GENETIC AND MOLECULAR ANALYSIS OF DELETIONS AT THE BROWN LOCUS ON MURINE CHROMOSOME 4

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PhD
University of Edinburgh
1994
Declaration

I declare

a. that this thesis is composed by myself and

b. that the work is my own, except where otherwise stated.
Abstract

The murine brown locus on chromosome 4 is one of seven loci involved in the specific locus mutagenesis test (SLT). For over forty years mutations at these loci have been induced by radiation or chemical mutagenesis and recovered using the SLT. These mutations, especially those due to deletions, have proved to be valuable tools in characterising the genes involved in the SLT and the regions surrounding them.

Radiation mutagenesis experiments carried out at Oak Ridge National Laboratories, Tennessee, generated a panel of over thirty deletions at the brown locus. These were analysed by E. M. Rinchik and most were lethal when homozygous. Deficiency mapping and complementation analysis of these deletions enabled a gross genetic map of the region to be generated. Various functional units involved in development were identified and localised on this map. Until relatively recently, molecular analysis of the region was not possible, however the mapping of the brown gene in 1988 permitted molecular access to this region.

The principal aim of this project was to characterise developmentally important genes located close to the brown gene, using both molecular and genetic techniques. Seven mice with radiation induced mutations at the brown locus were received from B. Cattanach. The phenotype of mice homozygous for one of these deletions, b57H, revealed the presence of a brown associated fitness or baf gene. These mice were much smaller than their littermates and frequently died soon after birth. Those that did survive were subfertile and commonly had litters of only one or two mice. The baf gene was identified concurrently by E.M. Rinchik, in compound heterozygote mice, with combinations of different deletions. All viable mice with compound deletions exhibited the baf phenotype indicating the baf gene was situated relatively close to brown. In an attempt to localise this gene further various molecular techniques were employed.

A collaboration with E.M. Rinchik enabled hybrid DNA from 25 of the deletions to be obtained, in which the deleted Mus musculus chromosome was heterozygous with a Mus spretus chromosome. The presence or absence of markers in different deletions can be detected by virtue of
polymorphisms between the two species. This enabled microsatellite markers and microdissection clones to be mapped to the region and to further classify deletions within a particular complementation group. PCR primers from the microdissection clones which mapped closest to the brown gene, as well as primers from the brown gene itself, were used to screen two YAC libraries. Seventeen YACs were isolated and will be used to generate a YAC contig of the region which contains the baf gene. This work has improved the physical map at this locus and will allow the isolation of a YAC contig and ultimately the baf gene itself.
Acknowledgements

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I am grateful to Bruce Cattanach for the mice with the b locus mutations and also to NATO. Their grant enabled a fruitful collaboration to be set up between Gene Rinchik and ourselves and funded visits between the two labs. I am indebted to Gene Rinchik for being able to use the deletion panel DNAs and also for showing us around on our visit.

My thanks go also to John Maule for his help with PFGE and Chris Boyd for his assistance in computer emergencies. I am also grateful to Kathy Evans for proof-reading this thesis. In addition, thanks to Norman, Sandy and Douglas from the photography department and Sheila for the excellent library service.

I would also like to thank Dougie Boyd for the support he has given me over the last three years. He has been there to celebrate with, when things go well and commiserate with, when things go wrong.
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<td>Adfp</td>
<td>adipocyte differentiation-related protein</td>
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<td>alf</td>
<td>albino lethality factor</td>
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<tr>
<td>b</td>
<td>brown</td>
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<tr>
<td>baf</td>
<td>brown associated fitness</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C57</td>
<td>strain C57BL/6/Ola (supplied by Harlan Olac Ltd)</td>
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<td>cM</td>
<td>centimorgens</td>
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<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
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<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
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<tr>
<td>dGTP</td>
<td>deoxyguanidine triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>dep</td>
<td>depilated</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid disodium salt</td>
</tr>
<tr>
<td>eed</td>
<td>embryonic ectoderm development</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
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<tr>
<td>EUCIB</td>
<td>European Collaborative Interspecific Backcross panel</td>
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<tr>
<td>exed</td>
<td>extraembryonic ectoderm development</td>
</tr>
<tr>
<td>Fah</td>
<td>fumarylacetoacetate hydrolase</td>
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<td>fig</td>
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<td>FISH</td>
<td>fluorescent in-situ hybridisation</td>
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<td>G6Pase</td>
<td>glucose-6-phosphatase</td>
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<td>hepatocyte-specific developmental regulation-1</td>
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<td>Hxb</td>
<td>Hexabrachian</td>
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<td>Ifα</td>
<td>Interferon α</td>
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<td>IRS PCR</td>
<td>Interspersed repetitive sequence polymerase chain reaction</td>
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<tr>
<td>kb</td>
<td>kilobases</td>
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<td>ld</td>
<td>limb deformity</td>
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<tr>
<td>Lv</td>
<td>delta-aminolevulinate dehydratase</td>
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<td>msd</td>
<td>mesoderm specific development</td>
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<td>Om</td>
<td>orosomucoid</td>
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<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
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<td>pid</td>
<td>preimplantation development</td>
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<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
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<td>RG</td>
<td>Research Genetics</td>
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<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<td>RFLV</td>
<td>Restriction fragment length variant</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SLT</td>
<td>Specific Locus Test</td>
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<tr>
<td>SSCP</td>
<td>Single stranded conformational polymorphism</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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<td>tyrp-1</td>
<td>tyrosinase related protein-1</td>
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<td>TAT</td>
<td>tyrosine aminotransferase</td>
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<tr>
<td>wi</td>
<td>whirler</td>
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<td>YAC</td>
<td>Yeast artificial chromosome</td>
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Chapter 1
Introduction

The study of mutations in laboratory mice has provided great insights into the function of the mammalian genome. Mutations affecting development have generated a valuable resource with which to study mammalian development and, in some cases, have provided a murine model of a human disease. Germ line mutagenesis techniques have generated mutations, especially deletions, which can be isolated and recovered. Such deletions are a valuable resource in the fine structure physical and functional analysis of specific regions. This thesis describes how deletions of the brown locus on murine chromosome 4 have been used to characterise and map this region.

1.1 Historical aspects

Mice have been used in experimental studies since the seventeenth century. However, they were not used to study heredity and evolution until the early twentieth century, when scientists turned to mice in order to test Mendel's laws of inheritance. An important figure in this work was W.E. Castle, whose initial studies had concentrated on the variable coat colour of mice (Castle and Allen, 1903). There was available, a considerable pool of mice with coat colour variants, which facilitated their initial studies. Over the years, unusual or fancy mice had been collected by mouse fanciers worldwide. Indeed, a mutant waltzing mouse was described as early as 80 BC in Japan (Morse, 1981). Since the nineteenth century, mice with unusual characteristics had been collected and bred; most prized were those with uncommon coat colours. Albino, pink-eyed dilution, chinchilla, dilute and spotted mutant mice had all arisen by the end of the nineteenth century and were analysed by W.E. Castle and his colleagues. During these studies, other spontaneously arising abnormalities were observed, for example, the short ear mutant (Lynch, 1921) and the shaker mutant (Lord and Gates, 1929). However, the accumulation of new spontaneously occurring genetic variants was a slow process.

Around this time, it was realised that in order to study the genetic effects of a particular disease, mice with a uniform genetic background were needed. This observation resulted from work by E.E. Tyzzer (1909), who found that the susceptibility to develop cancer varied in different strains of mice. This lead to the development of the inbred strains of laboratory mice used today. For
example, mice with the coat colour genes dilute, brown and non-agouti were first inbred by C.C. Little in 1909, and these mice were the progenitors of the DBA strain of laboratory mice. Studies using these mice first demonstrated that hereditary factors were important in the development of cancer (Little, 1916). The importance of inbred strains was now apparent and large breeding programs were established to generate more inbred strains. However, the inbreeding of a number of strains was found to cause lethality, due to homozygosity of pre-existing recessive mutations. During these programs, mice with developmental abnormalities, similar to those found in humans, were occasionally observed. The occurrence and detection of these rare mutations was principally due to the large numbers of mice now under study. Functional analysis and characterisation of these mutations lead to a greater understanding of mammalian developmental biology and, thus, confirmed the importance of mutational analysis in the study of mammalian systems. However, the low frequency at which these mutations arose, limited the knowledge which could be gained from such analysis. Clearly, if a method was developed which could generate heritable mutations in the mouse, it would facilitate studies on mammalian development and functional analysis of the complex mammalian genome.

1.2 The Specific Locus Mutagenesis Test (SLT)
The specific locus mutagenesis test (SLT) was developed by W.L. Russell in 1951. The SLT (fig. 1.1) involves using radiation or chemical mutagenesis to induce germ line mutations in mice. Mutagenised mice are then crossed to those from a tester stock, which carries homozygous recessive mutations at seven loci. Six of the seven loci affect coat colour and the other, the size of the external ear. The vast majority of the offspring produced from such crosses will be phenotypically wild type, as they will be heterozygous at these seven loci. However, mice carrying induced mutations at one of these seven loci can be recovered in the first generation, as they have visible phenotypes. The loci involved are: a (non-agouti), chromosome 2; b (brown), chromosome 4; p (pink-eyed dilution) and c<sub>ch</sub> (chinchilla), chromosome 7; d (dilute) and se (short ear), chromosome 9; s (piebald), chromosome 14. Chinchilla, an allele of the albino locus, is linked to the pink-eyed dilution locus (15cM) on chromosome 7, whereas the dilute and short ear loci are more closely linked (0.16cM) on chromosome 9 (Russell, 1951).
This breeding protocol was used to detect mutations which had been generated at the seven loci involved in the SLT. These loci were $a$, $b$, $p$, $c^{ch}$, $d$, $se$ and $s$. Chromosome markers are $+$ (wild type allele), $y$ (one of the seven loci listed above) and $y^{*}$ (represents an induced mutation at one of these loci). The protocol is explained in the text.
Mutations at these loci are first detected in mice which carry the viable recessive mutation from the tester stock and the newly induced mutation. This enables mice carrying newly induced viable, sub-lethal or lethal mutations to be isolated and propagated. The use of closely linked tester loci enables deletions at these loci to be detected. Some of the mice generated from early experiments contained presumed deletions of chromosome 9, as they had both a dilute coat colour and short ears (Russell et al., 1958).

When mice carrying newly induced mutations were recovered, the first step was to check that each mutation was allelic with the tester gene presumed to be involved. Mice homozygous for the newly induced mutations were then generated. In a high proportion of cases, these mutations were found to be lethal when homozygous and the developmental stage of death of these mice, was subsequently ascertained. As the seven loci involved in the SLT are not essential for survival, induced mutations which were lethal when homozygous, were presumed to involve other loci i.e. multilocus deletions or gross rearrangements. This has since been shown to be an accurate assumption (Rinchik et al., 1990). Lethal deletions were then analysed to see if they affected nearby genes, by mating them with mice known to contain mutations in these genes (reviewed by Russell, 1989). This type of complementation analysis generated information about the extent of the deletions. Lethal deletions were then analysed for their ability to complement one other, by mating mice carrying individual deletions. This allowed mutations at any given locus to be arranged into complementation groups. The offspring from such crosses included mice with compound deletions and their phenotype or gestational stage of death was determined. This enabled the identification and localisation of functional units in the surrounding regions. This type of analysis enabled functional and structural maps of these regions to be constructed (see below). These simple maps provided a basic framework for future molecular and physical analysis of these regions. They have proved to be a valuable resource in the mapping and identification of genes at these seven loci and have enhanced our understanding of the composition of the mammalian genome.

As well as isolating mice with mutations at the seven specific loci involved in the SLT, mice with other phenotypic abnormalities were also detected. It is now known that these dominant mutations primarily affected three loci: $S$, $I$, $J$. 

steel; \( W \), dominant spotting; \( T \), brachyury (reviewed by Rinchik et al., 1990a). Some spontaneously arising mutations were also recovered at much lower frequencies \( (1 \times 10^{-5} \text{ per locus}) \) (Schlager et al., 1971) from non-irradiated control stocks.

It was noted that the type of mutagenesis and the germ cell stage it affected, were important factors in determining the nature of the induced mutation (Sankaranarayanan, 1991). As analysis of deletions yields more information about particular chromosomal segments and loci associated with specific developmental phenotypes, mutagenesis protocols have been optimised to generate gross rearrangements, rather than intragenic mutations.

1.3 Mutagenic agents

For over forty years, specific locus mutagenesis tests have been carried out at various centres, providing the opportunity to study the effectiveness of different radiation or chemical mutagens. The majority of radiation induced mutations (68%), using either X-rays, gamma rays or neutrons, were found to be deletions or other gross rearrangements (Searle, 1974). These mutagens were found to be most effective when exposed to mature oocytes or in the postmeiotic stages of spermatogenesis, with mutation rates of \( 1.9 \times 10^{-4} \) and \( 3.3 \times 10^{-4} \) mutations per locus, respectively (Rinchik, 1991). Both the number and type of mutations which were induced, varied between the seven loci. For instance, the highest rates of radiation induced mutations occur at the \( s \) locus and the majority of them are homozygous lethals, whereas at other loci the proportion of homozygous lethals varies between 16 and 50% (Sankaranarayanan, 1991). The high proportion of lethal mutations at the \( s \) locus suggests there is a developmentally important gene close to the \( s \) gene.

Many of the alkylating agents used in the initial chemical mutagenesis experiments were found to be relatively ineffective. Since then many chemicals have been tested and chlorambucil, a chemotherapeutic agent, was found to be one of the most mutagenic (Rinchik et al., 1990b). It is most effective when exposed to early spermatids and induces mutations at a rate of \( 1.3 \times 10^{-3} \) per locus, considerably higher than the rates obtained with radiation mutagens (Rinchik, 1991). It primarily induces gross chromosomal rearrangements, principally deletions and translocations (Rinchik et al., 1993a). Another chemical mutagen, N-ethyl-N-nitrosourea (ENU), has proved
to be one of the most effective mutagens in mice (Russell et al., 1979a). In contrast to chlorambucil, ENU primarily induces small intragenic point mutations, at a rate of $1.5 \times 10^{-3}$ per locus in spermatogonial stem cells (Rinchik, 1991). Interestingly, it produces a different distribution of mutations when compared to other mutagens, with rates lowest at the $s$ locus. This may suggest that the gene involved in producing the piebald phenotype is relatively short in length. This type of mutagenesis has proved most useful in refining the gross functional maps generated by deletion analysis.

Genetic and more recent molecular analysis of induced mutations at the seven loci involved in the SLT, have resulted in the generation of functional and physical maps of these regions, as well as the identification of some previously unknown genes. The results of this type of analysis at the seven loci will be briefly reviewed, whilst the work involved in the generation of the albino locus map will be more comprehensively examined.

1.4 The albino ($c$) locus
By 1979, 119 radiation induced mutations involving the albino locus, on chromosome 7, had been isolated from over three million offspring (Russell et al., 1979b). Mutations at the albino locus affect the tyrosinase enzyme which is involved in the melanin biosynthesis pathway and, consequently, mice with mutations at this locus either have no pigment i.e. albino, or the amount of pigment is greatly reduced i.e. chinchilla (Silvers, 1979). A comprehensive study of 34 independent, $c$ locus mutations was carried out by Russell et al. (1982). This enabled a basic functional map of the region to be constructed, which provided the framework for subsequent mapping experiments. Three types of analysis, which are described below, were used to characterise the albino locus mutations.

1.4.1 Developmental stage of lethality
Mice homozygous for each of the 34 mutations were examined in order to ascertain their phenotype or their developmental stage of lethality (Russell et al., 1979c). Subvital mice which were homozygous for one of the mutations, were identified. These mice were smaller and generally less fit than their littermates. In seven cases, homozygous mutant mice were observed at birth, but died shortly after (neonatal lethals). In all other cases, no homozygous mutant mice were seen. Embryos from such crosses were then examined at
different stages of development. It was concluded that, in 13 cases, the mutants die before implantation (pre-implantation lethals) while the other 13 die at or shortly after implantation (implantation lethals). This analysis enabled the 34 mutations to be classified into four groups.

1.4.2 Deficiency mapping
Four genes had been genetically mapped to the albino region and each of the c locus mutations were analysed to determine whether the potential deletions incorporated these genes (Russell et al., 1982).

1. The taupe (tp) locus is a recessive coat colour mutation which lies 3cM proximal to c. Mice carrying the albino locus mutations were mated to taupe mice. In each case, all the offspring were wild type, indicating that none of the deletions extended to the taupe locus.

2. The gene encoding the metabolic enzyme Mod-2 (mitochondrial malic enzyme) has been mapped 2cM distal to the c locus. MOD-2 activity was measured in the heart mitochondrial fractions of mice carrying the c locus mutations, and compared to wild type levels. Twenty four of the deletion heterozygotes were found to have reduced activity, indicating that these deletions included the Mod-2 locus.

3. Shaker-1 (sh-1) is involved in the development of the inner ear and maps 4cM distal to Mod-2. Mutants exhibit a characteristic shaking phenotype and have difficulties with balance. Shaker mice were mated with mice carrying the c locus mutations and the offspring examined for the shaker phenotype. This phenotype was observed in the offspring from two crosses, thus two deletions extend as far as the sh-1 locus.

4. The gene encoding the haemoglobin β chain (Hbb) maps 2cM distal to sh-1. The levels of Hbb in mice carrying the two deletions that extended to sh-1, were compared to the levels found in wild type mice. No differences could be detected, indicating that these deletions did not extend to the Hbb gene.

This analysis enabled the albino locus mutations to be subdivided into three groups. Those which did not extend to any of the markers tested (10), those which incorporated Mod-2 only (22), and those which included both Mod-2
1.4.3 Complementation mapping
Mice carrying albino locus deletions were mated, in order to identify which pairs of deletions could complement one another (Russell et al., 1982). Albino offspring, hemizygous for different lethal deletions, were observed in some crosses and were classified into four groups: good survival; poor survival; neonatal death; prenatal death. Thirty deletions were characterised in this way.

Classification of the deletion homozygote phenotype and the deficiency mapping analysis yielded information on the extent of the deletions and the genes located within the deletion complex. These genes could be distinguished by their function and were subsequently referred to as functional units. The complementation analysis allowed these functional units to be ordered in a linear fashion, in such a way that conformed with the previous results. The resultant map (fig. 1.2) covers a 6-11cM region of chromosome 7 and shows the eight ordered functional units and the extent of the thirteen complementation groups of deletions (Russell et al., 1982).

1.4.4 Characterisation of c locus deletion phenotypes
The phenotypes of mice deleted for specific functional groups were then studied in more detail. Embryos were examined for developmental or enzymatic abnormalities and this information was used to speculate on the function of the gene present in these functional groups. For example, embryological studies of mice deleted for the distal implantation locus have indicated that there are two distinct types of abnormalities in these mice (Niswander et al., 1989). Some embryos have undifferentiated extraembryonic ectoderm in regions which normally contain mesoderm and the primitive streak. These embryos die at 7.5 days gestation. The locus involved in this phenotype has been termed extraembryonic ectoderm development (exed). In contrast, other embryos survive until 8.5 days gestation. In these embryos, extraembryonic ectoderm does develop into extraembryonic ectodermal structures. However, although some embryonic mesoderm is observed, it fails to develop into embryonic mesodermal structures (head process, notochord, somites). This has been termed the embryonic ectoderm development (eed) locus. These studies have shown
This figure was adapted from Russell et al., 1982. The map covers a 6-11cM region of chromosome 7 and the position of four mapped genes is shown. Analysis of c locus deletions (see text) enabled them to be divided into 13 complementation groups and allowed the eight functional units to be ordered on the chromosome.
that there are at least two genes at the distal implantation locus.

Analysis of mice with deletions of the proximal implantation locus have found that these mice fail to develop mesoderm (Rinchik et al., 1990a). Embryos of 7.5 days gestation show retarded growth, but defined extraembryonic and embryonic ectoderm, in contrast to embryos with mutations at the exed and eed loci. The ectoderm fails to differentiate into mesoderm and these embryos die at around 10 days gestation. The gene involved in this process has been termed msd (mesoderm development).

The phenotype of mice deleted for the preimplantation survival gene (pid) has been examined. Embryos stop dividing between the 2-6 cell stage and die before implantation (Lewis, 1978). A sequence with homology to cyclin B has been mapped close to the preimplantation region (Lock et al., 1992). As cyclin B is important in cell cycle regulation, it may be a candidate for all or part of the pid phenotype.

Mice containing deletions of the juvenile survival region, frequently die before weaning. Males that do survive are sterile, due to defects in spermatogenesis and females have a high frequency of spontaneous abortion (Lewis et al., 1978).

1.4.5 The hsd-1/alf locus

Biochemical studies of mice deleted for the postnatal survival region demonstrated that these mice die from hypoglycaemia, shortly after birth. These mice also exhibit abnormalities mainly of the liver, but also of the kidney and thymus (Erickson et al., 1968). This has been termed the hepatocyte-specific developmental regulation-1 locus (hsdr-1) (McKnight et al., 1989) or the albino lethality factor (alf) (Ruppert et al., 1990). Deletion of this locus leads to pleiotropic effects, caused by the abnormal activity and quantity of liver and kidney specific enzymes. The induction of various enzymes, principally those involved in gluconeogenesis [e.g. glucose-6-phosphatase (G6Pase), tyrosine aminotransferase (TAT)], fails to occur. Alterations in transcription and mRNA stability may account for some of these variations (Schmid et al., 1985; Loose et al., 1986). Subtractive cDNA hybridisation studies showed that expression of the affected genes was normally induced by glucocorticoids (Ruppert et al., 1990). In-vitro studies of
hsdr-1 deficient liver cells demonstrated that glucocorticoids were unable to
induce expression of TAT and G6Pase (Tonjes et al., 1992). This lead to the
proposal, which has now been disproved, that hsdr-1 encodes a regulatory
factor involved in inducing specific, hormone dependant gene expression
(Gluecksohn-Waelsch, 1987; Ruppert et al., 1990). Positional cloning was
used to map the gene involved (Kelsey et al., 1992; Klebig et al., 1992). The
hsdr-1/alf gene was localised to a 310kb region between two deletion end-
points. This region was subsequently shown to contain the fumarylacetoacetate
ydrolase (Fah) gene, which was proposed as a candidate for hsdr-1. In humans, FAH deficiency is responsible for hereditary
tyrosinemia type I. Individuals with this condition have very similar symptoms
to the hsdr-1 phenotype seen in mice. Transgenic mice, homozygous for
disruption of the Fah gene, have been produced from targeted embryonic
stem (ES) cells. These mice display the same phenotype as hsdr-1/alf
deficient mice (Grompe et al., 1993). Also the hsdr-1/alf phenotype of mice
with deletions at this locus has been rescued when Fah is expressed from a
transgene (Kelsey et al., 1993). Fah catalyses the final step of tyrosine
breakdown and a deficiency leads to an accumulation of toxic tyrosine
metabolites. These cause cellular damage in tissues which normally express
Fah and gene expression is affected, resulting in the multiple affects
associated with this phenotype, but the exact mechanism remains unclear.
Thus, the characterisation of mice with deletions at this locus has enabled the
gene responsible for postnatal survival to be identified and cloned.

1.4.6 ENU Mutagenesis of the c-locus
Although eight functional units have now been mapped to the c locus, others
that are concealed by earlier acting mutations, may also be present. ENU
mutagenesis is ideally suited to uncover such loci as it induces primarily
intragenic mutations and enables fine-structure mutational analysis of large
chromosomal regions (reviewed by Rinchik, 1991). This type of analysis
enables loci which are closely linked, and therefore usually inseparable by
deletion analysis, to be distinguished. Any new loci defined by ENU
mutagenesis can easily be mapped using the panel of deletions.

Mutations, induced on either chromosome, close to the albino locus were
detected using the breeding protocol shown in fig. 1.3. Albino male mice were
mutagenised and mated with wild type females. Any induced mutations in the
Figure 1.3: ENU mutagenesis of the c locus

This figure was adapted from Rinchik, 1991. This breeding protocol was used to detect ENU induced mutations close to the c locus. Chromosome markers are + (wild type allele), c (albino mutation), c<sup>ch</sup> (chinchilla mutation), * (induced mutation) and deletion Df<sup>26DVT</sup> is represented by a gap in the chromosome. The protocol is explained in the text.
offspring would be generated on chromosomes marked by the standard albino mutation. Females were then crossed to males heterozygous for the $c^{ch}$ mutation (an allele of the albino locus) and the $Df^{26DVT}$ deletion. This is one of the largest $c$ locus deletions, incorporating the $c$, Mod-2 and $sh$-$1$ loci. Albino progeny were examined for mutant phenotypes, which could be present if a mutation was generated within the limits of the $Df^{26DVT}$ deletion. The absence of albino mice in the offspring indicated that a lethal mutation had been induced. This mutation could then be recovered in the light chinchilla mice. An indication that saturation mutagenesis of this region was being achieved, was given by the fact that repeat mutations were observed at several loci (Rinchik, 1991).

Six independent lethal mutations, which map distal to $c$, have been recovered (Rinchik, 1991). Two have been mapped to the $eed$ interval by complementation analysis and both have the $eed$ lethal phenotype. These two mutations have been shown to complement each other, indicating that there are at least two genes present in the $eed$ functional unit (Rinchik et al., 1993c). The other four lethal mutations have been mapped in a similar way, distal to the $pid$ locus. They are lethal shortly after implantation, although the specific times varied (Rinchik et al., 1993d). Also a new locus, fit-1, was uncovered which maps between $eed$ and $exed$. Mice with mutations at fit-1 have a generalised runting syndrome (Rinchik, 1991). Thus, exhaustive point mutagenesis of this region has uncovered seven new loci.

### 1.4.7 Molecular access to the $c$-locus

Several strategies have been used to gain molecular access into the deletion complex, which would enable physical mapping studies to be initiated. This has involved physically isolating DNA sequences from the region, which can then be used as starting points for long range chromosome walking techniques. This would ultimately allow physical distances to be assigned to the functional map and enable the genes located there, to be isolated. This approach has been used to characterise the region surrounding the tyrosinase gene. The gene was cloned in 1987 by Kwon et al. and more than 70kb of surrounding sequence has now been characterised.
In 1985, Silver reported that in the mouse strain c58/Lw, a defective ecotropic
murine leukaemia provirus (Emv-23), was closely linked to the c locus. This
was cloned along with its flanking chromosome 7 sequences and the two
ends were then subcloned. They were mapped by backcross analysis and
positioned 0.5cM distal to the c locus, between c and Mod-2. A probe from
the flanking region was used to map the proviral integration site to the deletion
complex. It mapped between the c and the juvenile survival loci (Rinchik et
al., 1989). Another integration site for the polytropic murine leukaemia virus,
Pmv-31 was found to be closely linked to the c locus (Frankel et al., 1989). It
was mapped, using the deletion complex, proximal to c, between c and the
neonatal survival loci. Several viable albino locus deletions were shown to
remove both the Emv-23 and Pmv-31 integration sites (Rinchik et al., 1993b).
These have provided two additional molecular entry points close to the
tyrosinase gene.

Several anonymous clones have been mapped within the deletion complex.
These have generally been isolated from enriched cDNA libraries constructed
from chromosomes carrying albino locus translocations (Disteche et al.,
1984). Some have also been isolated from microdissection libraries of
metaphase chromosomes (Tonjes et al., 1991). Clones have been mapped
using the albino locus deletion panel. When DNA from homozygote lethals
has not been available, as in the case of early acting lethals, heterozygotes
have been crossed to M. spretus mice and DNA from the hemizygous
offspring has been used. Restriction fragment length variants (RFLV)
between the two species have enabled the clones to be mapped.

Molecular access to the deletion breakpoints would greatly enhance the
construction of a physical map. Recently this has been possible as useful
anonymous clones have been mapped close to deletion end-points, from
which chromosome walking or jumping studies can be initiated. Some clones
have also detected size altered restriction fragments carrying deletion
endpoints, which have then been cloned (Rinchik et al., 1993d).

Pulsed field gel electrophoresis (PFGE) analysis using markers which have
been mapped to the region, is being used to generate long range physical
maps of this region (Klebig et al., 1992). Neighbouring markers could then be
used to generate a YAC contig. Techniques such as exon amplification,
cDNA library screening, CpG island localisation and the identification of evolutionary conserved sequences, could lead to identification of possible candidate genes on YACs.

Molecular analysis of the c locus has enabled a more detailed map of this region to be constructed, with the physical distances of some regions known. Some of the viable deletions, \( c^{51} \text{DTD} \) and \( c^{31} \text{HATH} \), extend for at least 1.5Mb (Klebig et al., 1992) while \( c^{14} \text{CoS} \), the smallest deletion to remove the \( Fah \) gene, is at least 3.6Mb (Kelsey et al., 1992). Genes within the functional units have been further localised and others may be cloned in the very near future. However it is probable that some genes within this complex have still not been identified.

1.5 The dilute/short ear (d-se) locus
The dilute and short ear loci are closely linked (0.16cM) on chromosome 9. Many of the induced mutations were found to involve both loci and these have now been shown to be deletions of this region (Rinchik et al., 1986). Mice homozygous for the original d locus mutation, which arose spontaneously, have light coloured fur. This is now known to be due to the altered distribution of melanin within the melanocytes and hair shafts (Rinchik et al., 1985). However, most spontaneous mutations at this locus have an additional lethal phenotype, termed opisthotonus (Rinchik et al., 1985). This neurological disorder manifests at around three weeks when mice display convulsive arching of the head and back. Abnormal cartilage structures are seen in mice with homozygous mutations at the se locus. This results in a reduction in the size of the external ear as well as other cartilage defects (Rinchik et al., 1985).

