments, three (10%) contained inserts which did not hybridize to mouse DNA and yet were too large to be the internal phage sequences usually replaced by insert in the cloning process. Ten unique sequence phage (34%) were assigned to chromosomes other than mouse 7.

Two phage (7%), CLX18 and CLX23, were assigned to mouse chromosome 7. Figure 9 shows hybridization of these two recombinant phage to the somatic cell hybrid mapping panel. Lambda CLX18 contains a 7.5 kbp EcoRI insert and hybridizes to a 7.5 kbp EcoRI fragment in mouse genomic DNA. There is no hybridization to Chinese hamster DNA (Figure 9, lane 16). This sequence was assigned to mouse chromosome 7 on the basis of an 8% discordancy between chromosome 7 retention and CLX18 hybridization (Table 3). All other chromosomes show a greater than 23% discordancy. Lambda CLX23 contains a 3.5 kbp EcoRI insert and hybridizes to a 3.5 kbp EcoRI fragment in mouse genomic DNA. There is no hybridization to Chinese hamster DNA (Figure 9B, lane 16). This sequence was assigned to mouse chromosome 7 on the basis of no discordancy between CLX23 hybridization and mouse chromosome 7 retention in hybrids (Table 3). All other chromosomes show greater than 15% discordancy with hybridization to hybrids.

2. The EBS-11B6 Cosmid Library

Twelve clonal cell lines were obtained from the segregation of mouse chromosomes from somatic cell hybrid EBS-11, followed by selection against mouse chromosome X with 6-thioguanine. These cell lines were examined for the presence or absence of mouse chromosome 7 by starch gel

Figure 9. Hybridization of Lambda CLX18 and CLX23 to the EBS somatic cell hybrid mapping panel. 10 μg EcoRI digested DNA was loaded per lane. A. Probe is the 7.5 kbp EcoRI unique sequence insert fragment of CLX18. B. Probe is the 3.5 kbp EcoRI unique sequence insert fragment of CLX23. Lane 1. C57BL/6. Lane 2. BALB/c. Lane 3-17. EBS-1, -2, -3, -4, -5, -8, -11, -13, -15, -17, -18, -51, -74, -11B6. Lane 18. E36, Chinese hamster cells.

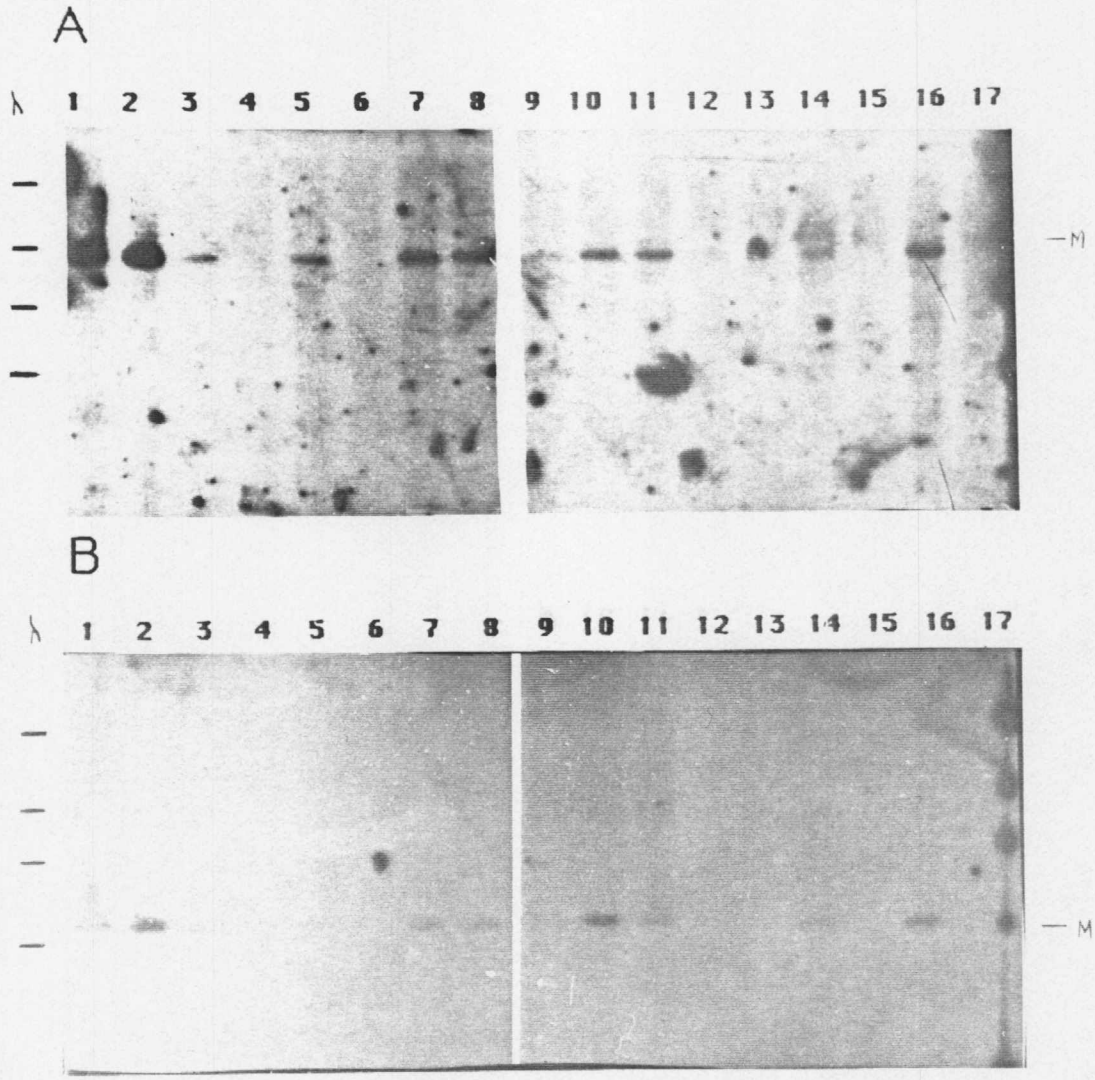

A
λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
M
B
λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
M

Table 3: Correlation Between Mouse Chromosomes and Hybridization of Lambda Clones CLX18 and CLX23 and Cosmid 11B6-9 to Somatic Cell Hybrids

Mouse Chromosome	Clones CLX18 and 11B6-9: Chromosome retention/sequence hybridization						Clones CLX23: Chromosome retention/sequence hybridization					
	Total	Number of hybrids with:				Percent discordancy	Total	Number of hybrids with:				Percent discordancy
		+/+	-/-	+/-	-/+			+/+	-/-	+/-	-/+	
1	13	2	4	7	0	54	13	2	5	0	6	46
2	13	6	2	3	2	38	13	7	2	3	1	31
3	13	4	2	5	2	54	13	4	3	1	5	46
4	13	5	4	0	4	31	13	5	5	0	3	23
5	13	1	3	2	7	69	13	2	4	1	6	54
6	13	4	2	3	4	54	13	4	3	3	3	46
7	13	8	4	0	1	8	13	8	5	0	0	0
8	13	5	4	0	4	31	13	5	5	0	3	23
9	13	3	3	2	5	54	13	3	3	2	5	54
10	13	5	4	0	4	31	13	5	5	0	3	23
11	13	0	4	0	9	69	13	0	5	0	8	62
12	13	6	2	2	3	38	13	6	3	2	2	31
13	13	6	2	3	2	38	13	6	2	3	2	38
14	13	2	2	2	7	69	13	2	3	2	6	62
15	13	8	1	4	0	31	13	8	1	4	0	31
16	13	5	2	2	4	46	13	5	3	2	3	38
17	13	6	3	1	3	31	13	6	4	1	2	23
18	13	4	4	0	5	38	13	4	5	0	4	31
19	13	6	4	0	3	23	13	6	5	0	2	15
X	13	8	0	5	0	38	13	8	0	5	0	38

Comparison of chromosome retention versus sequence hybridization is indicated as "+" for present and "-" for absent. Percent discordance is calculated as the number of hybrids for which +/- and -/+ correlations occur, divided by the total number of hybrids.

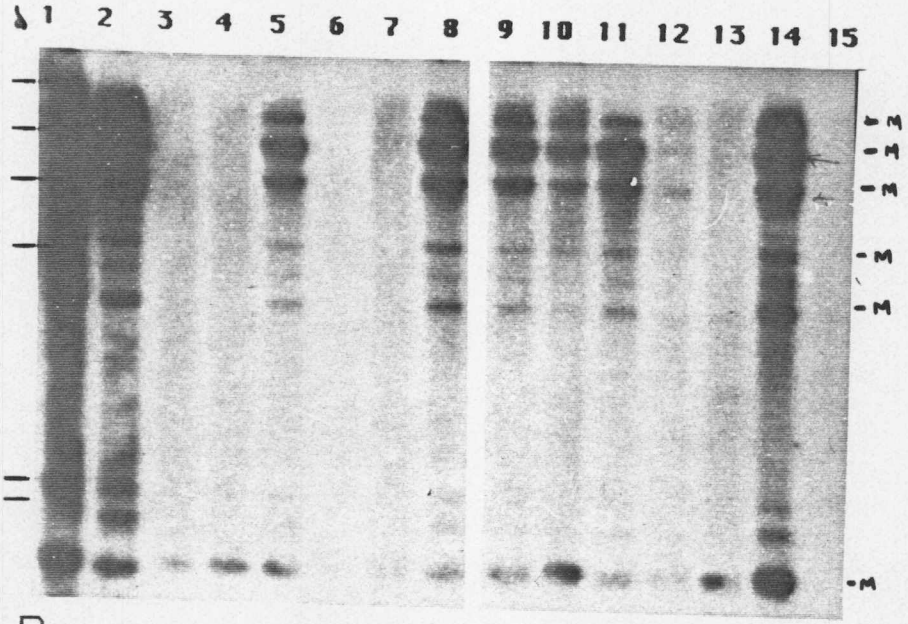
electrophoresis of cell extracts. The mouse isozyme of GPI was present in all 12 lines. However, the mouse isozyme of LDH-A was present in only one of the lines, EBS-1186 (data not shown). This isozyme pattern suggested that all clonal lines tested except EBS-11B6, had undergone chromosome rearrangement of at least the portion of 7 on which Ldh-A resides. EBS-11B6 was analyzed by additional starch gel electrophoresis. The results (shown in Table 1, page 22) indicated retention of the mouse chromosomes 7, 10, and 12 and loss of mouse chromosome 2 from the parental hybrid EBS-11. Southern blot hybridization of a cloned cDNA for the mouse hprt gene, pHPT5 (Nussbaum et al., 1983), indicated the absence of those sequences, and it is likely that segregation of the X-chromosome also occurred (data not shown). Southern hybridization to EBS-11B6 of an anonymous chromosome 15 sequence, CLX31, identified in the screening of the lambda CLX library (data not shown), gave positive hybridization, indicating that mouse chromosomes 15 was retained. Thus with the four mouse chromosomes (7, 10, 12, and 15) retained in a background of 22 Chinese hamster chromosomes, one out of 6.5 sequences (4/26) should originate from a mouse chromosome. The repetitive sequence TU96 should identify 9% of any mouse clones, therefore 72 colonies (6.5/0.09) must be examined to obtain one mouse sequence. At least one out of 4 mouse sequences should be on chromosome 7 and one out of ten chromosome 7 sequences would be involved in one of the larger c-locus deletions. Thus if 288 colonies (4 × 72) are examined roughly one sequence on mouse chromosome 7 should be obtained, and if 2880 colonies are examined approximately ten

sequences on mouse 7 should be obtained, one of which should be from a c-locus deletion.

From this hybrid EBS-11B6 enriched for mouse chromosome 7 by a factor of 1.5 over the parental hybrid, we constructed a phage and a cosmid recombinant DNA library. Screening of 3000 colonies of the cosmid library with the mouse-specific repetitive sequence TU96, yielded 11 positive colonies, or one out of 273 colonies. Examination of five cosmids revealed inserts of 35 to 40 kbp. Two cosmids contained repetitive sequences on all EcoRI fragments and were unsuitable for use as probes on the Southern blots containing somatic cell hybrid mapping DNAs or genomic mouse DNAs. Unique sequence EcoRI fragments were identified in three cosmids by hybridization of total mouse DNA to Southern blots of digested cosmid DNA. Of these, one sequence mapped to mouse chromosome 12 and one sequence still contained a repetitive sequence which gave greater than 30 hybri ization bands upon Southern blot hybridization to genomic mouse DNA. Hybridization of the third sequence, cosmid 11B6-9, is shown in Figure 10A. Two unique sequence EcoRI fragments were isolated from the cosmid and hybridize to the same size EcoRI fragments in genomic mouse DNA. This sequence was assigned to mouse chromosome 7 on the basis of 8% discordancy between hybridization of cosmid 11B6-9 to hybrids and retention of mouse chromosome 7. All other chromosomes show discordancies greater than 23% between hybridization and chromosome retention.

Figure 10. Hybridization of phage EMBL6-15 and cosmid 11B6-9 to the EBS somatic cell hybrid mapping panel. 10 μg EcoRI digested DNA was loaded per lane. A. Probe is the EcoRI insert fragment of EMBL6-15. Lane 1. C57BL/6. Lane 2. BALB/c. Lanes 3-14. EBS-1, -2, -3, -4, -5, -8, -13, -15, -18, -51, -74, -11B6. Lane 15. E36, Chinese hamster. B. Probe is the 9.4 kbp EcoRI fragment of cos 11B6-9. Lane 1. C57BL/6. Lane 2. BALB/c. Lanes 3-15. EBS-1, -2, -3, -4, -5, -8, -13, -15, -17, -18, -51, -74, -11B6. Lane 16. E36, Chinese hamster cells.

A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M
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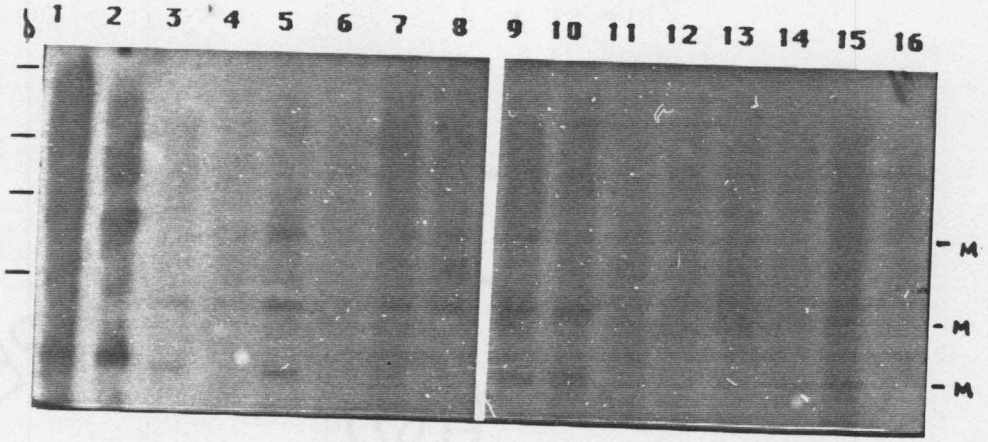
M

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
M
M
M

3. The EMBL Size-fractionated Phage Library

Hybridization of 10,000 plaques from the lambda EMBL4 recombinant library of EBS-11B6, to TU96 yielded 84 positive plaques. Ten positive plaques were examined with restriction enzymes HindIII and by double digestion with EcoRI and BamHI. Results showed that only six recombinant phage were unique. This redundancy is expected in a size-selected library. Four of the phage contained repetitive sequences in all EcoRI/BamHI and HindIII fragments and were unsuitable for further examination. Two of the phage contained at least one unique sequence fragment. One phage was assigned to chromosome 10. Southern blot hybridization of the other phage, lambda EMBL11B6-15, to the somatic cell hybrid mapping panel is shown in Figure 10. The 9.4 kbp EcoRI fragment of the phage insert hybridized to 16 kbp, 9.4 kbp, 7.0 kbp and 3.0 kbp EcoRI fragments of mouse DNA. It did not hybridize with Chinese hamster DNA. Analysis of discordancies for this cloned sequence assigns it to mouse chromosome Y.

4. Comparison of Recombinant Libraries

An evaluation of the usefulness of each of the recombinant libraries we worked with should be based on the actual recovery of chromosome 7 sequences and on the ease with which these sequences are obtained. With the lambda CLX library, 50% of the phage inserts were, potentially, unique sequences. Out of 29 such sequences, two sequences on mouse chromosome 7 were isolated. Thus one out of 15 clones were on chromosome 7, compared to the approximately one out of nine clones expected from the size of the chromosome 7 insert in the Cattanach

translocation and from the composition of the original recombinant library. This disparity is probably due to an 18% increase in non-recombinant phage and an 8% increase in inserts of unknown origin which occurred upon amplification of the library. As a result, the number of sequences that need to be analyzed in order to identify one involved in a c-locus deletion increases from 27, as previously expected, to 45. Thus, it is not surprising that none of the sequences assigned to chromosome 7 was involved in a c-locus deletion.

The effectiveness of using the somatic cell hybrid EBS-11B6 to enrich for mouse chromosome 7, and to construct libraries from which chromosome 7 sequences can be isolated, is reflected in the following results. Recovery of 2.25% of the mouse sequences present, or one mouse sequence out of 273 colonies, from the cosmid library with TU96 was decreased almost four fold from the expected results of 9%, or one out of 72 colonies. Recovery of 84 mouse sequences out of 10,000 recombinant phage in the EMBL11B6 library is 0.84%. This library was constructed from size-selected EcoRI fragments whose average length was 14,000 base pairs, therefore, representation of TU96 per insert is decreased by approximately one half over that for a cosmid library of average insert length 30,000. Thus recovery of 4.5% of the mouse sequences present from such a library using TU96 would be expected, but instead only one-fifth of this frequency were recovered. One explanation of the difference is that the frequency of occurrence of TU96-like sequences in the mouse genome was overestimated, since the 9% stated was based on a screening of only 100 mouse DNA colonies and may not be accurate. Or the hybridization and washing conditions used in the

screenings of the EBS-11B6 libraries may have been too stringent. Another explanation of the difference is that TU96-like sequences are dispersed unevenly through the mouse genome, such that any given region of the genome may have more or fewer copies of TU96 than any other region.

Weiss et al. (1984; 1986) have constructed a cosmid library from a metaphase chromosome transferrant of a neomycin resistance-marked murine major histocompatibility complex into hamster cells. They screened the library three times with TU96 as a hybridization probe, obtaining 32 TU96-hybridizing cosmids. These were separated into eight clusters of overlapping cosmids centered around the eight TU96-like sequences present in the parent transferrant line. The parent transferrant line had been estimated to contain approximately 1% of the mouse genome (roughly 10^6 - 10^7 base pairs of DNA) by dot blot hybridization to total mouse DNA and to TU96 (Nelson, 1984). From the results of those library screenings, Weiss et al estimate that TU96-like sequences occur every 150,000 base pairs in the mouse genome. That work suggests that TU96 is distributed fairly evenly in the mouse genome. In view of these results, it appears that the low yield of mouse sequences from EBS-11B6 libraries screened with the TU96 probe was probably not due to uneven dispersion of the sequence in the mouse genome.