Complementation analysis of lethal and sublethal deletions revealed that, like the c locus, there were loci involved in prenatal (six) and postnatal (two) development in this region (Rinchik et al., 1986). Several of the deletions appear to be non-linear, making analysis more difficult. The d and se loci were unable to be separated by complementation analysis.

The original d mutation was found to be due to insertion of the murine leukaemia provirus, \( Emv-3 \), whilst revertants had lost these viral sequences (Jenkins et al., 1981). The probe p0.3 was isolated from the proviral insertion
site, in the intron sequences of the d gene, allowing molecular access to this gene. This probe detected a breakpoint fusion fragment in one of the d locus deletions. Two other fusion fragments have now been cloned from deletions incorporating the d and se loci. A rat brain cDNA clone which had been genetically mapped to this region, was mapped close to the se locus using the deletion panel (reviewed by Rinchik et al., 1990a).

Subsequently, a cDNA from the dilute gene was isolated. This cDNA was estimated to encode a 215K protein (Mercer et al., 1991). The protein was present in all the adult tissues examined, however different tissues contained transcripts of different lengths. In embryos, the protein is localised to the spinal cord and ganglia. It has extensive homology with myosin heavy chains and contains sites for myosin light chains. Myosins are involved in contraction and movement, but the exact function of the dilute protein is not known. It has been suggested that it may be involved in the production or function of the dendritic processes of melanocytes and some neurons.

It is now thought that the dilute opisthotonus phenotype is the true null mutation of the d locus and the dilute phenotype, seen in the original mutation, is due to altered gene expression (Russell, 1989).

Utilising deletions generated at this locus, a directed chromosome walk to the short ear locus was undertaken (Kingsley et al., 1992). The gene Bmp-5 (bone morphogenetic protein-5) was found to be located in this region and it is disrupted by several short ear mutations. In mammals eight bone morphogenetic proteins are known, all of which are involved in bone and cartilage development. This evidence strongly suggests that the short ear locus encodes Bmp-5 (Kingsley, 1994).

These clones, along with others generated from breakpoint fusion fragments, will allow access to the functional units within this 3-9cM region and subsequently to the isolation of the genes contained within it.

1.6 The pink-eyed dilution (p) locus
The pink-eyed dilution locus lies 15cM proximal to the tyrosinase gene on chromosome 7. Mice with the original p locus mutation were hypopigmented, having a cream coat and pink eyes. The hypopigmentation phenotype results
from a reduction in mainly eumelanin pigment in the melanosomes.

Over forty mutations at this locus have been isolated and analysed by complementation analysis. Two other loci had been genetically mapped to the region, ruby-2 (ru-2) and lactate dehydrogenase-1 (Ldh-1). Only one deletion which removes both these genes and extends over 3cM has been isolated. Four functional units have been identified; PE (prenatal lethality), RJGS (runting, jerky gait, sterility), p locus and CP (cleft palate syndrome). Some mice with radiation induced p locus mutations, i.e. presumed deletions, have only the hypopigmentation phenotype with no additional abnormalities. This suggests that the null effect of the p locus affects only coat colour (Lyon et al., 1992).

The unstable p locus mutant (p\textsuperscript{un}) was found to contain duplicated sequences at the p locus. These were cloned along with the flanking sequences of the duplication, which were then used to screen a melanocyte cDNA library. A clone encoding a 833 amino acid polypeptide was isolated. This was predicted to be a membrane spanning protein with twelve hydrophobic domains. RNA analysis showed that this transcript was absent or altered in mice with p locus mutations. Mice with the p\textsuperscript{un} duplication have a large aberrant transcript whereas, in revertants, this duplication is lost and the normal transcript is seen. This indicates that this protein is the product of the p locus. It is known to be a membrane spanning protein which has been shown to be melanocyte specific. Alterations or absence of this protein affect melanosome structure and melanin content (Gardner et al., 1992).

Two clones from chromosome 7 enriched cDNA libraries have been mapped within the 3cM p locus deletion. The Ldh-1 gene was also localised using this deletion, and has subsequently been cloned (Mendel, 1987). Many more chromosome 7 specific clones are being characterised using interspecific hybrid crosses, in the hope that some will enable molecular access to the functional groups contained in this region.

The p locus exhibits synteny with the 15q11-q13 region in humans which is associated with the imprinted Prader-Willi and Angelman syndromes (reviewed by Brilliant, 1992). Although mental retardation and craniofacial abnormalities are common features of both syndromes, they have other
distinct clinical features. Because hypopigmentation is often associated
with both syndromes, a human melanoma cDNA library was screened with a probe
from the mouse \( p \) gene. Partial cDNA clones were isolated which displayed
homology to murine cDNAs. Deletions of the human homologue of the \( p \)
genome are associated, although not exclusively, with hypopigmentation in
individuals with Prader-Willi syndrome (Gardner et al., 1992). Another study
of five people with type II oculocutaneous albinism (OCA II), one of which also
had Prader-Willi syndrome, showed that all had alterations (either mutations
or deletions) in both alleles of the human \( P \) gene (Lee et al., 1993). Many
features of the Prader-Willi and Angelman syndromes are similar to those
seen in mice with deletions at the \( p \) locus and they may be useful as models
to study some aspects of these syndromes (reviewed by Brilliant, 1992).

1.7 The agouti (\( a \)) locus
The agouti locus on chromosome 2 is responsible for the black and yellow
pigment banding pattern seen in the individual hairs of wild type mice. It acts
within the hair follicle, regulating the alternate production of the two types of
pigment.

As this is the least mutable of the seven loci involved in the SLT, relatively low
numbers of deletions at the \( a \) locus were recovered. Nevertheless, many
alleles have now been recovered. Some cause only subtle effects on coat
colour, while others have a more drastic effect on the overall distribution of the
black and yellow pigments. For instance, lethal yellow (\( A_Y \)) mice contain only
yellow pigment when heterozygous for a deletion at this locus, which is a
preimplantation lethal in the homozygous state. Heterozygous mice also
display other dominant pleiotropic effects, such as reduced fertility in females,
non-insulin dependant diabetes, obesity and a tendency to develop tumours.
In black-and-tan mice, the distribution of the two pigments is altered,
generating mice with a black dorsal surface and a yellow ventrum.

Complementation analysis of eight lethal deletions revealed there were at
least three functional units at the \( a \) locus. The ecotropic murine leukaemia
provirus \( Emv-15 \), was found to be closely associated with the lethal \( A_Y \)
mutation (Copeland et al., 1983). However one strain of mice with the \( A_Y \)
mutation did not contain \( Emv-15 \), indicating that it was not responsible for the
\( A_Y \) phenotype. Unlike other loci, sequences isolated from the proviral
insertion site have not, as yet, proved useful in the molecular analysis of this region. However, the recent characterisation of a radiation induced inversion has permitted molecular access to the agouti gene and the complex itself. The end-points of the inversion involve two genes, agouti (a) and limb deformity (ld), which normally map 22cM apart. By utilising a probe from the ld gene, sequences from the agouti locus were isolated (Woychik et al., 1990). An evolutionary conserved region was identified in adjacent sequences and this was used to screen a cDNA library. One cDNA which was identified encoded a 131 amino acid protein. This transcript was either altered or abnormally expressed in all the agouti mutants examined. For instance, in black-and-tan mice the transcript is overexpressed in yellow ventral regions and absent in black dorsal regions (Bultman et al., 1992). The agouti gene product is a secreted protein which is expressed in the testes and neonatal skin, however its mode of action has not yet been ascertained.

The cloning of the agouti gene has enabled molecular analysis of the Ay mutation (Michaud et al, 1993). Lethal yellow mice have a larger than normal agouti transcript, which is due to a larger 5' region. This transcript is not localised to the skin and testes, but is present in all tissues examined. It is now thought that the Ay mutation is due to a small deletion, which places the agouti gene under the control of the promoter of a neighbouring gene. This gene, Raly, encodes a RNA binding protein which may be involved in mRNA splicing. The protein is normally expressed in the preimplantation embryo and in all adult tissues, but was not detected in lethal yellow mice. Thus, the dominant effects of the Ay mutation are thought to be due to the overexpression of the agouti gene product in all tissues. The recessive embryonic lethality may be due to the lack of the Raly gene product in Ay mice (Michaud et al, 1993).

The cloning of the agouti gene has already enabled the Ay mutation to be molecularly characterised and allows access to the agouti locus and the genes within it.

1.8 The brown (b) locus

The brown locus on chromosome 4 is involved in the production of the black wild type pigment, eumelanin. A mutation in this gene results in the production of brown eumelanin instead of black pigment. The original
recessive brown mutation used in the SLT, arose spontaneously and was first described by Little in 1913. Many mutant alleles both recessive and dominant to wild type have been isolated over the years.

Many more radiation or chemically induced mutations at the brown locus were isolated using the SLT. These were initially analysed by E. M. Rinchik to determine whether the mutations were lethal or viable in the homozygous state. This was determined using the complex breeding protocol detailed in section 4.1 and breeding stocks of the lethal mutations were established. However, as there was no way of distinguishing between mice homozygous for the original brown mutation (used in the SLT) and those with the newly induced viable mutations, these mutations were unable to be maintained.

Genetic analysis of \textit{b} locus mutations began effectively once the \textit{brown} gene itself was cloned. In 1986 Shibahara et al., isolated a full length cDNA clone, pMT4 from a mouse melanoma cDNA library. It was isolated using differential hybridisation techniques and was, therefore, pigment cell specific. This cDNA was originally thought to be derived from the mouse tyrosinase gene as it displayed similarities to the tyrosinase gene in \textit{Neurospora}. However, it was shown by recombinant inbred analysis to map within 2.8cM of the brown locus (Jackson, 1988). \textit{In vivo} experiments, in which cultured melanocytes isolated from brown mice were transfected with a retrovirus containing pMT4, showed that their brown phenotype is rescued by the pMT4 cDNA (Bennett et al., 1990). Thus, this cDNA is the product of the \textit{brown} gene and has been termed \textit{tyrosinase related protein 1} or \textit{Tyrp1}. Another tyrosinase related protein, TRP-2, has been shown to be the product of the slaty locus on murine chromosome 14, which is also involved in pigmentation (Jackson et al., 1992).

Comparisons of the \textit{Tyrp1} gene in wild type and brown mice revealed four nucleic acid changes, two of which lead to alterations in the protein sequence (Zdarsky et al., 1990). Only one of these, where a conserved cysteine is substituted by a tyrosine, is responsible for the brown phenotype. This was revealed by sequence analysis of the \textit{Tyrp1} gene from a brown cultured melanocyte which had reverted to wild type. The levels of \textit{Tyrp1} mRNA were found to be normal in mice with the original brown mutation, but the translated protein was non-functional. Other allelic mutants were found to have either reduced or absent amounts of \textit{Tyrp1} mRNA (Zdarsky et al., 1990).
possible functions of the TRP1 protein will be discussed in section 1.10.3.

One of the nucleic acid changes in the Tyrp1 mutants abolished a TaqI site and gave rise to a TaqI restriction fragment length polymorphism (RFLP) between the wild type gene and that present in mice with the original brown mutation used in the SLT. All strains of brown mice which were examined had lost this TaqI site, suggesting that they all arose from a common progenitor (Jackson, 1988). As it was now possible to distinguish between the original brown mutation and newly induced mutations, analysis of these mutations could now proceed.

Complementation analysis on a panel of 28 lethal b locus deletions was recently initiated by E. M. Rinchik. This lead to the identification of four functional units in this region, all of which affected development. One, lying proximal to the brown gene, is a neonatal lethal while the two lying distal to brown are embryonic lethals. The forth functional unit lies very close to the brown gene and has been termed the brown associated fitness gene or baf. Mice deleted for the baf gene were considerably smaller and less fit than their littermates. All the brown mice generated from the complementation tests, i.e. those with compound deletions, displayed the baf phenotype. This indicated that the baf gene must lie relatively close to the brown gene, but their relative orientation could not be established.

Two other genes were known to map close to the brown gene, whirler (wi) which maps 7cM proximal and depilated (dep), 2cM distal (Rinchik, 1994). The wi mutation causes deafness in adult mice as well as characteristic circling and head tossing behaviour. Mice carrying each deletion were mated to whirler mice and the offspring were examined for the whirler phenotype. Only one of the deletions was found to extend to this locus. Depilated mice have abnormalities in the skin epidermis which is associated with hair loss. Each deletion was tested, in the same way as above, to determine whether they extended to this locus. Nine of the deletions were found to be at least 2cM long and incorporate the depilated gene (Rinchik et al., 1994).

A complementation map of the region could now be constructed (fig. 1.4), in which the deletions could be grouped together. The cloning of the brown gene allows molecular access to this region, thus enabling molecular analysis
This figure was adapted from Rinchik, 1994. The map was constructed as a result of the complementation analysis of 28 lethal b locus deletions. This enabled them to be divided into six complementation groups. Three genes which have been mapped are indicated. Also shown are the approximate positions of the four other genes which were identified as a result of this analysis (see text).
studies to be commenced. This region of murine chromosome 4 displays synteny with human chromosome 9 and the human equivalents of the brown and several nearby genes have been mapped to chromosome 9 (Abbott et al., 1991). In humans, rearrangements of this region are frequently associated with different cancers, including melanoma (Fountain et al., 1992). Thus, characterisation of this region in the mouse may lead to a greater understanding of the role this region plays in human cancer.

1.9 The piebald (s) locus
The site of action of the piebald gene on chromosome 14 is thought to be the neural crest, where it affects two cell lineages (Russell, 1989). A mutation in the gene leads to an absence of melanocytes in discrete areas of the coat, resulting in patches of non-pigmented fur. This is known as white spotting. In some cases it also leads to the absence of enteric ganglia in the distal portion of the colon causing megacolon. Lethality usually occurs before the mice are weaned, at three weeks.

Two spontaneous alleles have been recovered, piebald (s) and piebald lethal (s1), which has a more severe phenotype. Many other alleles have now been isolated by the SLT.

Analysis of this region was restricted, until recently, because of the lack of any useful nearby markers. However the genetic mapping of six microsatellite markers to the s locus has enabled the piebald gene to be localised to within a 2cM interval between two markers.

These markers were also used to study nine induced mutations at the s locus. Six were found to be deletions incorporating at least one of the surrounding markers, although the others may contain smaller deletions. A more detailed fine structure map of this region will facilitate the isolation of the piebald gene. As this is one of the candidate genes for Hirschsprungs disease in humans, it will be interesting and useful to determine its function (Metallinos et al., 1994).

1.10 Melanogenesis
1.10.1 Embryology of pigmentation
Mice have two types of hairs, the overhairs, of which there are three kinds, and the underhairs (Silvers, 1979). All have a similar basic structure, an inner medulla surrounded by the cuticle and outer cortex. Most of the pigment
granules or melanosomes are located in the medulla, but some are also present in the cortex. The hair follicle develops from an epidermal invagination into the dermis. Melanoblasts, the melanocyte precursors, enter the hair follicle at this stage. These cells have migrated to the surface of the embryo from the neural crest. In the mature hair follicle, melanocytes secrete pigment granules or melanosomes into the hair cortex and medulla as it develops. Each hair follicle is thought to have around 20 melanocytes within it. In mice hair growth is cyclical and pigment granules are only produced during the period of active hair growth. In newborn mice this cycle lasts about 17 days and is followed by a resting period (Silver, 1979).

Melanocytes are also found in the retina. However, these are developmentally distinct from those found in the skin, as they are not derived from the neural crest.

1.10.2 Eumelanin and phaeomelanin
In wild type animals two types of pigment are produced, eumelanin which is black and the yellow phaeomelanin pigment. These pigment granules are visibly different, with the phaeomelanin granules having a uniform shape and colour, whereas the eumelanin granules are much more variable in both respects. Also, the density of pigment granules is five times higher in black hairs when compared with yellow. Each melanocyte can produce both types of pigment, however, they are not produced at the same time. In wild type agouti mice, each hair has a banded appearance due to alternating bands of yellow and black pigment. The synthesis of these pigments is controlled by the dermal cells in the hair follicle. Mutations at the agouti locus affect these dermal cells and affect the alternating production of the two pigments (reviewed by Jackson, 1985).

Both types of pigment are derived from tyrosine, although the metabolic pathway of their biosynthesis is not known. However, the first step in both pathways involves the two step conversion of tyrosine to dopaquinone by tyrosinase, the product of the albino locus. The pathways then diverge. As tyrosinase is the only enzyme common to both pathways, its absence results in pigment not being produced, as is seen in albino mice (the null phenotype). Other alleles of the albino locus also affect both types of pigment. For instance, in chinchilla mice, the normally black eumelanin is a dull slate colour.
and the yellow phaeomelanin is a creamy shade.

Most of the coat colour mutations used in the SLT affect pigment production within the melanocyte itself. Brown eumelanin is produced in mice with a mutation in the \textit{Tyrp-1} gene, whereas in mice with a mutation at the pink-eyed dilution locus, eumelanin synthesis is severely affected. Mice with mutations in two coat colour genes may show the cumulative effects of these mutations. For instance, chinchilla mice which also carry a mutation at the pink-eyed dilution locus have cream coloured coats. This is because the only pigment produced in these mice is phaeomelanin, which is cream in colour due to the effect of the chinchilla mutation.

1.10.3 Characteristics of the TRP-1 protein

Although the role of TRP-1 in melanogenesis is not known, speculation as to its function is possible, using information gained from sequence data, cellular localisation and enzymatic activities. The \textit{Tyrp1} gene is 15kb in length and contains eight exons and seven introns. Comparisons between the tyrosinase gene, \textit{Tyrp1} and \textit{Tyrp2} have been undertaken, in order to identify common melanocyte specific promoter elements. 5' cis acting regulatory elements (TATA and CCAAT boxes) have been identified in the tyrosinase gene but are not present in \textit{Tyrp1}. A common 11bp sequence, termed the M box, has been found in all three genes which may act as a positive regulatory element. Its sequence suggests it may be a binding site for a helix-loop-helix transcription factor. Two other upstream regulatory elements have been identified but their binding proteins are also not known (Jackson et al., 1991; Lowings et al., 1992).

The TRP-1 protein is 58,000 daltons and around 40% identical to tyrosinase, with the regions predicted to be functionally important being more highly conserved. Both proteins have a transmembrane domain near the C-terminus, signal sequences and the conservation of two copper binding sites. Both also have glycosylation sites and conservation of cysteine, tryptophan and histidine residues. The original brown mutation is known to be caused by substitution of one of the conserved cysteines in TRP-1 (Bennett et al., 1990), while substitution of another conserved cysteine in tyrosinase, leads to inactive tyrosinase and albino mice (Shibahara et al., 1990). The mutant TRP-1 protein is not fully glycosylated and is less stable than the normal
protein in melanoma cells. It has been suggested it may result from aberrant folding of the protein making some glycosylation sites inaccessible (Orlow et al., 1993). The cysteine rich regions have similarities to the EGF domains of other proteins and may be involved in protein to protein interactions (Jackson et al., 1992).

The tyrosinase protein and TRP-1 have three types of catalytic activity, however, tyrosinase has a much stronger catalytic potential (Hearing et al., 1992). The human equivalent of TRP-1, gp75, was thought to have catalase activity, but these experiments have never been repeated. It has been suggested that gp75 may be involved in the breakdown of byproducts produced in melanin biosynthesis. Both tyrosinase and TRP-1 are melanosomal transmembrane proteins, however their distribution patterns are different. TRP-1 is largely restricted to the early unmelanized melanosomes, whereas tyrosinase is primarily detected in latter stage melanized melanosomes (Orlow et al., 1993).

1.11 Physical mapping at the brown locus
The panel of deletions at the brown locus, generated by the SLT, provide an excellent resource with which to begin detailed genetic and physical mapping in this region. Studies of the deletion phenotype and complementation mapping have revealed the presence and position of functional groups which are important in development. Physical mapping by molecular means is possible, now that the brown gene has been cloned, which will enable the identification and cloning of the genes within these groups. It is probable that other genes exist in this region which have not been identified by genetic studies, or that more than one gene is present within a functional group. The generation of a physical map will enable these previously unidentified genes to be uncovered.

The mapping of DNA markers to this region constitutes the first step in the generation of a physical map and this can be achieved using the panel of deletions at this locus. These markers will then be used to isolate cloned DNA from the region and a contig can be established. Any gaps can be filled by chromosome walking techniques (these will be discussed further in section 1.14). Once the location of these genes has been further defined, the appropriate clones will be examined for transcribed sequences and candidate
genes identified. Thus, the generation of a contig spanning this region will facilitate the isolation of genes within the four identified function groups.

1.12 Generation of DNA markers
DNA markers have been generated by numerous and varied techniques and some of the most productive are detailed below. Emphasis will be placed on those which have generated DNA markers in the mouse, especially those which map to chromosome 4.

Libraries made from single, flow sorted chromosomes have proved useful in providing molecular access to some genes located on these chromosomes (Rommens et al., 1990). However, as clones are isolated from the whole chromosome, those closely linked to the region of interest must still be identified. This is commonly done by fluorescent in-situ hybridisation (FISH) or by mapping using hybrid or deletion panels. If libraries are made from smaller chromosomal fragments, such as those from radiation or fragmentation cell lines, then useful clones can be identified more easily. However, for many chromosomal regions, such cell lines are not available. Although these techniques have been useful in the field of human genetics, for technical reasons they have been of limited use in mouse genetics. There are few somatic cell hybrid lines containing mouse chromosomes and mapping markers by FISH is problematic because of the difficulty in identifying individual chromosomes in metaphase spreads.

The development of the polymerase chain reaction (PCR) (Saiki et al., 1985; Mullins et al., 1987), enabled specific target DNA sequences to be amplified from a complex mixture and this facilitated the development of many new approaches to DNA analysis. Discussed below are several techniques involving PCR, which enable markers to be generated from previously uncharacterised regions.

1.12.1 Microdissection and microcloning
The technique of microdissection was developed using the polytene chromosomes isolated from the salivary glands of Drosophila. It was first used on murine chromosomes by Rohme et al. (1984) and enabled large numbers of markers to be cloned from specific subchromosomal regions. The chromosome under study is identified in unstained metaphase spreads and
chromosomal material is physically dissected from the chromosome. Microdissected DNA is then digested with appropriate enzymes and cloned into a suitable vector. This approach has been used to characterise many chromosomal subregions involved in human disease. For example, the Prader-Willi region on chromosome 15 (Buiting et al., 1990) and the 22q12-q13 region which is involved in adenomatous polyposis coli (Fiedler et al., 1991).

In the mouse this technique is hindered somewhat, because of the difficulty in identifying specific chromosomes in unstained metaphase spreads. However the existence of Robertsonian translocations between certain chromosomes has enabled this technique to be of use in the analysis of some chromosomal regions.

Bahary et al., (1993) generated a microdissection library from murine chromosome 4 surrounding a locus involved in diabetes (db), which maps 10cM distal to the brown gene. Chromosomes from mice carrying a 4:15 Robertsonian translocation were used, thus enabling the conclusive identification of chromosome 4 in metaphase spreads, due to its increased length and centrally located centromere. The brown locus had previously been localised to the translocated chromosome by in-situ hybridisation and DNA was microdissected from this region. After EcoRI digestion, isolated DNA was cloned in the lambda vector, λgt10. Of an initial 200 clones isolated, 41 were genetically mapped by intercross analysis, to a 21cM region surrounding db. The microclones were on average 180bp in length and located 0.5cM apart. The remaining clones were either unable to be mapped, because of the presence of repeat sequences or the absence of an RFLV, or they were found to be unlinked to chromosome 4.

These microclones significantly increase the density of markers at the brown/db region and will assist in the positional cloning of the db gene, as well as in the future physical mapping of this region. As the microclones can be amplified by PCR, this will greatly contribute to their ease of use.

1.12.2 Microsatellites
Microsatellites are simple sequence tandem repeats which are present in all eukaryotic DNAs examined so far, except yeast. The repeat unit is usually
between 1-6bp long and most consist of 10-50 tandemly repeated units. They are frequently present and randomly distributed in the genome, unlike some other repeat families. The most common microsatellite is a CA dinucleotide repeat and it is estimated to be present, on average, every 18kb in the mouse and every 30kb in humans (reviewed by Hearne et al., 1992). Microsatellites are located in unique sequence DNA and this, along with their size, makes them amenable to study using PCR. Primers designed from flanking unique sequence DNA are used to amplify across the microsatellite and size polymorphisms, due to variation in the number of repeat units, can be detected using agarose or polyacrylamide gels. Initial studies revealed the highly polymorphic nature of microsatellites, which facilitates their use as genetic markers in linkage and mapping studies.

Studies have shown that 50% of microsatellites are variable amongst inbred strains (Love et al., 1990) and they are considerably more variable between species. This implies that microsatellites have a high mutation rate, which has been estimated at around 2x10^{-4} per locus per generation in the mouse (Dietrich et al., 1992). Preliminary investigations in the human male germline have indicated that most mutations occurring at the microsatellites studied, involve expansion of the number of repeat units. Some mutations appear to involve non-reciprocal interallelic exchange, which may be due to repair processes (Jeffreys et al., 1994). However, it is not known whether these events are ubiquitous and it is possible that many mutations occur simply as a result of slippage of the DNA polymerase during replication.

Polymorphic microsatellite repeats have been isolated by various means. Databases have been searched for the presence of such repeats in previously cloned sequences (Hearne et al., 1991). More recently, Dietrich et al. (1992) isolated over 300 microsatellite markers and used them to generate a microsatellite linkage map of the mouse. This involved screening a genomic library by hybridisation using a (CA)n oligonucleotide, in order to identify clones containing CA microsatellite repeats. Clones smaller than 500bp were then sequenced and PCR primers were designed from unique sequence flanking DNA. Those microsatellites which demonstrated allelic polymorphisms were then mapped by intercross analysis (discussed further in section 1.13). Thus, a microsatellite genetic map was constructed. This was then integrated with the existing genetic map, by utilising microsatellite
polymorphisms in previously mapped genes. The integrated map contains 317 randomly spaced markers which are on average around 4.3cM apart and will prove useful to many researchers (Dietrich et al., 1992).

Other approaches that enable the isolation of microsatellite markers from specific chromosomal regions have been employed. In the case of murine chromosome 4, a library made from a 4:6 Robertsonian translocation chromosome, isolated by flow sorting, was screened for CA and GT containing microsatellites (Cornall et al., 1992). Of the 20 that were analysed for polymorphisms, 70% were polymorphic in inbred strains and 85% showed interspecific variation. Thirteen of these clones were genetically mapped using both recombinant inbred (RI) panels and backcrosses.

Yeast artificial chromosomes (YACs), which have been isolated from a region under study, are suitable material for isolating useful microsatellites. An average sized YAC should contain several microsatellites which may be of use in detecting linkage with a specific disease. The role of YACs in physical mapping will be discussed in section 1.14.

1.12.3 Interspersed Repetitive Element PCR (IRS-PCR)
In the mouse, repeat elements from the long (LINE) and short (SINE) interspersed element families have been characterised. The L1Md repeat is the most abundant repeat element in the LINE family, there are between 70-100,000 copies in the genome. Individual elements are usually truncated at the 5' end and contain a conserved R repeat element at the 3' end. The most frequently found members of the SINE family are the B1 and B2 repeat elements. 130-180,000 copies of the B1 repeat are present in the murine genome. This element shares homology with the major human SINE element, the Alu repeat. However the B2 element which has 80-120,000 copies, does not share homology with any human repeat (Hastie, 1989).

Oligonucleotide primers have been designed from the conserved sequence present at the ends of these repeat elements (Cox et al., 1991). They are orientated with their 3' ends towards the end of the repeat element, thereby allowing the amplification of unique sequence DNA from between repeat elements. The PCR product generated from such reactions contains a small amount of DNA from the repetitive element at each end, with unique inter-
repetitive sequence DNA in the middle. However, PCR products will only be amplified when two repeat elements lie in the correct orientation and a suitable distance apart.

Cox et al. (1991) used this technique to generate inter-repeat element amplified products from genomic DNA and examined them in two different murine species, in an attempt to identify polymorphic products. These would be produced if the position of the repeat element or the primer binding site were altered. A number of polymorphic PCR products were identified and 13 were mapped using an interspecific backcross. However, the identification of polymorphic products was difficult, as many PCR products were produced when genomic DNA is used as the template. As L1Md repeat elements are found less frequently in the genome than other repeat elements, when primers from only this repeat are used, less PCR products are generated. Thus, the identification of polymorphic products on an agarose gel is simplified. When other primers or combinations of primers are used, the pattern of products is too complex to be resolved by conventional gel electrophoresis.

This technique has been adapted by using, as the template DNA, somatic cell hybrids containing specific murine chromosomes. The use of murine specific primers (from the B2 and R repeat elements) under stringent conditions, ensured that PCR products were amplified only from murine DNA. By reducing the complexity of the template DNA, a reduction in the number of IRS PCR products was achieved. This was demonstrated by Simmler et al. (1991) using radiation-fusion Chinese hamster cell hybrids containing the murine X chromosome. Also used were hybrids containing overlapping fragments of the murine X chromosome. Different hybrids had some PCR products in common. As these were amplified from common sequences, hybrids containing overlapping fragments of the X chromosome could be identified by common IRS PCR markers.

However, monochromosomal somatic cell hybrids are not available for many regions of the mouse genome. If YACs are available from a region under study, this method can be used to generate potentially polymorphic YAC markers or to characterise a YAC contig by the identification of overlapping YACs. When the template is restricted to smaller regions of DNA, such as
those contained in YACs, fewer IRS-PCR products will be formed. Although this simplifies analysis, the information gained from this type of study is restricted. However adaptations to the basic IRS-PCR technique, for instance IRS-bubble PCR, have been developed to overcome this problem (Munroe et al., 1994). This involves ligating linkers to the digested template DNA and using primers, specific to a repeat element and the linker, in the PCR reaction. Thus, a PCR product will be generated from fragments which contain only one repeat element, if it lies an appropriate distance from the linker.

Other related PCR based approaches have also been fruitful. For instance, randomly generated oligonucleotides have been used to amplify unique DNA sequences from the mouse (Woodward et al., 1992). If a polymorphism in these PCR products exists, either between or within species, they can be genetically mapped. These approaches have generated many new markers which have already proven their usefulness in genetic and physical analysis.

1.13 Mapping of DNA Markers

Genetic mapping of randomly generated markers is easier in the mouse than it is in man. Because of this difficulty, somatic cell hybrids containing single chromosomes and radiation hybrids with subchromosomal regions are commonly used in human analysis. However, these are not available for all regions and cannot always be used to map a marker precisely. FISH analysis is also a useful technique, but many markers isolated by PCR are simply too small to make this technique applicable.

In the mouse alternative techniques have been developed which enable random markers to be genetically mapped to specific chromosomal subregions. They involve using the progeny of either intraspecific (recombinant inbred strains) or interspecific backcrosses. These progeny are first typed for polymorphic anchor loci, whose chromosomal location has been previously identified. Commonly three or four loci which span the length of each chromosome are used. This enables recombinational events occurring between the progenitor chromosomes to be identified and localised between two anchor loci. These mice are then typed for random polymorphic markers and genetic linkage to an anchor loci can be established by analysing recombination frequencies. This technique allows the genetic distance from a marker to an anchor loci, to be determined.
In order to map a specific marker, a polymorphism must be present between the two alleles of the backcross mice. Linkage analysis was first carried out using inbred laboratory strains or those derived from these strains eg. recombinant inbred strains. However it was quickly realised that, because of extensive inbreeding, the levels of allelic variation were low. This problem was overcome by increasing the genetic variability by crossing the laboratory mice, *Mus musculus domesticus*, to another wild species (Avner et al., 1988). *Mus spretus* mice were commonly used as they show the most allelic variation. As such, the European Collaborative Interspecific Backcross is currently being developed, which will enable the generation of a high resolution genetic map. This will be invaluable to subsequent physical mapping of the murine genome. As comparative maps of the mouse and human genomes have been established in some regions, murine markers may be of use in human genetic analysis.

This mapping technique depends on the presence of a detectable polymorphism. Those which alter enzyme restriction sites, termed restriction length fragment polymorphisms (RFLP) can be easily identified by Southern blot analysis. PCR technology has lead to the isolation of many more polymorphic markers which do not depend on the alteration of a restriction site. Size polymorphisms in microsatellites amplified by PCR can be detected on Nusieve or Metaphor agarose gels (FMC Bioproducts). Alternatively, polyacrylamide gels can be used.

It is also possible to detect sequence differences in single or double stranded PCR products, even single base pair changes which do not alter the restriction pattern. Two commonly used techniques involve using single stranded conformational polymorphisms (SSCP) and heteroduplex analysis. SSCP was first used by Orita et al. in 1989. It is based on the principal that the migration of DNA fragments, when subject to electrophoresis in non-denaturing conditions, depends not only on their length, but also on their sequence. Even small sequence changes give rise to alterations in secondary structure, which affect motility and these can be resolved on non-denaturing polyacrylamide gels. The conformation a particular fragment adopts is sensitive to various factors and these can be altered to increase the resolution of such molecules. This technique has been used to map previously cloned sequences (Beier et al., 1992) and markers generated by
IRS-PCR (Hunter et al., 1993) have been mapped by RI and intercross analysis. Alternatively, heteroduplex analysis detects polymorphisms between two DNA fragments based on their double stranded secondary structure. One way of detecting such polymorphisms involves using non-denaturing Hydrolink gels (AT Biochem). The migration of annealed PCR products in the gel, again, depends on their secondary structure. Any polymorphic PCR products will form a heteroduplex, whose migration will be slower than that of any homoduplex products (Keen et al., 1991).

1.14 Isolation of cloned sequences

Once markers have been linked to a gene of interest or mapped to a specific region, they can be used to isolate cloned DNA sequences. These can then be used to isolate overlapping clones and generate a contig of the region. Thus, a physical map of a previously genetically characterised region can be established.

Different types of vectors have been used to generate genomic libraries, each with its own advantages and disadvantages. Cosmids have been used extensively and have a high cloning efficiency (Collins and Hohn, 1978). However their major limitation is that not more than 50kb can be cloned into a cosmid, making this type of vector more suitable for the examination of some individual genes or small gene clusters. The P1 bacteriophage cloning system is an improvement on this, enabling fragments of 100kb to be cloned (Sternberg, 1990). However, yeast artificial chromosomes (YACs) are the method of choice in the study of megabase regions of DNA. With this system, large DNA fragments can be cloned and maintained in a eukaryotic yeast cells. The source DNA is usually genomic, although flow sorted chromosomes can be used to generate chromosome specific libraries. The DNA is first digested with a rare cutting enzyme or partially digested with a more frequent cutter. The fragments are then cloned into a yeast vector, which contains all the functions needed for replication; centromere, telomeres, replication sequences as well as selectable markers. These are then transformed into yeast cells, where the cloned material is maintained as artificial chromosomes (Burke et al., 1987). The ability to clone such large continuous DNA fragments has revolutionised large scale genome mapping projects. It simplifies positional cloning strategies and enables larger genes or gene complexes to be studied more efficiently.
YACs from initial libraries commonly had average insert sizes of less than 200kb. Improved techniques have increased the average insert size to around 400kb (Imai and Olsen, 1990) and YACs over 1Mb have been isolated. The technical improvements include better handling techniques, size selection of the DNA before cloning and size fractionation before transformation.

YACs can be analysed by various means. Their size can be determined by pulsed field gel electrophoresis (PFGE), which can also be used for restriction mapping of the YACs. Markers from YACs can be generated by PCR based techniques. Isolation of the YAC ends is important for the establishment of contigs. This can be done using conventional cloning stratagies, but PCR based methods are quicker and more generally used. Inverse PCR involves the circularisation of digested YAC fragments and the specific amplification of end fragments, using primers derived from either arm of the vector (Arveiler and Porteous, 1991). The "vectorette" method involves ligating linkers to the digested YAC and amplifying the end-fragments using primers complementary to the vector and the linker (Riley et al., 1990). Once YAC end fragments have been isolated they can then be used to screen YAC libraries and identify overlapping clones. YAC libraries can be screened either by hybridisation or PCR.

Although YACs have considerable advantages over other cloning techniques in the field of genome analysis, they have disadvantages too. The main difficulties are the relatively high levels of chimaeric YACs in libraries and the instability or rearrangement of some YACs within yeast cells. Chimaeric YACs contain noncontiguous DNA and most are thought to be generated from co-ligation events in the cloning process. The chromosomal origin of the YACs can be confirmed in mice by mapping the end-clones using backcrosses. In humans fluorescence in-situ hybridisation (FISH) to metaphase chromosomes is commonly used or mapping of the end fragments using somatic cell hybrids. Studies have shown that over 40% of the YACs in some libraries are chimaeric (Selleri et al., 1992). Chimaeric YACs tend to be significantly larger than other YACs. Although it is possible to isolate the sequences of interest from such YACs, it is usually more straightforward to isolate another YAC. As most libraries have 2-5 fold coverage of the genome, this is usually easily achieved.
Some YACs have proved to be very unstable and rearrangements, usually deletions, have been detected. The high level of homologous recombination in yeast is thought to be responsible for this instability. Recombination between repeat elements on individual YACs has been detected (Green et al., 1991) and also between two YACs within the same yeast cell. YACs with a large number or tandem repeats are very unstable (Neil et al., 1990). In most cases the instability of YACs is thought to be sequence dependant, although size is also a factor in YACs under 100kb. The high level of homologous recombination in yeast has been utilised to generate larger YACs. Recombination between partially overlapping YACs has enabled a 2.3Mb YAC encompassing almost all of the human dystrophan gene to be generated (Den Dunnen et al., 1992).

The development of the YAC cloning system has enabled megabase regions of DNA to be physically characterised and the genetic and physical maps to be correlated. The recombinational distances of the genetic maps (1cM is approximately equivalent to 1Mb in humans and 1-2Mb in the mouse) can be directly compared to physical distances. YACs also simplify positional cloning strategies, thus enabling the rapid cloning of genes. Studies of gene function and regulation are also facilitated as large genes can be isolated in a single YAC clone. As YACs can be transferred into mammalian cell lines, functional and mutational analysis can be carried out. The possibility of YACs being used in gene replacement techniques is also being explored.
Chapter 2
Materials and Methods

2.1 Maintenance of mice stocks
Mice were kept at the Biomedical Research Facility at the Western General Hospital. They were cared for principally by V. Ranaldi and A. Wilson. The animal work in this project was authorised by Home Office licence number 60/01010.

2.1.1 Origin and strains of mice
The *M. musculus* strain CBA/Ca, was used to maintain stocks of mice carrying the Harwell deletions (see section 3.4). CBA/Ca is an inbred laboratory strain which has a wild type, black, agouti coat colour. The non-inbred laboratory strain *b<sup>Y</sup>* was also used to maintain stocks of the Harwell deletions. *b<sup>Y</sup>* mice have a brown coat colour, as they carry the original *brown* mutation. The *b<sup>Y</sup>* strain was derived from a Light stock of mice, which were originally obtained from the MRC Radiobiology Unit, Chilton, Didcot.

Seven brown mice were obtained from B. Cattanach at the MRC Radiobiology Unit, Didcot. These contained radiation induced mutations at the brown locus and were isolated by the SLT (W.L. Russell, 1951). Male offspring from a C3H/HeH x 101/H cross were irradiated with two doses of three Gray X-rays, with a 24 hour interval between each dose. The irradiation was confined to the lower half of the body. These mice were then mated with females from the tester stock (see section 3.1), and brown mice were identified in the offspring. Of the seven mice obtained from B. Cattanach, three (*b<sup>57H</sup>-*<sup>59H</sup>) were male and four female (*b<sup>60H</sup>-*<sup>63H</sup>). All were compound heterozygotes at the brown locus, containing the newly induced mutation over the original *brown* mutation.

Stocks of these mice were maintained as detailed in section 3.4. These mice were referred to as those which carried the Harwell deletions. Mice were weaned at three weeks and mated at six weeks.

Matings were set up between mice hemizygous for the *b<sup>63H</sup>* deletion and *M. castaneus* mice, which were a gift from K. Steel at the MRC Institute for Hearing Research, Nottingham.
2.1.2 Tail tipping procedure
In order to differentiate between mice carrying the newly induced mutation and those carrying the original brown mutation, the genotype of these phenotypically wild type mice was established. This was ascertained by extracting DNA from the tail tip and carrying out a PCR based assay (section 3.4).

The tail tipping procedure was performed by V. Ranaldi when the mice were at least three weeks old. Mice were anaethetised with fluothane gas (ICI Pharmaceuticals) using a Boyle's anaesthesia apparatus. Approximately 1cm was removed from the end of the tail using a sterile scalpel blade. The mice were then earmarked for subsequent identification. After they had recovered from the anaesthetic, they were returned, to their cages.

2.1.3 Isolation of b^{63H} homozygous embryos
To ascertain the stage of lethality of the b^{63H} homozygotes, embryos were examined at different stages of development. Females were checked every morning for plugs; if present, the gestation of the embryos was counted as 1/2 day. Embryos were routinely examined at 11.5-12.5 days gestation.

The females were killed and the uterus was removed and placed in PBS solution (140mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH7.4). The embryos were carefully removed from the uterus, then separated from the placenta, before being rinsed in PBS solution. This procedure was carried out with the aid of a dissecting microscope (Wild, M7). The embryo was examined and its stage of development ascertained, before DNA was made from the embryonic tissue.

2.2 Yeast cell culture and DNA isolation
2.2.1 Media and additives
All media was sterilised by autoclaving.

AHC broth and agar
AHC is a rich, selective medium which lacks uracil and tryptophan. It was used for selective growth of YAC recombinants prior to production of plugs and isolation of DNA.
1.7g yeast nitrogen base (without amino acids and ammonium sulphate) (Difco), 5g ammonium sulphate and 10g casein hydrolysate-acid (low salt) were added to 1 litre distilled water. The pH was adjusted to 5.8. 17-20g of Bacto agar (Difco) were added to each litre of broth, to make AHC agar. After autoclaving and allowing to cool, 50ml of filter sterilised 40% glucose and 10ml of 2mg/ml sterile adenine sulphate solution were added to the broth (or agar).

**YPD broth and agar**
YPD medium was used for growing yeast cultures.

20g bactopeptone (Difco), 5g yeast extract (Difco), 5g sodium chloride were added to 1 litre distilled water. 1.5% agar was added at this stage, for YPD agar. After autoclaving and allowing to cool, 50ml of sterile 40% glucose was added to the broth or agar.

**2.2.2 Yeast strains**
*Saccharomyces cerevisiae* strains YP148 (Jones et. al., 1989) and AB972 (Link and Olson, 1991) were used as size markers for pulsed field gel electrophoresis. Table 2.1 lists the sizes of the chromosomes of the two strains.

**2.2.3 Yeast DNA preparation**
This method was adapted from Hoffman and Winston (1987).

10ml yeast cultures were grown to saturation, and the cells collected by centrifugation at 3000 rpm. The cells were resuspended in 500μl of dH₂O, transferred to an eppendorf tube and collected by a brief centrifugation at 13,000 rpm. Most of the supernatant was removed and the pellet was briefly vortexed. 200μl lysis buffer (2% Triton X-100 (Sigma); 1% SDS; 100mM NaCl; 10mM Tris.HCl, pH8; 1mM EDTA, pH8), 400μl phenol/chloroform and 0.3g of 0.4mm glass beads (BDH) were added to the cells, which were then vortexed for 4 minutes. This process results in cell lysis and separation of the nucleic acids, which remain in the aqueous layer, from the cell debris which remains in the organic layer. After vortexing, 200μl TE was added and the tubes were centrifuged at 13,000 rpm for five minutes. The supernatant was decanted to a fresh tube and 1ml cold ethanol was added to the
supernatant (in order to precipitate the DNA). The tubes were inverted to mix and centrifuged at 13,000 rpm for 2 minutes. The pellet obtained was dried and resuspended in 40μl of TE (10mM Tris.HCl, pH8; 1mM EDTA, pH8). RNA was digested by addition of 30μg RNaseA (Sigma), followed by a 5 minute incubation at 37°C. The DNA was precipitated by addition of 10ml 4M ammonium acetate and 1ml ethanol (at -20°C). The tube was inverted to mix and centrifuged at 13,000 rpm for 2 minutes. Once dry, the pellet was resuspended in 50μl of TE.

<table>
<thead>
<tr>
<th>Chr. Number</th>
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<tr>
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<td>VII prox.</td>
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</tr>
<tr>
<td>XIII</td>
<td>930</td>
<td>XVI</td>
<td>1000</td>
</tr>
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<td></td>
<td></td>
<td>I</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VII dist.</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 2.1: Chromosomal size (in kb) of yeast size marker strains

2.2.4 Preparation of yeast DNA plugs for PFGE
This method allows the extraction of intact yeast chromosomes, including yeast artificial chromosomes (YACs), from cells. It was adapted from Maule (1994).
A 100ml yeast culture was grown, with shaking at 200 rpm, from a single colony, at 30°C for 24 hours, or until the number of cells reached approximately $10^8$ per ml. The culture was then chilled on ice for 15 minutes. The cells were then harvested, by centrifugation at 4000 rpm, for 10 minutes at 4°C. The supernatant was discarded and the pellet dispersed in 50ml of chilled 50mM EDTA, pH7.5. The cells were harvested, resuspended in fresh EDTA and harvested again, as described above. The pellet was resuspended in 3ml chilled 50mM EDTA, pH 7.5 and warmed to 37°C. Cell walls were disrupted by the addition of 1.2ml freshly prepared cell wall digestion solution (2ml SCE, pH8 (1M sorbitol; 0.1M sodium citrate; 60mM EDTA); 100μl 2-mercaptoethanol (Sigma); 2mg zymolyase (ICN Biomedicals). 6ml of 1% low melting temperature agarose (Gibco) in 0.125M EDTA, pH 7.5, at 50°C was added to this. After thorough mixing, 100μl aliquots of the solution were quickly dispensed into plug moulds and allowed to set on ice. Once solidified, the plugs were ejected into 25ml ETM solution (0.45M EDTA; 10mM Tris.HCl, pH8; 7.5% 2-mercaptoethanol) and placed in a 37°C waterbath for 24 hours. The ETM was replaced with 20ml of 1% NDS solution (0.45M EDTA; 10mM Tris.HCl, pH9; 1% SDS) containing proteinase K (Boehringer Mannheim) at 1mg/ml and the plugs incubated at 50°C for 24 hours. This step was repeated with fresh 1% NDS solution. Finally, the plugs were stored in 20ml of ETM at 4°C.

2.2.5 The ICRF YAC library
The ICRF YAC Library was screened by Southern hybridisation (Southern, 1975) (see sections 2.7-2.9). It consisted of YACs from two libraries, which had been gridded mechanically onto two filters, each containing 18,000 YACs. The libraries, constructed by Z. Larin et al. and S. Meier-Ewert, were both constructed in a similar way (Larin et al., 1991). Genomic murine DNA was partially digested using a combination of EcoRI and EcoRI methylase. This generated DNA molecules which ranged in size from 200-2000kb. These fragments were cloned into the EcoRI cloning site in yeast vector pYAC4, and transformed into S. cerivisiae AB1380 cells. Z. Larin's library contained 15,000 clones, with an average insert size of 700kb, which represented a three fold coverage of the mouse genome.
2.2.6 The RG YAC library
The Research Genetics library was screened by PCR. Screening was carried out in two stages, primary screening of the complex pools, followed by secondary screening of the appropriate pools.

2.2.7 European Collaborative Interspecific Backcross (EUCIB) panel
DNA was available from the EUCIB panel (European Backcross Collaborative Group, 1994) and this was used to map a microsatellite repeat isolated from YAC 2/1.

The DNA was isolated from mice derived from matings between *M. musculus* c57/B6 and *M. spretus*. PCR primers from the polymorphic microsatellite repeat were used to amplify over 50 DNAs from the EUCIB panel. The results of this typing were analysed using the MBx database and the chromosomal location of the microsatellite determined.

2.3 DNA isolation from murine tissues
2.3.1 DNA extraction from spleen
After dissection, spleens were snap frozen in liquid nitrogen and stored at -70°C, until required. The frozen spleen was placed in 10ml of 50mM Tris.HCl, pH8, 5mM EDTA and 100mM NaCl. It was homogenised briefly, using a Silverson homogeniser, and SDS was added to a final concentration of 0.5% and Proteinase K, to a final concentration of 100μg/ml. The homogenate was then incubated at 55°C for three hours.

The DNA was then phenol extracted and ethanol precipitated. An equal volume of phenol was added and the tube, gently mixed. It was then centrifuged for 10 minutes at 3000rpm and the aqueous layer was removed. This process was repeated, first with chloroform, and then with a 24:1 solution of chloroform:isoamyl alcohol. The DNA was then precipitated by adding two and a half volumes of ice cold ethanol and mixing thoroughly. The DNA was spooled out using a sealed glass pipette and dipped into 70% ethanol, before being left to dry. It was then resuspended overnight in 1.5ml of TE. (10mM Tris.HCl, pH8, 1mM EDTA).
2.3.2 DNA extraction from tail tips
This method was adapted from Laird et al., 1991. The lysis buffer has been adjusted to allow restriction digestion or PCR amplification of the DNA, without prior organic solvent extractions.

Once the tail tip had been obtained, it was immediately placed into 0.5ml of lysis buffer (100mM Tris.HCl, pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100μg/ml Proteinase K). It was then incubated overnight in a 55°C waterbath. The tubes were then briefly vortexed, before being spun in an eppendorf centrifuge for 10 minutes at room temperature. The supernatent was then removed and placed in a fresh eppendorf, along with 0.5ml of isopropanol. After mixing, the DNA was spooled out with a disposable yellow tip, dipped into 80% isopropanol and dissolved overnight in 200μl of TE.

2.3.3 DNA extraction from embryos
The method described above (section 2.3.2), was used to extract DNA from whole embryos. The only alterations to this were an increase in the amount of Proteinase K in the lysis buffer, from 100μg/ml to 300μg/ml, and the DNA was dissolved in 400μl of TE.

2.4 Enzymatic manipulation of DNA
2.4.1 Restriction enzyme digestion of genomic DNA
Digestions with restriction endonucleases (Boehringer Mannheim) were carried out in the appropriate buffer (supplied by the manufacturer), at the recommended temperature. Commonly, 8μg of DNA was digested in 60μl, with 10 units of restriction enzyme, for three hours. When longer incubation times were used, the amount of enzyme was reduced accordingly. If two different enzymes were used, both of which required the same buffer, the digests were carried out simultaneously. Otherwise, digestion with the enzyme requiring the lower salt buffer concentration was carried out first. The salt concentration was then altered, by addition of the appropriate amount of sodium chloride solution and the second digestion carried out. Reactions were terminated by heating to 68°C or 80°C for 15 minutes, according to the heat sensitivity of the enzyme (NEB catalogue, 1992), or alternatively, by phenol/chloroform extraction of the reaction. Restriction digests, which were to be run on agarose gels, were terminated by the addition of 1/10th of their volume of "stop mix" (100mM EDTA, pH8; 20%
Ficoll and orange G).

### 2.4.2 Restriction enzyme digestion of plasmid and pYAC4 DNA

The plasmid pMT4 (Shibahara et al., 1986) was digested with HindIII to release the cloned fragment, Trp1.6, which was to be used as a radioactive probe.

The digest was carried out as detailed above, with the following modifications. 10μg of plasmid DNA was digested in a total volume of 80μl, using 10 units of enzyme. Digests were incubated for two hours at 37°C.

10μg of the yeast vector pYAC4 was digested with EcoRI and BamHI, as described above, for 3 hours at 37°C. The 2.6kb fragment from the left arm and the 1.4kb band from the right arm were isolated, and used as hybridisation probes (section 2.6.3).

### 2.4.3 Restriction enzyme digestion of PCR products

Usually 20μl of the PCR reaction was digested in a total of 30μls, with 1-2 units of restriction enzyme, as detailed in section 2.4.1. The appropriate amount of 10x concentrated buffer was added to the digestion mix. When using enzymes which required a low salt buffer, the amount of added buffer was reduced accordingly, to take into account that which was already present.

### 2.5 Amplification of DNA by the Polymerase Chain Reaction (PCR)

PCR (Saiki et al., 1985; Mullis and Faloona, 1987) involves the enzymatic amplification of a specific DNA sequence. The sequence specificity is provided by a pair of oligonucleotide primers which direct amplification. The primers are complimentary in sequence to the 5' ends of the sequence to be amplified and are annealed to the template DNA under conditions where they hybridise only to their exact complement. The PCR reaction is a series of cycles, each of which consists of three steps. The first step is a high temperature step, which causes denaturation of the template. This is followed by a reduction in temperature (to that which is optimum for precise annealing of the primers) and finally an extension step at 72°C (which is the optimum temperature of the thermostable polymerase).
2.5.1 PCR from genomic DNA
Reactions were carried out in a 50μl volume which contained 5mM KCl; 10mM Tris.HCI, pH8.4; 0.1% Triton X-100 (buffer from Promega), 1.5mM MgCl₂ (Promega), 100ng of each primer, 0.2mM of each dNTP (Pharmacia), 100ng of template DNA and 1 unit of thermostable DNA polymerase (Promega). Reactions were overlaid with mineral oil (Sigma).

PCRs were carried out on a Techne PHC-2 machine. The temperature and length of the different steps were varied, according to the primer pair and the length of the fragment to be amplified. When published PCR conditions were available, these were used as the basis of the initial reaction. If no information was available, as was the case when primers were designed from sequence information, then the initial conditions were determined as follows. The length of the initial denaturation step was increased (generally 94°C for 3 minutes). This was followed by a series of cycles, the number of which was dependent on the efficiency of amplification (initially 30 cycles were performed). This series of cycles consisted of:

- denaturation step: 94°C for 30 seconds
- annealing step: for 1 minute
- extension step: 72°C for 1 minute per kb amplified

The temperature of the annealing step was 5°C lower than the melting temperature (Tm) of the primers, which was determined using the equation:

\[ T_m \text{ at } 1 \text{M Na}^+ \text{ concentration} = 4(G+C) + 2(A+T) \]

In a number of cases, the initial conditions required modification in order to promote optimum amplification. If no product was produced, then the annealing temperature was decreased by 2°C until a product was amplified, or the MgCl₂ concentration was altered. If non-specific amplification was seen, the annealing temperature was increased by 2°C, until a specific product was seen. If a small amount of product was amplified, then the number of cycles were increased, until a satisfactory amount of product was seen.

2.5.2 Primer design
When primers were designed from sequence information, they were generally designed "by eye" and checked with the Oligo4 programme (Hybaid).
<table>
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<th>Primer sequence</th>
<th>Reaction conditions</th>
<th>Reference</th>
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</thead>
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<td>Brown</td>
<td>5' TCT TTC TCC CTT CCT TAC TGG 3' 5' TCA TTT GGG TCT TAC TGT TAC 3'</td>
<td>94°C for 3 min. 92°C for 30 sec. 50°C for 45 sec. 72°C for 75 sec.</td>
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<td></td>
<td></td>
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<tr>
<td>Orm</td>
<td>5' TGG CCA ACC TCT GTG CTT CC 3' 5' ACA GTT GTC CTC TGA CAT CC 3'</td>
<td>94°C for 3 min. 92°C for 30 sec. 50°C for 45 sec. 72°C for 60 sec.</td>
<td>Dietrich et al., 1992</td>
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<td>Adfp</td>
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<td>λgt10</td>
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<td>94°C for 3 min. 92°C for 30 sec. 55°C for 60 sec. 72°C for 75 sec.</td>
<td>Huynh et al., 1984</td>
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<tr>
<td>GATA</td>
<td>5' GAA TCC TAT GAC TTT CAT GTA C 3' 5' TAT AGC ATG ACC CTA TAG CAT C 3'</td>
<td>94°C for 3 min. 92°C for 30 sec. 55°C for 45 sec. 72°C for 60 sec.</td>
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| left arm   | 5' GAA TTG ATC CAC AGG ACG GG 3'  
5' GCC AAG TTG GTT TAA GGC GC 3' | 94°C for 3 min.  
92°C for 30 sec.  
60°C for 45 sec.  
72°C for 2.5 min. | Arveiler & Porteous, 1991 |
| right arm  | 5' GGA AGA ACG AAG GAA GGA GC 3'  
5' GCC CGA TCT CAA GAT TAC G 3' |                                          |                                |
| IRS PCR    |                                                                                |                                          |                                |
| B1         | 5' GTC CGG CCG CCT GGA ACT CAC TCT GAA GAC 3'                                 | 94°C for 3 min.  
92°C for 30 sec.  
60°C for 60 sec.  
72°C for 3 min. | Cox et al., 1991 |
| B2A        | 5' TAG ACG CGG CCG CTC TTC TGG AGT GTC TGA AGA 3'                             |                                          | Cox et al., 1991 |
| B2B        | 5' TAG ACG CGG CCG CGA CTG CTC TTC CGA AGG TCC 3'                             |                                          | Cox et al., 1991 |
| L1         | 5' GGA CGC AGA TGT AGC TGT CTC TTG TGA GAC 3'                                 |                                          | Simmler et al., 1991 |
| R1         | 5' GCT GAT TAT GGG ATG GAT CC 3'                                             |                                          |                                |
| D4Nds7     | 5' GTT TTC TCC CAA GCA TAC TAA AC 3'                                         | 94°C for 3 min.  
92°C for 30 sec.  
55°C for 45 sec.  
72°C for 1 min. | Cornall et al., 1992 |
|            | 5' CTA GAT TTG ACA AGA TGC TGA AAC 3'                                        |                                          |                                |

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<td>52°C for 45 sec.</td>
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<td>D4Mit25</td>
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**Table 2.2: PCR reaction conditions and primer sequences**

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</tbody>
</table>
2.5.3 Oligonucleotide synthesis

Oligonucleotides were synthesised, in the form of ammonium stocks, on an Applied Biosystems 381A oligonucleotide synthesizer.

Oligonucleotides were precipitated from stocks by the addition of 1/10th volume 3M sodium acetate and 2.5 volumes ethanol, followed by a 1 hour incubation at -20°C. They were pelleted by a 15 minute centrifugation at 1300rpm, washed twice in 80% ethanol and dried under vacuum. The pellet was resuspended in 200μl TE. The concentration of the DNA obtained was determined by measurement of the optical density; where the absorbance at 260 nm (ABS$_{260}$) =1 at 25μg/ml single stranded DNA.

2.5.4 Oligonucleotide sequence

The primer sequences, conditions of amplification, size of the amplified fragment and the source references, are described in table 2.2.

2.5.5 PCR from yeast colonies

This method was adapted from Taylor (1991).

The yeast colony was picked, with a sterile wooden toothpick, into 100μl of dH$_2$O. This was then boiled for 10 minutes and spun down for 5 minutes in an eppendorf centrifuge. 3μl of the supernatent were used in each PCR reaction, which was set up as detailed in section 2.5.1.

2.5.6 Interspersed sequence (IRS) PCR

Adapted from Cox et al., 1991.