Of the three libraries we screened, the lambda CLX library gave the best results. Sequences from the lambda CLX library are easier to develop as unique sequence hybridization probes for mapping studies on mouse genomic DNAs. They are prescreened to eliminate repetitive-sequence-containing inserts, whereas, inserts from the EBS-11B6

libraries contain at least one copy of the repetitive sequence TU96, inherent to their selection. Many of them may be derived from chromosomal regions of high repeat density. Indeed, Weiss et al. (1986) have observed this phenomena in using TU96 to probe cosmid libraries of mouse into hamster transferrants. Such repetitive sequences must be identified in cloned insert DNA to prepare uniques sequences for hybridization. Although, in the case of the c-locus region of mouse chromosome 7, a flow-sorted mouse chromosome library did give the best results, it is not as yet possible to flow-sort all the mouse chromosomes. For constructing libraries of chromosomes that cannot be sorted, somatic cell hybrid enrichment and screening with mouse-specific repetitive sequences provide a feasible approach for obtaining cloned sequences from those chromosomes.

Two cloned sequences from mouse chromosome 7 were obtained. These sequences will be examined utilizing the systems developed in Chapters IV and VI, for involvement in a c-locus deletion. Corraborative evidence, for or against inclusion, will be obtained by examining the two sequences for linkage to the c and Hbb loci using recombination data from N1 interspecific mice. Thus not only will the c-locus region be better defined, but the genetic map of mouse chromosome 7 will be extended.

CHAPTER V

DEVELOPMENT OF *MUS MUSCULUS* × *MUS SPRETUS* PROGENY FOR ANALYSIS OF THE *c*-LOCUS REGION

INTRODUCTION

All the *c*-locus deletions that extend proximal to functional unit 3 and distal to functional unit 5 are recessive lethals (see Figure 1, page 5). Stocks of animals carrying these deletions must be maintained as heterozygotes. Examination of DNA sequences in these outside regions in heterozygotes must be done by quantitation of DNA hybridization on Southern blots, a tedious and inaccurate procedure. However, if DNA polymorphisms exist which can be introduced opposite these deletions on the normal chromosome 7, then each allele could be identified by a unique hybridization band on Southern blots. Sequences missing from the deletions would be missing the hybridization band for the deleted allele when hybridized to heterozygote DNA, simplifying the identification of deleted sequences considerably.

A high rate of natural polymorphisms exists between *M. musculus* and *M. spretus* on the protein level (Bonhomme et al., 1984). This is suggestive of a high degree of polymorphism on the DNA level. Indeed, Benoit et al. (1985) have shown examples of polymorphisms between these species for actin and the light chain of myosin. The potential of *M. spretus* for introducing useful DNA polymorphisms will be explored. *M. musculus* females heterozygous for representative members of complementation groups A, Dq, Fp and Fq (see Figure 1, page 5) were

crossed to male M. spretus. One-half the progeny from these matings is expected to carry the deletion (or less than one-half if viability is affected by the heterozygous deletion). However, because the wild-type coat color of M. spretus is dominant, all progeny have wild-type coats, and deletion carriers cannot be visually identified. To identify deletion-carrying F_1 animals, two strategies were developed: (1) a backcross of F_1 animals to M. musculus homozygous chinchilla tester stock; (2) Southern blot hybridization to clone 12A, a sequence known to be included in many of the c-locus deletions.

Only female progeny of a M. musculus × M. spretus mating can be genetically tested, since male progeny are sterile (Bonhomme et al., 1984; Verne Chapman, personal communication). One-half the backcross animals should receive the spretus chromosome 7 from their mother, with its dominant wild-type allele at the c-locus, and be wild-type in color with black eyes. One half of the backcross animals should receive the musculus chromosome 7 from their mother and have an informative coat color. If the mother is a deletion carrier, no musculus allele at c will be transmitted and offspring will be hemizygous for c^{ch} (medium gray coat) somewhat reduced eye pigment). From a non-deletion mother, the allele transmitted is chinchilla, resulting in a c^{ch}/c^{ch} offspring (dark gray coat and black eyes). Thus it was possible to identify deletion carriers among the female F_1 animals by the coat color of their backcross progeny.

To identify deletion carriers among the sterile male F_1 animals, we employed clone 12A as a hybridization probe on Southern blots of F_1 animals. The 12A sequence is deleted in mutants of the Dq, Fq and Fp

complementation groups. Upon Southern blot hybridization to F_1 animals from these stocks, clone 12A should give a hybridization band for the spretus allele and another band for the musculus allele whose absence identifies deletion carriers of both sexes. The 12A sequence is not included in complementation-group A mutants and therefore, hybridization to F_1 animals from such stocks would not result in any missing hybridization band by which deletion carriers are identified.

MATERIALS AND METHODS

All matings were done at the Oak Ridge National Laboratory under the supervision of Dr. Liane B. Russell and Clyde Montgomery. Male Mus spretus were provided by Dr. Verne Chapman of Roswell Park Memorial Institute. All other mice were provided by Dr. Liane B. Russell (Oak Ridge National Laboratory). The deletion stocks 26DVT (complementation group Fp), 12 FR60Hb (complementation group Fq), 146G (complementation group Dq), an 14CoS (complementation group A) were maintained as (c^*/c^{ch}) at the c locus on chromosome 7, where c* indicates the appropriate deletion. The general mating scheme followed for each deletion is outlined below:

Mus musculus (c^*/c^{ch}) × Mus spretus (+/+)

Expected progeny:

$c^*/+$: one half of progeny, wild-type with black eyes.

$c^{ch}/+$: one half of progeny, wild-type with black eyes.

F_1 ($\underline{c}^*/+$) × *Mus musculus* 2A ($\underline{c}^{ch}/\underline{c}^{ch}$)

Expected backcross progeny:

$\underline{c}^*/\underline{c}^{ch}$: one half of progeny, medium gray with somewhat reduced eye pigment.

$+/\underline{c}^{ch}$: one half of progeny, wild-type with black eyes.

F_1 ($\underline{c}^{ch}/+$) × *Mus musculus* 2A ($\underline{c}^{ch}/\underline{c}^{ch}$)

Expected backcross progeny:

$\underline{c}^{ch}/\underline{c}^{ch}$: one half of progeny, dark gray with black eyes.

$+/\underline{c}^{ch}$: one half progeny, wild-type with black eyes.

DNA was isolated from the liver or brain of mice by the following procedure. Tissue was minced in 5 ml of ice cold RSB (10 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$), set on ice for five min, homogenized with a Potter-Elbejheim homogenizer. Nuclei were pelleted twice with ice cold TNE, then resuspended in 2 ml ice-cold TNE. 2 ml of 1% SDS, 100 mM NaCl, and 300 μg/ml proteinase K were added to lyse the nuclei. The reaction was incubated at 50°C overnight. Extraction, twice against equal volumes of TE-saturated phenol and twice against equal volumes of chloroform was followed by precipitation in 50% isopropanol at room temperature. DNA was wound on a glass hook, dipped on the hook in two changes of 70% ethanol to get rid of residual phenol and chloroform, then air dried. DNA was resuspended in 1 ml of TE. Southern blot hybridization of mouse genomic DNA was carried as described in

Chapter II (page 15). Insert DNA from clone 12A was prepared from a complete EcoRI digestion run on a 1% low gelling temperature agarose gel. The insert band was excised from the gel and labelled by the random primer method described in Chapter II (page 16).

RESULTS AND DISCUSSION

The pedigrees of animals obtained from the *M. musculus* × *M. spretus* matings are shown in Figure 11. We obtained sixteen F_1 animals from crosses of A-group *c*-deletion animals to *M. spretus*, four from an Fp-group cross, four from a D cross, and five from an Fq cross. Close to the expected 1:1 ratio of female to male progeny was obtained. Out of 29 F_1 animals, 15 were female and 14 were male.

The results of the backcross matings of these female F_1s are listed in Table 4. Females number 3, 6, 10, 11, 16, 18, 19, 35 and 42 bore progeny of $+/c^{ch}$ (wild-type coat) and c^{ch}/c^{ch} (chinchilla coat) genotypes. This indicates that these females carry the chinchilla allele and not the *c*-locus deletion on their *musculus* copy of chromosome 7. Females number 14, 15, 20, 41 and 43 bore progeny of $+/c^{ch}$ (wild-type coat) and c^{ch}/c^{*} (chinchilla-albino coat) genotypes. They carry the *c*-locus deletion on their *musculus* copy of chromosome 7. Those females identified as deletion carriers by their backcross progeny are indicated by solid black circles in Figure 11.

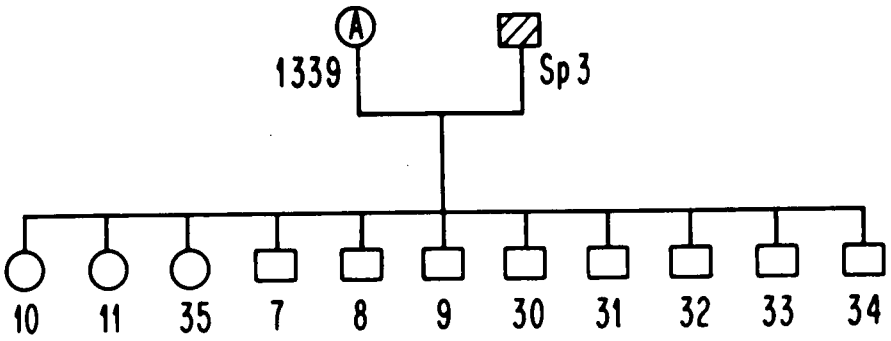
Figure 12 shows the results of hybridization of clone 12A to Southern blots of *M. spretus*, *M. musculus* and F_1 animal DNAs digested with five different restriction enzymes to reveal DNA polymorphisms

Table 4: Results of Backcross Matings for c-Deletion *M. musculus* × *M. spretus* F_1 Females

F_1	Total Progeny	Female/Male	Genotype (Coat Color)		
			+/+ (wt) Female/Male	c^{ch}/c^{ch} (chin) Female/Male	c^{ch}/c^{*} (chin-alb) Female/Male
3	4	2/2	0/2	2/0	0/0
6	1	0/1	0/0	1/0	0/0
5	Sterile	---	---	---	---
10	8	3/5	3/2	0/3	0/0
11	5	2/3	1/2	1/1	0/0
14	9	3/6	0/6	0/0	3/0
15	6	3/3	2/2	0/0	1/1
16	4	1/3	0/1	1/2	0/0
18	5	0/5	0/2	0/2	0/0
19	7	4/3	3/1	1/2	0/0
20	11	6/5	3/1	0/0	3/4
35	2	0/2	0/1	0/1	0/0
41	5	1/4	0/3	0/0	1/1
42	3	3/0	1/0	2/0	0/0
43	5	2/3	0/1	0/0	2/2

F_1 animals are identified by their unique animal number. wt, wild-type coat; chin, chinchilla coat; chin-alb, chinchilla-albino coat. Genotypes given are at the c locus.

Figure 11. Pedigrees of c-locus deletion stock to M. spretus. Circles indicate females, squares indicate males. M. spretus males are indicated by cross-hatched squares. Letters inside circles of female parents indicate the complementation group of which stock is representative of: A--stock is 14CoS; Dq--stock is 146G; Fp--stock is 26DVT; Fq--stock is 12FR60Hb. F_1 animals identified as deletion carriers by backcrosses to tester stock are indicated by black circles. Animals identified as carriers by hybridization with clone 12A are indicated by a line under that animal's symbol. S--indicates sterile. Progeny of female 1339 and spretus male 3 include two litters. All other pedigrees are for a single litter.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Sp 3
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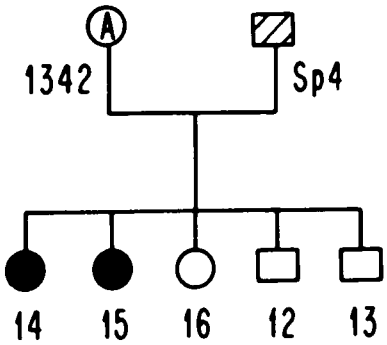
33
34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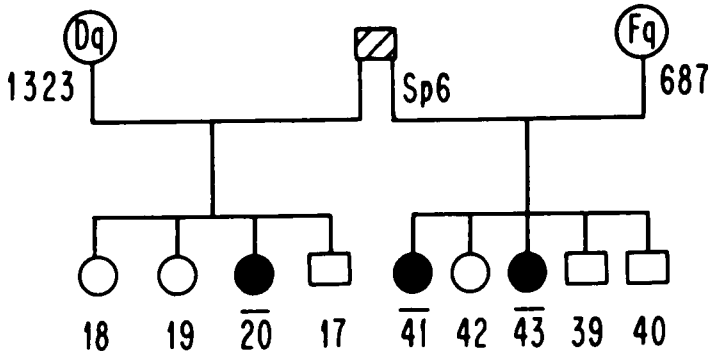
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Sp6
Fq
687
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42
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40

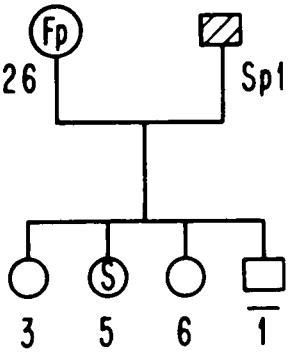
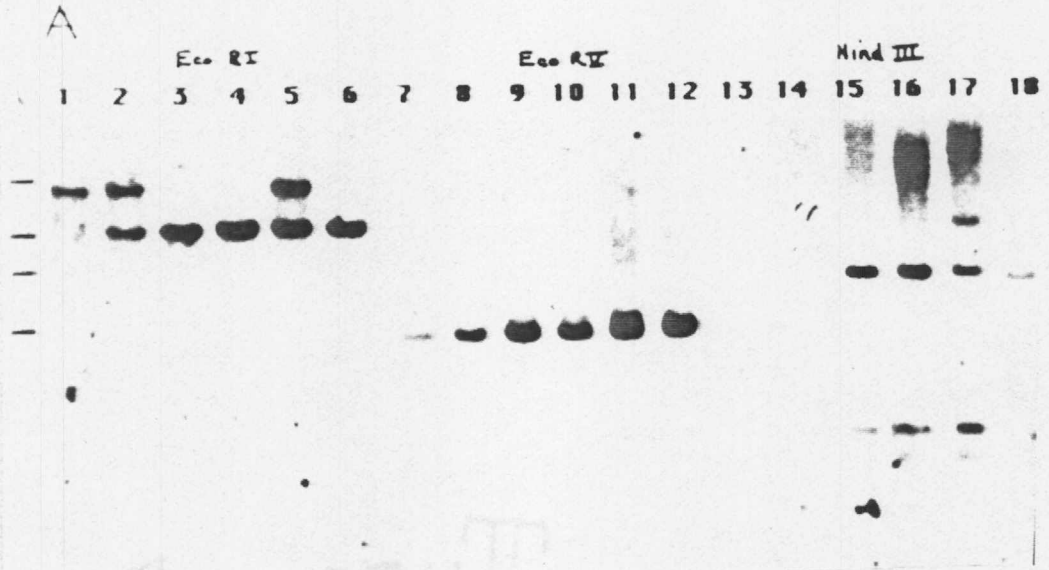

Fp
1226
Sp1
S
3
5
6
1

Figure 12. Hybridization of clone 12A sequence to M. musculus and M. spretus to identify RFLPs. Probe is random primer labelled plasmid 12A. 10 μg DNA was digested with the restriction enzyme indicated, then loaded in a single lane. DNAs are loaded in identical sets of six in the order male spretus, male spretus, C57BL/6, BALB/c, F1-19 (normal sib of the 146G c-deletion stock × M. spretus), and F1-44 (C57BL/10 × M. spretus).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Eco RI
Eco RII
Hind III
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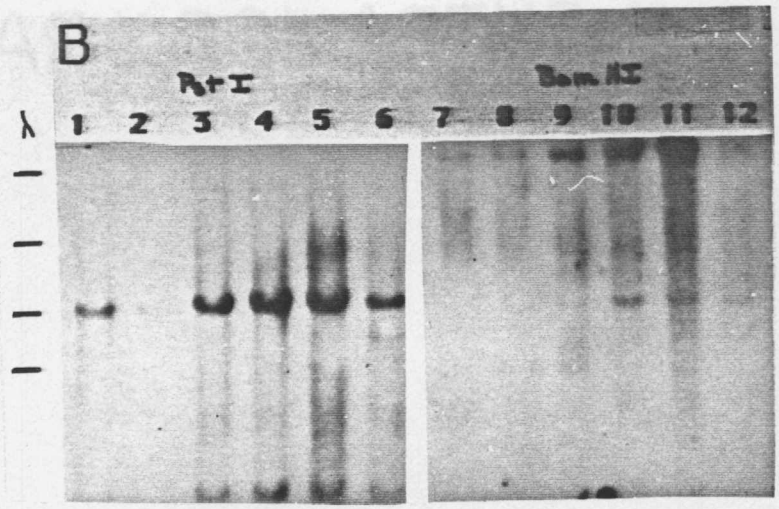

B
PstI
BamHI
λ
1 2 3 4 5 6 7 8 9 10 11 12

between the musculus and the spretus alleles. Hybridization to EcoRI digested DNAs gave two hybridization bands (Figure 12, panel A, lanes 1-6). The upper band is present only in spretus mice. The lower band is present in one of the spretus mice and in C57BL/6 and BALB/c musculus mice. Thus both alleles visualized by the EcoRI restriction fragment length polymorphism (RFLP) are present in the spretus population. But only the allele visualized by the lower band is present in the musculus population. As can be seen in lanes 5 and 6, depending on the paternal spretus allele inherited, musculus × spretus F_1 animals will be either heterozygous or homozygous. This RFLP can identify deletion carrying F_1 animals only when the animal is a heterozygote. It is uninformative in homozygous animals.