Different combinations of primers, from characterised murine repeats, were used to amplify unique sequence DNA from between repeat elements in murine genomic DNA. IRS PCR was carried out essentially as detailed in section 2.5.1.

2.5.7 Inverse PCR

Adapted from Arveiler and Porteous, 1991.

This was used to isolate DNA fragments from the ends of the YACs. 2μg of YAC DNA was digested individually with TaqI, SacI and RsaI (see section
2.4.1), in a total volume of 20μl. The digestion products were then gene cleaned (Bio 101), resuspended in 20μl of H₂O and ligated under dilute conditions, to generate monomeric circles.

Ligation reactions were carried out in a 10μl volume, which contained 0.1 unit of T4 DNA ligase (Boehringer Mannheim), 1μl of the digested DNA and 1μl of ligase buffer (Boehringer Mannheim) (which gave a final volume of 50mM Tris·HCl, pH7.4; 10mM MgCl₂; 10mM DTT; 1mM spermidine; 1mM ATP; 100g/ml BSA).

The reaction was incubated overnight at 16°C. The enzyme was then heat inactivated by incubation at 68°C for 20 minutes.

2.5.8 PCR using end-labelled oligonucleotides
This was carried out when species specific size polymorphisms in PCR products, were too small to be resolved on agarose gels. A PCR reaction was carried out, using in addition to the normal concentration of unlabelled primer, a relatively low concentration of end labelled primer. The PCR reactions were then run on a 6% denaturing polyacrylamide gels (section 2.6.4)

One of the PCR primers was end labelled in a 20μl volume (section 2.8.3). The PCR reaction was set up as detailed in section 2.5.1, with the addition of 1μl (1.5ng) of the end labelled oligonucleotide. Before loading onto the gel, 5μl of the PCR reaction was mixed with 5μl of stop mix (95% deionised formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cincinol FF) and denatured at 80°C for 10 minutes, then chilled on ice. 4μl were then loaded onto the gel.

2.5.9 PCR using end labelled oligonucleotides for SSCP analysis
Fifteen microclones, which had been genetically mapped to the brown locus (Bahary et al., 1993), were received from J. Friedman. The microclones had been cloned into λgt10 and they were subsequently sequenced (see section 2.11). Primers were designed which amplified across the cloned DNA (see section 2.5.2). These primers were used to amplify DNA from M. spretus and M. musculus and polymorphisms detected by SSCP analysis.
PCR reactions with end labelled oligonucleotides were set up essentially as detailed in section 2.5.8. The only alteration being that, either one or both of the end labelled primers were used in these PCR reactions (as detection of an SSCP can vary between complementary strands, Sheffield et al., 1993). If an SSCP was detected, DNA from the panel of deletions was then amplified using the appropriately labelled oligonucleotide. When both the labelled oligonucleotides were used in a PCR reaction, 1μl of each of the labelled primers were used in each PCR reaction.

The samples were denatured before being run on 5% non denaturing polyacrylamide gels (section 2.6.4). 1.5μl of the PCR reaction was mixed with 8.5μl of stop buffer (section 2.5.8), this was denatured at 80°C for 10 minutes, then chilled on ice before loading.

2.6 DNA electrophoresis
2.6.1 Electrophoresis solutions
20 X TBE
1M Tris.HCl, pH8, 20mM EDTA, pH8 and 1M boric acid pH8.3.

20 X TAE
0.8M Tris.HCl, pH8, 20mM EDTA, pH8 and 0.4M acetic acid.

10 X DNA Loading buffer ("stop mix")
20% ficoll, 100mM EDTA, orange G.

6% Denaturing Polyacrylamide
57g of acrylamide, 3g of bis-acrylamide and 460g of urea were dissolved in 1 litre of 1 X TBE. This was stored at 4°C in foil covered bottles. 60ml of this 6% acrylamide solution was used for each polyacrylamide gel. 75μl of N,N,N,N'-tetramethylethlenediamine (TEMED) (Sigma) and 0.5ml of 10% ammonium persulphate were added to this, just before use.

5% Non-denaturing Polyacrylamide
50g of acrylamide, 0.5g of bis-acrylamide were dissolved in 1 litre of 1 X TBE. When glycerol was required, either 5 or 10% glycerol was added at this stage. 60ml of this 5% acrylamide solution was used for each polyacrylamide gel. 75μl of N,N,N,N'-tetramethylethlenediamine (TEMED)
(Sigma) and 0.5ml 10% ammonium persulphate were added to this, just before use.

**Size Markers used in Agarose Gel Electrophoresis.**
The size markers used were either \( \lambda \) DNA digested with HindIII (Gibco BRL), or \( \Phi X174 \) digested with HaeIII (Promega), depending on the size of the DNA fragments. The sizes of the markers are shown below.

**Table 2.3**

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<th>( \lambda )Hind III</th>
<th>( \Phi XHaeIII )</th>
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Table 2.3: Sizes (in bp) of DNA markers

**2.6.2 Agarose gel electrophoresis**
DNA molecules were separated, according to size, in horizontal agarose gels by electrophoresis. The percentage of agarose (Type II medium EEO) (Sigma) in the gel, varied according to the range of size separation required. Restricted genomic DNA was run on large 300ml, 0.8% agarose gels. Smaller DNA fragments, such as plasmids or PCR products were run on 25ml 1-2 % gels, with fragments smaller than 200bp being run on either 4% 3:1 Nusieve or 4% Nusieve GTG agarose gels (FMC Bioproducts). All agarose gels were made from and run in either 1 X TBE or 1 X TAE. Ethidium bromide was added to all agarose gels and buffer at a concentration of 1μg per ml buffer. 1/10 of the sample volume of 10 X "stop mix", was added to DNA samples prior to loading on the gel.
Gels were run in Northumbria Biologicals Limited gel boxes at 50 volts when TAE buffer was used and 100 volts when TBE was used. Genomic digests, for Southern blotting, were generally run in TAE at 50 volts overnight.

DNA fragments were visualised on a UV transilluminator at 305nm and photographed using a video copy processor (Mitsubishi).

2.6.3 Preparative agarose gel electrophoresis
DNA fragments were run in low melting point agarose gels (Ultrapure LMP agarose) (Gibco BRL) in 1 X TBE. Gels were viewed on a UV transilluminator at 305nm and the required fragment was cut out of the gel using a sterile scalpel blade. Care was taken to insure that the minimum size of gel slice was excised.

DNA was isolated from the gel slice using agarase (Boehringer Mannheim), according to the manufacturers instructions.

DNA fragments which were to be used only as hybridisation probes, were not isolated from the gel, but prepared as follows. The gel slice was weighed and 1-3 X the weight of water was added. This mixture was then dispensed into 20μl aliquots. These were then used directly in labelling reactions (each 20μl aliquot contained 25-50ng DNA).

2.6.4 Polyacrylamide gel electrophoresis
Denaturing polyacrylamide gels were used to visualise DNA sequencing reactions and labelled PCR reactions.

The glass plates were prepared by washing thoroughly with detergent and rinsing with water and then 100% ethanol. The front plate was coated in dimethyldichlorosilane solution and both plates were left to dry. Spacers were placed on either side of the back plate and the front plate was placed on top. The plates were then taped together at the sides and the bottom. The freshly prepared polyacrylamide was then poured between the plates, a sharkstooth comb was inserted and clamps applied. The gel was allowed to set for at least 1 hour at room temperature before use.
Gels were run in 1 X TBE on vertical slab gel apparatus (Gibco BRL) at 55 volts for 3-5 hours.

Non denaturing polyacrylamide gels were made as described above. Gels containing glycerol were run at 40 volts at room temperature for 4-7 hours, depending on the size of the PCR product. Gels without glycerol were run at 40 volts at 4°C, to minimise temperature increases.

After electrophoresis, the tape and the front plate were removed and the gel was blotted onto 3MM Whatman paper, covered in Saran wrap (Dow Chemical Company) and dried at 80°C on a Drygel Slab Gel Dryer (Hoefer) for one hour. The Saran wrap was removed and the gel exposed to film or a phosphorimage screen (see section 2.11.4).

2.6.5 Pulsed field gel electrophoresis
The agarose plugs were loaded into the wells of a 1% agarose, 0.5 X TAE gel and the wells were sealed with 0.5% LMP agarose. The gel was placed into the apparatus (constructed by John Maule) (Maule and Green, 1990), which contained sufficient buffer, such that the gel was submerged 3mm below the surface. The buffer had previously been cooled and was kept at a constant temperature of 10°C (by circulation of the buffer through a cooling unit).

Pulsed field times depended on the size of the DNA fragments which were being separated. The running conditions for particular gels are shown in the figure legends.

After electrophoresis, the DNA was visualised by staining in buffer containing ethidium bromide at 5μg/ml for 40 minutes.

2.7 Transfer of DNA to membranes
2.7.1 Southern transfer protocols
DNA which had been run on TAE agarose gels was transferred onto nylon membranes by capillary blotting. This method was adapted from Southern (1975).
Gels were photographed next to a ruler to allow future sizing of DNA fragments.

An additional step was included when blotting pulsed field gels. To aid the transfer of the large yeast chromosomes and YACs, pulsed field gels were UV irradiated on a transilluminator (at 305 nm) for 5 minutes, after being photographed.

The DNA was denatured by gentle shaking of the gel in denature solution (0.5M NaOH, 1.5M NaCl) for 45 minutes. The gel was then neutralised by gentle shaking in neutralising solution (1M Tris.HCl; 2M NaCl, pH5.5) for 45 minutes. A large strip of 3MM filter paper (Whatman) was soaked in 20 X SSC (3M NaCl, 0.3M Na$_3$C$_6$H$_5$O$_7$.2H$_2$O, pH 7) and placed on a board. The ends of the paper were placed into a reservoir of 20 X SSC, generating a wick. The gel was placed on top of the wick and a piece of nylon membrane (Hybond-N, Amersham), which had been cut to the size of the gel and prewetted in 2 X SSC, was placed directly onto the gel, avoiding bubbles. On top of this was placed two pieces of 3MM blotting paper, also cut to size and prewetted in 2 X SSC, followed by two dry pieces of 3MM paper. Any exposed wick was covered in Saran-wrap (Dow Chemical Company) and a 12cm stack of paper towels and a weight (approximately 1kg), were placed on top of the gel.

The length of blotting time varied according to the size of fragments which were being transferred. Pulsed field gels were blotted for two days, genomic digests overnight and smaller fragments for four hours.

The membranes were then rinsed briefly in 2 X SSC and blotted dry. The DNA was bound to the filter by exposing the filter to 1200μJoules of UV radiation (Stratagene's UV Stratalinker). Nylon membranes were stored in Saran-wrap (Dow Chemical Company) at room temperature.

2.8 Radiolabelling of DNA
2.8.1 Random priming of DNA probes

Probes were labelled with $^{32}$P$d$CTP by random priming from hexadeoxyribonucleotides with the Klenow fragment of E. coli DNA polymerase I (Feinberg and Vogelstein, 1983, 1984).
25-50ng DNA (either in the form of a gel slice or in solution) in a volume of 20μl, was denatured by incubation at 100°C for 5 minutes. If the DNA was in solution, the reaction was cooled rapidly on ice, or if a gel derived probe was used, the reaction was cooled to 37°C. A Boehringer Mannheim Random Priming kit was used to label the probe. 3μl of 10X reaction buffer, 1μl each of dATP, dGTP and dTTP (0.5mM), 2 units of Klenow and 30μCi [32P] CTP were added to the probe, which was then incubated at 37°C for a minimum of 45 minutes. After this time, the level of [32P]dCTP incorporation was measured by trichloroacetic acid precipitation (Sambrook et.al., 1989). Once a sufficiently high level of incorporation (at least 50%) had been achieved, unincorporated nucleotides were removed from the probe. The nucleotides were removed by running the probe through a Nick Column (Pharmacia). The storage buffer was removed from the column, which was then equilibrated with 800μl TNE (10mM Tris.HCl, pH8, 1mM EDTA, pH8, 0.2M NaCl). The probe was then added to the column in a volume of 100μl. 300μl of TNE was added and the probe was eluted in a further 400μl. 500μg of denatured sonicated salmon sperm DNA was added to the probe and this mixture was denatured at 100°C for 10 minutes. After this time, the probe was chilled on ice, to prevent reannealing, before being added to the hybridisation mix.

2.8.2 Preannealing of repetitive sequences
Probes which contained repetitive sequences were annealed, after labelling, to repeat containing DNA. This was carried out in order to prevent the repetitive element of the probe taking part in the hybridisation reaction. This procedure was adapted from Sealey et al. (1985).

The probe was labelled and incorporation tested, as described in 2.8.1, but the probe was not purified. After incorporation had been checked, the probe was incubated at 100°C with 1mg sonicated mouse DNA, for 10 minutes. The probe was then incubated, in a prewarmed lead pot, at 68°C for 30-45 minutes. After this time, the probe was added to the hybridisation mix.

2.8.3 End Labelling of DNA oligonucleotides
Oligonucleotides were labelled by the transfer of the 32P-labelled phosphate from [32P] onto the terminal 5'-OH group.
30ng oligonucleotide was labelled in a 20μl volume which contained 1 X Polynucleotide kinase buffer (5mM Tris.HCl, pH8, 1mM MgCl₂, 0.5mM DTT) (Boehringer Mannheim), 10 units of Polynucleotide kinase (Boehringer Mannheim) and 30μCi of [³²P]γATP (Amersham). Reactions were incubated at 37°C for 30-40 minutes and were then added to the hybridisation mix.

2.8.4 Nick translation of genomic DNA
Genomic DNA was labelled with [³²P]dCTP by nick translation, using a combination of DNase I and DNA polymerase I.

A Nick Translation kit from Gibco BRL was used to label the DNA. The reaction, in a total volume of 10μl, consisted of 100ng of genomic DNA, 0.02mM GTP, TTP and ATP, 30μCi [³²P] CTP, 0.4 units of DNA polymerase I, 40pg DNase I, 5mM Tris.HCl, pH8 and 1mM MgCl₂. The reaction was incubated at 15°C for 60 minutes and then 1μl of stop buffer (300mM Na₂ EDTA, pH8) was added. The labelled DNA was was denatured at 100°C for 10 minutes. After this time, the probe was chilled on ice, to prevent reannealing, before being added to the hybridisation mix.

2.9 Hybridisation protocols
2.9.1 Hybridisation solutions
Hybridisation mix
6 X SSC, 10% dextran sulphate, 0.1% sodium pyrophosphate, 0.5% sodium dodecyl sulphate, 4 X Denhardts solution. (100 X Denhardt's solution: 2% BSA; 2% polyvinylpyrrolidone; 2% ficoll; 1mM EDTA, stored at 4°C).

Oligo hybridisation mix.
5 X SSC, 0.05% BSA, 0.05% ficoll, 0.1% sodium dodecyl sulphate, 0.05% polyvinylpyrrolidone, 0.1% sodium pyrophosphate.

Hybridisation mix used in YAC screening
7% sodium dodecyl sulphate, 0.5M sodium phosphate, pH7.2, 1% BSA, 1mM EDTA.
2.9.2 Pre-hybridisation protocols
When an end labelled oligonucleotide was used, the pre-hybridisation of filters was carried out in oligo hybridisation mix at 5°C below the Tm of the oligonucleotide. For random primed probes, pre-hybridisation took place at 68°C in hybridisation mix. Filters were placed between two gauze sheets in a hybridisation bottle (Hybaid). 10ml of hybridisation mix was used with the small bottles and 20ml with the larger bottles. Bottles were placed in a Hybaid hybridisation oven with a rotating spindle for at least 1 hour (end labelled oligonucleotides) and at least 4 hours (random primed probes). Denatured sonicated salmon sperm was added to the hybridisation mix at a concentration of 100μg/ml.

2.9.3 Hybridisation and washing protocols
End labelled oligonucleotide hybridisations were carried out for 4-16 hours at 5°C below the Tm of the oligonucleotide. Filters were then removed from the bottles and given three 5 minute washes at room temperature. They were washed in 4 X SSC, 0.1% SDS, which had been heated to the same temperature as the hybridisation reaction. Random primed hybridisations were incubated for 16 hours at 68°C. Filters were then washed in the hybridisation bottles with 2 X SSC, 0.1 % SDS for 20 minutes at 68°C. They were then removed from the bottles and washed with increasing stringency to 1 X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS or 0.1x SSC, 0.1% SDS at 68°C.

2.9.4 Screening the ICRF YAC library by hybridisation
This protocol, recommended by ICRF, was used to screen the ICRF YAC library. Filters were prehybridised at 68°C for 4 hours, as detailed in sections 2.9.1 and 2.9.3. Purified random primed probes were added to the prehybridisation mix (section 2.8.1) and the hybridisation reactions incubated at 68°C for 16 hours. The filters were then removed from the bottles and washed as follows; two 5 minute washes in 40mM NaPO₄ (pH7.2), 0.1% SDS at room temperature; two 15 minute washes in 40mM NaPO₄ (pH7.2), 0.1% SDS at 65°C.
2.9.5 Removal of hybridisation signal
Probes were removed from Southern blots according to the manufacturer's instructions. A solution of 0.1% SDS was prepared and boiled. This solution was then poured onto the membrane and allowed to cool to room temperature.

2.10 Detection of hybridisation
2.10.1 Autoradiography
Filters were exposed to Kodak X-OMAT film in cassettes with intensifying screens. Filters hybridised to $^{32}$P labelled probes were exposed at -70°C and $^{35}$S reactions at room temperature. Films were exposed for a period of 30 minutes to 2 weeks (depending on the signal strength), before being developed using an automatic X-ray film processor RGII (Fuji).

2.10.2 Phosphorimaging
A Molecular Dynamics PhosphorImager was also used for detecting and quantifying hybridisation. Filters were exposed on a phosphor screen for periods of 30 minutes to 48 hours. They were then scanned on the PhosphorImager, where a laser beam converts the radioactive signal into a digital image, with variations in the pixel values proportional to the amount of radioactivity present. The image was displayed on a grey scale, the upper and lower limits of which were adjusted to give good image visualisation. The image was then printed out on a grey scale laser printer.

2.11 Sequencing of PCR products
Adapted from Sanger et al., 1977, by Winship, 1989. The USB DNA sequencing kit (Sequenase Version II) was used to sequence PCR products.

The PCR product was isolated by running in a 1.4% low melting point gel as detailed in section 2.6.3. The DNA was extracted from the gel slice using Geneclean (Bio 101) and eluted in 15μls of H$_2$O.

Two reactions were set up per PCR fragment, each with different primers. 3 μl of the eluted DNA (approximately 60ng) was added to 150ng of primer in 40mM Tris.HCl, pH7.5, 20mM MgCl$_2$, 100mM NaCl and 10% DMSO. This was heated to 100°C for 3 minutes and snap frozen in liquid nitrogen (to denature the template and minimise renaturation). The labelling mix for
each reaction, (dGTP) contained 5μCi of [35S] dATP (Amersham), 1.5μM of dGTP, dCTP, dTTP, 25mM DTT and three units of T7 DNA polymerase (Sequenase, USB) (the enzyme had previously been diluted 1:7 with enzyme dilution buffer). This was added to the thawed DNA and the extension reaction were incubated at room temperature for one minute. The extension reactions were then terminated by addition of 3.5μl of the reaction mix to 2.5 μl of the four dideoxynucleotide termination mixes (containing either ddATP, ddGTP, ddTTP or ddCTP in 10% DMSO), which had previously been warmed at 37°C for 5 minutes. The termination reactions were incubated at 37°C for 5 minutes, after which time 4μl stop mix (USB) was added. The reactions were then stored at -20°C prior to polyacrylamide electrophoresis.
Chapter 3
Analysis of mouse strains with radiation induced mutations at the brown locus

3.1 Introduction

*M. musculus* mice containing radiation induced mutations at the brown locus were obtained from Bruce Cattanach at the MRC Radiobiology Unit, Didcot. These had been generated by the specific locus mutagenesis method, (section 2.1.1) developed by W. L. Russell (1951). These will subsequently be referred to as the Harwell mutations.

Brown mice had been isolated by mice fanciers many years ago. Once the *brown* gene had been cloned, sequence analysis revealed four nucleic acid differences between wild type and brown mice (Jackson, 1988). One of these nucleic acid changes inactivated a TaqI restriction site in brown mice. This TaqI polymorphism can be detected in two ways, by hybridisation and by PCR. Fig. 3.1 shows a Southern blot of TaqI digested DNA from wild type (c57) and brown mice (b\(^Y\)). This has been probed with a 1.6kb fragment of the *brown* gene (Trp1.6) (Jackson, 1988), which spans the polymorphic TaqI site. This probe hybridises to a 4.9kb fragment in brown mice and a 3.7kb fragment in wild type mice. The TaqI polymorphism can also be detected by PCR. Primers spanning the polymorphic TaqI site were designed from published sequence (Shibahara et al., 1986). These primers amplified a 227bp fragment from wild type (c57) mice, which was digested into two fragments of 121 and 106bp by TaqI digestion. In brown (b\(^Y\)) mice, the amplified 227bp fragment was unaltered by TaqI digestion (fig. 3.2). These different haplotypes have been defined, with respect to the polymorphic TaqI site (Jackson, 1988).

- **B haplotype** - presence of TaqI site. (all wild type strains)
- **D haplotype** - absence of TaqI site. (all brown strains)

As it was now possible to differentiate between the original and newly induced mutations at the brown locus, analysis of mice with mutations at the brown locus was possible.
Figure 3.1: Detection of the TaqI polymorphism by Southern blotting

figure legend overleaf
Figure 3.1 legend

Figure 3.1 shows a Southern blot of TaqI digested genomic DNA, from wild type (c57) and brown (bY) mice, hybridised with the Trp1.6 probe from the brown gene. Genomic DNA was digested with TaqI and separated on a 0.8% agarose gel (sections 2.4.1 and 2.6.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, to the radio-labelled probe (sections 2.7, 2.8.1 and 2.9). Hybridisation was detected by a Molecular Dynamics Phosphorimager after a 16 hour exposure (section 2.10.2).

The Trp1.6 probe hybridised to a polymorphic fragment of 4.9kb in bY mice and 3.7kb in C57 mice. It also hybridised to other polymorphic fragments, of either 12.8 or 9kb, which were derived from a pseudogene, unlinked to the brown locus. The sizes of the fragments, in kb, were already known (Jackson, 1988) and are shown on the left of the figure, with the haplotype designation shown on the right.
DNA from wild type (c57) and brown (By) mice was analysed by PCR, in order to detect the polymorphic TaqI site. The PCR reaction was carried out using primers which amplified across the polymorphic TaqI site in the brown gene (section 2.5.1 and table 2.2). The amplified fragments were digested with TaqI (section 2.4.3) and analysed on 4% 3:1 Nusieve agarose gels (section 2.6.2). The size marker is øXHae III (section 2.6.1) and the sizes (in bp) of the PCR fragments are shown. The results demonstrate that the amplified fragment generated from c57 wild type mice, contains a TaqI site, whereas that from the By brown mice, does not.
This chapter describes the analysis of mice with radiation induced mutations at the brown locus, which were obtained from B. Cattanach. The first step in this analysis was to determine which of the mutations at the brown locus were due to deletions, as these might also remove other nearby genes. These genes would be identified if their loss gave rise to a phenotypic effect. Subsequent analysis involved determination of which brown locus deletions were lethal when homozygous, the stage of development this lethality occurred and the cause of lethality. Viable mice, which were homozygous for a particular deletion, were examined for any abnormal phenotype.

3.2 Deletion analysis of the brown mutations
The mice obtained from B. Cattanach were heterozygous for the brown mutation. They contained the original mutation over the newly induced brown mutation, i.e. their haplotype was B-D (where B denotes the newly induced mutation on a B haplotype chromosome). To ascertain whether the mutation was a deletion, DNA was extracted from the tail tip of each mouse. This was then subjected to PCR, using primers from the brown gene which amplify across the polymorphic TaqI site (table 2.2). The 227bp product was then digested with TaqI. The results (fig. 3.3) show that in mice with the D haplotype (b''), the 227bp amplified fragment was not digested with TaqI, but in B haplotype mice (c57), it was digested into two fragments, of 121 and 106bp, as expected. In mice which were heterozygous for the b58H, b59H and b60H mutations, all three fragments were observed. This indicates that a PCR product was generated from both chromosomes and, therefore, the mutation at the brown locus was not a deletion. Only the 227bp fragment from the D haplotype chromosome was observed in mice carrying the b57H, b61H, b62H and b63H mutations, indicating that no product was generated from the B haplotype chromosome. Thus these mice contain deletions at the brown locus.

The result of this haplotype analysis was confirmed by Southern blotting. After breeding lines had been established from these mice, DNA was extracted from their spleen. This was digested with TaqI and hybridised with the Trp1.6 probe. The results are shown in fig. 3.4. Mice carrying the b58H, b59H and b60H mutations have both 4.9 and 3.7kb bands from the D and B haplotype chromosomes, suggesting that these mutations are not deletions. Whereas mice carrying the b57H, b61H, b62H and b63H mutations
Figure 3.3: PCR analysis of the mice obtained from Harwell

These mice were heterozygous for the brown mutation, containing a copy of the newly induced mutation and a copy of the original brown mutation. DNA isolated from tail tips (section 2.3.2) was analysed, in order to detect any deletions which had been generated at the brown locus. The PCR reaction and subsequent analysis was performed as detailed in the legend of fig. 3.2. The size marker is øXHaeIII (section 2.6.1) and the sizes (in bp) of the PCR fragments are shown.

The results demonstrate that a PCR product was amplified from only one chromosome, in mice carrying the b^{57H}, b^{63H}, b^{61H} and b^{62H} mutations, indicating that these mutations are deletions.
have only the 4.9kb band from the D haplotype chromosome, indicating that these mutations are deletions.

To summarise, mice with the mutations b^{58H}, b^{59H} and b^{60H} do not contain deletions at the brown locus, whereas those with mutations b^{57H}, b^{61H}, b^{62H} and b^{63H} contain deletions at the brown locus.

### 3.3 Phenotype of mice homozygous for the brown mutations

The next step was to determine which of the deletions were lethal when homozygous. This was carried out by breeding mice carrying the deletions to homozygosity. Mice containing non-deletional mutations at this locus were also analysed in this way, in order to check that they were viable.

All seven mouse strains were analysed using the breeding protocol shown in fig. 3.5. The B^{-}D mice were mated with wild type CBA mice (haplotype BB), and the offspring were analysed to determine which were carrying the deletion. 50% of the mice from this cross were expected to have the haplotype BD and 50% were expected to be B^{-}B. Mice carrying deletions were identified by PCR analysis of the polymorphic TaqI site, using DNA isolated from tail tips. Those mice which carried the D haplotype were not deletion carriers. The results from a typical litter are shown in fig. 3.6. A sib-mating was then carried out between two mice which carried the deletion. If the mutation was non-lethal, we would expect 25% of the offspring to be phenotypically brown, whereas if it were lethal, no brown mice would be seen. The results are shown in table 3.1.
Figure 3.4: Southern blot analysis of the mice obtained from Harwell

figure legend overleaf
Figure 3.4 legend
These mice were heterozygous for the brown mutation, containing a copy of
the newly induced mutation and a copy of the original brown mutation. DNA
isolated from these mice, was analysed by Southern blotting, in order to
detect any deletions which had been generated at the brown locus. The
Southern blotting and hybridisation protocol were as previously detailed
(legend of fig. 3.1). Fragment sizes (in kb) are shown.
The results show that the Trp1.6 probe hybridises only to the 4.9kb fragment
from the D haplotype chromosome, in mice with mutations b⁵⁷H, b⁶³H, b⁶¹H
and b⁶²H. Therefore these mice have deletions of the brown locus on their B
haplotype chromosome.
Figure 3.5: Breeding Protocol to detect homozygous lethal mutations

figure legend overleaf

[Diagram]

1. Identify deletion carriers by PCR:
   - Cross: $b^D_B \times +B_b$
   - Result: $b^D_B \times B_B$
   - Identify deletion carriers.

2. Sib-mate deletion carriers:
   - Cross: $+b^D_B \times +B_B$
   - Result: $+b^D_B \times B^D_B$
   - Sib-mate deletion carriers.

3. Absence of brown offspring indicates lethal deletion:
   - Cross: $B^D_B \times B^D_B$
   - Result: $B^D_B \times B^D_B$
   - Absence of brown offspring indicates lethal deletion.

Legend:
- Brown mice
- Wild type heterozygotes
- Wild type homozygotes
Figure 3.5 legend
Mice carrying the Harwell deletions were mated according to this protocol, in order to determine which of the deletions were lethal when homozygous. Chromosomal 4 markers are $b$ (brown gene), + (wild type trp1 gene) and deletions are represented by a gap in the chromosome. The haplotype of each mouse is also shown (where $B^{-}$ represents a brown locus deletion on the B haplotype chromosome). The protocol is explained in the text.