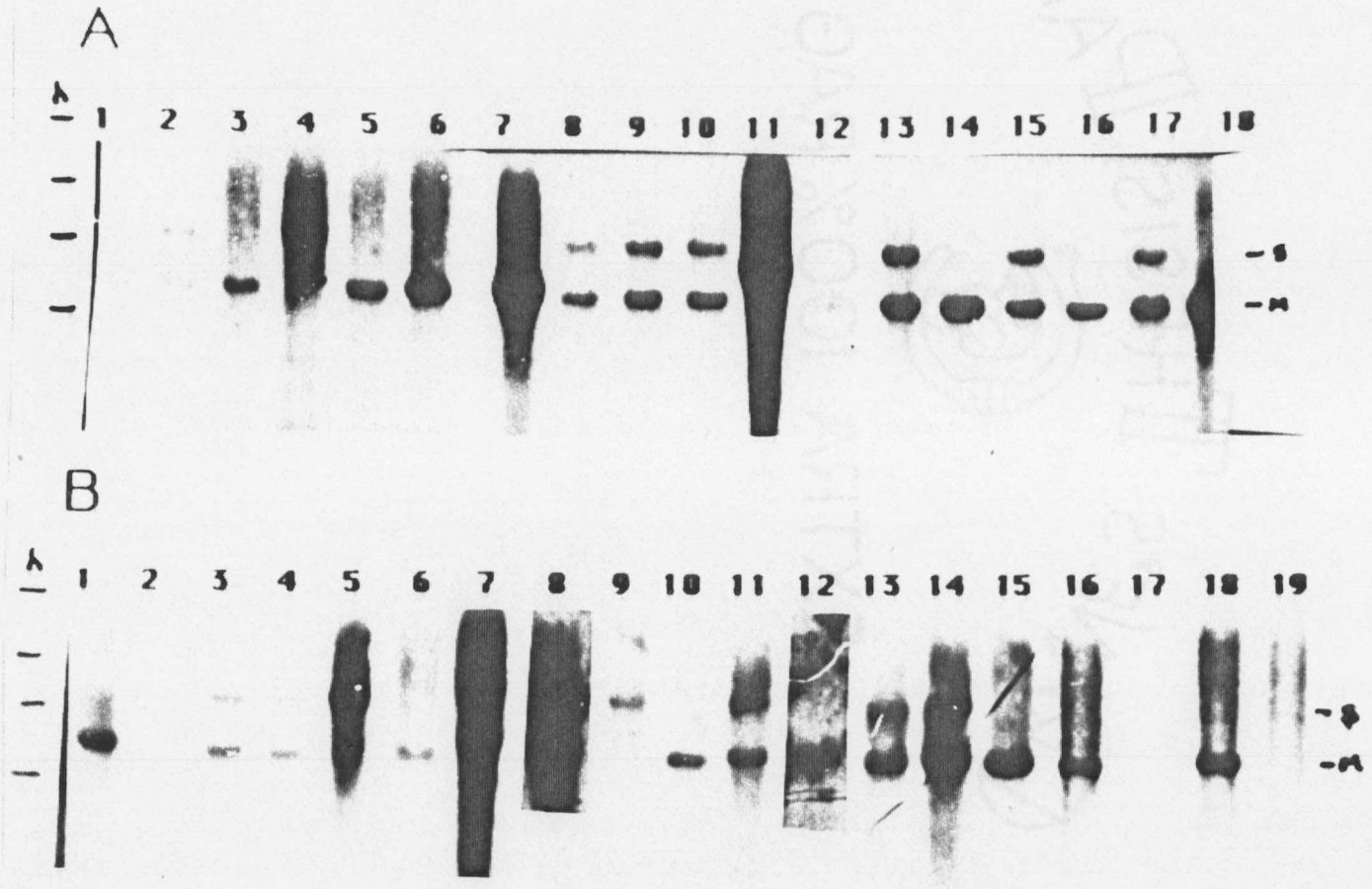
Hybridization of clone 12A to HindIII-digested DNAs gave four hybridization bands, as shown in Figure 12, panel A, lanes 7-12. The lower three bands, representing the a allele, are present in the spretus and the musculus populations. The upper band, representing the b allele, is unique to the spretus population. Lanes 11 and 12 show hybridization to F_1 animals. As with the RFLP for EcoRI, this HindIII polymorphism may or may not be present in F_1 animals, depending on which paternal spretus allele was inherited. Thus this RFLP can only be used to identify deletion carrying F_1 animals from among the heterozygotes. No RFLPs were found with restriction enzymes EcoRV, PstI and BamHI (Figure 12A, lanes 13-18 and 12B, lanes 1-12).

Since none of the restriction enzymes gave restriction fragments polymorphic throughout the M. musculus and M. spretus populations examined, the EcoRI RFLP was used to analyze c-deletion musculus

× spretus F_1 animals. Figure 13 shows the results of hybridization of clone 12A to Southern blots of the F_1 animals. Females 20, 41 and 43 and male 1 have only the top band characteristic of the b allele (Figure 13, lanes 32, 11, 10, and 4, respectively). Since this allele is unique to the spretus population, we know that it represents the paternal chromosome 7. Indeed, this is further born out by the hybridization pattern of the c-deletion heterozygous mothers of these F_1s. They are all homozygous for the a allele characterized by the lower fragment and so could not contribute the b allele characterized by the upper fragment (Figure 13, lanes 28, 7 and 3, respectively). Thus animals 20, 41, 43 and 1, where the maternal allele, a, is missing, are indicated as carriers of their respective c-locus deletion. Females 20, 41 and 43 were also identified as deletion carriers by the coat colors of their backcross progeny. Male 1 was sterile, as are all F_1 males, and so could not be tested for the presence of a c-locus deletion by a backcross. Thus hybridization of the known c-deletion sequence in clone 12A to F_1 animals did identify a male carrier of the Fp-group deletion, 26DVT, which would not otherwise have been identified.

These initial trials at identifying c-deletion carriers by Southern blot hybridization to the sequence in clone 12A reveal some drawbacks. The first drawback is that since this sequence is located in the overlap region of complementation groups B and E, which is not included in complementation group A (Disteche and Adler, 1984), it will not be absent from animals carrying deletions from complementation groups V, A, or Ai. Thus it cannot be used to identify deletion carriers among musculus × spretus F_1 animals produced from stocks of deletions

Figure 13. Hybridization of clone 12A to c-deletion *M. musculus* × *M. spretus* families. M-indicates *Mus musculus* fragments; S-indicates *Mus spretus* fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). 10 μg DNA was digested with EcoRI and loaded in a single lane. A. Lane 1. C57BL/6. Lane 2. Male *spretus*. Lane 3. 26DVT carrier female 1226, mother of F_1-1, 3, and 6. Lane 4. F_1-1, 26DVT c-deletion carrier. Lane 5. F_1-3. Lane 6. F_1-6. Lane 7. 14CoS-carrier female 1339, mother of F_1-7 through F_1-11 and F_1-30 through F_1-35. Lane 8. F_1-7. Lane 9. F_1-8. Lane 10. F_1-9. Lane 11. F_1-10. Lane 12. F_1-11. Lane 13. F_1-30. Lane 14. F_1-31. Lane 15. F_1-32. Lane 16. F_1-33. Lane 17. F_1-34. Lane 18. F_1-35. B. Lane 1. 146G-carrier female 1323, mother of F_1-17 through 20. Lane 2. F_1-17. Lane 3. F_1-18. Lane 4. F_1-19. Lane 5. F_1-20, 146G c-deletion carrier. Lane 6. 12FR60Hb-carrier female 687, mother of F_1-39, 40, and 43. Lane 7. F_1-39. Lane 8. F_1-40. Lane 9. F_1-43, 12FR60Hb c-deletion carrier. Lane 10. 14CoS-carrier female 1342, mother F_1-12 through 16. Lane 11. F_1-12. Lane 12. F_1-13. Lane 13. F_1-15, 14CoS c-deletion carrier. Lane 14. F_1-16. Lane 15. F_1-14, 14CoS c-deletion carrier. Lane 16. A × C. Lane 17. B × E. Lane 18. A × A. Lane 19. C × E.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

belonging to any of these complementation groups. Indeed, lanes 11-15 of Figure 13A and 2-12 of Figure 13B, show hybridization of clone 12A to sixteen F_1 animals from the cross 14CoS (A-group) × spretus. All of the animals possess both maternal and paternal copies of the clone 12A sequence. Notable among these heterozygotes is female 15. Female 15 was shown to be a deletion carrier by the coat colors of her backcross progeny, and yet she has both the paternal upper band and a maternal lower band. This verifies that the clone 12A sequence cannot identify deletion carriers from the A-group deletions.

The previous assignment of clone 12A to overlapping-deletion offspring of the B × E cross was verified by Southern hybridization, as shown in Figure 13. There is no hybridization of clone 12A to the B × E animal, indicating its absence in that animal. There is hybridization to the A × A animal. These data place the clone 12A sequence somewhere within functional groups 4 or 5. When this work was initiated, it was not known whether the clone 12A sequence was deleted in animals carrying C-group deficiencies. Lanes 16 and 18 show hybridization of clone 12A to offspring from A × C and to E × C crosses, which carry overlapping deletion. There is hybridization of the sequence to the DNA of A × C, indicating that the clone 12A sequence lies outside of functional group 3, the overlap region of the A and C complementation groups. Absence of hybridization to E × C and B × E animals suggests that the sequence lies within functional group 4, previously characterized only by juvenile survival. Table 5 summarizes the results of the Southern blot hybridization to clone 12A. It shows that there is no discordancy with assignment of the 12A sequence to functional group 4. All other groups

Table 5: Correlation Between Hybridization and c-Locus Functional Group Deletion

Functional Group	Functional Group/ Hybridization +/+	-/-	+/-	-/+	Percent Discordancy	Hybridization of Clone 12A to:	
1	3	2	0	3	37.5	R5D Hybrids	-
2	1	3	2	2	50	14CoS	+
3	0	5	3	0	37.5	146G	-
4	3	5	0	0	0	12FR60Hb	-
5	3	4	0	1	12.5	A × A	+
6	3	3	0	2	25	E × C	-
7	3	3	0	2	25	A × C	+
8	3	2	0	3	37.5	B × E	-

Functional group numbers follow the designation illustrated in Figure 1 (page 5). "+" indicates presence of the functional group sequences and presence of hybridization to clone 12A; "-" indicates the absence of the functional group and absence of hybridization to clone 12A. Percent discordancy is calculated as the total number of +/- and -/+ divided by the total number of hybridizations examined. Data are based on hybridization to the eight animals and cell lines listed in the right side of the Table.

give a greater than 12.5% discordancy between presence of the functional group and presence of clone 12A.

Another drawback to identification of deletion-carrying F_1 animals by hybridization to clone 12A, is demonstrated by the hybridization pattern obtained in females 3, 6, 10, 14, and 35, and in males 13, 31, and 33 in Figure 13. All these animals are homozygous a/a for 12A. In them the maternal musculus allele cannot be distinguished from the paternal spretus allele and therefore, a deletion of the musculus allele would not be detected. This points out the difficulty arising from heterozygosity within the spretus population for the RFLP of clone 12A. If the a allele, represented by the lower hybridization band, were not shared between musculus and spretus, then only heterozygous a/b F_1 animals would exist and all deletion carriers of the complementation groups that include functional group 5 would be identifiable by hybridization to clone 12A. Thus, this difficulty could be avoided if a prescreening of DNA isolated from the tail or ears of the spretus males were done in order to identify homozygous b/b individuals for use in the matings to deletion-carrying musculus females. This would assure that all F_1 animals were heterozygous a/b for clone 12A. We were fortunate in that all the F_1 males from the Fp, Dq and Fq groups, were heterozygous for clone 12A. Therefore, they were classified as deletion or non-deletion carriers on the basis of hybridization to clone 12A. Not all the F_1 females from the Fq, Dq and Fp groups were heterozygous. Females 3 and 6 were homozygous for the allele represented by the lower hybridization band and, were it not for the backcross progeny data, they would not have been typed.

Genetic identification of deletion carriers from among the F_1 animals by a backcross also has its drawbacks. First, it is time consuming to allow the F_1 females to mature so that they can be mated and bear progeny that reveal the maternal genotype. Second, the method only works for fertile F_1 animals and, therefore, cannot identify carriers among the male F_1 and the sterile female F_1 animals (such as female 5). The difficulties in identifying deletion-carrying F_1 animals with both methods (hybridization to deletion clone 12A and backcross progeny) point out the importance of utilizing both methods simultaneously to ensure identification of a maximum number of carriers and verification of carrier status from two independent sources for those animals in which both methods work.

Mus musculus × *Mus spretus* F_1 animals carrying the $\underline{c^{14CoS}}$, $\underline{c^{146G}}$, $\underline{c^{26DVT}}$, and $\underline{c^{12FR60Hb}}$ deletions were obtained. These deletions are missing functional units 2 and 3, 1 through 7, 2 through 8 and 1 through 8, respectively. In these interspecies deletion carriers, the outlying regions of functional units 1, 2, 6, 7 and 8 are accessible. Examination of these animals by Southern blot hybridization to the mouse chromosome 7 sequences described in Chapter IV for which an RFLP between *M. musculus* and *M. spretus* was identified, will reveal the involvement of any of those sequences in any of the deletions. Each allele is characterized by a specific hybridizing fragment. Sequences absent from one of the deletions would be missing the hybridization band characteristic of the deleted maternal *M. musculus* allele, thus identifying that sequence as a part of the region deleted in the mutation.

A genetic approach to provide access to recessive lethal mutations for molecular studies has been developed. This approach can be applied to not only the c-locus deletions as presented here, but also to other recessive lethal mutations available in mice. A general scheme would consist of mating female Mus musculus heterozygous for the mutation of interest, to male Mus spretus. Deletion animals among the resulting F_1s would be identified by a serological, biochemical or phenotypic assay if available, or by Southern blot hybridization to a sequence from the region. Deletion carrying female F_1s would also be identified by a backcross to a Mus musculus strain whose genetic background allows detection of heterozygotes for the mutation of interest either by the phenotype or by the biochemical/serological assay by which the mutation was originally characterized. Using this approach, many recessive lethal mutations which cannot be studied at present on the molecular level, could be made accessible. This approach should be especially beneficial for studying early developmental recessive lethal mutations, such as are available at the c, p and d loci.

CHAPTER VI

ANALYSIS OF CHROMOSOME 7 SEQUENCES FOR INCLUSION IN c-LOCUS DELETIONS

INTRODUCTION

Two approaches for identifying sequences missing from c-locus deletions were developed in this work. The first approach, outlined in Chapter II, utilizes mouse-Chinese hamster somatic cell hybrids in which the only mouse chromosome-7 sequences retained reside on a T(X;7def)5RL7 chromosome, where the deficiency present is the Fp-group c-locus deletion, 26 DVT. The second approach, outlined in Chapter V, utilizes *Mus musculus* × *Mus spretus* F_1 animals that carry various c-locus deletions. Both approaches are designed to place the deletion-carrying *musculus* chromosome 7 in a polymorphic background so that all *musculus* alleles native to chromosome 7 can be visualized as unique hybridization bands on Southern blots. Then the absence of any sequence from the c-deletion can be identified immediately by an absence of the hybridization band that represents the *musculus* allele. In Chapters III and V, the legitimacy of both approaches was demonstrated by utilizing a known c-locus deletion sequence contained in plasmid clone 12A. In Chapter IV, the basis and methods of acquisition were described for nine chromosome 7 sequences for which a map position was not known. In this chapter the two approaches developed were applied to these unmapped chromosome-7 sequences to determine which, if any, are included in a c-locus deletion.

Unmapped chromosome-7 sequences were hybridized to Southern blots of the R5D and R6 somatic cell hybrids. The hybridization pattern of the bands associated with the musculus copy of a sequence was compared to that of the c-deletion clone 12A. Concordance between the two sequences' hybridization indicates the inclusion of that sequence in the large Fp-group c-locus deletion, 26DVT. Any sequence indicated by the somatic cell hybrids as being deleted was hybridized to Southern blots of Mus musculus, Mus spretus and two typical F_1 animal DNAs digested with six different restriction enzymes in order to detect a restriction fragment length polymorphism (RFLP). Once an RFLP for the sequence was identified, the sequence was hybridized to Southern blots of genomic DNA from c-locus deletion × M. spretus F_1 animals. Absence of the musculus allele of the sequence examined from an F_1 animal indicates inclusion of that sequence in the c-locus deletion carried by the F_1 animal.

A summary of the functional units expected to be absent from the various cell lines and animals utilized is shown in Table 6. Since the 26DVT deletion spans functional units 2 through 8 (Figure 1, page 5), Southern blot hybridization to the deletion-carrying somatic cell hybrids should identify sequences originating in any of these groups. However, the somatic cell hybrids do not provide data that would determine to which of the series of functional units the sequence belongs. Also sequences originating in functional unit 1 would not be identified by the somatic cell hybrids. Southern blot hybridization to the F_1 animals would allow assignment of a deleted sequence to functional unit 1 or 8. Hybridization data from the F_1 animals alone would not distinguish between sequences in units 2 and 3, or between

Table 6: Distribution of c-Locus Functional Units in the Cell Lines and Animals Examined

	Functional Unit:							
	1	2	3	4	5	6	7	8
R5D-1A	+	-	-	-	-	-	-	-
R5D-1B	+	-	-	-	-	-	-	-
R5D-5	+	+	+	+	+	+	+	+
R5D-9A	+	-	-	-	-	-	-	-
R5D-9B	+	-	-	-	-	-	-	-
R5D-14	+	-	-	-	-	-	-	-
R6	+	+	+	+	+	+	+	+
EBS-11B6	+	+	+	+	+	+	+	+
C57,BALB/c,DBA	+	+	+	+	+	+	+	+
A × A	+	-	-	+	+	+	+	+
A × C	+	+	-	+	+	+	+	+
B × E	+	+	-	-	-	+	+	+
C × E	+	+	-	-	+	+	+	+
F1(26DVT)	+	-	-	-	-	-	-	-
F1(12FR60Hb)	-	-	-	-	-	-	-	-
F1(146G)	-	-	-	-	-	-	-	+
F1(14CoS)	+	-	-	+	+	+	+	+

Functional units are as shown in Figure 1 (page 5).

Contribution of functional units is derived from the presence of a normal mouse chromosome 7 present in these somatic cell hybrids.

Contribution of functional units is derived from the presense of an intact T(X;7)6RL7 mouse chromosome in this somatic cell hybrid.

"+"--presence of Mus musculus copy of functional unit. "-"--absence of Mus musculus copy of functional unit.

sequences in units 4, 5, 6, and 7. To distinguish between these units, sequences were hybridized to Southern blots of offspring from A × A, C × E, E × C, A × C, and B × E crosses. When hybridization data from the M. musculus × M. spretus F_1 animals is supplemented by data from these animals, it is possible to distinguish between the sequences of each of the functional units, except between those of units 6 and 7.

MATERIALS AND METHODS

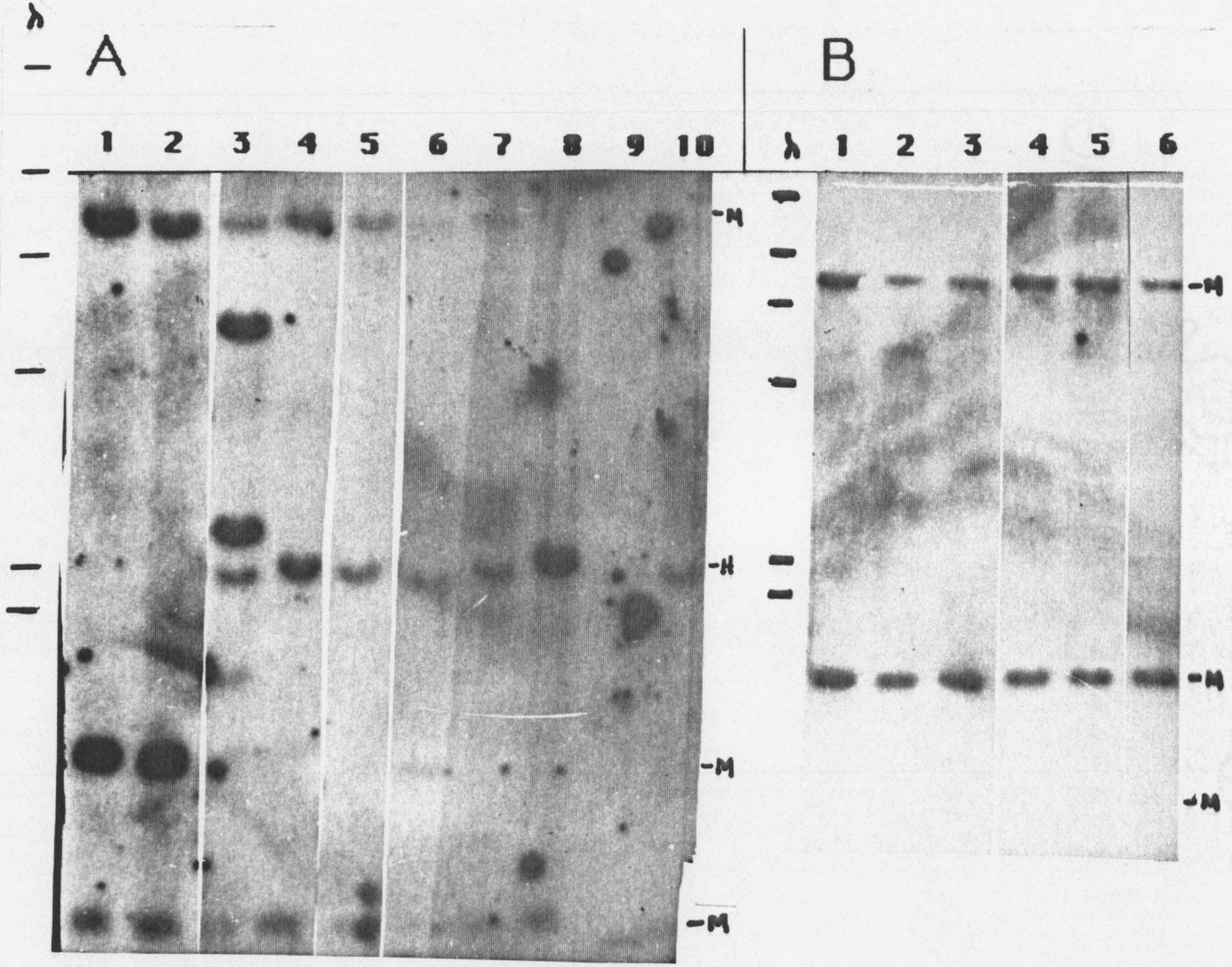
The albino-region deletion mice were provided by Dr. Liane B. Russell (Oak Ridge National Laboratory). Mus spretus males for matings were provided by Dr. Verne Chapman (Roswell Park Memorial Institute). All matings were performed under the supervision of Dr. Liane B. Russell and Clyde Montgomery at the Oak Ridge National Laboratory. Mouse-Chinese hamster somatic cell hybrid lines R6 and R5D-1A, -1B, -5, -9A, -9B, and -14 were as described in Chapter II. DNA from somatic cell hybrids was prepared from the pelleted nuclei during preparation of cell extracts, as described in Chapter II (page 15). F_1 animals from c-locus deletion Mus musculus × Mus spretus matings were derived as described in Chapter V. These include the progeny of 14CoS (complementation group A), 146G (group Dq), 26DVT (group Fp) and 12FR60Hb (group Fq) females. The A × A animal was derived from the 1FaFyh deletion stocks. The C × E an E × C animals were derived from the 20FATw (group C) and the 65K (group E) deletion stocks. The A × C animals were derived from the 14CoS (group A) and the 20FATw (group C) deletion stocks. The B × E animal was derived from the 5FR60Hg (group Bi) and the 112K (group E)

deletion stocks. DNA from F_1, Mus spretus and Mus musculus deletion animals was isolated as described in Chapter IV (page 57). Southern blot hybridization to restriction enzyme digested genomic DNAs was performed as described in Chapter II (page 16). DNA sequences used as hybridization probes on Southern blots were as described in Chapter IV.

RESULTS AND DISCUSSION

Figure 14 shows the hybridization of plasmid clone pI47 (insulin cDNA) to the R5D and R6 somatic cell hybrid lines, to Mus musculus and Mus spretus animals and to viable homozygous and overlapping deletion animals. Plasmid clone pI47 was known to hybridize to three EcoRI restriction fragments in the mouse. Chirgwin et al. (1983) mapped a 8.4 kbp EcoRI fragment to mouse chromosome 7, a 1.48 kbp fragment to mouse chromosome 6, and a 0.9 kbp fragment to mouse chromosome 15, using somatic cell genetics. We followed the hybridization pattern for the 8.4 kbp chromosome 7 locus, Ins-2. Hybridization of pI47 to the 8.4 kbp EcoRI fragment is present in R5D-1A, -1B, -5, and -14, and in R6 and EBS-11B6 (Figure 14A) and absent in R5D-2 and EBS-4. This hybridization pattern is consistent with the assignment of this sequence to mouse chromosome 7, but is inconsistent with its inclusion in the 26DVT c-locus deletion. These data are in agreement with hybridization of pI47 to homozygous A-group and overlapping A × C and C × E animals (Figure 14B). The presence of the 8.4 kbp EcoRI fragment in all of these animals is consistent with a mapping of that sequence outside these c-locus deletions. The deletions of the Fp (26DVT), A, C and E

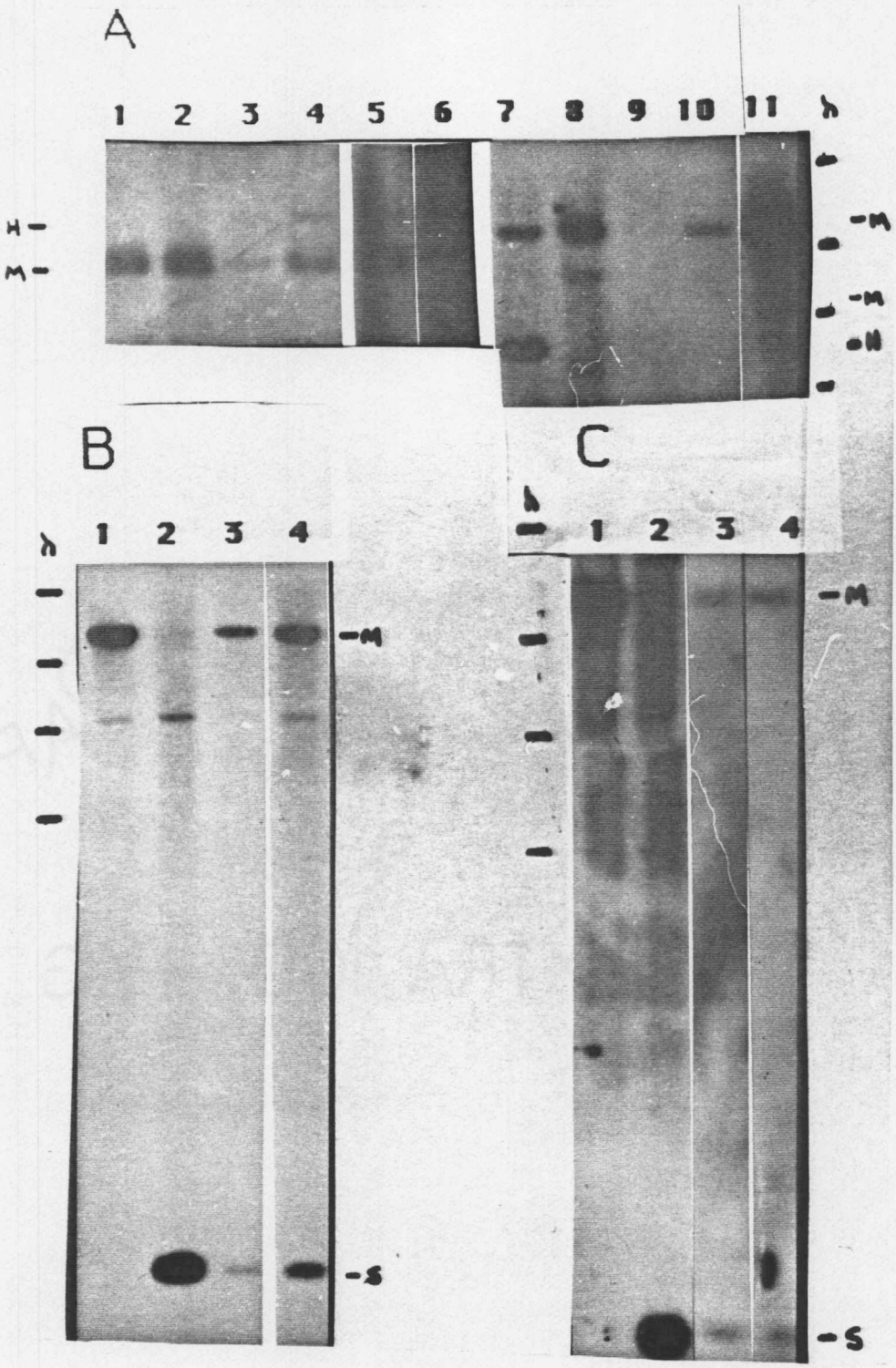
Figure 14. Hybridization of the insulin cDNA clone pI47 to somatic cell hybrids, interspecies hybrid animals and viable homozygous and overlapping c-deletion animals. Probe is random primer labelled plasmid pI47. M-indicates Mus musculus fragments; S-indicates Mus spretus fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. C57BL/6. Lane 2. DBA. Lane 3-6. R5D-1A, R5D-1B, R5D-5, R5D-14, respectively, somatic cell hybrids from the fusion of spleen cells of a male mouse carrying a T(X;7def)5RL7 chromosome to E36 Chinese hamster cells. Lane 7. R6, a somatic cell hybrid from a fusion of spleen cells of a male mouse carrying a T(X;7)6RL7 chromosome to E36 Chinese hamster cells. Lane 8. E36, an HPRT deficient Chinese hamster cell line used as the parental cell line for the R5D hybrids and the R6 hybrid. Lane 9. EBS-4, a somatic cell hybrid from the fusion of BALB/c spleen cells to E36, retaining mouse chromosomes 13, 15, 17 and X. Lane 10. EBS-11B6, derived from EBS-11, a BALB/c XE36 somatic cell hybrid, by back-selection in 6-thioguanine. B. Viable homozygous and overlapping c-deletion animals. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. Tester stock 22A. Lane 2. C57BL/6. Lane 3. DBA. Lane 4. A × A. Lane 5. A × C. Lane 6. C × E.


λ
A
1 2 3 4 5 6 7 8 9 10
-M
-M
-M
B
λ 1 2 3 4 5 6
-M
-M
-M

complementation groups examined include all the functional units defined for the c-locus deletions except functional unit 1. Thus none of the above data are informative for unit 1. In order to obtain data on the inclusion or exclusion of a sequence from functional unit 1, the interspecific F_1 146G (complementation group Dq) and F_1 12FR60Hb (complementation group Fq) deletion carriers were examined. The first step in obtaining data from these animals is to identify an RFLP for the sequence in question. The hybridization pattern for pI47 with genomic *Mus musculus*, *Mus spretus*, and *musculus* × *spretus* F_1 DNAs digested with six different restriction enzymes: EcoRI, HindIII, BamHI, EcoRV, PstI and TaqI. None of the six enzymes showed any difference in hybridization pattern between *M. musculus* and *M. spretus* for the fragment corresponding to the *Ins-2* locus on mouse chromosome 7. Thus it appears that the chromosome 7 sequence identified by pI47 is highly conserved between the two species. Lacking an RFLP, no deletion data for functional unit 1 was obtainable for the *Ins-2* locus. A more extensive search of restriction enzymes might yield an RFLP which could be used to obtain data for unit 1.

Figure 15A shows the hybridization pattern for the somatic cell hybrids with plasmid H19. This sequence hybridizes to a 10 kbp EcoRI fragment and 10 kbp and 6.5 kbp BamHI fragments in mouse DNA. It hybridizes weakly to a 24 kbp EcoRI fragment in Chinese hamster DNA. The pattern of hybridization is the same as for the insulin clone pI47--hybridization to R5D-1A, -1B, -5, and -14, to R6, EBS-11B6 and C57. In addition, data for the hybrid R5D-9A was obtained for H19, to which there was also hybridization. These data are consistent with a

Figure 15. Hybridization of H19 to somatic cell hybrids and interspecies hybrid animals. Probe is random primer labelled 350 bp PstI insert fragment of clone H19. M-indicates Mus musculus fragments; S-indicates Mus spretus fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with EcoRI in lanes 1-6 and with BamHI in lanes 7-10, then loaded in a single lane. Lane 1. C57BL/6. Lane 2. Tester stock 22A. Lane 3-7. R5D-1A, R5D-1B, R5D-5, R5D-14, R5D-9A, respectively, somatic cell hybrids from the fusion of spleen cells of a male mouse carrying a T(X;7def)5RL7 chromosome to E36 Chinese hamster cells. Lane 8. R6, a somatic cell hybrid from a fusion of spleen cells of a male mouse carrying a T(X;7)6RL7 chromosome to E36 Chinese hamster cells. Lane 9. E36, an HPRT deficient Chinese hamster cell line used as the parental cell line for the R5D hybrids and the R6 hybrid. Lane 10. EBS-11B6, derived from EBS-11, a BALB/c × E36 somatic cell hybrid, by back-selection in 6-thioguanine. Lane 11. C57BL/6. B. Identification of an RFLP. 10 μg DNA was digested with BamHI and loaded in a single lane. Lane 1. Male spretus. Lane 2. C57BL/6. Lanes 3 and 4. M. musculus × M. spretus F_1 animals. C. 12FR60Hb c-deletion carrier. 10 μg DNA was digested with BamHI and loaded in a single lane. Lane 1. Male spretus. Lane 2. C57BL/6. Lane 3. F_1 female 41, 12FR60Hb c-deletion carrier. Lane 4. F_1 female 42, normal sib.


A
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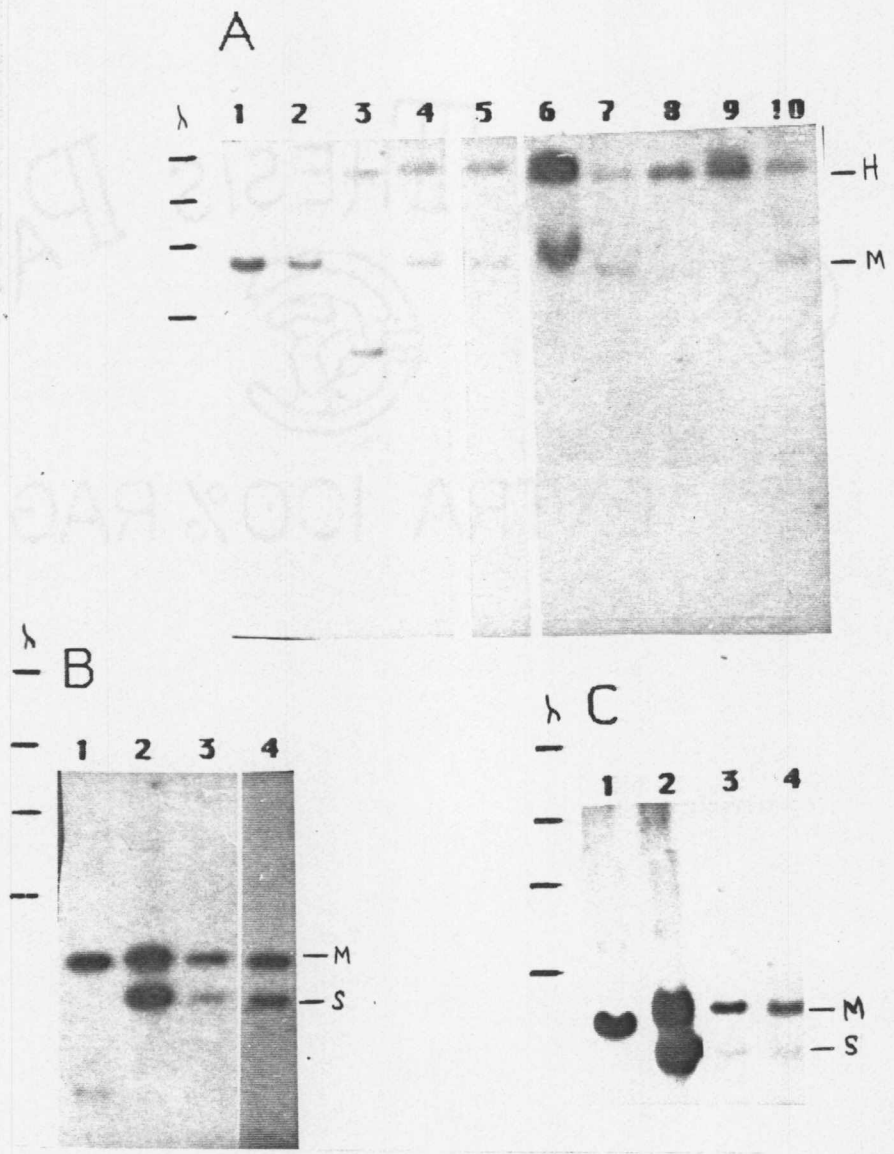
mapping of the H19 sequence outside the 26DVT deletion and therefore outside functional units 2 through 8. To obtain data for functional unit 1, we hybridized H19 to Southern blots of the genomic DNAs of Mus musculus and Mus spretus digested with six different restriction enzymes. A suitable RFLP was identified with the restriction enzyme BamHI (Figure 15B). Mus musculus carries a unique 10 kbp BamHI fragment hybridizing to H19, designated as the a allele. Mus spretus has a unique 0.8 kbp BamHI fragment hybridizing to H19, designated the b allele. Hybridization of clone H19 to Southern blots of BamHI digested genomic DNA of F_1 animals from the Fq complementation group, represented by its sole member 12FR60Hb, is shown in Figure 15C. Female 41 was shown by her backcross progeny and by hybridization to deletion clone 12A, to be a c-locus deletion carrier. Her sib, female 42, was shown to carry an intact musculus chromosome 7. Clone H19 hybridizes to the 10 kbp BamHI fragment of the a allele and the 0.8 kbp BamHI fragment of the b allele in both female 41 and 42. This is consistent with the exclusion of the H19 sequence from functional group 1. Thus the data map the H19 sequence outside all of the known c-locus deletions.

Hybridization of plasmid clone BS9, containing Harvey murine sarcoma virus sequences, to the somatic cell hybrids is shown in Figure 16A. BS9 hybridizes to a 4.7 kbp HindIII fragment in mouse DNA and a 14.5 kbp HindIII fragment in Chinese hamster DNA. The mouse fragment is present in R5D-1A, -1B, -5, and -14, and in R6, EBS-11B6 and C57BL/6 DNA. This is consistent with a mapping of Hras-1 outside functional units 2 through 8. A search for an RFLP to be used with the F_1 animals to obtain data on group 1, yielded the BamHI polymorphism

Figure 16. Hybridization of Hras-1 sequence to somatic cell hybrids and interspecies hybrid animals. Probe is random primer labelled plasmid BS9. M-indicates Mus musculus fragments; S-indicates Mus spretus fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with HindIII and loaded in a single lane. Lane 1. C57BL/6. Lane 2. Tester stock 22A. Lanes 3-6. R5D-1A, R5D-1B, R5D-5, R5D-14, respectively, somatic cell hybrids from the fusion of spleen cells of a male mouse carrying a T(X;7def)5RL7 chromosome to E36 Chinese hamster cells. Lane 7. R6, a somatic cell hybrid from a fusion of spleen cells of a male mouse carrying a T(X;7)6RL7 chromosome to E36 Chinese hamster cells. Lane 8. E36, an HPRT-deficient Chinese hamster cell line used as the parental cell line for the R5D hybrids and the R6 hybrid. Lane 9. EBS-4, a somatic cell hybrid from the fusion of BALB/c spleen cells to E36, retaining mouse chromosomes 13, 15, 17 and X. Lane 10. EBS-11B6, derived from EBS-11, a BALB/c × E36 somatic cell hybrid, by back-selection in 6-thioguanine. B. Identification of an RFLP. 10 μg DNA was digested with BamHI and loaded in a single lane. Lane 1. Male spretus. Lane 2. C57BL/6. Lanes 3 and 4. M. musculus × M. spretus F_1 animals. C. 12FR60Hb c-deletion carrier. 10 μg DNA was digested with BamHI and loaded in a single lane. Lane 1. Male spretus. Lane 2. C57BL/6. Lane 3. F_1 female 41, 12FR60Hb c-deletion carrier. Lane 4. F_1 female 42, normal sib.


A
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M
S

shown in Figure 16B. The unique 3.5 kbp BamHI fragment in the C57BL/6 lane represents the a allele, while the unique 3.0 kbp BamHI fragment in the M. spretus lane represents the b allele. Both alleles can be seen in the F_1 animals. Hybridization of BS9 to the 12FR60Hb deletion-carrying F_1 female 41, and her normal sib, female 42, showed the presence of the musculus a allele, as well as the spretus b allele (Figure 16C). This is consistent with exclusion of Hras-1 from not only functional units 2 through 8, but also from unit 1. Thus the concensus from the data is that Hras-1 lies outside the known c-locus deletions.