Figure 3.6: Identification of deletion carriers by PCR analysis

Deletion carriers in the offspring from the first cross, detailed in fig. 3.5, were identified by PCR analysis. The offspring were heterozygous at the brown locus. All contained a wild type B haplotype chromosome and either a D or a $B^{-}$ haplotype chromosome. DNA was extracted from tail tips (sections 2.1.2 and 2.3.2) and analysed by PCR and subsequent TaqI digestion, as detailed in the legend of fig. 3.2. The results show that, in tracks 1, 2 and 7, a PCR product was amplified only from one chromosome. Therefore these mice carry the deletion chromosome and were used in subsequent crosses.
Table 3.1:

<table>
<thead>
<tr>
<th>deletion</th>
<th>number of brown offspring</th>
<th>number of non-brown offspring</th>
<th>percentage of brown offspring</th>
<th>$\chi^2$ value &amp; significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>b^{57H}</td>
<td>25</td>
<td>146</td>
<td>14.6%</td>
<td>9.83; P&lt;0.01</td>
</tr>
<tr>
<td>b^{58H}</td>
<td>13</td>
<td>44</td>
<td>22.8%</td>
<td>0.15</td>
</tr>
<tr>
<td>b^{59H}</td>
<td>15</td>
<td>56</td>
<td>21.1%</td>
<td>0.57</td>
</tr>
<tr>
<td>b^{60H}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>b^{61H}</td>
<td>15</td>
<td>48</td>
<td>23.8%</td>
<td>0.05</td>
</tr>
<tr>
<td>b^{62H}</td>
<td>13</td>
<td>45</td>
<td>22.4%</td>
<td>0.21</td>
</tr>
<tr>
<td>b^{63H}</td>
<td>0</td>
<td>78</td>
<td>-</td>
<td>26; P&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3.1: Summary of breeding data

3.3.1 b^{58H}, b^{59H} and b^{60H} homozygotes
The results showed that mutations b^{58H} and b^{59H}, which are not deletions, were not lethal. Brown mice accounted for 22.8% of the offspring in the b^{58H} crosses and 21.1% in the b^{59H} crosses, and they were found to be fully viable. The female mouse carrying the b^{60H} mutation was infertile and, on dissection, the uterus was found to be abnormal. It was swollen as a result of being filled with fluid.

3.3.2 b^{61H}, b^{62H} and b^{63H} homozygotes
Of the mutations which were due to deletions of the brown locus, only b^{63H} was lethal when homozygous. In a total of 78 offspring, no brown mice were observed.

b^{61H} and b^{62H} deletions were not homozygous lethal, as brown mice were seen in the offspring of these crosses. Mice homozygous for deletions b^{61H} and b^{62H} were found to be viable and were present at the frequency expected (23.8% and 22.4% respectively). These mice were also fertile.

DNA isolated from the brown offspring was used to confirm that both alleles of the brown gene were deleted. A Southern blot using TaqI digested DNA was carried out, with Trp1.6 as the probe. This probe, as well as hybridising to DNA from the brown gene, also hybridises to a pseudogene located
outwith the brown locus (Jackson, 1988). The pseudogene is polymorphic in
different strains of mice and Trp1.6 hybridises to either a 12.8 or a 9kb
fragment, depending on the origin of the DNA. The results show (fig. 3.7)
that the probe binds only to the pseudogene, confirming that the mice are
indeed homozygous deletion mutants.

3.3.3 b^{57H} homozygotes
Southern blot analysis of DNA isolated from b^{57H} homozygotes (fig. 3.7),
confirmed that both alleles of the brown gene were deleted in these mice.
Mice homozygous for the b^{57H} deletion were observed at a lower frequency
than expected. The brown mice were also much smaller than their
littermates and frequently died within the first week of life. Fig. 3.8 compares
the size of the b^{57H} homozygotes with their wild-type littermates when four
days old. Some of the mice that did survive this period, died when they were
three weeks old, just after they had been weaned. However the small
proportion of mice that did survive, had almost reached the size of their
littermates, by the time they were six weeks old. When mice homozygous for
the b^{57H} deletion were mated together, they did produce offspring, but these
were reduced in both number and fitness. Commonly litter sizes of one or
two mice were observed and they too were very small and often died before
weaning.

One factor which complicated analysis of these crosses was that the mice,
apart from carrying the mutation at the brown locus, also carried recessive
mutations at other coat colour loci. These were inherited from their mother,
who came from the tester stock used in the SLT, which carried homozygous
recessive mutations at six coat colour loci. These mutations are transmitted
to the offspring and the recessive phenotype can appear in subsequent
generations. Therefore mice of varying coat colours can be seen in the F2
generation (see fig. 3.5) and this can lower the rate of detection of mice
homozygous for the brown gene. Although we would expect 25% of the
mice in the F2 generation to be brown, the number was often lower than that,
because of the presence of mice with other coat colours. For example, mice
homozygous for the chinchilla mutation are grey and those homozygous for
chinchilla/pink eyed dilution are almost white. It was not possible to visually
ascertain whether these mice were also homozygous for the brown mutation
and so they could not be scored as brown. However mice which were
Figure 3.7: Southern blot analysis of mice with homozygous deletions of the brown locus

figure legend overleaf
Figure 3.7 legend
Brown mice were observed in the offspring from matings between deletion carriers. DNA was isolated from these mice in order to confirm that both alleles of the brown gene were deleted. DNA, isolated from the spleen (section 2.3.1), was analysed as detailed in the legend of fig. 3.1. Fragment sizes (in kb) are shown. The Trp1.6 probe hybridises only to the polymorphic pseudogene (12.8 or 9kb) in DNA isolated from these mice. This confirms that mice homozygous for deletions b57H, b61H and b62H are viable.

Figure 3.8: Mice homozygous for the b57H deletion

The two mice on the right are homozygous for the b57H deletion, while the two on the left are wild type littermates. This photograph was taken when the mice were four days old, when it was first possible to visually identify the brown mice. The photograph shows that the b57H homozygotes are smaller than their wild type littermates.
homozygous for the brown mutation and also for either agouti, dilute/short ear, piebald or pink-eye could be identified.

If only those mice that were scorable were taken into account, then, out of a total of 171 mice from four crosses, 14.6% of the offspring were brown, i.e. homozygous for the $b^{57H}$ deletion. This figure is significantly lower ($\chi^2 = 9.83; P<0.01$) than the 25% expected. It is not known whether less brown offspring were born or whether they died before being scored for their coat colour. This could not be scored for at birth, but only after four to five days, when their coat started to grow. Of the 25 mice scored for brown, a further six died before they were six weeks old. Mice which were homozygous for the $b^{57H}$ deletion appeared to have a reduced fitness, as compared to their littermates. However, no other specific abnormalities were seen.

3.4 Breeding Protocol

Stocks of mice carrying each of the deletions were maintained as outlined in fig. 3.9. Mice carrying the deletion had previously been identified in the F1 generation (see fig. 3.5) and these BB$^{-}$ mice were crossed to brown mice (DD haplotype). On average, 50% of the offspring from this cross would be phenotypically wild-type (BD) and 50% brown (DB$^{-}$). The brown mice were then crossed to wild-type CBA mice (BB haplotype) and two classes of offspring were produced. These were BB$^{-}$ and BD, both of which were phenotypically wild-type. Litters were analysed by extracting the DNA from tail tips and the haplotype was determined by carrying out PCR and subsequent TaqI digestion. The results from a typical litter are shown in fig. 3.10. In this way, mice carrying the deletion were identified (tracks 3, 5 and 6) and used in following matings.

Initially, mice stocks carrying each of the deletions were maintained in this way. After three generations, when mice homozygous for the $b^{61H}$ and $b^{62H}$ deletions were observed to be fertile, matings between deletion homozygotes were used to maintain stocks.

The $b^{57H}$ deletion was maintained in its hemizygous form, as the homozygous mice were not fully viable. However after five generations it was discovered, when haplotyping the litters by PCR, that crosses had been set up between mice carrying the D haplotype and not the $b^{57H}$ deletion.
This could be traced back to an error in haplotyping two generations previously. This may have occurred because of a mix up with the DNA samples or the mice could have been wrongly identified from their ear markings, as their ears were occasionally torn from scratching or fighting. Whatever the cause, the net result was that stocks of mice carrying the $b^{57H}$ deletion had been lost. This was unfortunate as it would have been very interesting to discover at what stage of gestation, their development was affected and if it could be rescued by complementation with any of the other deletions at this locus. However some DNA from $b^{57H}$ homozygous mice had been saved, so molecular analysis of this mutation could be undertaken. Furthermore, this phenotype was also found in mice with deletions at the brown locus which were generated at Oak Ridge National Laboratories (discussed in chapter 4).
Stocks of mice carrying each of the deletions were maintained using this breeding protocol. Chromosome 4 markers and haplotypes are as detailed previously (fig. 3.5 legend). The protocol is explained in the text.
Deletion carriers in the offspring from the first cross, detailed in fig. 3.9, were identified by PCR analysis. The offspring were heterozygous at the brown locus. All contained a wild type B haplotype chromosome and either a D or a B^- haplotype chromosome. DNA was extracted from tail tips (sections 2.1.2 and 2.3.2) and analysed by PCR and subsequent TaqI digestion, as detailed in the legend of fig. 3.2. The size marker is øXHaeIII (section 2.6.1) and the sizes (in bp) of the PCR products are shown.

The results show that, in tracks 3, 5 and 6, a PCR product was amplified only from one chromosome. Therefore these mice carry the b^63H deletion and were used in subsequent crosses.
3.5 Determining the stage of lethality of \(b^{63H}\) homozygous mice

\(b^{63H}\) is the only deletion which is homozygous lethal and I wanted to determine at what stage of development this lethality occurred. Crosses were set up between mice hemizygous for the deletion, i.e. \(B^{+}B \times B^{-}B\). The females were checked each morning for plugs and then dissected at different stages of pregnancy. Embryos of 11.5-12.5 days gestation were dissected from the uterus and the placenta was carefully removed. No moles were seen at this stage, indicating that no embryos had died recently. There was some variation in the size of the embryos, but no gross abnormalities were seen.

DNA was extracted from the embryos and digested with TaqI. A Southern blot was carried out using Trp1.6 as the probe. Thus, if any of these embryos were homozygous for the \(b^{63H}\) deletion, the Trp1.6 probe would hybridise only to the pseudogene. The results from a typical litter are shown in fig. 3.11. This shows that the Trp1.6 probe has hybridised to DNA from the brown locus in each of the eight embryos (6.6, 3.7, 3.3 2.65, 1.5 and 1.2kb bands). Therefore none of these embryos are homozygous for \(b^{63H}\), indicating that this deletion, when homozygous, causes lethality at an earlier stage of development. We would expect 25% of these embryos to be \(b^{63H}\) homozygotes and none were identified in a total of 27 embryos. This result was statistically analysed using a \(\chi^2\) test (fig. 3.12). The probability that this result occurs by chance is less than 1%, therefore this data suggests that \(b^{63H}\) homozygotes die before 11.5 days gestation.

Embryos were then examined at an earlier stage of development. Pregnant females were killed and dissected 7.5 days after fertilisation. However, at this stage, it was difficult to effectively remove the placenta from the embryos, as they were so small. If the embryo was contaminated with material from the maternally derived placental tissue, then the results would be invalid. To test this a cross was set up between a heterozygous female, haplotype BD, and a wild-type male, BB. Embryos were carefully dissected from the uterus at 7.5 days gestation, DNA was extracted and a Southern blot was carried out as before. We would have expected half of the embryos to have a BD haplotype and half BB. If there was DNA present of maternal origin, then the Southern blot would reveal the presence of a band corresponding to the D haplotype in all tracks. The results are shown in fig.
Mice carrying the b^{63H} deletion were mated and the litters analysed to determine at what stage of gestation, the lethality of b^{63H} homozygotes occurred. DNA was extracted from embryos at 11.5-12.5 days gestation (sections 2.1.3 and 2.3.3). It was analysed by Southern blot analysis using the Trp1.6 probe (as detailed in the legend of fig. 3.1). Fragment sizes (in kb) are shown.

The results show that all of the eight embryos in this litter, contained DNA from the brown gene.
A $\chi^2$ test was used to determine whether the absence of $b^{63\text{H}}$ homozygote offspring, from $b^{63\text{H}}$ heterozygote crosses at 11.5 days gestation, was significant. In a total of 27 embryos, no $b^{63\text{H}}$ homozygotes were observed. The $\chi^2$ value was calculated to be 9. At the 1% significance level, with one degree of freedom, the $\chi^2$ table value is 6 (Eason et al., 1986). As the calculated $\chi^2$ value exceeds this figure, this data suggests that $b^{63\text{H}}$ homozygotes die before 11.5 days gestation.
A female brown mouse (haplotype DD) was mated with a wild type male (haplotype BB). DNA was extracted from embryos at 7.5 days gestation and analysed, as detailed in the legend of fig. 3.11. Fragment sizes (in kb) are shown. The Trp1.6 probe hybridised to a 4.9 kb fragment in all seven embryos, indicating that the DNA samples were contaminated with maternally derived DNA.
3.13. They show the presence of both a 3.7kb band in each track, corresponding to the B haplotype and a 4.9kb band, indicative of the D haplotype. This shows that the DNA extracted from each embryo also contains maternal DNA, arising from the placenta. For this reason younger litters were not examined for the presence of b<sup>63H</sup> homozygote embryos. It is likely that the homozygote embryos die at an early stage in development, as no moles were found when examining the 11.5-12.5 day litters, therefore the embryos must have been reabsorbed by this stage.

3.6 Mapping the extent of the deletions

The extent of the four deletions that spanned the brown locus was investigated by molecular means, using markers which had been mapped to the region (see chapter 4). DNA was available from the three deletion strains which were homozygous viable.

Three microclones had been mapped close to the brown gene, D4 Rck4, 9 and 122 and two microsatellite repeats, D4Nds7 and D4Mit7 (see chapter 4). PCR analysis was carried out, using primers spanning the markers, on the three homozygote deletion DNAs. The results are shown in fig. 3.14a-e. PCR products, which had been amplified from these markers, were generated from each deletion DNA. These results show that none of the non-lethal deletions extend as far as the closest mapped markers.

However, DNA was only available from b<sup>63H</sup> hemizygotes, as this deletion was lethal when homozygous. In order to characterise this deletion, b<sup>63H</sup> hemizygotes were crossed to M. castaneous mice. Polymorphisms between the two strains enabled this deletion to be characterised using two microsatellite markers which mapped to this region. The offspring generated from these crosses were analysed, in order to identify the deletion carriers. TaqI polymorphisms at the brown locus were identified between the M. musculus and M. castaneous strains by hybridising a Southern blot with the Trp1.6 probe. Fig. 3.15 shows the differing hybridisation patterns between the two species. Also shown are results from the analysis of two offspring from this cross. Both of the offspring can be clearly identified as being deletion carriers, and were used in the PCR analysis.
Figure 3.14: Molecular analysis of YACs and Harwell deletions

A

B
Figure 3.14: Molecular analysis of YACs and Harwell deletions
Five markers which map close to the *brown* gene (details in chapter 4), were used to analyse the extent of deletions $b^{57}H$, $b^{61}H$ and $b^{62}H$ and also another homozygous viable deletion, 5ChLo$^-$ (generated at Oak Ridge, Tennessee). Yacs which contained the *brown* gene were also analysed in this way and this is discussed in section 5.3. DNA was isolated from the spleens (section 2.3.1) of mice homozygous for the above deletions. PCR reactions, using primers which amplified the DNA markers, were analysed on 4% 3:1 Nusieve gels (section 2.5.1 and table 2.2). The size marker used was øXHaeIII.

Fig. 3.14a-e shows the results of PCR reactions when the following primers were used. Fig. 3.14a-D4Rck4; fig. 3.14b-D4Mit7 (with primers from the *brown* gene as a positive control); fig. 3.14c-D4Rck9; fig. 3.14d-D4Rck122; fig. 3.14e-D4Nds9. A PCR product is amplified from each sample with every set of primers, indicating that the deletions do not incorporate these markers.
Figure 3.15: Identification of $b^{63H}$ deletion carriers by Southern blot analysis

figure legend overleaf
Figure 3.15 legend
The offspring of an interspecific cross between *M. castaneous* and *M. musculus* b$^{63H}$ deletion carriers, were examined by Southern blot analysis (as detailed in the legend of fig. 3.1). Fragment sizes (in kb) are shown. The Trp1.6 probe detects a Taqi polymorphism, which enables b$^{63H}$ deletion carriers to be identified.

Figure 3.16: Molecular analysis of the b$^{63H}$ deletion

Two polymorphic microsatellite markers (D4Mit7 and D4Mit25) were used to analyse the extent of the b$^{63H}$ deletion. The offspring of the interspecific cross which carried the b$^{63H}$ deletion had previously been identified (fig. 3.15). DNA was isolated from spleens (section 2.3.1) and amplified using primers from the microsatellite repeats (section 2.5.1 and table 2.2) (tracks 1 and 2). PCR reactions were analysed on 4% 3:1 Nusieve gels. The D4Mit7 PCR reaction is shown on the left of the øXHaeIII size marker and D4Mit25, on the right. The results show that a PCR product was amplified from both chromosomes, therefore the lethal b$^{63H}$ deletion does not extend to markers D4Mit7 and D4Mit25.
Figure 3.16 legend (continued)
Primers from the polymorphic microsatellite repeats D4Mit7 and D4Mit25 were used to amplify the hemizygous DNA (both markers lie centromeric of brown, with D4Mit7 lying closer to the brown gene). The results, shown in fig. 3.16, show that a PCR product is amplified from both the M. musculus and M. castaneous chromosomes. Therefore the b\textsuperscript{63H} deletion does not extend as far as these microsatellite markers.

3.7 Discussion
The screening protocol, used in the SLT, enabled the identification of any mutation at the brown locus which gave rise to a visible phenotype. This could range from large rearrangements at this locus, for instance deletions, to small intragenic mutations. These could be point mutations which inactivate the brown gene product. Intragenic mutations were probably the cause of the b\textsuperscript{58H}, b\textsuperscript{59H} and b\textsuperscript{60H} mutations, as the Trp1.6 probe from the brown gene showed a pattern of hybridisation comparable with that of DNA from wild type mice. However, the possibility of a deletion in another region of the gene cannot be excluded, but as the mice are viable, any possible deletion cannot extend to nearby vital genes. As these mutations are not useful in mapping this region, no further analysis of them was carried out.

Of the remaining four mutations which were shown to be deletions, two were viable, one was lethal and one subviable. There are at least three lethal genes in this region which have been genetically mapped by E. M. Rinchik at Oak Ridge (see section 4.2) (Rinchik, 1994). It is probable that b\textsuperscript{63H} is deleted for one of these. The viable deletions b\textsuperscript{61H} and b\textsuperscript{62H} obviously do not encompass these lethal genes. The mapping data shows that they do not extend to the nearest mapped markers.

Mice homozygous for the b\textsuperscript{57H} deletion are smaller and have a reduced fitness when compared to their littermates. This suggests that the b\textsuperscript{57H} deletion removes a gene involved in embryonic growth and fitness, which is present in the viable b\textsuperscript{61H} and b\textsuperscript{62H} deletions. This gene has been termed the brown associated fitness gene or baf and a future aim is to isolate and clone this gene.
The unfortunate loss of mice carrying the b\textsuperscript{57H} deletion was due to an error in the typing of such mice. Further analysis of the deletion phenotype would have been carried out. Weight measurements of newly born mice would have been taken and compared to their littermates. The mice would have been marked at birth, in order to identify those which developed a brown coat colour. Even by the time the coat colour could be determined, at around five days, the difference in size was less pronounced. This would also have given a better idea of the survival rates of brown mice in the first few days after birth. Embryonic studies would then have been carried out to determine at what stage of development their growth was affected. This would have given an indication on the stage of embryonic development when the \textit{baf} gene product was required. The mice would also have been examined for any other developmental abnormalities.

However DNA was available from b\textsuperscript{57H} homozygous mice and this enabled molecular characterisation of the deletion. The \textit{baf} phenotype was also found in mice with deletions at the brown locus which had been generated at Oak Ridge National Laboratories (see chapter 4). These mice are being sent to us and they will be used in developmental studies, which will enable more extensive characterisation of the "\textit{baf}" phenotype.

In order to map the \textit{baf} gene, the deletion end-points must first be determined. This will enable the position of the \textit{baf} gene to be localised with higher resolution. The viable b\textsuperscript{61H} and b\textsuperscript{62H} deletions do not incorporate the \textit{baf} gene whereas the b\textsuperscript{57H} deletion does. However, all of the markers which have previously been mapped to this region lie outwith these deletions. More useful markers which lie closer to the \textit{brown} gene are needed. Markers have been genetically mapped to this region, but their exact distance from the \textit{brown} gene is not known. In order to map these markers, the panel of deletions generated at Oak Ridge National Laboratories was utilised. This work is discussed in chapter 4.
Chapter 4
Investigation of the panel of brown locus deletions from Oak Ridge National Laboratories

4.1 Introduction
The specific locus mutagenesis test (SLT) was developed by L.B. Russell in 1951 at the Oak Ridge National Laboratories in Tennessee. For the past 40 years these experiments have continued and many mutations have been isolated at the seven loci involved in the SLT.

The protocol followed at Oak Ridge Laboratories is detailed below (Rinchik et al., 1994). Male *M. musculus* mice from a C3H x 101 cross were mutagenised and mated to the Oak Ridge tester stock. Mutations were occasionally generated at the brown locus and these brown mice were identified in the F1 generation. As these mutations were generated before the brown gene was cloned, there was no molecular means of distinguishing the newly induced mutations from the original brown mutation. Therefore a breeding protocol was developed which would enable identification of those newly induced mutations that were homozygous lethals (fig. 4.1) (Rinchik et al., 1994). The brown mice isolated by the SLT were initially crossed to wild type mice (BB). New lines were established from twelve phenotypically wild type male offspring [on average, half would carry the newly induced mutation (BB*) and half the original mutation (BD)]. They were then crossed to wild type females (BB) and the resultant offspring crossed to brown mice, in order to identify which carried a mutation at the brown locus (which could be either B* or D). These mice were then crossed back to the male from the F1 generation, to produce third generation progeny. A quarter of the mice from this cross were expected to be brown, either homozygous for the original mutation or the newly induced mutation. If brown mice were seen in all twelve lines, it was concluded that mice homozygous for the new mutation were viable. If some lines did not generate brown mice, it was concluded that the brown mice had died, i.e. the mutation was a homozygous lethal. Absence of brown gene product itself, is not fatal, these lethal mutations must remove another gene important in development, i.e. they were assumed to be deletions. Twenty eight of the homozygous lethal deletions at the brown locus, detected using this breeding protocol, were examined and these were used to characterise the surrounding region in more detail. Many more non-lethal mutations were generated at this locus, but as they had identical
Figure 4.1: Breeding protocol to detect homozygous lethal deletions

This figure was adapted from Rinchik et al., 1994. This breeding protocol enabled homozygous lethal mutations at the brown locus, generated by the SLT, to be distinguished from the original viable brown mutation. The haplotypes of the mice are shown (where B* represents a newly induced mutation on a B haplotype chromosome). The protocol is explained in the text.
phenotypes to the original brown mutation, there was no way of distinguishing between them and consequently, of studying them.

At Oak Ridge, a genetic map was generated, based on the results of complementation studies between the 28 deletions described previously (fig. 1.4). Mice hemizygous for each deletion were mated to one another and the presence of any brown offspring was noted. The brown mice contained two different deletions which were able to complement one other, and these mice were termed compound heterozygotes. This gave some indication about the extent of overlap of the deletions and indicated that there were at least three lethal genes within the complex, one proximal and two distal to brown.

The brown mice which were generated from these crosses were not fully viable mice, they were smaller than their littermates and had a reduced survival rate. This phenotype is similar to mice homozygous for the b57H deletion, suggesting that these mice are also deleted for the baf gene. The brown mice generated from the complementation studies are deleted for the brown gene and probably, for a relatively small region surrounding it. The observation that no fully viable mice were generated, indicates that no deletion end-point lies between the brown and the baf gene, i.e. they must be linked.

Once the brown gene had been cloned, it was possible to map the deletions by molecular means. DNA from interspecific crosses can be used to map anonymous clones to deletion complexes, utilising RFLVs, size and sequence polymorphisms. This approach has already been used to map random markers to the albino locus on chromosome 7 (Johnson et al., 1989). The panel of deletions at the brown locus is a valuable resource in the mapping of markers to this region. For this reason, a collaboration was set up with G. Rinchik at Oak Ridge. Markers which lie close to brown will prove useful for localising the baf gene, the proximal and distal lethal genes and dep and wi.

4.2 Preliminary map of the deletion complex
This work was carried out by G. Rinchick's group at Oak Ridge, Tennessee. The initial complementation map (fig. 1.4) was expanded to include two genes which were known to be linked to the brown gene. The wi gene lies 6cM distal to brown (Fleming et al., 1994) and each deletion carrier was mated
with mice showing the recessive whirler phenotype. If any whirler mice were detected in the F1 generation, then the deletion must extend to this locus. Only one deletion, 11R30M extended this far. The dep gene, which lies 2cM distal to brown, was also tested in this way, and seven deletions were found to extend to this locus (Rinchik, 1994).

The panel of deletions was then used to physically map markers which had been genetically localised in this region. Mice which were hemizygous for each deletion were mated to M. spretus mice and DNA was isolated from the offspring of each cross. Deletion carriers were identified by Southern blotting, using a probe from the brown gene which detected a RFLV between M. musculus and M. spretus. This also confirmed that 26 of the 28 prenatal lethals contained deletions of the brown gene (mice heterozygous for two of the deletions failed to produce offspring with the M. spretus mice).

Four microdissection clones (D4Rck4, 9, 52 and 140) had been genetically mapped to this region of chromosome 4 (Bahary et al., 1993). Southern blot analysis revealed which deletions included these loci. These clones could now be mapped to the panel of deletions and consequently, the clones were able to be ordered along the chromosome.

A collaboration with E. M. Rinchik at Oak Ridge, Tennessee, made available DNA from the offspring of twenty five interspecific crosses (listed in table 4.1) and also from mice with compound deletions. These DNAs were used to map more markers to the deletion panel and consequently, to build up a more detailed map of the region. Markers which mapped close to brown would be important in characterising the Harwell deletions and mapping the baf gene. This work is described below.

4.3 Mapping of genes and microsatellite repeats to the deletion complex
4.3.1 Mapping known genes
Seven genes have been placed on the genetic map of this region of chromosome 4. Four lie at the proximal end of the complex. The L\_v gene lies 6.8cM proximal to b, wi about 6cM proximal and Hxb about 5cM proximal. Orm lies between Hxb and L\_v. Ifa, dep and Adfp lie at the distal end of the complex. Ifa is around 6cM distal to b and dep and Adfp are around 2cM distal (fig. 4.16) (Pilz et al., 1992; Fleming et al., 1994; Beier et al., 1992).
Table 4.1: Oak Ridge deletion panel (deletion numbers correspond to those used in the following figures)

<table>
<thead>
<tr>
<th>Deletion Number</th>
<th>Deletion Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ITHO-IV</td>
</tr>
<tr>
<td>2</td>
<td>IDF1OD</td>
</tr>
<tr>
<td>3</td>
<td>47DThWb</td>
</tr>
<tr>
<td>4</td>
<td>33IK</td>
</tr>
<tr>
<td>5</td>
<td>13DT</td>
</tr>
<tr>
<td>6</td>
<td>26R60L</td>
</tr>
<tr>
<td>7</td>
<td>3YPSh</td>
</tr>
<tr>
<td>8</td>
<td>37DTD</td>
</tr>
<tr>
<td>9</td>
<td>12Pu</td>
</tr>
<tr>
<td>10</td>
<td>55Cos</td>
</tr>
<tr>
<td>11</td>
<td>3YPSc</td>
</tr>
<tr>
<td>12</td>
<td>11R30M</td>
</tr>
<tr>
<td>13</td>
<td>173G</td>
</tr>
<tr>
<td>14</td>
<td>11Pu</td>
</tr>
<tr>
<td>15</td>
<td>9Pu</td>
</tr>
<tr>
<td>16</td>
<td>37Pub</td>
</tr>
<tr>
<td>17</td>
<td>10Z</td>
</tr>
<tr>
<td>18</td>
<td>33G</td>
</tr>
<tr>
<td>19</td>
<td>51DThWb</td>
</tr>
<tr>
<td>20</td>
<td>8Pub</td>
</tr>
<tr>
<td>21</td>
<td>49HATH Th</td>
</tr>
<tr>
<td>22</td>
<td>5CHLe</td>
</tr>
<tr>
<td>23</td>
<td>46UTHhc</td>
</tr>
<tr>
<td>24</td>
<td>9R75VH</td>
</tr>
<tr>
<td>25</td>
<td>13R75M</td>
</tr>
</tbody>
</table>
Polymorphisms between *M. musculus* and *M. spretus* had been detected in these genes and these were utilised to map the extent of the deletions.

PCR primers from the *Orm* gene were generated, for details see table 2.2. Details of primer sequences had been published (Dietrich et al., 1992). These primers amplify a microsatellite repeat from the *Orm* gene, which is also known as D4Mit17. A 130bp fragment is amplified from *M. spretus* and a 170bp fragment from *M. musculus* DNA (sizes are approximate, being estimated from the gel). All the hemizygote DNAs were amplified and the PCR products run out on a 4% Nusieve gel, shown in fig. 4.2. When a PCR product from both chromosomes was generated, then both copies of the *Orm* gene were present. In only one deletion DNA, 11R30M (track 12), was the PCR product from the *M. musculus* chromosome absent, indicating that this deletion extends as far as the *Orm* gene. Predictably 11R30M is the only deletion which encompasses the *whirler* gene.

I.J. Jackson obtained a probe from C. Abbott which was derived from the *Hxb* gene (Pearson et al., 1988). He carried out a Southern blot which also showed that this gene was excluded from only the 11R30M deletion. He also obtained a probe from the *Lv* gene (Bishop et al., 1986) and found that the 11R30M deletion does not extend to this gene (data not shown). Thus the proximal end point of the 11R30M deletion lies between *Lv* and the *wi*.

PCR primers, whose sequence had been published (Abbott et al., 1992), were generated from the *Iftα* gene (details in table 2.2). These primers amplified a 170bp band in *M. spretus* and one of around 150bp in *M. musculus*. Even on a 4% Nusieve gel, the size difference was difficult to visualise, so a polyacrylamide gel was used in order to improve separation of the polymorphic PCR products. One primer was end-labelled and the PCR reactions were run on a 6% denaturing polyacrylamide gel. The results are shown in fig. 4.3. The *M. spretus* and *M. musculus* PCR products were easily distinguished on this gel. Also evident was a polymorphism between the two strains of *M. musculus* mice, the 101 PCR product (160bp) being slightly larger than the C3H product (150bp) (the mutagenised mice were the offspring from a C3H/101cross, therefore the mutation could have been
A polymorphic microsatellite marker (D4Mit17) from the *Orm* gene was mapped to the deletion panel by PCR analysis. DNA from each deletion was amplified with D4Mit17 primers (section 2.5.1 and table 2.2) and analysed on 4% Nusieve GTG gels. The size marker used was øXHaeIII and estimated sizes of PCR products (in bp) are shown. The results show that a product was amplified from both chromosomes in all tracks, except track 12. Thus, only deletion 11R30M extends to the *Orm* gene.
A polymorphic marker from the \textit{Ifa} gene was mapped to the deletion panel by PCR analysis. End-labelled primers were used in the PCR reactions (section 2.5.8 and table 2.2), which were analysed on 6\% polyacrylamide gels (sections 2.6.1 and 2.6.4). The dried gels were exposed to film for 16 hours at -70\textdegree C (section 2.10.1).
A polymorphism was detected in products amplified from both the \textit{M. spretus} and \textit{M. musculus} (C3H and 101) chromosomes (see text). An amplified fragment, from either C3H or 101 \textit{M. musculus} DNA, was detected in each deletion DNA. Therefore the deletions do not extend to the \textit{Ifx} gene.

generated on either chromosome). A polymorphism was also identified in \textit{M. spretus}. The most common product was 170bp in length, but in some DNAs (tracks 4, 15, 16 and 21), a 165bp product was produced (all sizes are approximate). In all of the deletion DNAs, a PCR product was generated from both chromosomes, i.e. the deletions do not extend as far as the \textit{Ifx} gene.

### 4.3.2 Mapping anonymous markers

Microsatellite markers which mapped to this region of chromosome 4, were also available. Six polymorphic markers from Research Genetics, the D4Mit MapPair series, had previously been genetically mapped to the region (Dietrich et al., 1992). Three microsatellite markers (D4Mit7, 25 and 44) had been mapped to between \textit{b} and \textit{Lv} and three others to between \textit{b} and \textit{Ifx} (D4Mit15, 27 and 45) (Fleming et al., 1994). Two microsatellite markers (D4Nds7 and D4Nds9), isolated by Cornall et al., 1992, had also been mapped to this region. PCR primers which amplify these polymorphic repeats were obtained from Research Genetics (D4Mit series) and the others (D4Nds series) were a gift from K. Steel. The products of these PCR reactions were generally between 100-250bp. In order to detect size polymorphisms, the labelled PCR products were run on denaturing 6% polyacrylamide gels.

The D4Mit7 primers amplify a fragment of approximately 160bp from \textit{M. spretus} DNA and a slightly larger one from \textit{M. musculus}. Once again, there was again a polymorphism between the C3H and 101 \textit{M. musculus} strains (fig. 4.4). A product from the \textit{M. spretus} chromosome was seen in all tracks, but a \textit{M. musculus} product was absent from tracks 4, 5, 12, 15, 16 and 21, corresponding to deletions 33IK, 13DT, 11R30M, 9Pu, 37Pub and 49HATH. Thus six of the deletions included the D4Mit7 locus.
The polymorphic microsatellite marker, D4Mit7, was mapped to the deletion panel by PCR analysis (as detailed in the legend of fig. 4.3). A polymorphism was detected between the C3H and 101 *M. musculus* DNAs as well as with *M. spretus* DNA. An amplified product from the *M. musculus* chromosome was detected in all DNAs, except tracks 4, 5, 12, 15, 16 and 21. Thus deletions, 33IK, 13DT, 11R30M, 9Pu, 37Pub and 49HATh, include the D4Mit7 marker.
The polymorphic microsatellite marker, D4Mit25, was mapped to the deletion panel by PCR analysis (as detailed in the legend of fig. 4.3). An amplified product from the *M. musculus* chromosome was detected in all the deletion DNAs (not shown), except tracks 4, 12 and 21. Thus deletions 33IK, 11R30M and 49HATH extend to the D4Mit25 marker.
The D4Nds7 microsatellite marker was analysed as described in the legend of fig. 4.3. A polymorphism, between *M. spretus* and *M. musculus* C3H, was not detected, therefore, D4Nds7 could not be mapped to the deletion panel.
The polymorphic microsatellite marker, D4Nds9, was mapped to the deletion panel by PCR analysis (as detailed in the legend of fig. 4.3). When *M. musculus* DNA was present, it was preferentially amplified and a PCR product from the *M. spretus* chromosome was not observed. An amplified product from the *M. musculus* chromosome was detected in all the deletion DNAs (not shown), except tracks 7, 12, 20 and 25. Thus deletions 33IK, 11R30M, 37Pub and 49HATH extend to the D4Nds9 marker.
Primers from the D4Mit15 locus amplified a band of around 330bp from \textit{M. musculus} DNA. No PCR product was generated from the \textit{M. spretus} chromosome. The primers were generated from \textit{M. musculus} sequence and there may be a polymorphism at the primer binding site in \textit{M. spretus}. The annealing temperature of the PCR reaction was reduced from 55°C to 50°C, thereby making the primer binding step less stringent, but still no PCR product was observed. If this microsatellite was to be mapped to the deletion complex, the absence of any PCR product from the \textit{M. musculus} chromosome would infer that this microsatellite was deleted. However, without the PCR product from the \textit{M. spretus} chromosome acting as a positive control for the PCR reaction, the results would be invalid. For this reason primers from another locus, were added to the reaction as a positive control. However, this locus was preferentially amplified and no product was seen from the D4Mit15 locus. Consequently the microsatellite D4Mit15 could not be mapped to the deletion panel.

Primers from the D4Mit25 microsatellite amplify a 222bp fragment from \textit{M. spretus} and a 210bp product from \textit{M. musculus}. Fig. 4.5 shows that a PCR product was not amplified from the \textit{M. musculus} chromosome in tracks 4, 12 and 21, corresponding to deletions 331K, 11R30M and 49HATH. Therefore, these three deletions extend as far as the D4Mit25 locus. No other deletions were found to extend to the D4Mit25 locus (data not shown).

The other three D4Mit microsatellites were present in all the deletion complex DNAs and, consequently, must map outwith the deletion complex.

D4Nds7 primers amplify an 85bp fragment from \textit{M. musculus} DNA and a similar sized fragment from \textit{M. spretus} DNA. When the labelled reactions were run on a polyacrylamide gel (fig. 4.6), it was apparent that there was a polymorphism between the \textit{M. musculus} C31-1 and 101 DNAs. Unfortunately no polymorphism was detectable between C31-1 and \textit{M. spretus}, the PCR products are indistinguishable. Therefore, the D4Nds7 microsatellite could not be mapped to the deletion complex.

Primers from D4Nds9 amplify a 90bp fragment from \textit{M. musculus} and a slightly larger one from \textit{M. spretus} DNA. Fig. 4.7 shows that in the heterozygous control (track sp/C3H), the \textit{M. musculus} DNA is preferentially
amplified. A product from the *M. musculus* chromosome is seen in tracks 9 and 14, corresponding to deletions 13DT and 9Pu. Only the *M. spretus* PCR product is observed in tracks 7, 12, 20 and 25, corresponding to deletions 33IK, 11R30M, 37Pub and 49HATH, indicating that these deletions extend to the D4Nds9 locus.

I.J. Jackson obtained a probe from the polymorphic D4Ler2 locus (LeRoy et al., 1992) and hybridised it to the deletion complex DNAs. He found that the locus was absent in deletions 33IK, 13DT, 3YPSc, 11R30M, 9Pu, 37Pub, 49HATH and 13R75M (data not shown).

In all, four microsatellite markers were mapped within the deletion complex by virtue of their polymorphic nature and a further three map outwith the complex. This data is summarised in table 4.2. The microsatellite markers can be placed, along with those genes analysed, on the complementation map generated by E. M. Rinchik (1994). This will be discussed in section 4.5.

<table>
<thead>
<tr>
<th>Microsatellite Marker</th>
<th>Mapping Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4Mit7</td>
<td>Absent in deletions 33IK, 13DT, 11R30M, 9Pu, 37Pub and 49HATH.</td>
</tr>
<tr>
<td>D4Mit15</td>
<td>Unable to be mapped, no product amplified from <em>M. spretus</em> chromosome.</td>
</tr>
<tr>
<td>D4Mit25</td>
<td>Absent in deletions 33IK, 11R30M and 49HATH.</td>
</tr>
<tr>
<td>D4Mit27</td>
<td>Maps outwith the deletion complex.</td>
</tr>
<tr>
<td>D4Mit44</td>
<td>Maps outwith the deletion complex.</td>
</tr>
<tr>
<td>D4Mit45</td>
<td>Maps outwith the deletion complex.</td>
</tr>
<tr>
<td>D4Nds7</td>
<td>Unable to be mapped, no polymorphism between <em>M. spretus</em> and <em>M. musculus</em> C3H.</td>
</tr>
<tr>
<td>D4Nds9</td>
<td>Absent in deletions 33IK, 11R30M, 37Pub and 49HATH.</td>
</tr>
<tr>
<td>D4Ler2</td>
<td>Absent in deletions 33IK, 13DT, 3YPSc, 11R30M, 9Pu, 37Pub, 49HATH and 13R75M.</td>
</tr>
</tbody>
</table>

Table 4.2: Summary of microsatellite mapping data
4.4 Mapping markers by SSCP analysis

4.4.1 The D4Rck series

A microdissection library of the mid-distal region of chromosome 4 was generated by Bahary et al., 1993. The microclones, termed the D4Rck series, had been genetically mapped to the region. This section describes the mapping of fifteen microclones, which mapped closest to the brown gene, using the deletion complex.

The aim was to sequence each of the microclones (which had been cloned into λgt10), then design locus specific primers, which could be used to amplify the heterozygous deletion DNAs.

λgt10 phage stocks of the fifteen microclones were obtained from J. Friedman and the microclones were amplified by PCR using λgt10 primers (details in table 2.2). The size of the microclones varied between 150-300bp, 78bp of this being λgt10 sequence. The PCR products were purified from agarose gels (section 2.6.3) and sequenced from both ends using the amplifying primers (see section 2.11). Fig. 4.8 shows a typical sequencing gel with the junction between the λgt10 sequence and the microclone highlighted.

Primers were designed from the microclone sequence, as close to the ends as possible, in order to generate a PCR product as large as possible. The chosen primer sequences were checked on the OLIGO4 (Hybaid) program before being synthesised (sections 2.5.2 and 2.5.3). In all, PCR primers were generated from thirteen of the fifteen microclones (table 2.2). The sequence of two microclones (D4Rck49 and D4Rck52), was unable to be determined. These PCR primers were then used to amplify M. spretus, M. musculus and the heterozygous DNAs, and the PCR products were analysed on Nusieve gels. This was carried out in order to check that the primers did amplify a fragment of the expected size, and to see if any size polymorphisms could be identified. All of the primers generated a fragment of the expected size, based on the sequence data, but no size polymorphisms between M. spretus and M. musculus DNAs were observed. This result was expected as the PCR products are of a relatively small size. Sequence polymorphisms were therefore detected by SSCP analysis (Orita et al., 1989a). This technique relies on the observation that single stranded DNA molecules, run in non-denaturing conditions, will adopt a specific, stable secondary structure.
The D4Rck117 microclone had been cloned into λgt10 (Bahary et al., 1993). It was amplified using λgt10 PCR primers (section 2.5.1 and table 2.2) and the PCR product was isolated and sequenced (section 2.11). The sequencing reactions were analysed on 6% polyacrylamide gels (sections 2.6.1 and 2.6.4) and the dried gels were exposed to film for 16 hours at room temperature (section 2.10.1).
This figure shows the sequencing of microclone D4Rck117 using primers from both arms of λgt10 (gels A and B). The junction of λgt10 and the start of the microclone sequence have been arrowed.

Potentially, even single base pair differences between two DNA strands can alter their secondary structure, and therefore their migration in non-denaturing polyacrylamide gels.

The conformation a DNA molecule adopts, and therefore, SSCP detection, is dependant primarily on its sequence, but is also markedly affected by the local environment. Gels made with low cross linking acrylamide afford better separation (reviewed by Hayashi, 1991). 5% polyacrylamide gels were used, with a 99:1 ratio of acrylamide to N,N'-methylenebisacrylamide. The presence of 5 - 10% glycerol can also improve separation (Orita et al., 1989b). Glycerol has a weak denaturing action on nucleic acids, but it is not known how this alters the detection of SSCPs. The conformation of the DNA molecules is also affected by temperature, being more stable at lower temperatures (reviewed by Hayashi, 1991). The gels were run on BRL gel sequencing apparatus, which contained an aluminium plate to distribute the heat evenly. They were run at 40 volts in 1x TBE at 4°C to minimise heat build up. However, the presence of glycerol in the gel inhibits mobility of the DNA fragments and this is exacerbated at 4°C, presumably due to its increased viscosity. Gels containing glycerol were run at room temperature to avoid extended running times. SSCP detection is often increased in one strand (Michaud et al., 1992), when compared to its complementary strand. It has been suggested that this may be because DNA fragments with a higher purine content, show more striking gel shifts (Sheffield et al., 1993).

The running conditions of the gels were optimised for SSCP detection, for each set of primers, before amplifying the 25 heterozygous DNAs from the deletion panel. Initially *M. spretus, M. musculus* and hybrid DNAs were amplified, with either or both primers end-labelled. They were analysed on 5% non-denaturing polyacrylamide gels run at 4°C, or at room temperature when either 5 or 10% glycerol was added. For instance, fig. 4.9 shows the D4Rck117 PCR with either one or both of the primers labelled. When the
upper primer is labelled, PCR products from the two species, each form two stable conformations. An SSCP can be detected in both of these conformations, thus it was possible to map D4Rck117 to the deletion complex.

This protocol enabled SSeps to be detected in eight of the thirteen sequenced microclones. In the others, a polymorphism was not observed, regardless of the running conditions of the gel. Fig 4.10 shows the D4Rck140 PCR run on a 5% non-denaturing polyacrylamide gel. A different band pattern is observed when different primers are labelled and when both are labelled, a combined pattern is seen. However no differences in the band pattern are seen between *M. musculus* and *M. spretus*, regardless of which primer is labelled, so SSCP analysis of this microclone could not be carried out.

When an SSCP was detected between *M. musculus* and *M. spretus*, the 25 deletion panel DNAs were then amplified. Fig. 4.11 shows the D4Rck4 PCR of the deletion complex DNAs using upper labelled primer. The PCR product from the *M. spretus* chromosome migrates faster through the gel, giving the appearance of a smaller band than that from the *M. musculus* chromosome. In most of the tracks only the *M. spretus* band was amplified, indicating that these deletions incorporate this locus. A doublet was seen in tracks 7, 14, 17, 18, 20, 23 and 24, corresponding to deletions 3YPSH, 11Pu, 10Z, 33G, 8Pub, 46UTHc and 9R75VH, indicating that they do not extend as far as D4Rck4. This microclone was one of the four which E. M. Rinchick had mapped by Southern blot analysis to the deletion panel. The SSCP results correlate exactly with those already obtained, thus confirming the position of the D4Rck4 locus on the map.

Initially only six deletion DNAs, that spanned the entire length of the deletion complex, were analysed with the PCR primers from D4Rck122. Fig. 4.12 shows that once again, the PCR product amplified from *M. spretus* migrates faster than the *M. musculus* PCR product. D4Rck122 is deleted in five of these deletion DNAs and only present in track 20, deletion 8Pub. All 25 of the deletion DNAs were then amplified (fig. 4.13) and a PCR product from the *M. musculus* chromosome was observed only in tracks 17, 20, 23 and 24, corresponding to deletions 10Z, 8Pub, 46UTHc and 9R75VH. In the
Figure 4.9: SSCP detection of the D4Rck117 microclone

M. musculus specific

M. spretus specific
Figure 4.10: SSCP detection of D4Rck140
Figure 4.9 legend
The microclone marker D4Rck117 was amplified by PCR and polymorphisms detected by SSCP analysis. Both primers were end-labelled and used separately and in combination, in the PCR reactions (section 2.5.9 and table 2.2). The reactions were analysed on a 5% non-denaturing polyacrylamide gel, run at 40 volts, at 4°C for 6 hours (sections 2.6.1 and 2.6.4). The SSCPs were detected by a Molecular Dynamics Phosphorimager (section 2.10.2) after a 16 hour exposure.
The results show that when the upper primer was labelled, the PCR products from either M. musculus C3H or M. spretus DNA form two stable conformations (as indicated by the two intense bands in each track, which are arrowed). In each conformation an SSCP is apparent in the PCR products from the two species. When both primers were labelled, a similar pattern was observed. The results when the lower primer was labelled, cannot be analysed, as two of the PCR reactions failed. As an SSCP can be detected when the upper primer is end-labelled, this was used in further analysis.

Figure 4.10 legend
The microclone marker D4Rck140 was amplified by PCR and the products were analysed by SSCP analysis. The protocol was described in the legend of fig. 4.9, except that the samples in this figure were analysed on a 5% non-denaturing polacrylamide gel with 5% glycerol, run for 4 hours at room temperature.
Although different band patterns are observed when different primers are labelled, no SSCPs were detected between M. musculus and M. spretus PCR products. D4Rck140 could not be mapped to the deletion complex by SSCP analysis.
The microclone marker D4Rck4 was amplified from deletion panel DNAs by PCR and polymorphisms detected by SSCP analysis. The protocol was as previously described in the legend of fig. 4.9, except the upper primer was labelled in these reactions, which were analysed on a 5% non-denaturing polyacrylamide gel, run at 4°C for 5 hours. The results show a product was amplified from both chromosomes in tracks 7, 14, 17, 18, 20, 23 and 24. Therefore deletions 3YPSH, 11Pu, 10Z, 33G, 8Pub, 46UTHc and 9R75VH do not extend to the D4Rck4 marker.
The microclone marker D4Rck122 was initially amplified by PCR from six deletion panel DNAs (which spanned the length of the deletion complex) and polymorphisms detected by SSCP analysis. The protocol was as previously described in the legend of fig. 4.9, except the lower primer was labelled in these reactions, which were analysed on a 5% non-denaturing polyacrylamide gel, run at 4°C for 5 hours. The results show that a product was amplified from both chromosomes in track 20 only (deletion 8Pub). Therefore D4Rck122 was amplified from the rest of the deletion panel DNAs (fig. 4.13).
The microclone marker D4Rck122 was amplified from deletion panel DNAs by PCR and polymorphisms detected by SSCP analysis (as detailed in the legend of fig. 4.12).

The results show that a product was amplified from both chromosomes in tracks 17, 20, 23 and 24. Therefore deletions 10Z, 8Pub, 46UTHc and 9R75VH do not extend to the D4Rck122 marker.
Figure 4.14: Mapping of D4Rck32 by SSCP analysis

figure legend overleaf
Figure 4.14 legend

The microclone marker D4Rck32 was initially amplified by PCR from six deletion panel DNAs (which spanned the length of the deletion complex) and polymorphisms detected by SSCP analysis. The protocol was previously described in the legend of fig. 4.9, except that the upper primer was labelled in these reactions, which were analysed on a 5% non-denaturing polyacrylamide gel with 10% glycerol, run at room temperature for 5 hours. The results show that a product was amplified from the *M. spretus* chromosome only, in track 12. Therefore deletion 11R30M extends to the D4Rck32 marker. The other deletion panel DNAs were then analysed and all were found to include D4Rck32 (data not shown).

heterozygote control DNA, where both *M. spretus* and *M. musculus* DNA are present, the PCR product from the *M. musculus* chromosome was preferentially amplified. This phenomena was also observed in the four deletion DNAs which contained the D4Rck122 locus. This locus is deleted in 21 of the 25 DNAs and therefore must map close to the *brown* gene.

Fig 4.14 shows the D4Rck32 PCR run on a 5% non-denaturing polyacrylamide gel with 10% glycerol. The six deletion DNAs, whose deletions span the entire deletion complex, were amplified. The PCR product from the *M. spretus* chromosome was present in all the DNAs. The three bands which represent the *M. musculus* PCR product are present in all deletion DNAs, except track 12, deletion 11R30M. The other 19 deletion DNAs were then analysed and the D4Rck32 locus was found to be present in them all (data not shown).

Of the eight microclones which exhibited an SSCP between *M. spretus* and *M. musculus*, three mapped within the deletion complex. A further five were present in all the 25 DNAs from the deletion panel, i.e. they mapped outwith the deletion complex. This data is summarised in table 4.3. The three microclones that mapped within the deletion complex can be incorporated into the map of this region (discussed in section 4.5).
4.4.2 The *Adfp* gene

The *Adfp* gene has been mapped between *Ifx* and the *brown* gene. The sequence of primers which amplify a 170bp fragment from the gene, have been published (Beier et al., 1992) and SSCP analysis of this fragment was carried out. When both primers are labelled a polymorphism is apparent, with the *M. musculus* product migrating faster in a 5% non-denaturing polyacrylamide gel. Fig. 4.15 shows that a PCR product from the *M. musculus* chromosome is seen in all the deletion complex DNAs. In some tracks the *M. spretus* product is absent, indicating that the *M. musculus* PCR product is preferentially amplified. Therefore none of the deletions extend as far as the *Adfp* gene.

**Table 4.3.**

<table>
<thead>
<tr>
<th>Microclone Marker</th>
<th>Mapping Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4Rck4</td>
<td>Absent from all deletions except 3YPSH, 11Pu, 10Z, 33G, 8Pub, 46UTHc and 9R75VH.</td>
</tr>
<tr>
<td>D4Rck9</td>
<td>No SSCP identified.</td>
</tr>
<tr>
<td>D4Rck28</td>
<td>No SSCP identified.</td>
</tr>
<tr>
<td>D4Rck32</td>
<td>Absent from deletion 11R30M only.</td>
</tr>
<tr>
<td>D4Rck49</td>
<td>Unable to be sequenced.</td>
</tr>
<tr>
<td>D4Rck52</td>
<td>Unable to be sequenced.</td>
</tr>
<tr>
<td>D4Rck60</td>
<td>No SSCP identified.</td>
</tr>
<tr>
<td>D4Rck117</td>
<td>Maps outwith deletion complex.</td>
</tr>
<tr>
<td>D4Rck119</td>
<td>Maps outwith the deletion complex.</td>
</tr>
<tr>
<td>D4Rck122</td>
<td>Absent in all deletions except 10Z, 8Pub, 46UTHc and 9R75VH.</td>
</tr>
<tr>
<td>D4Rck138</td>
<td>Maps outwith deletion complex.</td>
</tr>
<tr>
<td>D4Rck140</td>
<td>No SSCP identified.</td>
</tr>
<tr>
<td>D4Rck193</td>
<td>No SSCP identified.</td>
</tr>
<tr>
<td>D4Rck196</td>
<td>Maps outwith deletion complex.</td>
</tr>
<tr>
<td>D4Rck205</td>
<td>Maps outwith deletion complex.</td>
</tr>
</tbody>
</table>

Table 4.3: Summary of microclone mapping data
Primeers from the *Adfp* gene were used to amplify the deletion panel DNAs and polymorphisms were detected by SSCP analysis. The protocol was as previously described in the legend of fig. 4.9, except that both primers were end-labelled in these reactions, which were analysed on a 5% non-denaturing polyacrylamide gel, run at 4°C for 4 hours. The results show that a product was amplified from the *M. musculus* chromosome in all tracks. Therefore none of the deletions extend to the *Adfp* gene.
4.5 Generation of a more detailed map of the brown locus
Markers which proved to be informative could now be placed on the map generated by E. M. Rinchick. Some will determine the extent of the deletion complex as a whole and others can be placed within it. Markers which map close to the brown gene will be helpful in characterising the Harwell deletions and useful in mapping the baf gene itself.

Only two of the markers mapped to the telomeric side of the deletion complex. The deletions do not extend as far as Ifα gene, which has been genetically mapped 6cM distal to the brown gene. Furthermore, no deletions incorporated the Adfp gene, which can be placed between dep and Ifα. Therefore, deletions 8Pub, 3YPSH, 11Pu, 13R75M, 12Pu, 5CHLe and 46UTHc have an end-point between dep and Adfp, which is around 2cM distal to the brown gene.

The rest of the markers which could be mapped, were positioned on the centromeric side of the brown gene. The D4Rck122 microclone is only present in deletions 10Z, 8Pub, 46UTHc and 9R75VH, placing it between the brown gene and D4Rck4. D4Rck52 and the lethal proximal gene also map to this interval, but their order is not known.

Deletions 33IK, 13DT, 3YPSc, 11R30M, 9Pu, 37Pub, 49HATH and 13R75M, include the D4Ler2 locus placing it between D4Rck4 and Hxb. The D4Mit7 locus is deleted in six of the above deletions, indicating that it lies centromeric to D4Ler2. This means that two of the deletions have their end point in the D4Ler2/D4Mit7 interval.

Similarly, D4Nds9 is deleted in the 33IK, 11R30M, 37Pub and 49HATH deletions, placing it centromeric to D4Mit7, therefore two deletions have their end-points in the D4Mit7/D4Nds9 interval. The D4Mit25 locus includes three of the above deletions and can be placed centromeric to D4Nds9. Lastly D4Rck32 is deleted only in 11R30M, and as this deletion is the only one to include wi, Hxb and Orm, the order of these loci cannot be determined from this data.

In this way, a more detailed map of the brown locus (shown in fig. 4.16) was generated. It is evident that most of these newly mapped markers lie in the
Figure 4.16 legend

The genes and polymorphic markers which had been mapped to this region, were incorporated into the existing genetic map, and a more detailed map was generated. The genes and markers which are boxed, were mapped as previously detailed, whereas the others were mapped by E. M. Rinchik at Oak Ridge. The approximate location of baf and the three lethal genes are shown. The genetic map is shown below, with the distances in cM. The extent of the deletions is shown, however, the end points of the two arrowed deletions (with regard to D4Rck9) have not been determined.

D4Rck4/wi interval. When compared to the original map of the brown locus, there are many deletion end-points in this region, suggesting that D4Rck4 and wi are a considerable distance apart. These end-points have now been characterised more fully and the deletions split into more subgroups. The overall extent of the deletion complex has been determined; it is bordered proximally by Lv and distally by Adfp.

As baf is thought to be located very near the brown gene, the markers which will be of most use in its further localisation will be those between D4Rck4 and D4Rck140. Unfortunately, only one additional marker has been mapped to this area. However, this does suggest that this region is relatively small. All of these markers will be of use in screening YAC libraries, in order to generate a YAC contig of the region, and this is described in chapter 7.

4.6 Discussion

Mapping of these markers to the brown locus, using the deletion panel, has enabled the construction of a more detailed map of the region (fig. 4.16). Positioning of the markers on the map, depends on the presence of a polymorphism between M. musculus and M. spretus DNAs. Microsatellite markers are highly polymorphic between species, due to an increased rate of spontaneous mutation which alters their length (Dallas, 1992). All of the eight microsatellite markers used in this study, demonstrated a size polymorphism which enabled them to be mapped to the deletion panel.

Mapping of the microclones also depended on polymorphisms being present between the two species. These polymorphisms are likely to be single base
pair substitutions, rather than size polymorphisms. Such sequence polymorphisms were detected by SSCP analysis. Single stranded DNA molecules adopt a specific, stable, secondary structure under non-denaturing conditions. A single base pair substitution can alter the conformation of a DNA molecule. This can be detected if it alters the mobility of the fragment. The conformation they adopt, depends primarily on their sequence, but is also affected by their local environment.

As there is considerable sequence variation between *M. spretus* and *M. musculus* (reviewed by Avner et al., 1988), we would expect most of the microclones to contain a sequence polymorphism. In five of the thirteen microclones sequenced, no SSCP was identified. Either these microclones do not contain a polymorphism, or it was not detected. One of the most important factors in detecting SSCPs is the length of the fragment. A study by Sheffield et al., 1993 showed that 97% of single base pair changes can be detected by SSCP in 155bp DNA fragments. This rate decreases when the fragment length is increased (72% detected in 212bp fragments), or decreased (80% in 135bp fragments). This study had not been published when the PCR primers were designed. Previously, it was believed that fragments of around 300bp were optimal for SSCP analysis (reviewed by Hayashi, 1991). Of the five microclones which did not display a SSCP, four were between 90-120bp, only D4Rck9 at 170bp was close to the optimal size. Perhaps, in some cases, if different primers had been designed, more SSCPs would have been detected. However, for some clones this was not possible, as the microclones themselves were under 150bp in size. In addition, primers were designed, if possible, so that they contained mostly guanine (G) and cytosine (C) nucleotides, especially at their 3' end. Such primers can anneal to the template DNA at a higher temperature than less GC rich primers, thereby increasing the specificity of the PCR reaction (Taylor, 1991). The regions of microclone sequence which were suitable for designing a complementary GC rich primer, were limited. Therefore the position of the primers and thereby the size of the amplified PCR product, were also dependant on the sequence of the microclone itself.