Figure 17 shows hybridization of the anonymous human chromosome 11p sequence (D11S14) contained in plasmid pDs-1 to the somatic cell hybrids. This sequence hybridizes to an 11 kbp EcoRI fragment in mouse DNA, and to 8.25 and 3.9 kbp EcoRI fragments in Chinese hamster DNA. Hybridization to the 11 kbp EcoRI mouse fragment is present in DBA and R5D-1B but absent in R6 and EBS-11B6. These data suggest that the mouse homolog of D11S14 does not reside on mouse chromosome 7. Indeed, hybridization of clone Ds-1 to the somatic cell hybrids from Dr. Peter Lalley was consistent with assignment of the sequence to mouse chromosome 2 (Glaser et al., 1985). These data agree with the hybridization results to the R5D and R6 somatic cell hybrids since R5D-1B retained mouse chromosome 2, while R6 and EBS-11B6 segregated it.

The parathyroid hormone genomic sequence in clone prPths-1 hybridizes to the somatic cell hybrids as shown in Figure 18A. It hybridizes to 14 kbp and 1.8 kbp EcoRI fragments in C57BL/6, R5D-1A, -5 and -14 and R6, and to a 7 kbp EcoRI fragment in Chinese hamster DNA.

Figure 17. Hybridization of D11S14 sequence to somatic cell hybrids. Probe is nick-translated plasmid pDs-1, containing an anonymous DNA sequence assigned to the D11S14 locus on human chromosome 11p. M-indicates Mus musculus fragments; S-indicates Mus spretus fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. DBA. Lane 2. R5D-1B, somatic cell hybrids from the fusion of spleen cells of a male mouse carrying a T(X;7def)5RL7 chromosome to E36 Chinese hamster cells. Lane 3. R6, a somatic cell hybrid from a fusion of spleen cells of a male mouse carrying a T(X;7)6RL7 chromosome to E36 Chinese hamster cells. Lane 4. E36, an HPRT-deficient Chinese hamster cell line used as the parental cell line for the R5D hybrids and the R6 hybrid. Lane 5. EBS-11B6, derived from EBS-11, a BALB/c × E36 somatic cell hybrid, by back-selection in 6-thioguanine.

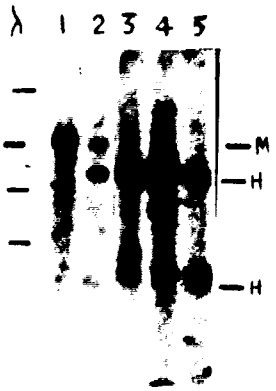
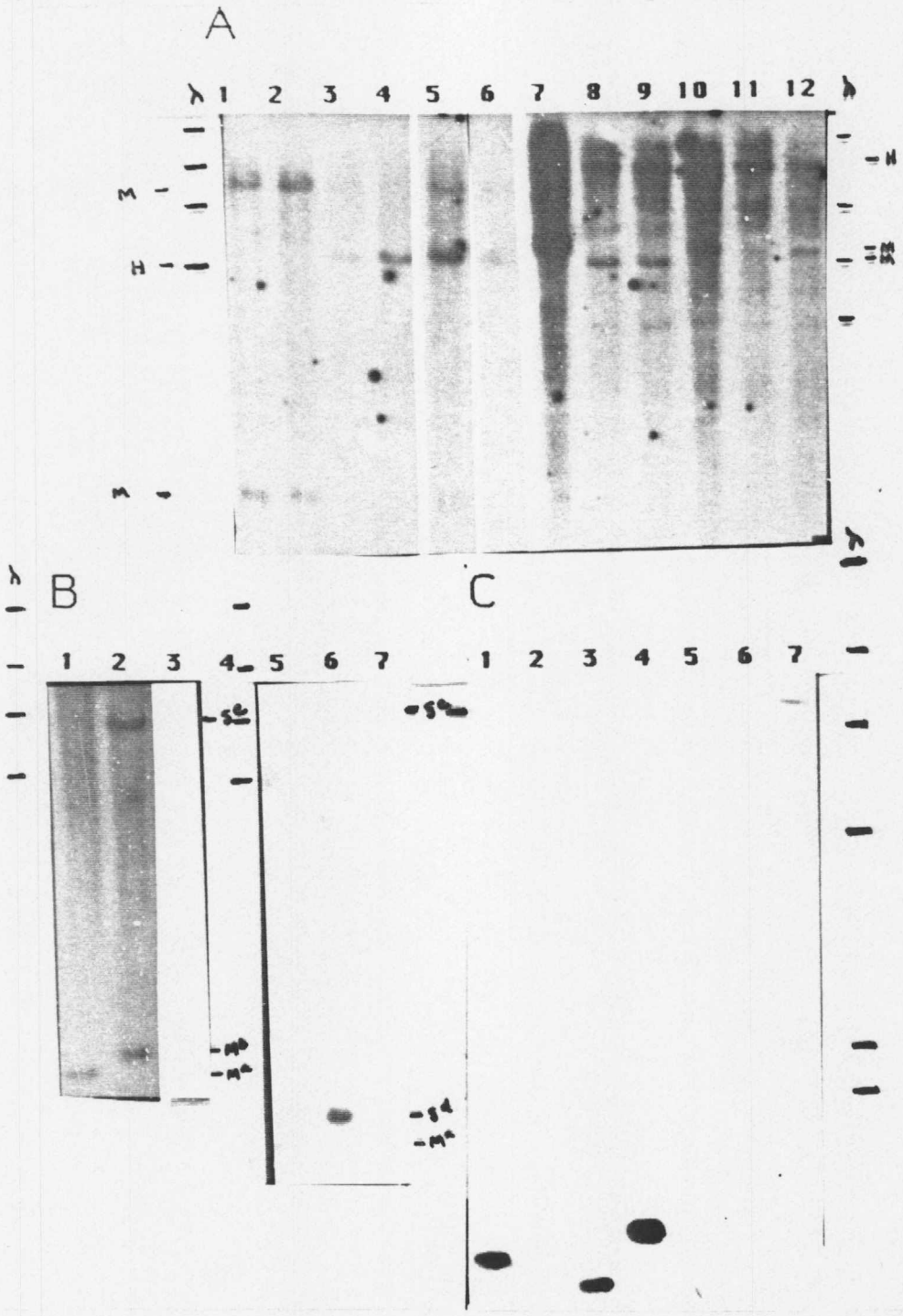

λ 1 2 3 4 5
M
H
H

Figure 18. Hybridization of *Pth* sequence to somatic cell hybrids and interspecies hybrid animals. Probe is random primer labelled 500 bp XbaI/NcoI insert fragment of clone prPths-1. M-indicates *Mus musculus* fragments; S-indicates *Mus spretus* fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with EcoRI in lanes 1-6 and with BamHI in lanes 7-12, and with HindIII in lanes 13 and 14, then loaded in a single lane. Lane 1. C57BL/6. Lane 2. Tester stock 22A. Lanes 3-6. R5D-1A, R5D-1B, R5D-5, R5D-14. Lane 7. C57BL/6. Lane 8. R5D-1A. Lane 9. R5D-9A. Lane 10. R6. Lane 11. E36, the HPRT-deficient Chinese hamster cell line used as the parental cell line. Lane 12. EBS-11B6. B. Identification of an RFLP. 10 μg DNA was digested with HindIII in lanes 1-4 and with BamHI in lanes 5-8, then loaded in a single lane. Lane 1 and 2. Male *spretus*. Lane 3. C57BL/6. Lane 4. *M. musculus* × *M. spretus* F_1 animal. Lane 5. Male *spretus*. Lane 6. C57BL/6. Lane 7. *M. musculus* × *M. spretus* F_1 animal. C. 26DVT and 12FR60Hb *c*-deletion families. 10 μg DNA was digested with HindIII and loaded in a single lane. Lane 1. C57BL/6. Lane 2. Male *spretus*. Lane 3. Female 1226, *M. musculus* 26DVT *c*-deletion stock, mother of F_1-1, 3 and 6. Lane 4. F_1-1, 26DVT *c*-deletion carrier. Lane 5. F_1-3. Lane 6. F_1-6. Lane 7. Female 687, *M. musculus* 12FR60Hb *c*-deletion stock, mother of F_1-39, 40, 43. Lane 8. F_1-39. Lane 9. F_1-40. Lane 10. F_1-43, 12FR60Hb *c*-deletion carrier.


A
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7
C
1 2 3 4 5 6 7

In R5D-1B, there is hybridization only to the 7 kbp hamster fragment - the mouse fragments are absent. Hybridization of prPths-1 to BamHI digested DNAs yielded an RFLP between mouse sequences. C57BL/6, BALB/c (as represented by the BALB/c derived hybrid EBS-11B6) and R6 contain a 6.2 kbp BamHI mouse fragment, while the R5D-1A and R5D-9A hybrids contain a 5.7 kbp BamHI mouse fragment hybridizing to prPths-1. Hybridization to HindIII digested C57BL/6 and R5D-9B DNA gave 0.8 kbp and 1.0 kbp mouse fragments, respectively. Surprisingly, all the data, except hybridization to R5D-1B, are consistent with mapping of Pth outside the 26DVT deletion. In order to test further for inclusion of the sequence in a c-locus deletion, prPths-1 was hybridized to M. musculus and M. spretus DNAs digested with six different enzymes. RFLPs between musculus and spretus were identified with restriction enzymes HindIII and BamHI (Figure 18B). Two M. musculus alleles are found with each enzyme, and the allele characterized by the 0.8 kbp HindIII and the 6.2 kbp BamHI fragments in C57BL/6 were designated the a allele, while the allele characterized by the 1.0 kbp HindIII and 5.7 kbp BamHI fragments in the deletion stocks was designated as the b allele. In M. spretus, two HindIII alleles were identified -- the c allele characterized by a 6 kbp HindIII fragment and the d allele represented by a 0.9 kbp HindIII fragment. Figure 18C shows hybridization of prPths-1 to HindIII digested F_1 animals from the 26DVT × M. spretus and 12FR60Hb × M. spretus matings. The 1.0 kbp HindIII fragment of the musculus b allele and the 6 kbp HindIII fragment of the spretus c allele are both present in the 26DVT deletion carrier, male 1,

and the 12FR60Hb deletion carrier, female 43. This is consistent with mapping of Pth outside the known c-locus deletions.

The question arose as to the source of the DNA polymorphism between the M. musculus deletion stocks, which are not inbred. RFLPs can be the result of several phenomena, one of which is the deletion or insertion of DNA sequences within the fragment to which a particular sequence hybridizes. Two possible explanations for the RFLP are of immediate interest to these studies: (i) Pth is located just outside the edge of the 26DVT and 12FR60Hb deletions such that the HindIII fragment and the BamHI fragment include the deletion endpoints; (ii) Pth is situated so near to the breakpoint of the T(X;7)5RL7 chromosome that the breakpoint is included on the HindIII and BamHI fragments. Either explanation is in line with the data thus far obtained, but the first possibility is less likely since it would require the coincidence of the sum of the distance from Pth to the deletion endpoint and the distance from the endpoint to the next restriction site across the deletion, to be the same for both the 26DVT and the 12FR60Hb deletions with two different enzymes. If either possibility is correct, then hybridization to the mouse stock that is the source of the polymorphism should give the characteristic b allele fragments. Figure 18C shows hybridization of prPths-1 to female 1226, a 26DVT deletion stock animal, and to female 687, a 12FR60Hb deletion stock animal. A single 1.0 kbp HindIII fragment is present. Thus it does not appear that the presence of the deletion endpoints is responsible for the b allele. Hybridization of prPths-1 to four male translocation carriers of the T(X;7)5RL7 stock, 1SaSd, revealed that all the animals were homozygous for the a allele

(data not shown). This suggests that the presence of the breakpoint of the T(X;7)5RL7 chromosome is not the source of the b allele.

Hybridization of plasmid clone C89, containing rat calcitonin sequences, to somatic cell hybrids is shown in Figure 19A. C89 hybridizes to a 6.9 kbp EcoRI fragment is present in R5D-1A, -5, -9B, and -14, in R6, and in EBS-11B6. It is absent from R5D-1B. With the exception of R5D-1B, these results suggest that Ct maps outside in 26DVT deletion. Further evidence to that effect was obtained through a BamHI RFLP which we detected for clone C89 between M. musculus and M. spretus (Figure 19B). We found two alleles for Ct - the a allele represented by a 12.5 kbp BamHI fragment found uniquely in M. musculus and the b allele characterized by a 14.5 kbp BamHI fragment found uniquely in M. spretus. Figure 19C shows hybridization of clone C89 to F_1 animals from the 26DVT and the 12FR60Hb deletion matings. All F_1 animals are heterozygous, including the deletion carriers, male 1 and female 41. These results are consistent with mapping of Ct outside all known c-locus deletions.

Lambda CLX18 hybridizes to a 7 kbp EcoRI mouse fragment and does not hybridize to Chinese hamster DNA (Figure 20A). The pattern of hybridization - positive hybridization to R5D-1A, -5, and -14 and R6 and EBS-11B6 - is in agreement with mapping of the sequence outside the 26DVT deletion. The exception to this agreement is the absence of hybridization to hybrid R5D-1B. Lambda CLX18 is the third DNA sequence demonstrated to be absent from R5D-1B but not from the other R5D hybrids. This suggests that sequences additional to 26DVT have become deleted from the T(X;7def)5RL7 mouse chromosome. We identified two alleles with an EcoRI RFLP for CLX18 between M. musculus and M. spretus

Figure 19. Hybridization of calcitonin sequences to somatic cell hybrids and interspecies hybrid animals. Probe is random primer labelled plasmid C89. M-indicates Mus musculus fragments; S-indicates Mus spretus fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with HindIII and loaded in a single lane. Lanes 1-5. R5D-1A, R5D 1B, R5D-9B, R5D-14, and R5D-5, somatic cell hybrids a mouse T(X;7def)5RL7 chromosome. Lane 6. R6, a somatic cell hybrid carrying a mouse T(X;7)6RL7 chromosome. Lane 7. E36, the HPRT deficient Chinese hamster parental cell line. Lane 8. EBS-11B6. Lane 9. BALB/c. B. Identification of an RFLP. 10 μg DNA was digested with BamHI, then loaded in a single lane. Lane 1. C57BL/6. Lane 2. Male spretus. Lanes 3 and 4. M. musculus × M. spretus F_1 animals.

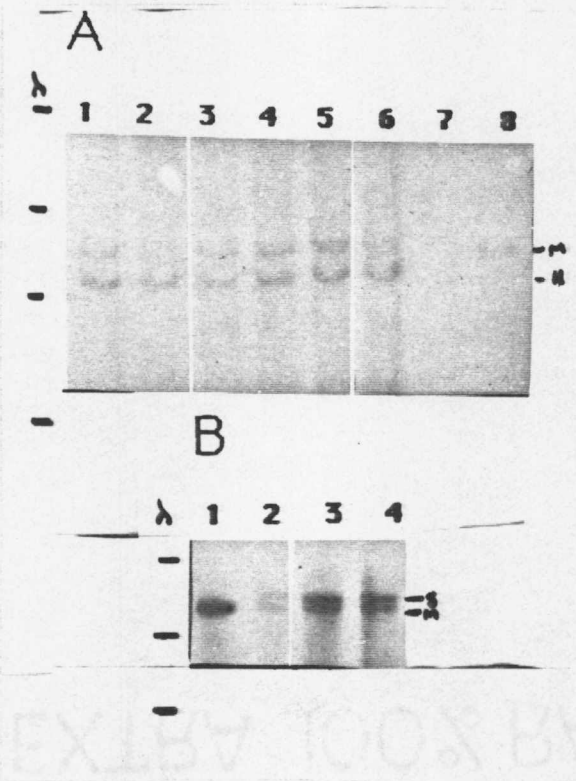
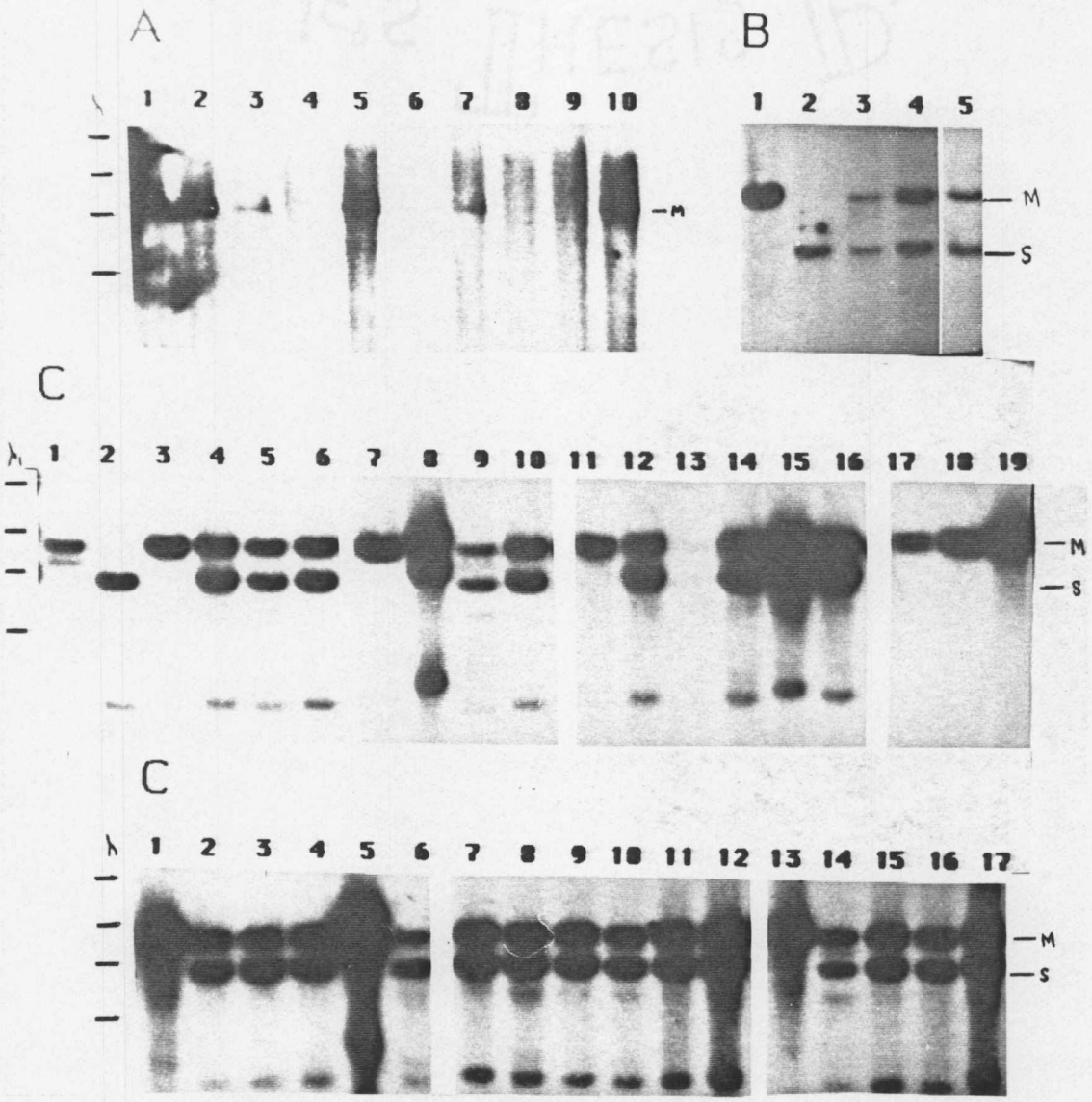

A
λ
1 2 3 4 5 6 7 8
B
λ 1 2 3 4

Figure 20. Hybridization of lambda CLX18 sequences to Southern blots. A. Somatic cell hybrids. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. C57BL/6. Lane 2. Tester stock 22A. Lanes 3-6. R5D-1A, R5D-1B, R5D-5, R5D-14, respectively. Lane 7. R6. Lane 8. E36, Chinese hamster. Lane 9. EBS-4. Lane 10. EBS-11B6. B. Identification of an RFLP. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. C57BL/6. Lane 2. Male *spretus*. Lanes 3, 4 and 5. C57BL/6. Lane 4. *M. musculus* × *M. spretus* F_1 animal. C and D. Interspecies *c*-deletion hybrids and viable homozygous and overlapping *c*-deletion animals. 10 μg DNA was digested with EcoRI and loaded in a single lane. C. Lane 1. C57BL/6. Lane 2. Male *spretus*. Lane 3. 26DVT-carrier female 1226, mother of F_1 animals 1, 3, and 6. Lane 4. F_1 male 1, 26DVT *c*-deletion carrier. Lane 5. F_1 female 3. Lane 6. F_1 female 6. Lane 7. 12FR60Hb-carrier female 687, mother of F_1 animals 39, 40, and 43. Lane 8. F_1 male 39. Lane 9. F_1 male 40. Lane 10. F_1 female 43, 12FR60Hb *c*-deletion carrier. Lane 11. 14CoS-carrier female 1342, mother of F_1 animals 12 through 16. Lane 12. F_1 male 12. Lane 13. F_1 male 13. Lane 14. F_1 female 15, 14Cos *c*-deletion carrier. Lane 15. F_1 female 16. Lane 16. F_1 female 14, 14CoS *c*-deletion carrier. Lane 17. A × C. Lane 18. B × E. Lane 19. E × C. D. Lane 1. female 1339 14CoS-carrier, mother of F_1 animals 7 through 11 and 30 through 35. Lane 2. F_1 male 7. Lane 3. F_1 male 8. Lane 4. F_1 male 9. Lane 5. F_1 female 10. Lane 6. F_1 female 11. Lane 7. F_1 male 30. Lane 8. F_1 male 31. Lane 9. F_1 male 32. Lane 10. F_1 male 33. Lane 11. F_1 male 34. Lane 12. F_1 female 35. Lane 13. female 1323, 146G-carrier, mother of F_1 animals 17 through 20. Lane 14. F_1 male 17. Lane 15. F_1 female 18. Lane 16. F_1 female 19. Lane 17. F_1 male 20, 146G carrier.