Some of the SSCP gels showed complex band patterns, for example D4Rck117 (fig. 4.9). There appear to be many faint bands in each track and three more intense band regions, which are arrowed. The faint bands are
probably due to incomplete denaturation of the samples before they were run on the gel (Beier et al., 1992). DNA fragments can adopt more than one stable conformation (Beier et al., 1992), and the three intense bands are probably different stable conformations of the same DNA fragments. In this gel, an SSCP can be detected, when the upper primer is labelled, in more than one of the stable conformations. Most of the other SSCP gels had simpler band patterns which were easier to analyse.

In some microclone PCR reactions, *M. musculus* DNA was amplified in preference to *M. spretus*. This was apparent in the D4Nds9 SSCP gel (fig. 4.7). When only *M. spretus* DNA was amplified (track labelled sp), three intense bands were seen, which run slower than the *M. musculus* PCR product (tracks C3H or 101). However when both DNAs are present (track sp/C3H), only the *M. musculus* PCR product was seen. This, however, does not hinder the mapping of this microsatellite, because if the locus was absent from the *M. musculus* chromosome, the *M. spretus* PCR product was always seen.

The sequence of both the microclones and the microsatellite primers was generated from *M. musculus* sequence. The absence of an amplified PCR product from the *M. spretus* chromosome, when *M. musculus* DNA was present, may be due to a polymorphism at the primer binding site. If the primer is not fully complementary to the *M. spretus* sequence, it would affect amplification of this locus. Thus, when *M. musculus* DNA is present, the primer will preferentially amplify from this chromosome, as it has a sequence fully complimentary to the primer. The annealing temperature of the PCR reaction determines the stringency of primer binding. This is most critical in the first few PCR cycles. If the primer does bind to the *M. spretus* DNA in the first few cycles, then products will be generated from this chromosome, which have sequence complementary to the primers at each end. These will then act as templates for further amplification and a *M. spretus* PCR product will be visualised on a SSCP gel. However similar PCR reactions can give varying results, in this respect. When D4Rck122 primers were initially used to amplify the six deletions DNAs (fig. 4.12), a PCR product was generated from both the *M. musculus* and *M. spretus* chromosomes. In a subsequent PCR reaction, when all the deletion DNAs were amplified (fig. 4.13), only a PCR product from the *M. musculus* chromosome was seen when both DNAs were
present. Although the PCR conditions were identical, the PCR machine used may have been different. Slight variations in the plate temperature, between machines, may have been enough to favour the preferential amplification from the *M. musculus* chromosome.

No product was able to be amplified from *M. spretus* DNA with the D4Mit15 primers. This suggests that there is a polymorphism at the primer binding site, perhaps at the critical 3' end, which inhibits the primer binding. Research Genetics subsequently published an update, giving the sizes of D4Mit15 product amplified in different species. This confirmed that no PCR product was generated from *M. spretus* DNA.

The PCR products generated from the *Ifα* locus showed a polymorphism between the two *M. musculus* strains, C3H and 101. The original mice that were mutagenised were C3H/101 *M. musculus* mice, therefore the mutation could have occurred on either chromosome. These results enable the mutagenised chromosome at this locus to be identified. Table 4.4 shows which chromosome carries the mutation in each of the 25 deletions, based on *Ifα* typing.

A polymorphism between C3H and 101 is also seen at the D4Mit7 locus. The mutagenised chromosome at this locus can, therefore, also be identified (table 4.4). However, six of the deletions extend to this locus, so their mutagenised *M. musculus* chromosome cannot be identified. When these results are compared with those at the *Ifα* locus, there are discrepancies. Deletions IDF1OD and 37DTD have C3H DNA at the *Ifα* locus, and 101 DNA at the D4Mit7 locus. Conversely 5CHLe has 101 DNA at the *Ifα* locus, and C3H DNA at the D4Mit7 locus. These results can be explained by a recombinational event occurring in this interval, between the C3H and 101 chromosomes. This must have occurred in at least three deletion carrying mice, before they were crossed to *M. spretus*.

Recombinational events occurring in this region meant that the D4Nds7 locus could not be mapped to the deletion panel. A polymorphism was present at this locus between C3H and 101, but not between C3H and *M. spretus*. Theoretically it was possible to map D4Nds7 to those deletions which have 101 DNA at this locus. However, because of the possibility of recombination,
Table 4.4: Origin of *M. musculus* allele at *Ifα* and D4Mit7 loci

<table>
<thead>
<tr>
<th>Deletion</th>
<th>If α</th>
<th>D4Mit7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C3H</td>
<td>101</td>
</tr>
<tr>
<td>ITHO-IV</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>IDF1OD</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>47DThWb</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>33IK</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>13DT</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>26R60L</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>3YPSh</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>37DTD</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>12Pu</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>55Cos</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>3YPSc</td>
<td>✓</td>
<td></td>
</tr>
<tr>
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<tr>
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<tr>
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<td>5CHLe</td>
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<td>46UTHc</td>
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</tr>
<tr>
<td>13R75M</td>
<td>✓</td>
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</tbody>
</table>

In summary, many more markers have now been mapped to the region and this has enabled greater characterisation of the deletion end points (fig 4.16). The markers which map close to the *brown* gene will be of most use in characterising the Harwell deletions and in the mapping of the *baf* gene. The markers lying close to *brown* will be used to screen YAC libraries and generate a YAC contig of the region (described in chapters 6 and 7). As the *baf* gene lies close to *brown*, it should be contained on one of these YACs.
Chapter 5
Analysis of YAC recombinants isolated from the ICRF library

5.1 Introduction

YACs from the brown locus will be a valuable tool in the physical characterisation of this region and in the mapping of the genes within it. They will be useful for isolating the sequences from around the brown gene, which are removed in both the Harwell and Oak Ridge deletions.

The ICRF YAC library (Larin et al., 1991) was screened for YACs containing the brown gene, using the Trp1.6 probe. The screening was carried out by L. Stubbs at the ICRF. Three YACs were isolated, termed YACs 2/1, 2/2 and 3/4. Fig. 5.1 (received from L. Stubbs) shows a Southern blot of a pulsed field gel, which has been probed with Trp1.6. This gives an indication of the sizes of the YACs, which range from 200kb-1.1Mb. YAC 2/1 is considerably larger than the average YAC in this library and may be very useful, as long as it is not chimaeric.

5.2 Initial characterisation of the YACs

Stabs of the three YACs, in YPD medium, were received from L. Stubbs. The YACs were grown in selective medium, and DNA was extracted using the yeast midi-prep protocol (section 2.2.3). The YACs were first checked to confirm that they contained sequences from the brown gene. A Southern blot of the EcoRI digested YAC DNA was probed with Trp1.6 (shown in fig. 5.2). This analysis showed that Trp1.6 hybridises to three bands in each YAC, verifying that all three YACs contain DNA from the brown locus.

The YACs were then characterised by their CA repeat content. Mouse genomes contain around $10^5$ CA$_{(n)}$ sequences, which are generally between 9-30 repeat units in length (Hamada et al., 1982). YACs were digested with BamHI, EcoRI and HindIII, and the Southern blot was probed with a CA$_{(10)}$ oligonucleotide. The results are shown in fig. 5.3a. By comparing the hybridisation pattern or fingerprint of each YAC, an idea of the extent of overlap between them can be inferred. However, these results showed that the yeast strain AB1380 also contains many CA repeats, and this obscures the pattern of YAC specific CA repeats. Therefore, no information about the extent of YAC overlap was gained from analysis of their CA repeat content.
Figure 5.1: PFGE analysis of YACs containing the brown gene

The three YACs isolated by L. Stubbs from the ICRF YAC library were analysed by PFGE. A Southern blot of the pulsed field gel was probed with the Trp1.6 probe from the brown gene. The estimated sizes of the YACs (in kb) are shown in the figure. This analysis was carried out by L. Stubbs.
The YACs received from L. Stubbs were analysed to confirm that they contained DNA from the *brown* gene. YACs were digested with EcoRI (section 2.4.1) and the DNA analysed on a 0.8% agarose gel (section 2.6.2). The DNA was transferred to a nylon membrane (section 2.7.1) and hybridised with the Trp1.6 probe from the *brown* gene (sections 2.8.1 and 2.9). Hybridisation was detected after exposure to film for 16 hours at -70°C (section 2.10.1).

The results confirm that all three YACs contain DNA from the *brown* gene.
However, other microsatellite repeats present in the mouse genome are not as abundant in yeast. The filter was reprobed with a $\text{GA}_{12}$ oligonucleotide and the results are shown in fig. 5.3b. There are far fewer GA repeats in the yeast strain AB1380, making the results easier to interpret. They show that all three YACs have some common GA repeats (small arrow), and nearly all those in present YAC 2/2 are also in YAC 3/4 (large arrows). This indicates that YACs 2/2 and 3/4 have a high degree of overlap, whereas YAC 2/1 contains a lot of unique DNA.

Although this gives an indication about the extent of overlap, other techniques were employed to map the YACs more precisely.

5.3 Mapping the YACs using mapped markers
The YACs were analysed to see if they contained any of the markers which had been mapped, using the Oak Ridge deletion panel, to the region surrounding the *brown* gene. These markers were microclones D4Rck4, 9, 122 and the microsatellite repeats, D4Mit7 and D4Nds7. Although D4Nds7 had not been physically mapped to the region, it had been mapped by backcross analysis. In 26 mice, no recombinants between D4Nds7 and *brown* were detected (Cornall et al., 1992).

PCR primers which amplified the five markers described above, were used to amplify YAC DNA. The results are shown in fig. 3.14a-e. In fig. 3.14b, where primers from the *brown* gene were used as a positive control, a 227bp product from the *brown* gene is observed as expected. However no PCR product was amplified from the D4Mit7 locus in any of the YACs. DNA from the Harwell deletions was also examined in this way (discussed in section 3.5). In the other PCR reactions, primers from the *brown* gene could not be used as a positive control, because of differing PCR reaction conditions. In these PCR reactions, DNA from the Harwell deletions acted as a positive control. No PCR product was amplified from any of the YACs, with primers from any of these markers. This shows that these YACs do not extend to the closest mapped markers.

5.4 Isolation of YAC end fragments by inverse PCR
Isolation of DNA fragments from both ends of the YACs would enable further characterisation of both the YACs themselves, and also the Harwell deletions. These "end clones" or end fragments, could then be hybridised to the YACs,
Figure 5.3: Fingerprint analysis of YACs

figure legend overleaf

A
The CA and GA dinucleotide repeat content of the YACs was examined in order to assess the extent of YAC overlap. YAC DNA was digested with BamHI, EcoRI and HindIII and analysed by Southern blotting (as described in the legend of fig. 5.2). The filter was hybridised with an end-labelled CA\textsubscript{(10)} oligonucleotide (fig. 5.3a), and then rehybridised with an end-labelled GA\textsubscript{(12)} oligonucleotide (fig. 5.3b) (sections 2.8.3 and 2.9). Hybridisation was detected after exposure to film for 4 hours at -70°C (section 2.10.1). The results are discussed in the text.
thereby generating information on the extent of overlap between the YACs and allowing a contig of the region to be assembled. Hybridisation of the end fragments to DNA from mice carrying the Harwell deletions, may also be useful in determining the extent of these deletions.

DNA fragments from the ends of all three YACs were isolated using the technique of inverse PCR (Arveiler and Porteous, 1991). The YAC arm which carries the TRP1 complementation gene was termed the left arm and that which carries the URA3 selectable marker, the right. YAC end fragments are specifically amplified using primers derived from each YAC vector arm, orientated with their 3' end towards the insert. This technique involved digesting the YAC DNA with various enzymes which were known to have recognition sequences near the SUP4 selectable marker in pYAC4, which contains the EcoRI cloning site. All the digestion products were ligated, under dilute conditions, to generate monomeric circles. These were then used as the template in an inverse PCR reaction, using primers derived from either in or near the SUP4 gene in pYAC4. The only DNA which will be able to act as a template will be that from the YAC ends. The ability of the end-fragments to be amplified depends on the size of the ligated monomeric circle. This, in turn, depends on how far the restriction site is from the end of the cloned DNA. If it is further than 3kb, no PCR product will be generated. Because of this, various restriction enzymes were used.

PCR products were obtained from both ends of the three YACs using TaqI, Rsal and Sacl to isolate ends from the left arm, and Rsal for the right arm. The amplified end fragments ranged in size from 500bp-2kb, a proportion of which was vector DNA. The PCR products were checked to confirm that they did indeed contain DNA from the YAC ends, by hybridising with a probe from the appropriate arm of pYAC4. Fig. 5.4 shows the results of this hybridisation. Amplified fragments which hybridise to the YAC vector probe, and were, therefore, generated from the YAC ends, are described in table 5.1. An end fragment was amplified from both ends of all three YACs.

The amplified end fragments were then used as hybridisation probes in Southern blots of YAC DNA. This would generate information about overlapping YACs and could also be used to check these probes were generated from the YAC ends (see below). Vector DNA was removed from the probes by digesting the PCR products with EcoRI and the enzyme used
Amplified fragments from the end of each YAC, were isolated using inverse PCR, were analysed to confirm they contained DNA from the YAC vector arms. Amplified end fragments (section 2.5.7) were analysed on a 1.2% agarose gel (fig. 5.4a) (section 2.6.2). The restriction enzymes used to digest the YAC DNA are shown in the figure. The amplified products in the four tracks on the left of the figure, were generated from the left arms of the YACs, and those on the right, from the right arms. Size markers used were λHindIII and ϕXHaeIII. The DNA was then transferred onto a nylon filter (section 2.7.1) and each half of the filter was hybridised to probes from the left or right YAC vector arms (sections 2.8.3 and 2.9). Hybridisation was detected after exposure to film for 16 hours at -70°C (section 2.10.1).

The results confirm that these amplified fragments were generated from the YAC ends and this data is summarised in table 5.1 (the result was verified after a longer exposure).
Table 5.1

<table>
<thead>
<tr>
<th>YAC</th>
<th>Arm</th>
<th>Enzyme</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/1</td>
<td>left</td>
<td>TaqI</td>
<td>1000 (90)</td>
</tr>
<tr>
<td>2/2</td>
<td>left</td>
<td>TaqI</td>
<td>2000 (90)</td>
</tr>
<tr>
<td>3/4</td>
<td>left</td>
<td>Sacl</td>
<td>1100 (360)</td>
</tr>
<tr>
<td>2/1</td>
<td>right</td>
<td>Rsal</td>
<td>600 (280)</td>
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<tr>
<td>2/2</td>
<td>right</td>
<td>Rsal</td>
<td>500 (280)</td>
</tr>
<tr>
<td>3/4</td>
<td>right</td>
<td>Rsal</td>
<td>1400 (280)</td>
</tr>
</tbody>
</table>

Table 5.1: YAC end fragments isolated by inverse PCR. Sizes of the PCR products are shown in bp, with the vector component shown in brackets.

in the inverse PCR protocol. YAC DNA was digested with

1. EcoRI - this was the cloning site used in pYAC4, so any fragment the probe binds to should be the same size in each YAC.
2. BglII - a restriction site lies upstream of the cloning site in the left arm of pYAC4. A probe from the left arm of a YAC will bind to a differently sized fragment in its YAC of origin, when compared to that of other YACs, if it is present.
3. Smal - a restriction site lies downstream of the cloning site in the right arm of pYAC4. A probe from the right arm of a YAC will bind to a differently sized fragment in its YAC of origin, when compared to that of other YACs, if it is present.

Fig. 5.5 shows the results of the hybridisation, when the YAC 2/2 left arm fragment was used as the probe. The probe does not bind to any sequences in yeast AB1380 DNA, showing that it is mouse specific. However, it binds to many fragments in the YACs, instead of the single band expected. It hybridises to fewer fragments in YAC 3/4, but this is probably due to the smaller size of this YAC. In the Smal YAC digest, a smear of high molecular weight DNA is seen. This is not unexpected, as this enzyme digests mammalian DNA relatively infrequently. In the EcoRI and BglII digests, the probe hybridises to many fragments. These results suggest that the end-fragment contains repetitive sequences. This was confirmed by hybridising the probe to murine genomic DNA, which had been digested with the same three restriction enzymes (fig. 5.6). Smears were seen in all tracks, with
YAC DNA was digested with Smal, EcoRI and BglII and analysed as detailed in the legend of fig. 5.2, except that filter was hybridised with the amplified, left arm fragment of YAC 2/2 (probe prepared as detailed in the text and section 2.6.3). The filter was exposed to film for 2 hours at -70°C. The results show that this probe contains repetitive sequences.
Genomic *M. musculus* c57/B6 DNA was digested with Smal, EcoRI and BglIII and analysed as detailed in the legend of fig. 5.5. The results confirm that the probe contains repetitive sequences (see text for discussion).
The filter shown in fig. 5.5 was hybridised with the amplified, right arm fragment of YAC 2/1 (as detailed in the legend of fig. 5.5). The results show that this probe also contains repetitive sequences, which are also present in yeast DNA.
The filter shown in fig. 5.6 was hybridised with the amplified, right arm fragment of YAC 2/1 (as detailed in the legend of fig. 5.5). The results confirm that this probe also contains repetitive sequences.
intense bands of 1.3kb in the EcoRI digest and 0.8kb in the BgIII digest. These intense bands are also seen when the probe is hybridised to YAC DNA. The intense band of 1.3kb seen in the EcoRI digest, suggests that the probe contains repeat sequences from the L1 LINE repeat sequence. There are between 70-100,000 copies of the L1 repeat in the human genome and the repeat unit contains EcoRI sites positioned 1.3kb apart (Hastie, personal communication).

A similar pattern was seen when the probe used was the left arm of YAC 2/1, or either arm of YAC 3/4. This suggests that all four YAC end fragments contain L1 repeat sequences. Consequently, these end-fragments would not be useful in characterising the YACs or Harwell deletions.

When the two right arm end-fragments from YACs 2/1 and 2/2 were used as probes, they gave similar results. Fig. 5.7 shows the hybridisation pattern when the right arm fragment of YAC 2/1 was used as a probe. Similar band patterns were seen in all tracks including the control, which contained genomic yeast DNA. When hybridised to genomic mouse DNA (fig. 5.8), smears of signal were seen. This indicates that the probe contains a repeat element which is not mouse specific.

To summarise, all the end fragments isolated from each YAC contained repeat elements. Therefore they could not be used to characterise the extent of the Harwell deletions or to determine YAC overlap. For this reason another technique, in which unique internal sequences can be isolated, was used to generate markers from the YACs.

5.5 Analysis of YACs using Interspersed Repetitive Sequence (IRS) PCR

5.5.1 IRS PCR of the YACs.

Various dispersed repetitive mouse DNA sequences have been isolated and characterised (discussed in section 1.12.3). PCR primers have been designed from conserved sequence at the ends of these repeat elements (Cox et al., 1991). Using such primers, it is possible to amplify unique sequence DNA which lies between two repeat elements, from YACs. Whether a PCR product can be generated depends on the orientation of and distance between the repeat elements. By using combinations of primers from different repeat elements in a PCR reaction, the chances of a PCR product being generated are optimised. As approximately 20% of the mouse
genome is composed of dispersed repetitive elements (Herman et al., 1992), IRS PCR products should be generated from most YACs. Common PCR products, generated from different YACs would indicate that the YACs overlap in this region. Thus information about YAC overlap can be generated. Such PCR products could also be used to characterise the Harwell deletions.

Primers derived from the L1, R1, B1 and B2 repeat elements were generated from published sequences (Cox et al., 1991 and Simmler et al., 1991). Two primers were generated from the B2 repeat element, which were derived from either end of the repeat and had different orientations. Sequences and PCR conditions are shown in table 2.2. All possible combinations of primers were used to amplify DNA from each YAC. Table 5.2 describes the results of these PCR5. More PCR products were generated when primers from different repeat elements were used. Presumably, this is because repeat elements from the same family lie further apart or in the same orientation, in these YACs. Fig. 5.9 shows some of these PCR reactions analysed on a Nusieve gel. Some common products are generated from all three YACs. For instance, in the R1xB2B PCR reaction, the YACs have a 200bp product in common. This indicates that the PCR product was generated from a region present in all YACs, and that this may lie relatively close to the brown gene.

From this data it should be possible to determine the relative orientation of the YACs. However, all of the YACs contain regions of DNA which are not present in the other YACs. This is shown by the presence of unique PCR products from each YAC. For example, YAC 2/1 has unique products generated in the B1xR1 PCR, YAC 2/2 has a unique product in the B1xB2B PCR and YAC 3/4 has a unique product in the L1xB2B PCR. It is not possible to orientate the YACs, taking into account their size, in a manner where each contains a region which does not overlap with any of the other YACs. This suggests that at least one of the YACs either contains DNA from elsewhere in the genome, or has undergone a rearrangement. Therefore, the relative orientation of the YACs could not be determined from this data. Nevertheless, common PCR products are likely to be derived from the brown locus, so these were analysed further.
IRS PCR reactions (section 2.5.6) were analysed on 1.2% agarose gels (section 2.6.2). The YAC DNA and the combinations of primers used in the PCR reactions are shown in the figure. Size markers were λHindIII and φXHaeIII (section 2.6.1). The results are discussed in the text.
Table 5.2: YAC IRS PCR products (sizes shown in bp).

<table>
<thead>
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<th>PCR Primers</th>
<th>YAC 2/1</th>
<th>YAC 2/2</th>
<th>YAC 3/4</th>
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<td>B1 x L1</td>
<td>1000</td>
<td>600</td>
<td>1000</td>
</tr>
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<td></td>
<td>600</td>
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<td>600</td>
</tr>
<tr>
<td>B1 x R1</td>
<td>1400</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 x B2</td>
<td>200</td>
<td>200</td>
<td>90</td>
</tr>
<tr>
<td></td>
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<tr>
<td>B1 x B2B</td>
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<td>L1 x R1</td>
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<td>L1 x B2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>L1 x L1</td>
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</table>

5.5.2 Mapping using IRS PCR products

IRS PCR products, which were amplified from more than one YAC, were used as hybridisation probes. They were hybridised to the YACs, to confirm the PCR results, and also to DNA isolated from mice homozygous for the Harwell deletions, b57H and b61H.

Probes were made from three PCR fragments generated from

1. B1xB2 PCR - 200bp product in YACs 2/1 and 2/2.
2. B1xR1 PCR - 600bp product in YACs 2/1 and 2/2.
3. R1xB2B PCR - 200bp product in all YACs.

Fig. 5.10a shows the results of a Southern blot when the B1xB2 PCR product was used as a probe. It hybridises strongly to genomic DNA, resulting in a smeared signal, and very weakly to YAC DNA. The probes do contain a small amount of repeat sequence DNA at either end and this may be binding to the repeats in genomic DNA. The probes were preannealed with either
Figure 5.10: Analysis of YAC 2/1 B1xB2 IRS PCR product

figure legend overleaf
YAC DNA and DNA isolated from mice homozygous for the Harwell deletions b$^{57H}$ and b$^{61H}$ were digested with EcoRI and HindIII and analysed by Southern blot analysis (as detailed in the legend of fig. 5.2). The filter was hybridised with the B1xB2 IRS PCR product from YAC 2/1 (sections 2.6.3, 2.8.1 and 2.9). Hybridisation was detected after exposure to film for 4 hours at -70°C (section 2.10.1). Fig. 5.10a shows that this probe contains repetitive sequences. The probe was preannealed with murine Cot1 DNA (section 2.8.2) and the hybridisation repeated (fig. 5.10b). Although the hybridisation signal was reduced, a smear of signal was still seen in genomic DNA.
Three IRS PCR products from YAC 2/1 (the primers used are shown in the figure) were analysed run on a 1.4% agarose gel and analysed by Southern blot analysis (as detailed in the legend of fig. 5.2). The filter was hybridised with nick translated genomic DNA (sections 2.6.2, 2.8.4 and 2.9) and hybridisation detected after exposure to film for 1 hour at -70°C. The results show that all the IRS PCR products contain repetitive sequences.
sonicated genomic mouse DNA or murine Cot1 DNA (Gibco) (section 2.8.2). Fig. 5.10b shows the results of the hybridisation when both the probe (B1xB2 PCR product) and filter had been preannealed to murine Cot1 DNA. However, although the preannealing step reduced the smearing seen in genomic DNA, no distinct bands were seen.

A similar pattern of hybridisation was seen with all three probes. The repetitive content of the probes was analysed by hybridising nick translated total genomic DNA to the three PCR fragments. Fig. 5.11 shows that the genomic DNA hybridises strongly to all three fragments, indicating that they have a high repetitive content. It is likely that they contain other repeat sequences, apart from the small region at either end. They were, therefore, not of use in characterising the Harwell deletions.

5.5.3 Analysis of the microsatellite content of IRS PCR products
Polymorphic microsatellite repeats have already been useful in mapping the Oak Ridge deletion panel. Any microsatellite repeats isolated from these YACs would be useful mapping tools. As these repeats may lie closer to the brown gene than those already analysed, they would, therefore, be of more use in mapping the deletions from Harwell. As internal fragments have already been isolated from the YACs by IRS PCR, these were analysed for their microsatellite content.

A CA\(_{10}\) oligonucleotide was labelled and hybridised to the IRS PCR products. The results are shown in fig. 5.12a. The probe hybridises to five IRS PCR products.

1. 1.4 kb product of the B1xL2 PCR from YAC 2/1
2. 0.8kb product of the B1xR1 PCR from YAC 2/1
3. 0.5kb product of the L1xB2B PCR from YAC 2/1
4. 0.6kb product of the B1xB1 PCR from YAC 2/1
5. 0.25kb product of the B1xB2B PCR from YAC 2/2

The filter was then reprobed with the GA\(_{12}\) oligonucleotide and the results are seen in fig. 5.12b. Two of the PCR products contain GA repeats.

1. 0.8kb product of the B1xR1 PCR from YAC 2/1
2. 0.25kb product of the L1xR1 PCR from YAC 2/1

Previous results (section 5.2) demonstrate that yeast DNA contains many CA repeats, so the IRS PCR products containing GA repeats were analysed first. These are more likely to be of use in mapping both the Harwell deletions and
Figure 5.12: Analysis of dinucleotide repeat content of IRS PCR products
The IRS PCR products were analysed by Southern blot analysis as detailed in the legend of fig. 5.11. The filter was hybridised to a CA\(_{10}\) oligonucleotide (fig. 5.12a) and then a GA\(_{12}\) oligonucleotide (fig. 5.12b) (as detailed in the legend of fig. 5.3). The results show that the CA oligonucleotide hybridises to five IRS PCR products and the GA oligonucleotide to two products (this result was confirmed after a longer exposure).
The YAC 2/1 B1xR1 IRS PCR product was sequenced (section 2.11) and analysed on a 6% polyacrylamide gel (sections 2.6.1 and 2.6.4). The gel was exposed to film for 16 hours at room temperature (section 2.10.1). A GATA repeat was identified in this sequence (arrowed) which contains 23 copies of the tetranucleotide repeat unit.
Primers which amplified the GATA repeat (table 2.2) were used to amplify DNA from *M. spretus, M. musculus* strains C3H, 101, and Ts (BALB/c *M. musculus* strain with the tail short mutation) (section 2.5.1). The PCR reactions were analysed on Nusieve GTG gel (section 2.6.2) (fig. 5.14a). A size polymorphism was apparent between *M. musculus* C3H and *M. spretus* DNAs, but not between *M. musculus* 101 and *M. spretus*. For this reason labelled PCR reactions (section 2.5.8) were analysed on 6% polyacrylamide gels (sections 2,6,1 and 2,6,4) (fig 5.14b). However a size polymorphism between *M. musculus* 101 and *M. spretus* DNAs was still not detected.
The YACs.

The 0.8kb product of the B1xR1 PCR was analysed. Over 600bp of this fragment were sequenced. A repeat was identified (fig. 5.13), however it was not the GA repeat as expected, but a GATA repeat. The GA oligonucleotide hybridised to this repeat despite the mismatch in the target sequence. The repeat contains 23 copies of the GATA repeat unit. PCR primers (sequence and conditions shown in table 2.2) were designed from the unique sequence DNA which bordered the repeat. The length of the GATA repeat in different species was investigated, to determine whether it was polymorphic, and could therefore be mapped to the Oak Ridge deletion panel. The primers amplified a fragment of 180bp from *M. spretus* DNA and a smaller fragment from *M. musculus* C3H DNA (fig. 5.14a). However, the fragments generated from *M. spretus* and *M. musculus 101* DNAs were indistinguishable on a Nusieve gel. Therefore, PCR reactions using end-labelled oligonucleotides were analysed on 6% polyacrylamide gels (fig. 5.14b). In the *M. spretus* track, two bands were seen, one of which was the same size as the *M. musculus* 101 PCR product. A similar sized fragment was also seen in C3H DNA, although the major PCR product was smaller. As there was no polymorphism between *M. spretus* and *M. musculus 101* DNA, it was not possible to map this microsatellite to the Oak Ridge deletion panel.

This microsatellite repeat was specific to YAC 2/1 and, as this YAC is so large, the possibility that it may be chimaeric must be considered. Analysis of the GA repeat content of the YACs (fig. 5.3b), indicates that there is very little overlap of YAC 2/1 with the other YACs. This would be consistent with YAC 2/1 being chimaeric. Analysis of the IRS PCR products produced from each YAC, also show that at least one of the YACs is chimaeric. Before further work was carried out, this possibility was investigated. The most straightforward technique would be that of FISH analysis, hybridising YAC 2/1 to metaphase chromosomes. However this was not possible due to problems caused by YAC instability (see discussion). Nevertheless, it was possible to check whether IRS PCR fragments isolated from YAC 2/1, were linked to the *brown* gene.