A
1 2 3 4 5 6 7 8 9 10
M
B
1 2 3 4 5
M
S
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
M
S
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
M
S

(Figure 20B) -- the a allele characterized by a 7 kbp EcoRI fragment found in M. musculus and M. spretus, and the b allele characterized by a 6.5 kbp EcoRI fragment found uniquely in M. spretus. Hybridization of CLX18 to Southern blots of F_1 animals is shown in Figure 20C. Deletion carriers, male 1 (26DVT), females 41 and 43 (12FR60Hb), female 20 (146G), and females 14 and 15 (14Cos), all show hybridization to the 7 kbp a allele as well as to the 6.5 kbp b allele, thus indicating that CLX18 maps outside all known c-locus deletion.

Lambda CLX23 hybridizes to a 3 kbp EcoRI mouse fragment in the somatic cell hybrids R5D-1A, -5, and -14 and R6 and EBS-11B6 (Figure 21). There is no hybridization to R5D-1B or to Chinese hamster DNA. This pattern of hybridization is identical to that of CLX18 and is consistent with mapping of the CLX23 sequence outside the 26DVT deletion and involvement of the sequence in the "expanded" deletion of hybrid R5D-1B. An examination of five restriction enzymes yielded no RFLP, so that we were unable to obtain data from musculus-spretus F_1 animals about possible involvement of CLX23 in functional unit 1.

Hybridization of cosmid clone 11B6-9 to somatic cell hybrids is shown in Figure 22A. The hybridization pattern is consistent with mapping of the sequence outside the 26DVT deletion, and the cos9B1 sequence joins the other sequences affected by the rearrangement in hybrid R5D-1B. Because cosmid 11B6-9 still contained some repetitive sequences in it and gave poor hybridization on Southern blots, interpretation of hybridization patterns obtained with mouse genomic DNAs was difficult. No musculus-spretus RFLP was identified. Hybridization data for homozygous A, A × C and C × E animals was

Figure 21. Hybridization of lambda CLX23 to somatic cell hybrids. Probe is random primer labelled 3.5 kbp EcoRI insert fragment. M-indicates Mus musculus fragments; S-indicates Mus spretus fragments; H-indicates hamster fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 µg DNA was digested with EcoRI and loaded in a single lane. Lane 1. C57BL/6. Lane 2. Tester stock 22A. Lanes 3-6. R5D-1A, R5D-1B, R5D-5, R5D-14, respectively, somatic cell hybrids carrying a mouse T(X;7def)5RL7 chromosome. Lane 7. R6, a somatic cell hybrid carrying a mouse T(X;7)6RL7 chromosome. Lane 8. E36, Chinese hamster. Lane 9. EBS-4, a somatic cell hybrid, retaining mouse chromosomes 13, 15, 17 and X. Lane 10. EBS-11B6.

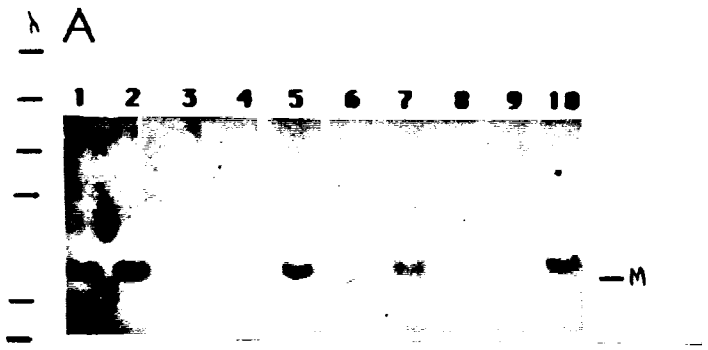
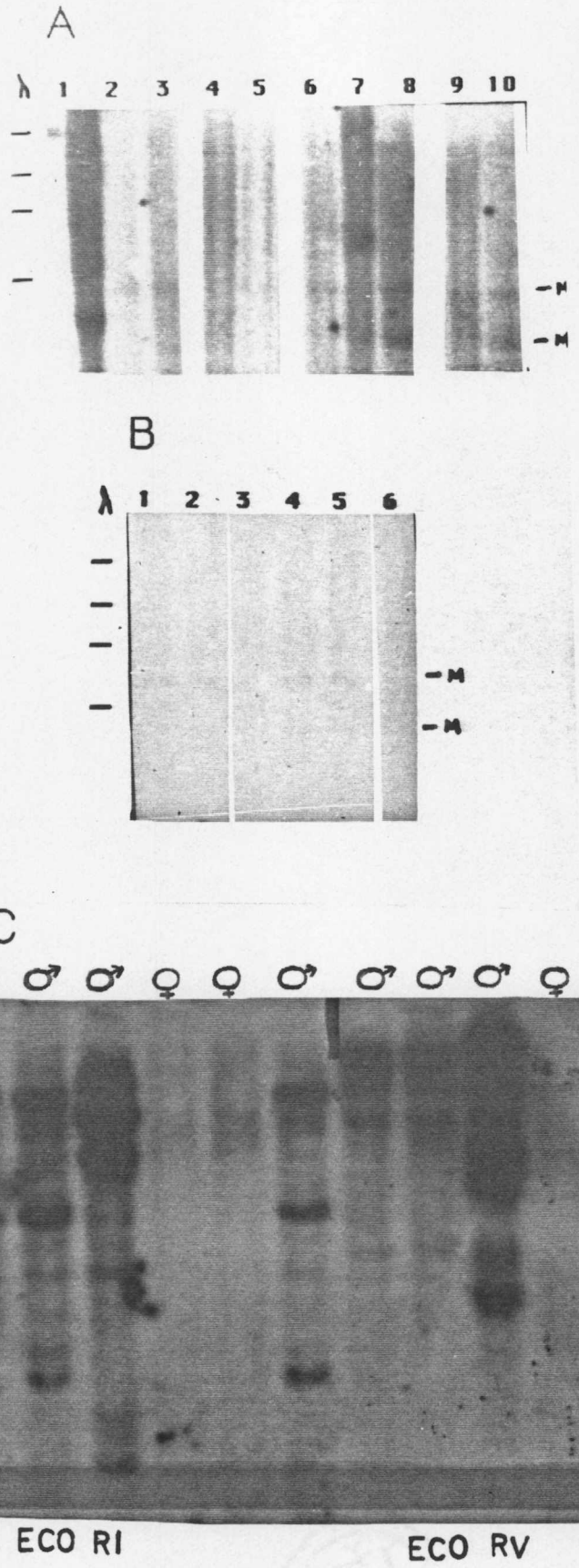

A
1 2 3 4 5 6 7 8 9 10
M

Figure 22. Hybridization of cosmid cos9B1 to somatic cell hybrids and viable homozygous and overlapping c-deletion animals. Probe is random primer labelled EcoRI insert fragment. M- indicates Mus musculus fragments; S-indicates Mus spretus fragments. Size standard generated by a lambda cI87 HindIII digestion in kilobase pairs (kbp). A. Somatic cell hybrids. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. C57BL/6. Lane 2. DBA. Lane 3. Tester stock 22A. Lane 4-7. R5D-1A, R5D-1B, R5D-9A, R5D-9B, respectively, somatic cell hybrids carrying a mouse T(X;7def)5RL7 chromosome. Lane 8. R6, a somatic cell hybrid carrying a mouse T(X;7)6RL7 chromosome. Lane 9. E36, Chinese hamster cells. Lane 10. EBS-11B6. B. Viable homozygous and overlapping c-deletion animals. 10 μg DNA was digested with EcoRI and loaded in a single lane. Lane 1. DBA. Lane 2. R6. Lane 3. E36, Chinese hamster. Lane 4. A × A. Lane 5. A × C. Lane 6. C × E. C. Phage EMBL6-15 search. Lanes are labelled with the sex of the animal and appear in sets of six DNAs digested with the indicated restriction enzyme.


A
λ 1 2 3 4 5 6 7 8 9 10
M
M
B
λ 1 2 3 4 5 6
M
M
C
♂ ♂ ♂ ♀ ♀ ♂ ♂ ♂ ♂ ♀ ♀ ♂
ECO RI
ECO RV

obtained (Figure 22B). The 11B6-9 sequence was present in all three types of animals, verifying a mapping of it outside these deletions.

Phage clone EMBL6-15, assigned to mouse chromosome Y (Chapter IV, page 70), gave an interesting hybridization pattern when hybridized to Southern blots of M. spretus and M. musculus to identify RFLPs. An EcoRI RFLP was identified and EMBL6-15 was hybridized to Southern blots of spretus, musculus, and hybrid animals (Figure 22C). The lanes in Figure 22C are labelled with the sex of the animal from which the DNA originated. When the lanes are labelled this way it becomes immediately apparent that the sequence hybridizes only to the three bands originally detected, in male M. musculus. There is weak hybridization to two new EcoRI fragments in male spretus and still weaker hybridization to at least one new EcoRI fragment in female M. musculus. This supports assignment of the sequence in EMBL6-15 to the Y-chromosome.

Table 7 presents a summary of the data obtained for hybridization of each of the nine unmapped chromosome 7 sequences examined, and for c-locus deletion clone 12A, to Southern blots of the hybrids and mice listed. As the data show, none of the sequences was included in a c-locus deletion. However the effectiveness of the approaches presented in this work to identify c-locus deletion sequences from among mouse chromosome 7 sequences was demonstrated. The new and modified genetic materials developed can be used in future searches for c-locus deletion sequences.

Table 7: Summary of Southern Blot Hybridization Results for Chromosome 7 Sequence

	Clone 12A	Ins-2	Hras-1	H19	pDS-1	Pth	Ct	CLX 18	CLX 23	Cos 9BL
R5D-1A	-	+	+	+	ND	+	+	+	+	+
R5D-1B	-	+	+	+	+	-	-	-	-	-
R5D-5	+	+	+	+	ND	+	+	+	+	ND
R5D-9A	-	ND	ND	+	ND	+	ND	ND	ND	+
R5D-9B	-	ND	ND	ND	ND	+	+	ND	ND	+
R5D-14	-	+	+	+	ND	+	+	+	+	ND
R6	+	+	+	+	-	+	+	+	+	+
EBS-11B6	+	+	+	+	-	+	+	+	+	+
C57,BALB/c,DBA	+	+	+	+	+	+	+	+	+	+
F1(26DVT)	-	ND	+	+	ND	+	+	+	ND	ND
F1(12FR60Hb)	-	ND	+	+	ND	+	+	+	ND	ND
A × A	+	+	ND	ND	ND	ND	ND	ND	ND	+
A × C	+	+	ND	ND	ND	+	ND	+	ND	+
B × E	-	ND	ND	ND	ND	+	ND	+	ND	ND
C × E	-	+	ND	ND	ND	+	ND	+	ND	+

"+" indicates presence of the mouse restriction fragment corresponding to that sequence; "-" indicates absence of the mouse restriction fragment corresponding to that sequence; ND indicates no data.

CHAPTER VII

DEVELOPMENT OF RECOMBINATION DATA FOR THE GENETIC MAPPING OF MOUSE CHROMOSOME 7 SEQUENCES: A COMPARISON WITH THE GENETIC MAP OF HUMAN CHROMOSOME 11P

INTRODUCTION

In experiments described in Chapter VI, it was shown that all of the eight mouse chromosome 7 sequences mapped outside of the c-locus deletions. This chapter describes attempts to localize these sequences on mouse chromosome 7. The traditional method for determining the frequency of meiotic recombination has been modified to include typing of progeny on the DNA level for restriction fragment length polymorphisms (RFLPs) that exist between inbred strains of M. musculus (Taylor, 1978). In this method recombinational data are obtained by comparing the haplotypes of two inbred strains, between which an RFLP has been identified, to the haplotypes of recombinant inbred (RI) lines that have been developed between the two inbred strains. Data are obtained for the unmapped sequence and compared to the computer-stored haplotypes of the RI lines with regard to sequences and loci whose map positions are well-established. Linkage to two or more of the reference loci is established and map position assigned from this comparison.

The use of RI lines for genetic mapping has certain drawbacks. First, it requires the identification of RFLPs between two inbred strains for which RI lines are available. This limits the useful materials, as RI lines are available only between certain inbred

strains. Second, the number of data points obtained is limited by the number of RI lines that have been developed for the two inbred strains between which the RFLP has been identified. And third, those sequences for which no RFLP can be identified cannot be mapped by this method. This is the case for the Ins-2 and the Hras-1 sequences (Cilia Blatt and Adrian Heyday, respectively, personal communications). Extensive searches with many restriction enzymes are quite often required before an RFLP is identified. These searches are both time consuming and expensive. The potential of an alternative method for obtaining frequencies of recombination and linkage data for genetic mapping that would overcome the drawbacks of RI lines will be explored in this thesis.

Benoit et al. (1985) reported the use of backcross progeny of a Mus musculus (DBA) to Mus spretus mating, as a source of meiotic recombination data. This method relies on the high degree of protein- and DNA-level polymorphisms between the two species (Bonhomme et al., 1984) to provide an abundance of RFLPs, and of isozyme and other measurable phenotypic differences for analysis. Theoretically, it should be easier in this cross to find an RFLP for sequences that have proved recalcitrant to analysis by RI lines (eg Ins-2 and Hras-1). Another advantage is that the reliability of the estimates obtained is limited only by the number of backcross progeny that is available. This progeny can be generated in only two generations, instead of the 20 generations required to develop a set of new RI lines. This chapter reports and discusses the results of a study of 44 such backcross progeny to analyze the five unmapped chromosome 7 sequences for which

RFLPs were identified (Chapter IV), as well as three sequences and one phenotypic marker that have been previously mapped to specific loci on chromosome 7.

A valid criticism of this alternative method is that during the divergence of the two species, Mus musculus and Mus spretus, significant chromosomal rearrangements may have occurred. These rearrangements might interfere with the normal meiotic processes, including crossing-over, in interspecies hybrid animals. If this occurred then the frequencies of recombination observed in the backcross progeny of such hybrid F_1 animals would not reflect the true frequencies within Mus musculus or Mus spretus. The low ratio of successful Mus musculus to Mus spretus matings (approximately 50%) obtained, and the sterility of F_1 males are likely to be evidence of such chromosomal rearrangements. However, the frequencies found by Benoit et al. (1985) did not show unexpected discrepancies and were in good agreement with data for the same loci using RI lines. It may be that only a small number of rearrangements occurred in species divergence, such that suppression of crossingover will occur between a limited number of markers. If this is the case, then this method should provide valid frequencies of recombination for the majority of markers on the linkage maps of Mus musculus and Mus spretus.

MATERIALS AND METHODS

The Mus musculus animals from C57BL/10, the tester stock 22A and the 146G, 12FR60Hb and 14CoS c-locus deletion stocks were provided by

Dr. Liane B. Russell (Oak Ridge National Laboratory). Mus spretus males were provided by Dr. Verne Chapman (Roswell Park Memorial Institute). All mating were performed under the supervision of Dr. Liane B. Russell and Clyde Montgomery at the Oak Ridge National Laboratory. F_1 animals were obtained from the following crosses, where the genotype given is for the c-locus:

♀♀ C57BL/10(+/+) × ♂♂ Spretus 1 (+/+)

♀♀ 146G)c^{ch}/c*) × ♂♂ Spretus 6 (+/+)

♀♀ 14CoS(c^{ch}/c*) × ♂♂ Spretus 4 (+/+)

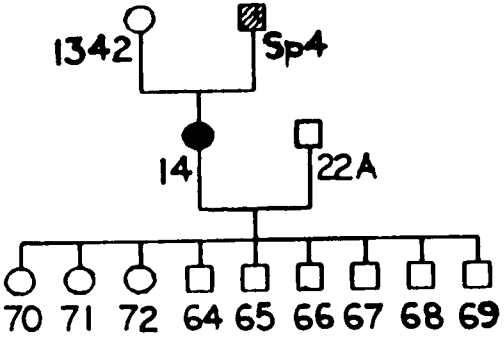
♀♀ 14CoS(c^{ch}/c*) × ♂♂ Spretus 3 (+/+)

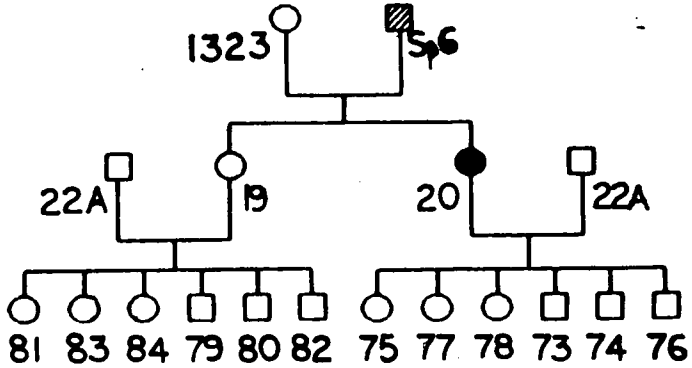
♀♀ 12FR60Hb(c^{ch}/c*) × ♂♂ Spretus 6 (+/+). F_1 females from the C57/BL10 × spretus mating were backcrossed to C57BL/10 males. F_1 females from the deletion stocks × spretus matings were backcrossed to the 22A($c^{ch}c^{ch}$) tester stock. DNA from all animals was prepared as described in Chapter IV (page 58) and submitted to Southern blot hybridization as described in Chapter II (page 16). Sequences used are as described in Chapters II (page 17) and IV (page 55) except for the feline sarcoma virus (c-fes) sequence in clone pN26 (Franchini et al., 1982).

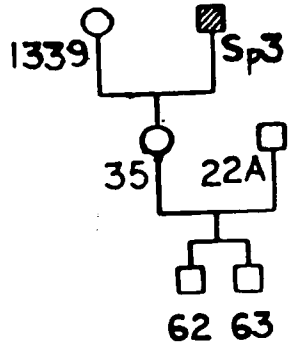
RESULTS AND DISCUSSION

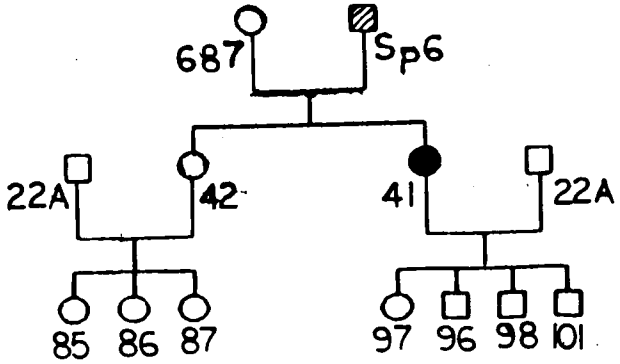
Figure 23 shows the pedigrees of the 44 backcross progeny obtained; there were 25 males and 19 females. Fourteen of the backcross animals are derived from the C57BL/10 strain, which is inbred. Thus, the F_1 mother was uniform with regard to the musculus alleles. Thirty

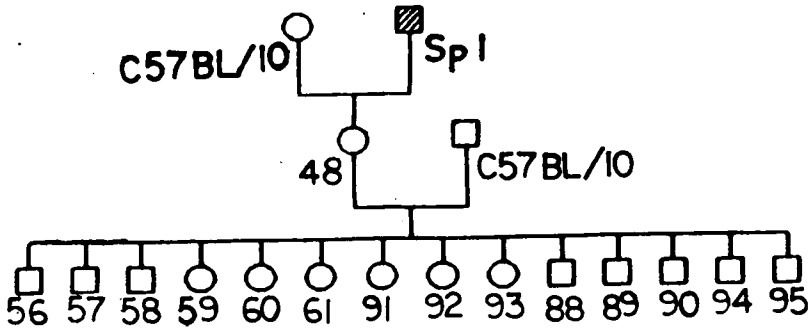
Figure 23. Pedigrees of Backcross Progeny. Circles indicate females, squares indicate males. M. spretus males are indicated by cross-hatched squares. Progeny of female 48 and her C57BL/6 mate includes two litters. All other pedigrees are for single litters. F_1 animals carrying c-locus deletions are indicated by black circles - females 14 and 35 of the 14CoS stock, females 19 and 20 of the 146G stock, and females 41 and 42 of the 12FR60Hb stock.


1342
Sp4
14
22A
70 71 72 64 65 66 67 68 69


1323
Sp6
22A
19
20
22A
81 83 84 79 80 82 75 77 78 73 74 76


1339
Sp3
35
22A
62 63


687
Sp6
22A
42
41
22A
85 86 87
97 96 98 101


C57BL/10
Sp1
48
C57BL/10
56 57 58 59 60 61 91 92 93 88 89 90 94 95

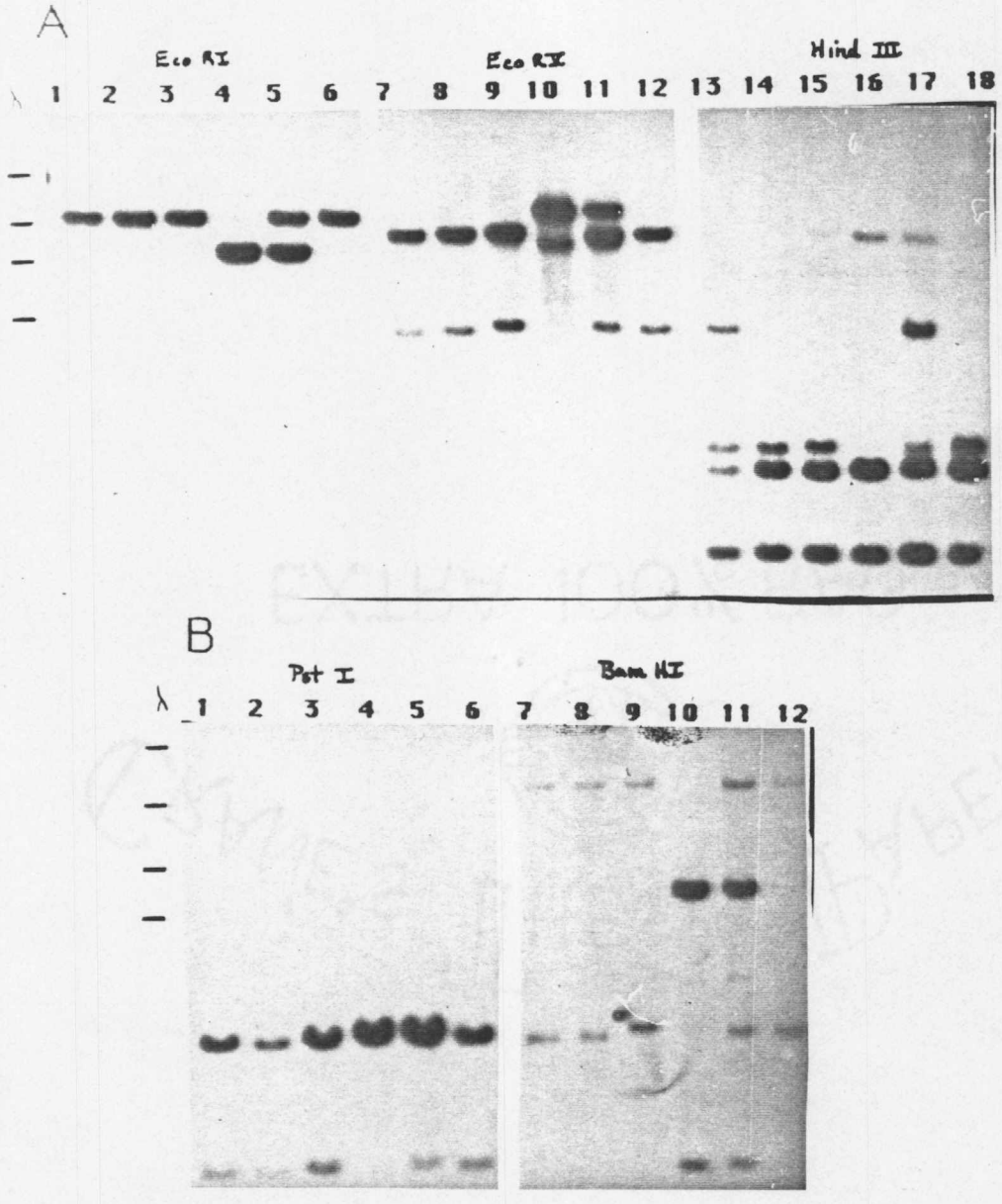
backcross animals were derived from the non-inbred deletion stocks. These animals could potentially demonstrate the disadvantage of using a heterogeneous genetic background -- the introduction of possible diverse musculus alleles, some of which might be shared with the Mus spretus population, producing F_1 animals that are homozygous for one of the shared alleles and whose backcross progeny would thus be uninformative. However, they had two major advantages: (i) the status of the c-locus in the M. musculus chromosome 7, namely chinchilla or deletion, allows these animals to be typed at the c-locus; (ii) deletion stock F_1 animals were produced and backcrossed to the 22A tester stock to detect deletion carriers for the work described in Chapters V and VI, so that it was both convenient and expedient to utilize this abundant source of N_1 offspring.

All backcross progeny were typed using the following convention: "+" indicates homozygous for the allele derived from the Mus musculus chromosome, and "-" indicates heterozygous for the allele derived from the Mus musculus chromosome and the allele derived from the Mus spretus chromosome. Backcross progeny are referred to as "BC", followed by the unique animal number assigned at birth. The typing at the well-mapped c-locus, for use as a genetic reference point, of the thirty backcross animals derived from the c-locus deletion stocks is taken directly from their coat colors, which are listed in Table V (page 88). The typing at all other loci was taken from the hybridization of the corresponding sequence to Southern blots of the backcross progeny. The RFLPs utilized for the five unmapped sequences were identified and initially used in Chapter VI.

The second reference point we used was the well-mapped betahemoglobin locus (Hbb). In Mus musculus laboratory strains and wild-type strains, an inversion that occurred early in evolution resulted in two alleles, called Hbb^d (diffuse) and Hbb^s (single) (Hutton et al., 1962). In a given Mus musculus strain one or the other allele has become fixed. The two alleles are characterized by the following restriction enzyme fragments: (i) Hbb^s (typified by C57BL/6 and C57BL/10) - 11 kbp doublet EcoRI fragments, 2.3 and 9.5 kbp BamHI fragments, and 2.3, 2.0 and 1.2 kbp HindIII fragments; (ii) Hbb^d (typified by BALB/c and DBA) - 7.2 and 12.5 kbp EcoRI fragments, 4.0 and 1.1 kbp BamHI fragments, and 8.4, 2.1 and 1.2 kbp HindIII fragments. Detection of a new allele in Mus spretus which would give an RFLP for use in typing the backcross progeny.

The genomic Hbb sequence in plasmid pMSeHb2 was hybridized to Mus musculus and Mus spretus DNA digested with five different restriction enzymes (Figure 24). Hybridization of the sequence to two different Mus spretus animals is identical to that of C57BL/6 for EcoRI, EcoRV, PstI and BamHI. These data indicate that the Hbb^s allele is present in the spretus population. Indeed, additional hybridization to seven more M. spretus animals showed that all were homozygous for the Hbb^s allele (data not shown). The consequence of this shared allele between C57BL/10 and M. spretus is that all F_1 animals will be homozygous, and thus uninformative. This expectation is borne out by hybridization of the Hbb sequence to female 48, the mother of all the C57BL/10 derived backcross progeny (Figure 24). She is homozygous for Hbb^s, and so all her backcross progeny will be uninformative homozygotes. However,

Figure 24. Hybridization of the Hbb sequence to M. musculus and M. spretus to identify RFLPs. Probe is random primer labelled 1.8 kbp EcoRI/BamHI fragment of pMSEHbb2. 10 μg DNA was digested with the restriction enzyme indicated, then loaded in a single lane. DNAs are loaded in identical sets of six in the following order: male spretus, male spretus, C57BL/6, BALB/c, F_1 female 19 (derived from a cross of the 146G c-deletion stock × M. spretus), and F_1 male 44 (from C57BL/10 × M. spretus).

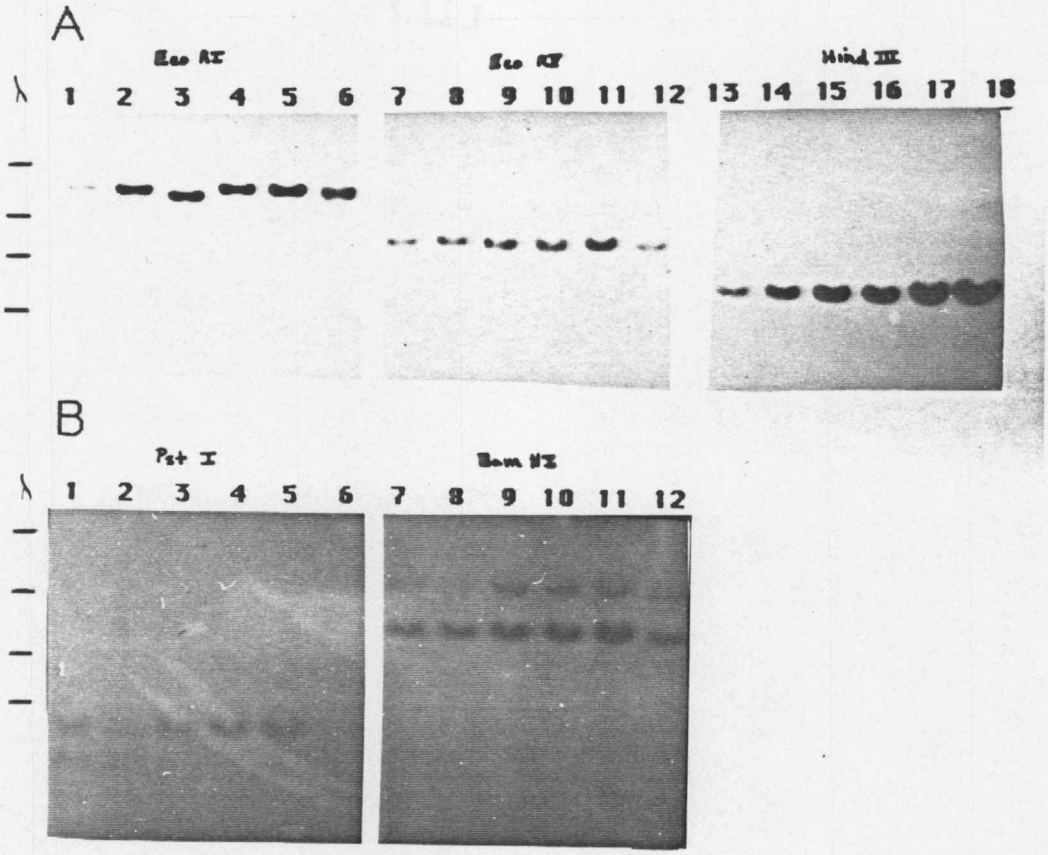

A
Eco RI
Eco RI
Hind III
λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
Pst I
Bam HI
λ 1 2 3 4 5 6 7 8 9 10 11 12

hybridization to HindIII digested DNA from two spretus males (one shown in Figure 24) showed them to be heterozygous at Hbb; They carried Hbb^s and a unique allele characterized by a 4.0 kbp HindIII fragment. The possibility that female 48 might also be heterozygous by use of HindIII. Hybridization of the Hbb sequence with HindIII digested DNA from female 48, showed only the three characteristic Hbb^s fragments (Figure 24). She did not carry the unique polymorphic 4.0 kbp fragment; therefore no recombination data for the Hbb locus could be obtained for her 14 backcross progeny.

Since the two genetic reference points examined, c and Hbb, are 6 cM apart on the standard map (Davisson and Roderick, 1985) data for a reference point far out was sought in order to increase mapping capability. The cellular feline sarcoma virus sequence that corresponds to the c-Fes locus which has been mapped between Gpi-1 and Pep-4, about 23 cM proximal to c (Blatt et al., 1984; Figure 7, page 46). A RFLP search disclosed an EcoRI RFLP between M. spretus and C57BL/6 or C57BL/10 (as represented by F_1 male 44), but no RFLP between M. spretus and BALB/c or deletion stock animals for any of five enzymes examined (Figure 25). Thus recombination data was obtained only for the C57BL/10 derived backcross progeny at the c-Fes locus.

Another outside marker examined in the Ldh-1 locus, 16 cM proximal to c (Davisson and Roderick, 1985). A portion of the genomic sequence for LDH-A (lactate dehydrogenase-A) which recognizes a unique 14 kbp EcoRI fragment in Mus musculus has recently been isolated by Jane Mendel (unpublished data) in the plasmid clone pUCLD14. She has also identified an EcoRI RFLP between M. musculus and M. spretus with this

Figure 25. Hybridization of the c-Fes sequence to M. musculus and M. spretus to identify RFLPs. Probe is random primer labelled insert. 10 μg DNA was digested with the restriction enzyme indicated, then loaded in a single lane. DNAs are loaded in identical sets of six in the following order: male spretus, male spretus, C57BL/6, BALB/c, F_1 female 19 (derived from the cross of the 146G c-deletion animal × M. spretus), and F_1 male 44 (from C57BL/10 × M. spretus).


A
Eco RI
Eco RV
Hind III
λ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
Pst I
Bam HI
λ
1 2 3 4 5 6 7 8 9 10 11 12

sequence. Southern blot hybridization revealed a HindIII RFLP between M. musculus and M. spretus. Both RFLPs were present in all backcross progeny, allowing acquisition of recombination data from the entire N_1 generation with two different restriction enzymes.

Hybridization of the five unmapped chromosome-7 sequences and of plasmid clones 12A, pMSeHbb2, pUCLD14, and pN26 to the backcross progeny was used to evaluate the genotype of the backcross progeny. Mice that lack the spretus-specific band are scored as homozygous musculus (+); mice that exhibit the spretus-specific band are scored as heterozygous (-). A summary of the typing of the backcross animals for all the sequences and loci examined, except pN26, appears in Table 8. This display of the haplotypes allows a direct visualization of the segregation of each of the alleles. Linkage relationships determined in this manner allow a most probable gene order to be established.

The most probable gene order was inferred by arranging the sequences in all possible permutations and determining the number of obligatory double crossovers for each order. The most likely order minimizes obligatory double crossovers. In Table 8, the sequences and loci have been arranged in the order that requires no double crossovers. The gene order suggested by these data is:

Centromere - Ldh-1 - Hras-1 - c - 12A - Hbb - (Ct,Pth) - CLX18 - H19

The deletion data presented in Chapter V (page 89) for 12A placed that sequence within the c-locus functional group 4. In addition, the standard map order is Ldh-1 - c - Hbb (Davisson and Roderick, 1985). When these data are included the suggested gene order becomes:

Table 8: Haplotypes of the *M. musculus*-*M. spretus* N_1 Animals

N_1	H19	CLX18	Ct	Pth	c	Clone 12A	Hbb	Hras-1	Ldh-1
70	-	+	+	+	+	ND	+	+	+
71	+	+	+	+	+	ND	+	-	-
72	+	+	+	+	+	ND	+	-	-
64	-	-	-	-	-	ND	-	-	ND
65	+	-	-	-	-	ND	-	-	ND
66	+	-	-	-	-	ND	-	-	-
67	+	-	-	-	-	ND	-	-	ND
68	-	-	-	-	-	ND	-	-	ND
69	-	-	-	-	-	ND	-	-	ND
81	-	+	+	+	+	ND	+	+	+
83	-	-	-	-	-	ND	-	-	-
84	+	+	-	-	-	ND	-	-	-
79	+	+	+	+	+	ND	+	+	+
80	+	+	+	+	+	ND	+	+	+
82	+	-	-	-	-	ND	-	-	-
75	+	+	+	+	+	ND	+	+	+
77	-	-	-	-	-	ND	-	-	-
78	+	-	-	-	-	ND	-	-	-
73	-	-	ND	+	+	ND	+	+	+
74	-	-	-	-	+	ND	+	+	+
76	-	-	-	-	-	ND	-	-	+
62	-	-	-	-	-	ND	-	-	-
63	+	+	ND	+	+	ND	+	+	-
97	+	+	+	+	+	+	+	+	+
96	+	+	+	+	+	+	+	+	+
101	-	-	-	-	-	-	-	-	-
85	-	+	+	+	+	+	+	+	+
86	-	-	-	-	+	ND	+	+	+
87	-	-	ND	-	-	-	-	-	-
98	-	-	ND	-	ND	-	-	-	-
56	+	+	+	+	ND	ND	ND	+	+
57	+	ND	-	-	ND	ND	ND	-	-
58	+	+	+	+	ND	ND	ND	-	-
59	+	+	+	+	ND	ND	ND	+	+
60	-	-	-	-	ND	ND	ND	-	-
61	-	+	+	+	ND	ND	ND	+	+
91	+	+	+	+	ND	ND	ND	+	+
92	-	-	-	-	ND	ND	ND	-	-
93	-	-	-	-	ND	ND	ND	-	-
88	-	-	-	-	ND	ND	ND	-	-
89	-	-	ND	+	ND	ND	ND	+	ND
90	-	-	-	-	ND	ND	ND	-	-
94	-	-	-	-	ND	ND	ND	-	-
95	+	+	+	+	ND	ND	ND	-	-

"+" indicates animal was hetrozygous for the *M. musculus* allele at that sequence; "-" indicates animal was homozygous for the *M. musculus* allele at that sequence; ND indicates no data.

Centromere - Ldh-1 - Hras-1 - c - 12A - Hbb - (Ct,Pth) - CLX18 - H19

Table 9 shows the analysis of the segregation of alleles in the backcross progeny and the percent recombination between loci, calculated as the number of progeny recombinant between two markers, divided by the number of progeny examined at both loci. The segregation data demonstrate close linkage between the c and Hbb loci (no recombination in 29 progeny), as previously known, and between calcitonin and parathyroid hormone (no recombination in 39 progeny). Linkage of all the markers tested is apparent from sequential pairwise companions. Although the terminal sequences, H19 and Hras-1, show significant recombination (19/44) no single successive step through the gene order shows recombination greater than the 21% (9/43) between CLX18 and H19. This is well within the 30 cM limit of detection of linkage for the 43 backcross progeny examined between CLX18 and H19 (Taylor, 1978).

How reliable are the recombination data from this backcross analysis? The standard error of our percent recombination estimates varied with the total number of progeny examined for each pair of sequences, from ±2.3% with 44 progeny examined to ±3.5% with 29 progeny examined in common. The 95% confidence interval for a binomial distribution, which varies with sample size and with the number of observed recombinants, is given in Table 9 below the percent recombination. These recombination data do not give reliable enough percent recombination to place the loci on the genetic map precisely, but they do provide good evidence in support of the linkage and gene order derived above.

Table 9: Analysis of the Segregation of Alleles in the Backcross Progeny

	H19	CLX18	Ct	Pth	c	Hbb	Hras-1	Ldh-1
H19	----	9/43	9/39	13/44	12/30	12/29	19/44	18/38
CLX18	21% (10-35%)	----	1/39	3/43	4/29	4/29	9/43	10/37
Ct	23% (12-39%)	2.5% (0-13%)	----	0/39	2/30	2/29	6/39	7/34
Pth	30% (17-43%)	7% (1-19%)	0 (0-6%)	----	2/30	2/29	6/44	8/38
c	40% (23-60%)	13% (4-31%)	7% (1-22%)	7% (1-22%)	----	0/29	2/30	4/25
Hbb	41% (25-56%)	14% (4-31%)	7% (0.7-22%)	7% (0.7-22%)	0 (0-12%)	----	2/29	4/24
Hras-1	43% (30-54%)	21% (10-35%)	15% (6-30%)	14% (6-27%)	7% (1-22%)	7% (0.7-22%)	----	2/38
Ldh-1	47% (32-61%)	27% (14-43%)	21% (9-38%)	21% (9-36%)	16% (5-35%)	17% (5-37%)	5% (0.8-17%)	----

Data appearing to the upper right are the number of recombinant progeny per total number of progeny examined in common for the two given sequences. Data to the lower left of the diagonal are the percents recombination calculated from the corresponding data in the upper right. The 95% confidence interval for a binomial distribution is given in parenthesis below each percent recombination.

No recombination was observed between Hbb and c. Since c and Hbb are 6 cM apart on the standard map, two recombinant animals were expected out of the 29 analyzed. This disparity is due either to statistical variation, or to inhibition of crossing over by the c-locus deletions in the musculus chromosome 7 of three of the F_1 mothers. The latter would result in a "shrinkage" of the genetic map between c and Hbb. This suppression of recombination would effect the data obtained from the 19 backcross progeny of the deletion carriers. Thus, only 10 of the progeny examined would be progeny of non-deletion carriers and thus unaffected, and this data set is too limited to see recombination between c and Hbb.

The gene order derived is consistent with data from the somatic cell hybrid R5D-1B. It was suspected that R5D-1B contained a rearrangement, which had occurred in culture and was not present in the parental mouse, that deleted the mouse sequences of CLX18, Pth, and Ct. Since the gene order derived shows that these sequences are clustered, the rearrangement in R5D-1B can be viewed as a simple deletion of one segment of the mouse T(X;7def)5RL chromosome.

In this chapter interspecific Mus musculus × Mus spretus backcross progeny were used for genetic mapping. One of the assets of this approach to genetic mapping is the saving of effort and expense in the identification of RFLPs. In practice, how valid is this statement and what factors affect the acquisition of useful RFLPs?

A search with six restriction enzymes, chosen on the basis of economic price and reputation for detecting RFLPs in mouse and man (Daniela Gerhard, personal communication), yielded at least one RFLP for

each of ten out of thirteen sequences examined. Of the same thirteen sequences, only five have RFLPs with the same six enzymes between the inbred strains C57BL/6, C57BL/10 and BALB/c. Thus the use of M. spretus to introduce DNA-level polymorphisms onto a M. musculus background appears to be effective and shows great promise for future mapping studies, especially for sequences resistant to analysis by RI lines.

An important factor for the successful revelation of RFLPs is the choice of M. musculus strain to be crossed to M. spretus. Recently, Guenet et al. (1985) reported on their progress with interspecific recombinant offspring. They have found that the inbred strain DBA gives the highest rate of RFLPs with M. spretus; in fact, it showed RFLPs for every probe they examined with "relatively few" restriction enzymes. In the work presented here no single Mus musculus strain was clearly superior to all others. With C57BL/10 and the BALB/c-like deletion-stock mice, alleles were shared completely with various Mus spretus animals at two loci, Hbb and c-Fes. That occurred with C57BL/10 for the Hbb RFLPs and with BALB/c and the deletion-stock mice for the c-Fes RFLP. Thus these data show that strain choice is a factor in the revelation of useful RFLPs for the thirteen sequences examined; but they also point out that the best strain to use may well be sequence dependent.

These results demonstrate that another factor can affect not the revelation of an RFLP, but its usefulness in following the segregation of the given allele in backcross populations. That factor is the abundant representation within M. spretus of alleles shared by the M. musculus and M. spretus populations. This heterogeneity can result

in a large number of F_1 animals being homozygous for the shared allele, and thus the loss of the RFLP. Segregation data for clone 12A was lost because 30 of the backcross progeny were derived from homozygous F_1 mothers. One way to avoid homozygosity of F_1 animals, is to prescreen male *spretus* (using tail- or ear-derived DNA) with the sequences to be mapped. Then only males that are homozygous for the unique *spretus* allele would be used to father F_1 animals. This method may not suit all situations, as it might prove difficult to identify a male *spretus* who was homozygous for the *spretus* allele at all the loci that are being examined, and who will interbreed successfully with *M. musculus*. An alternative would be to prescreen female F_1 animals to eliminate homozygous individuals before backcross progeny are produced.

The results presented in this thesis extend the linkage map of mouse chromosome 7 to include five previously uncharacterized sequences. With these additions, the linkage map of mouse chromosome 7 can be compared with that of human chromosome 11p at three new loci: (i) *Pth* and *PTH*, (ii) *Ct* and *CALC1*, (iii) *Hras-1* and *HRAS1*. The gene order for the five common loci does not appear to be the same in mouse and man:

Mouse *Ldh-1* - *Hras-1* - *Hbb* - *Ct*, *Pth*

Human 11p *LDHA* - *CALC1* - *PTH* - *HBBC* - *HRAS1*

This gene order suggests a minimum of one rearrangement has occurred between the two species in the region defined by these five loci. At least one more rearrangement has occurred between the two species which resulted in the gene for mitochondrial isocitrate dehydrogenase being

located on human chromosome 15, but located in the mouse on chromosome 7 between Ldh-1 and c (Davisson and Roderick, 1985). The human homolog of the c-Fes locus on mouse chromosome 7, also resides on human 15 (Kozak et al., 1985). Thus, there are at least two regions on mouse 7 homologous to human 15, separated by a region of homology of undetermined length between human 11p and mouse 7. This suggests a minimum of one rearrangement has occurred between the two species to bring about this order. In addition, the results reported here suggest that the mouse homolog of D11S14, proximal to LDHA on human 11p, is not on chromosome 7. This locus then defines another break in the homology between mouse 7 and human 11p that suggests at least one more rearrangement has occurred between the two species. Thus a minimum of four rearrangements has occurred in the divergence between mouse and man to bring about the gene order derived in this thesis.

An alternative method of obtaining frequencies of recombination and linkage data was explored. The method follows the segregation of alleles as revealed by analysis of RFLPs in the backcross progeny of a Mus musculus to Mus spretus mating. 44 offspring of such matings were examined at six defined loci and with three anonymous DNA sequences: Ct, Pth, c, Hbb, Hras-1, Ldh-1, H19, CLX18, and clone 12A. The segregation of alleles for these markers demonstrated linkage relationships from which a most probable gene order of:

Centromere - Ldh-1 - Hras-1 - c - 12A - Hbb - (Ct,Pth) - CLX18 - H19

was established. The linkage map of the mouse was compared with the human linkage map. An analysis of the status of the maps suggests a

minimum of four rearrangements has occurred in the divergence between mouse and man.

CHAPTER VIII

CONCLUSIONS

A basic goal of this thesis was to develop a detailed map of a large region of a mammalian chromosome. The chromosomal region chosen for study was the region of the c locus on mouse chromosome 7. This choice takes advantage of the wealth of genetic material involving the c-locus region. A number of DNA sequences have been isolated from mouse chromosome 7 and three additional DNA sequences were isolated in this thesis. Translocation chromosomes are available facilitating the construction of somatic cell hybrids for mapping DNA sequences (Russell and Montgomery, 1970). A large number of c-locus deletions have been characterized by complementation mapping and placed in eight functional units (Russell et al., 1982). It was hoped that one or more of the mouse chromosome 7 DNA sequences would be identified, using these materials, as missing in the c-locus deletions and could be assigned by deletion mapping to one of the eight functional units.

DNA sequences for calcitonin (Ct), parathyroid hormone (pth), insulin (Ins-2), Harvey sarcoma virus oncogene (Hras-1), and an anonymous sequence, H19, were obtained from other laboratories. To expand the sequences available from mouse chromosome 7, three new cloned sequences, CLX18, CLX23, and 11B6-9 were selected from recombinant DNA genomic libraries enriched for mouse chromosome 7, as described in Chapter IV. In the course of isolation of new sequences from mouse chromosome 7, three mouse-specific repetitive sequences were isolated

which can be used in future screening of mouse sequences from interspecific recombinant libraries.

These cloned mouse-specific repetitive sequences have been useful in other experimental work. Housman et al. (1983) used two of the repetitive sequences to evaluate the amount of mouse DNA stably integrated into monkey cells following chromosome transfer from mouse donors. In collaboration with Dr. Robert Levenson (Yale University), another cloned repetitive sequence was used to obtain a cloned sequence conferring ouabain resistance in a secondary transferrant of mouse into human cells (Levenson et al., 1984). This study demonstrated the utility of cloned mouse repetitive sequences as hybridization probes to isolate nearby sequences selected for in transfection experiments.

Homozygous deletion of a significant region of a chromosome is likely to result in the deletion of a gene vital for organism development and survival and hence be lethal. This phenomena is observed at the c-locus on mouse chromosome 7. Russell et al. (1982) showed that only animals of classes V, A, C, and E survive to birth when the deletions are homozygous. To circumvent the problems of lethality of large c-locus deletions two approaches were explored: (1) Construction of somatic cell hybrids carrying a large, lethal deletion of the c-locus and (2) Generation of F_1 animals from Mus musculus deletion stocks mated to the wild-type feral mouse Mus spretus.

Construction of the somatic cell hybrids carrying the large, recessive lethal c^{26DVT} deletion was described in Chapter II. These somatic cell hybrids were then used to establish a primary screening system for detection of DNA sequences included in functional units 2

through 8 of the c-locus deletions (Figure 1, page 5). Southern blot hybridization of DNA sequences to the somatic cell hybrids allows analysis of the DNA sequences removed in c-locus deletions. Five of the somatic cell hybrids segregated the normal mouse chromosome 7, uncovering the sequences deleted in c^{26DVT} on the T(X;7def)5R1 chromosome retained. Southern blot hybridization of a known c-locus deletion sequence, clone 12A, demonstrated the ability of the somatic cell hybrids to reveal dequences missing in the c-deletion. Any given DNA sequence from mouse chromosome 7 can now be examined by Southern blot hybridization to the somatic cell hybrids to determine if it is missing in the c^{26DVT} deletion.

The somatic cell hybrids can also be used to map sequences to regions other than the c-locus region of mouse chromosome 7. Southern blot hybridization to the hybrids will identify sequences from the region of mouse chromosome 7 that are proximal to the breakpoints of the longer R51 and R61 translocation chromosomes. It will also identify sequences from the region of chromosome 7 between the breakpoints of the longer 5R1 and 6R1 translocation chromosomes. Southern blot hybridization of a mouse chromosome X sequence to the somatic cell hybrids will allow assignment of that sequence to the regions proximal, distal and between the respective breakpoints of the two translocation chromosomes. Thus the somatic cell hybrids developed in this work provide a source of genetic mapping information for both mouse chromosome 7 and the X chromosome.

Generation of F_1 progeny from c-locus deletion stock Mus musculus to Mus spretus matings was described in Chapter V. A high rate of

natural polymorphisms exists between M. musculus and M. spretus on the protein level (Bonhomme et al., 1984). This is suggestive of a high degree of polymorphism on DNA level. Benoit et al. (1985) have shown examples of polymorphisms between these species for actin and for the light chain of myosin. The potential of M. spretus for introducing polymorphisms for analysis of a deletion was explored for the c-locus. M. musculus heterozygote deletion females of representative members of complementation groups A, Dq, Fp, and Fq (Figure 1, page 5) were crossed to male M. spretus.

M. musculus × M. spretus F_1 animals carrying the c^{14Cos}, c^{146G}, c^{26DVT}, and $c^{12FR60Hb}$ deletions were obtained. In these interspecies deletion carriers, the far outside regions of functional units 1, 2, 6, 7, and 8 (Figure 1, page 5) are accessible. Upon Southern blot hybridization of these interspecies hybrid deletion carriers to a polymorphic DNA sequence, each of the musculus and spretus allele will be characterized by a specific hybridizing fragment. Sequences absent from one of the deletions would be missing the hybridization band characteristic of the deleted M. musculus allele. This would identify sequences missing in the deletion carried by that animal. Southern blot hybridization of these animals to the mouse chromosome 7 sequences, described in Chapter IV, for which a restriction fragment length polymorphism (RFLP) between M. musculus and M. spretus was identified, was performed. Only the known c-locus deletion sequence, clone 12A, was missing the M. musculus hybridization fragment in the deletion carrying animals (Chapter VI). This suggests that none of the other sequences examined are missing in any of the c-locus deletions.

The genetic approach of generating interspecies deletion-carrying hybrid animals from M. musculus to M. spretus matings can be applied to not only the c-locus deletions as presented here but also to other recessive lethal mutations available in mice. This method of uncovering sequences missing in deletions was developed by Jane Mendel (University of Tennessee at the Oak Ridge National Laboratory and Massachussetts Institute of Technology) in collaboration with Dr. Liane B. Russell (Oak Ridge National Laboratory) for homozygous recessive p-locus deletions (Jane Mendel, personal communication).

In general female M. musculus heterozygous for the mutation of interest can be mated to male M. spretus. Deletion animals among the resulting F_1 progeny would be identified by a serological, biochemical or phenotypic assay, if available, or by Southern blot hybridization to a sequence from the region. Deletion carrying female F_1 animals would also be identified by a backcross to a M. musculus strain whose genetic background allows detection of heterozygotes for the mutation of interest. This could be assayed either by the phenotype or by the biochemicl/serological assay by which the mutation was originally characterized. Using this approach, many recessive lethal mutations which cannot be studied at present on the molecular level, could be made accessable. This approach should be expecially appropriate for studying early developmental recessive lethal mutations, such as are available at the c, p, and d loci.

In experiments described in Chapter VI, it was shown that all of the eight mouse chromosome sequences mapped outside of the c-locus deletions. Chapter VII describes attempts to localize these sequences

on mouse chromosome 7. The traditional method for determining the frequency of meiotic recombination has been modified to utilize analysis of segregation of restriction fragment length polymorphisms (RFLPs) existing between inbred strains of Mus musculus (Taylor, 1978). This method has certain disadvantages. It requires the identification of RFLPs between two inbred strains for which recombinant inbred (RI) lines of mice are available. This can prove limiting if no RFLP is identified in these inbred strains. The informative meiosis available for analysis are limited to the number of RI lines developed between those inbred strains which display the RFLP. The potential of an alternative method for obtaining frequencies of recombination for genetic mapping without the disadvantages of RI lines was explored.

An alternative method of obtaining frequencies of recombination was demonstrated by Benoit et al. (1985). The method follows the segregation of alleles as revealed by analysis of RFLPs in the backcross progeny of a Mus musculus to wild-type feral mouse, Mus spretus, mating. The method relies on the high degee of protein- and DNA-level polymorphisms between the two species (Bonhomme et al., 1984) to provide an abundance of RFLPs and other assayable phenotypes for analysis. Theoretically, it should be easier in this cross to find an RFLP for a given sequence. Since these offspring can be generated in only two generations, instead of the 20 generations required to develop a set of new RI lines, a very large number of informative meiosis can be examined.

In Chapter VII, 44 offspring of such matings were examined at six defined loci and with three anonymous DNA sequences: Ct, Pth, c, Hbb, Hras-1, Ldh-1, H19, CLX18, and clone 12A. The segregation of alleles

for these markers demonstrated linkage relationships from which a most probable gene order of:

Centromere — Ldh-1 -- Hras-1 -- c -- 12A -- Hbb -- (Ct,Pth) -- CLX18 — H19

was established.

The linkage map of the mouse was compared with the human linkage map. For the gene order derived, the loci shared between mouse chromosome 7 and human chromosome 11p suggest that a minimum of one rearrangement has occurred in the divergence of the two species. At least two more rearrangements in the divergence are suggested by the two separate regions of mouse chromosome 7, characterized by c-Fes and Idh-2, homologous to human chromosome 15. An additional rearrangement is suggested by the identification of the break in the region of homology between mouse 7 and human 11p characterized by the mouse homolog of human D11S14. Thus, an analysis of the status of the two linkage maps now suggests a minimum of four rearrangements has occurred in the divergence between mouse and man.

This alternative method holds great promise to provide large amounts of data on frequencies of recombination in the mouse. Analysis of the segregation of alleles can be performed for any polymorphic sequence or assayable polymorphic protein or phenotype. In collaboration with Dr. David Housman (Massachusetts Institute of Technology) and Dr. Liane B. Russell (Oak Ridge National Laboratory), 52 additional backcross progeny will be examined at the loci described above. The set of 96 offspring are also being used by Tom Glaser and Dr. David Housman

(Massachusetts Institute of Technology) to improve the linkage map of mouse chromosome 2 in the region of homology with human 11p characterized by the mouse homolog of D11S14 and the gene for the beta subunit of follicle stimulating hormone (FSHB). This syntenic region on human 11p may include the Wilm's tumor-anaridia complex (WAGR). Thus, that study may reveal a model system in the mouse for those human genetic disorders. Similar refinements of regions of mouse chromosomes homologous to human chromosomal regions that contain loci involved in human genetic disorders should aid greatly in increasing the mouse model systems identified for the disorders.

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