The GATA repeat, isolated from YAC 2/1, was genetically mapped using the European Collaborative Interspecific Backcross (EUCIB) panel (European Backcross Collaborative Group, 1994), in order to determine its chromosomal
location. The mice in this panel were derived from interspecific crosses between *M. musculus*, strain c57/B6, and *M. spretus* mice. The GATA repeat in these two strains was found to be polymorphic, enabling it to be mapped. Over 50 mice were typed for the YAC 2/1 specific GATA repeat. The chromosomal location of this repeat was determined using the MBx database (European Backcross Collaborative Group, 1994). The GATA repeat was mapped to chromosome 12, between the two anchor loci D12Mit54 and D12Mit4. This data proves that YAC 2/1 is chimaeric, as in addition to chromosome 4 sequences, it also contains DNA from at least one other region of the genome. Therefore analysis of the other IRS PCR products from YAC 2/1 was not carried out.

5.6 Discussion

Three YACs containing the *brown* gene, which were isolated by L. Stubbs from the ICRF YAC library, have been characterised. The YACs range in size from 200kb to 1.1Mb, but none extend to the closest mapped markers to *brown*. The results of the IRS PCR show that unique PCR products were obtained from each YAC, indicating that they all contain sequences which were not present in the other YACs. It was not possible to position the YACs in such a manner, when their size was taken into consideration. This shows that at least one of the YACs is chimaeric. Analysis of the GA repeat content of the YACs, shows that YACs 2/2 and 3/4 have a very similar pattern of GA repeats, indicating a high degree of overlap. Of the three YACs, it was therefore most likely that YAC 2/1 was chimaeric. The mapping of a polymorphic marker from YAC 2/1, using the EUCIB panel, indicated that YAC 2/1 was indeed chimaeric, as it contained DNA from chromosome 12, in addition to that from chromosome 4.

It would have been more straightforward to determine which YACs were chimaeric by performing FISH analysis. This involves isolating the YAC from yeast genomic DNA and then hybridising it to metaphase chromosomal spreads. YACs are isolated from the yeast chromosomes by PFGE. However, when these YACs were run on pulsed field gels, no band corresponding to any of the YACs was ever visible. Southern blots of the pulsed field gels, probed with Trp1.6 from the *brown* gene, were also negative. DNA for these gels was prepared in plugs of agarose as detailed in section 2.2.4. The YACs, in yeast strain AB1380, were grown in selective medium and it was noticed that these cultures grew very slowly. Different
selective mediums were tested and YAC containing yeast cells were found to grow best on AHC medium (section 2.2.1), but their growth rate was still reduced. Cultures should be grown until the concentration reaches \(10^8\) cells per ml, before extracting DNA. However cultures containing these YACs only reached around \(10^6\) cells per ml. If the cultures were left growing for longer, most of the yeast cells died. This observation, together with the absence of visible YACs on pulsed field gels, suggests that most of the cells are losing their YACs. The possible instability of these YACs in yeast cell cultures, would explain why they were not observed on pulsed field gels. When YAC cultures were grown in non-selective medium, the growth rate of the yeast cells improved, but again no YACs could be visualised on a pulsed field gel.

It is not known why these YACs are unstable in culture, although it has previously been reported that some YACs, especially those containing tandem repeats, are unstable in \(S.\ cer"evisiae\) strain AB1380 (Chartier et al., 1992).

These difficulties limited the type of analysis which could be carried out, as FISH analysis and restriction mapping of the YACs, both require PFGE analysis. However, PCR based approaches could still be used.

Fragments from both ends of each YAC were isolated using inverse PCR and internal fragments were isolated by IRS PCR. However, these were not useful in mapping the Harwell deletions, as the markers contained repeat sequences. It was unfortunate that all the YACs contained repetitive sequences close to their ends. This may be due to the way in which the library was constructed. Genomic DNA was partially digested with EcoRI, using a combination of EcoRI and EcoRI methylase. It may be that repeat sequences provide a local environment which favours the action of the restriction enzyme, rather than the methylase. Also, many of the EcoRI sites in the mouse genome lie in repetitive sequences, especially in the L1 LINE repeats. Whatever the reason, the end-fragments isolated from each YAC were not of use in mapping the deletions.

The problems encountered with YAC instability meant that future analysis of these YACs would be very difficult and, for this reason, analysis of these YACs was not continued. However YACs would still be of great use in the molecular characterisation of this region. Now that many more markers have been mapped to this region, using the Oak Ridge deletion complex, these can
be used to screen YAC libraries (described in chapter 6). It may be possible to build a YAC contig of the region and isolate a YAC containing the *baf* gene.
Chapter 6
Screening YAC libraries

6.1 Introduction

More useful markers have now been mapped to the brown locus, using the Oak Ridge deletion panel. The markers which lie closest to the brown gene were used to screen YAC libraries. These included D4Rck9 which lies distal to brown, D4Rck122 and D4Rck4 which lie proximal to brown, as well as the brown gene itself. The isolated YACs were examined in order to determine the extent of their overlap, as this information could be used to generate a YAC contig of this region. As the baf gene lies close to brown, it may be present on one of these YACs.

The ICRF YAC library was to be screened by hybridisation, therefore, the three microclone markers were first checked to see that they did not hybridise to YAC vector or genomic DNA. D4Rck122 was found to contain a repetitive sequence, as it gave rise to a smeared signal, when hybridised to mouse genomic DNA (data not shown). This marker was, therefore, not suitable for screening YAC libraries by hybridisation. As PCR primers which amplify this microsatellite marker have been generated, it was possible to screen another YAC library with them. The Research Genetics (RG) library was screened by PCR, using primers from D4Rck122 and the brown gene. This work was carried out by S. Hodge.

6.2 Screening the ICRF YAC library

Two filters were obtained from ICRF, which contained YACs from two libraries (section 2.2.5). The YACs had been mechanically gridded onto filters and there were around 18,000 YACs per filter. Each YAC had been plated out twice, to allow more accurate identification of positive YACs.

The filters were hybridised to all three probes (D4Rck4, 9 and a probe from the brown gene) simultaneously (section 2.9.4). The probe from the brown gene was the 227bp PCR product generated using the primers which amplify across the polymorphic TaqI site (table 2.2). The results of this screening are shown in fig. 6.1.

Positive YACs were only identified on one filter, which contained the YAC library constructed by Z. Larin. The YACs isolated by L. Stubbs, which had
The ICRF YAC library (section 2.2.5) was screened by hybridisation. Three probes, from microclones D4Rck4, D4Rck9 and the brown gene, were hybridised to the filters simultaneously (section 2.9.4). Hybridisation was detected after exposure to film for five days at -70°C (section 2.10.1). These probes hybridised to sixteen potential YACs (numbered in the figure), which had been gridded out in duplicate.
been previously analysed (chapter 5), were also from this library. The identification of positive YACs was made easier by the fact that all the YACs had been gridded out twice on the filter. 16 positive YACs were identified. However the YACs numbered 11-15 looked a little odd, as they all lay next to each other on the filter and in the same orientation. This pattern of hybridisation may have resulted from contamination during the gridding procedure and there was a possibility that these colonies all contain the same YAC.

The co-ordinates of each positive YAC were obtained and the YACs were ordered from ICRF.

6.3 Screening the RG YAC library
This library was screened by PCR, using the primers from the brown gene and those which amplified microclone D4Rck122 (table 2.2). Four YACs containing the brown gene and three YACs containing D4Rck122 were isolated from this library (data not shown). This library was screened by S. Hodge.

Analysis of these YACs is described in chapter 7.
Chapter 7
Analysis of YAC recombinants containing DNA from the brown locus

7.1 Introduction
Sixteen YACs were isolated from the ICRF library, using three probes which map to the brown locus. Four YACs containing the brown gene and three containing microclone D4Rck122, were also isolated from the Research Genetics library. The first step in their analysis was to determine which markers were present in each of these YACs.

The extent of overlap between the YACs was then be compared, using IRS PCR. Although YACs may not overlap at the marker loci studied, they may overlap in other regions, as indicated by common IRS PCR products. This type of information is useful in establishing YAC contigs.

The baf gene is located close to the brown gene and may be present in the brown positive YACs. Once the position of the baf gene is more accurately mapped, using markers isolated from these YACs, the appropriate YACs will then be examined for the presence of protein coding sequences (discussed further in chapter 8). A YAC contig would facilitate the isolation of other genes which have been genetically mapped to this region.

7.2 Initial Analysis
7.2.1 Analysis of YACs isolated from the ICRF Library
Stabs of the sixteen YACs were received from ICRF in YPD medium (details shown in table 7.1). They were grown in YPD medium and streaked out onto AHC agar plates. As the YAC stabs had not been grown from single colonies, two colonies were picked from each plate and amplified with primers from D4Rck4, D4Rck9 and the brown gene. Fifteen of the sixteen YACs were found to contain DNA from one of these three loci. Although colonies containing YAC 3 (A0880) were screened, none were found to contain DNA from these three loci. No further analysis of this YAC was carried out.

DNA was isolated from each of the YAC cultures and analysed by PFGE (sections 2.2.4 and 2.6.5) (fig. 7.1). Some YACs were clearly visible on this gel. For instance in track 4, YAC B0330 (arrowed in fig. 7.1) migrates
between AB972 yeast chromosomes IX at 445kb and V and VIII, which co-
migrate at 585kb. Other YACs, which were later found to co-migrate with a
yeast chromosome, were not so visible. A Southern blot of this gel was first
probed with the 2.6kb fragment, from the left arm of pYAC4 (section 2.4.2)
(fig. 7.2a). This shows that only one YAC was present in each track. Sizes
of the YACs were estimated from this Southern blot and are shown in table
7.1. YAC E0494 in track 5, was the largest YAC at around 1.1Mb. As this
was the same library that YAC 2/1 was isolated from, it was possible that
YAC E0494 and YAC 2/1 were the same, as they are of a similar size. The
smallest YACs were around 100kb. The pBR322 sequences in the probe,
also hybridised to chromosome VII in yeast strain YP148, which was used as
a size marker. In this strain, chromosome VII was split into two molecules of
90kb and 1035 kb by a technique involving pBR322, and the 90kb fragment
still contains some residual pBR322 sequence (Hieter et al., 1988). The
YACs in tracks 11-15 are all the same size and may be clonal. This is
especially likely as these YACs lay close to each other and in the same
orientation on the ICRF library filter.

The Southern blot was then sequentially hybridised with probes from the
brown locus, in order to determine the marker content of each YAC. The
Trp1.6 fragment from the brown gene hybridised to seven YACs (fig. 7.2b),
indicating that these YACs contain the brown gene. When the D4Rck9
microclone was hybridised to this filter (fig. 7.2d), two YACs which contained
this locus were identified. When this filter was reprobed with the D4Rck4
microclone (fig. 7.2c), five YACs were found to contain this locus. As
described above, it was suspected that these five YACs were identical.
These YACs all contain the same marker and are of the same size, therefore
it was assumed these YACs were clonal. For this reason only one of these
YACs, YAC 12 (B1125) was used in future analysis. YAC D1225 did not
appear to contain DNA from any of these markers and was not analysed
further.
Figure 7.1: PFGE analysis of YAC recombinants from the ICRF YAC library

YAC DNA (tracks 1-16) was resolved on a pulsed field gel (sections 2.2.4 and 2.6.5). The pulsed field gel was run at 200 volts, with a 70 second pulse time for 22 hours, then for a further 14 hours with a pulse time of 90 seconds. The size markers were *S. cerevisiae* strains YP148 and AB 972.
Figure 7.2: Determination of YAC size and marker content by PFGE analysis

figure legend overleaf
Figure 7.2a legend
YAC DNA resolved on the gel shown in fig. 7.1, was transferred to a nylon membrane (section 2.7.1) and then hybridised to a probe from the left arm of pYAC4 (fig. 7.2a) (sections 2.4.2, 2.8.1 and 2.9). Hybridisation was detected by a Molecular Dynamics Phosphorimager (section 2.10.2) after a 4 hour exposure.
The sizes of the YACs were estimated, by comparing them to the chromosomes of the YAC markers, whose sizes were known (table 2.1). Estimated YAC sizes are shown in table 7.1.

Figure 7.2b: Identification of YACs containing the brown gene
The filter shown in fig. 7.2a, was rehybridised with the Trp1.6 probe from the brown gene (as detailed in the legend of fig. 7.2a). The probe hybridised to the YACs in tracks 1, 2, 4, 5, 8, 9 and 10, therefore YACs D0138, B1256, B0330, E0494, G0538, G0436 and H0689 contain the brown gene.

Figure 7.2c: Identification of YACs containing D4Rck4
The filter shown in fig. 7.2a, was rehybridised with a probe from microclone D4Rck4 (as detailed in the legend of fig. 7.2a). PCR primers from the microclone were used to amplify D4Rck4 from genomic DNA (section 2.5.1 and table 2.2) and the probe was prepared from the amplified product (section 2.6.3). The probe hybridised to the YACs in tracks 11, 12, 13, 14 and 15, therefore YACs B1225, B1125, B1025, B0925 and B0825 contain the microsatellite marker D4Rck4 (see text for discussion).

Figure 7.2d: Identification of YACs containing D4Rck9
The filter shown in fig. 7.2a, was rehybridised with a probe from microclone D4Rck9 (as detailed in the legend of fig. 7.2c). The probe hybridised to the YACs in tracks 6 and 7, therefore YACs D0670 and B0548 contain the microsatellite marker D4Rck9.
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<th>Track</th>
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Table 7.1: Estimated sizes (in kb) of YACs isolated from the ICRF and RG libraries

Table 7.2

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Table 7.2: Marker content of YACs isolated from the ICRF and RG libraries
The microclones D4Rck52, 122 and 140, which map close to the brown gene (fig. 4.16), were also hybridised to the YACs. However, the YACs did not contain DNA from these loci. Each YAC contained DNA from only one of the three markers used to screen the library. This data indicates that there was no overlap between any of the YACs at these marker loci. However, the YACs may overlap in other regions.

These results are summarised in table 7.2. Seven YACs containing the brown gene, one containing D4Rck4 and two containing D4Rck9, were isolated from the ICRF library.

7.2.2 Analysis of YACs isolated from the RG library
The YACs isolated from this library were analysed in a similar way by S. Hodge. The sizes of the YACs were estimated from a Southern blot of a pulsed field gel probed with the 2.6kb fragment from the left arm of pYAC4. The results are shown in fig. 7.3 and the estimated sizes of the YACs are listed in table 7.1.

The filter was then reprobed with the Trp1.6 fragment of the brown gene and then microclones D4Rck4, 9, 52 and 122 (data not shown), in order to ascertain if the YACs contained DNA from other loci. Trp1.6 hybridised to the four YACs which had been isolated from the library with primers from the brown gene (YACs A-D). D4Rck122 also hybridised only to the three YACs which had been isolated with primers from this locus (YACs E-G). These YACs did not contain any of the other markers which had been mapped to this region (summarised in table 7.2).

7.3 Fingerprinting of the ICRF and RG YACs
Each of these YACs contain only one of the markers which have been mapped to this region. However as they may overlap in other areas, alternative techniques were employed to detect this.
YAC DNA (tracks A-G) was resolved on a pulsed field gel (sections 2.2.4 and 2.6.5). The running conditions were as detailed in the legend of fig. 7.1. The YAC DNA was transferred to a nylon membrane (section 2.7.1) and then hybridised to probe from the left arm of pYAC4 (as detailed in the legend of fig. 7.2a). The sizes of the YACs were estimated, by comparing them to the chromosomes of the YAC markers, whose sizes were known (table 2.1). Estimated YAC sizes are shown in table 7.1.
7.3.1 IRS PCR
This technique had previously been used to fingerprint the three YACs containing the *brown* gene, which were isolated by L. Stubbs from the ICRF library (see section 5.5). Certain combinations of primers from different repeat elements had proved more informative than others. Six of the most informative combinations were used to amplify DNA from the ten ICRF YACs and the seven isolated from the Research Genetics library.

The combinations of primers used in these PCR reactions were

1. B1xL1
2. B1xR1
3. B1xB2
4. L1xR1
5. L1xB2B
6. R1xB2B

The results of some of the IRS PCR reactions are shown in fig. 7.4. Common fragments were amplified from different YACs, e.g. YACs 113C6, 112F11 and 56E7 (tracks B, C and D), all contain a 1.4kb product generated in the B1xL1 PCR. This indicates that these three YACs overlap at this locus. However it was already known that these YACs overlap, as they all contain DNA from the *brown* gene. Common PCR products were not generated from YACs which contained different markers. Therefore no information about YAC overlap could be ascertained using IRS PCR.

7.3.2 Analysis of GA repeat content
An indication of the extent of overlap between the YACs, can also be obtained by analysis of the GA repeat content of the YACs. A Southern blot of EcoRI digested YAC DNA was probed with a GA\(_{(12)}\) oligonucleotide and the results are shown in fig. 7.5. It was apparent that YACs containing the brown locus had regions which overlapped, as they contained common GA repeats. This can be observed in tracks A, B, C and D. Likewise, YACs containing the D4Rck9 locus, also share some common GA repeats (tracks 6 and 7). Common GA repeat sequences were not detected in YACs containing different markers.
YAC DNA was amplified using combinations of primers derived from different repeat elements (section 2.5.6 and table 2.2). The amplified products were analysed on 1.2% agarose gels (sections 2.6.1 and 2.6.2). AB1380 was used in PCR reactions as a negative control, and in addition, the PCR reactions run in tracks labelled -, contained no DNA. *M. musculus* CBA genomic DNA was used as a positive control and øXHaelIII, as the size marker on these gels. The YACs can be identified by comparing the track number or letter with those shown in table 7.1. The results show that common PCR products were amplified from different YACs (see text).
YAC DNA was digested with EcoRI and analysed on 0.8% agarose gels (sections 2.4.1 and 2.6.2). The DNA was transferred to a nylon filter (section 2.7.1) and hybridised to a GA\textsubscript{12} oligonucleotide (sections 2.8.3 and 2.9). Hybridisation was detected using a Molecular Dynamics Phosphorimager (section 2.10.2) after a 4 hour exposure. The YACs can be identified by comparing the track number or letter with those shown in table 7.1. The results are discussed in the text.
7.4 Analysis of YACs containing the brown gene

The eleven YACs containing the brown gene were analysed to verify that they contained DNA from other regions of the brown gene. Analysis of the YACs from the Research Genetics library was carried out by S. Hodge. DNA fragments from two regions of the brown gene were used as probes, Trp1.6 and Trp1.0, which lies further upstream. These were hybridised to a Southern blot of TaqI digested YAC DNA. The results of the Trp1.6 hybridisation are shown in fig. 7.6a and 7.6b. When Trp1.6 was probed to the ICRF YACs (fig. 7.6a), the pattern of hybridisation was the same as that seen in wild type DNA. When probed to the YACs isolated from the Research Genetics library (fig. 7.6b), all the expected bands were seen, except in YAC 56E7 (track D). In this YAC, the probe hybridises only to one band of 1.2kb. This suggests that the YAC may have an end point in the brown gene itself. The filter was rehybridised to the Trp1.0 probe, which lies 5' to Trp1.6. The three bands seen in wild type DNA, can also be observed in all YACs (fig. 7.7a and 7.7b), except YAC 56E7 (track D), where only two bands were observed. These results suggest that either YAC 56E7 has an end-point within the brown gene itself, or it contains a rearrangement, which has resulted in the loss of some DNA from the brown gene. This will be determined by mapping the end fragments of this YAC to the deletion complex. In the phosphorimage prints shown in fig. 7.6-7.7, no hybridisation to wild type CBA DNA is seen. However hybridisation is apparent, if the density measurements are altered, but this leads to an overexposure of the YAC signal. This is due to the higher complexity of genomic DNA, when compared to the YAC DNA.
Figure 7.6: YAC analysis using the Trp1.6 probe

legend overleaf
Figure 7.7: YAC analysis using the Trp1.0 probe

legend overleaf
Figure 7.6 legend
YAC DNA from the ICRF library (fig. 7.6a) and RG library (fig. 7.6b) was digested with TaqI and analysed on 0.8% agarose gels (sections 2.4.1 and 2.6.2). The DNA was transferred to a nylon filter (section 2.7.1) and hybridised to the Trp1.6 probe from the brown gene (sections 2.8.1 and 2.9). Hybridisation was detected by a Molecular Dynamics Phosphorimager (section 2.10.2) after a 4 hour exposure. The YACs can be identified by comparing the track number or letter with those shown in table 7.1. The sizes (in kb) of the fragments are shown.

The Trp1.6 probe hybridises to DNA in all the YACs known to contain the brown gene. In track D, YAC 56E7, the probe hybridises only to a 1.2kb (discussed in text).

Figure 7.7 legend
The filter shown in fig. 7.6 was rehybridised to another probe from the brown gene, Trp1.0. The protocol was the same as that detailed in the legend of fig. 7.6. The probe hybridised to three bands in all YACs, except YAC 56E7 (track D) (discussed in text). Fragment sizes (in kb) are shown.
7.5 Discussion
Seventeen YACs containing DNA from the *brown* gene and surrounding markers, have been isolated from two libraries. Their size and marker content have been established. However regions of overlap have not been detected in YACs containing different markers. This suggests that these YACs do not overlap and therefore cannot be ordered into a YAC contig. YAC end fragments will be used to isolate YACs that bridge the gaps (discussed in chapter 8).

At the present time, the orientation of the *brown* gene on chromosome 4 is not known. If YAC 56E7 does have an end-point within the *brown* gene, it would be useful in orientating the *brown* gene on chromosome 4. Markers isolated from YAC 56E7 could be mapped either proximal or distal to the *brown* gene, thus orientating the gene on chromosome 4. These markers could be mapped, either by using the deletion panel DNAs, or by using YAC 56E7 markers to isolate overlapping YACs, enabling establishment of physical linkage with one of the proximal or distal markers.

The generation of a YAC contig is a crucial step in the physical analysis of this region and also in the localisation of *baf* and the other genes which are situated within it. The approaches which could be used to fulfill this aim are discussed in chapter 8.
Chapter 8
Discussion

8.1 Summary of results
This project has enabled the brown locus on murine chromosome 4 to be extensively characterised. The analysis of mice with deletions at this locus enabled the *baf* (*brown associated fitness*) gene to be identified. DNA markers, which had been genetically mapped to the brown locus, have now been physically mapped to the region. These markers have been used to isolate YACs, which are being used as the foundation on which to build a YAC contig. This will facilitate the isolation of genes known to map in this region.

Seven mice containing mutations of the *brown* gene were received from B. Cattanach, MRC Radiobiology Unit, Didcot. These mutations were generated by radiation mutagenesis and isolated using the SLT. It was determined that four of these mutations were deletions which incorporated the *brown* gene. The other three mutations were probably caused by small intragenic changes within the *brown* gene, as deletions were not detected. Of the four deletions, two (b⁶¹H and b⁶²H) were found to give rise to viable brown mice, when homozygous. b⁶³H deletion homozygotes died before 11.5 days gestation, indicating that this deletion incorporates a gene essential in development. Mice homozygous for the b⁵⁷H deletion were subviable and subfertile. When litters were examined (at four days, when b⁵⁷H homozygotes could first be distinguished from their littermates), lower numbers of b⁵⁷H homozygotes were observed, than expected (14.6% compared to the 25% expected). The mortality rate of b⁵⁷H homozygotes was increased, especially in the first week after birth and also at three weeks, when they were weaned. The main phenotypic abnormality in these mice was their size. Mice homozygous for the b⁵⁷H deletion were smaller than their littermates. This size difference was more pronounced at birth and decreased with age. The gene responsible for this phenotype has been termed *brown associated fitness* or *baf*. It is not known at the present time, if the *baf* gene affects only embryonic growth or whether it has any other developmental effects. It is possible that the increased neonatal death rate is due, primarily, to a runting effect (see section 8.2). The Harwell deletions did not extend to any of the nearby markers which had been mapped to this locus.
In order to characterise the brown locus and determine the extent of the deletions, DNA markers, which had been genetically mapped to the region, were physically mapped to the brown locus. A collaboration with E. M. Rinchik enabled DNA, from a panel of mice with deletions at this locus, to be obtained. Complementation analysis of these deletions had enabled a simple map of the region to be generated (fig. 1.4) and three lethal genes had been identified (Rinchik, 1994). Mice carrying these b locus deletions had been mated with M. spretus, enabling DNA markers to be mapped by virtue of polymorphisms between the two species. By establishing the presence or absence of a particular marker in each of the deletions, it was possible to order these markers on the chromosome. Six microsatellite markers and 15 microclones had been mapped to this region of chromosome 4. Polymorphic markers from five genes, which mapped to the b locus, were also analysed. In total, nine markers which mapped within the deletion panel were identified. The extent of the complex could be defined distally by \(Lv\) (6.8cM from the brown gene) and proximally by \(Adf\) (2cM from the brown gene). These markers will prove useful in the generation of a YAC contig of this region.

The Harwell deletions did not extend to the nearest mapped markers and in order to define their deletion end points, markers which lay closer to the brown gene were needed. YAC libraries were screened using markers which had been mapped close to the brown gene. Although YACs containing the brown gene had been isolated from the ICRF library by L. Stubbs, these had not proved to be useful. In total, 17 YACs, which contained DNA either from the brown gene or three closely linked markers, were isolated from two YAC libraries. Their size and marker content have been established. However regions of overlap have not been detected in YACs containing different markers. The YACs can be used as a starting point in the construction of a YAC contig of this region, which will enable the genes situated within it to be physically isolated. As the \(baf\) gene is known to be closely linked to brown, DNA markers isolated from YACs containing the brown gene would prove useful in localising \(baf\) and this gene could be the first to be isolated from this region.
8.2 Future directions

In the immediate future, the focus of this work will be concentrated on characterisation of the $baf$ phenotype and isolation of the $baf$ gene. A longer term goal is to generate a YAC contig of this region which will facilitate the physical isolation of genes, including $dep$ and the three lethal genes, which have been mapped to this region.

The $baf$ gene was first detected in mice homozygous for the $b^{57H}$ deletion. Initial observations indicated that this gene was involved in embryonic growth and fitness. However stocks of mice which carried this deletion were lost before more in depth analysis of the $baf$ phenotype could be undertaken. Mice with the $baf$ phenotype were also observed by E. M. Rinchik, when carrying out complementation analysis of mice with one of 28 deletions at the brown locus. Ten combinations of deletions were found to be able to complement one another. The brown mice (with compound deletions), which resulted from these crosses, were all found to have the $baf$ phenotype. More recently, a mouse was isolated at Oak Ridge using the SLT, which contained a radiation induced non-lethal deletion ($1FCHLc$) of the brown locus. Mice homozygous for this deletion also displayed the $baf$ phenotype. These mice are being sent to us and this will enable the $baf$ phenotype to be more extensively characterised.

Characterisation of the $baf$ phenotype will involve accurately measuring the size differences in $1FCHLc$ deletion homozygotes. The offspring of matings between heterozygous $1FCHLc$ deletion carriers will be examined and marked at birth. Weight measurements will be taken and these can be used to determine the extent of the size difference between deletion homozygotes and their littermates at birth. (In contrast, $b^{57H}$ deletion homozygotes were examined at four days, when the size difference was already less pronounced). The survival rates of $1FCHLc$ deletion homozygotes will also be examined and compared to that of their littermates. DNA can be extracted from any neonatal lethality, in order to determine whether these mice were homozygous for the $1FCHLc$ deletion. These mice will also be examined in order to try to establish the cause of death, whilst also looking for any developmental abnormalities. It is not known whether the apparent increase in neonatal death rates in these mice, is due to any specific defect. It is possible that it may be an effect of their smaller size, as runted mice are
frequently not fed or killed by the mother, especially in large litters. Mice with
the *baf* phenotype are also subfertile and the cause of this will also be
investigated. The stage of gestation at which embryonic growth is affected
will also be determined and the possibility of an increase in prenatal death
rates examined. Such studies will generate valuable information on the
nature of the *baf* gene and the stage of gestation when the *baf* gene product
is required. This will be useful when screening candidate genes.

The positional cloning strategies that are being used to clone the *baf* gene are
detailed below. As YACs have now been isolated from the *b* locus, it will be
possible to localise the position of the *baf* gene using markers from these
YACs and isolate the gene on a YAC. Candidate genes will then be isolated
and examined.

All the compound heterozygote mice generated by E. M. Rinchik have the *baf*
phenotype, which indicates that there are no deletion end-points between
*brown* and *baf*, in the panel of 28 deletions examined. This suggests that the
*baf* gene is closely linked to *brown*. For this reason, end fragments from
YACs containing the *brown* gene have recently been isolated. The end
fragments have been used to examine DNA from mice with compound
deletions, with the aim of defining the boundaries of the region where the *baf*
gene is located. This work has been done in conjunction with R. Suffolk. Of
the YAC end fragments isolated so far, one end of YAC 56E7 was detected in
two of the compound deletion DNAs. YAC 56E7 is 580kb in size and is
thought either to end in the *brown* gene itself or contain a rearrangement
which has resulted in the loss of some DNA from the brown locus. This will
be confirmed by isolating and sequencing the other end of the YAC, to
determine whether it is derived from the *brown* gene. Five of the *b* locus
deletions, ITHO-IV, 47DThWb, 37DTD, 173G and 33G, also contain the end
fragment of YAC 56E7. These deletion end points lie distal to the *brown*
gene, indicating that YAC 56E7 extends in this direction. Therefore, if the *baf*
gene lies distal to brown, it must be within 580kb. As it must lie closer to
brown than the five deletion end points also in this 580kb region, the distance
between *baf* and *brown* is likely to considerably less than 580kb (if *baf* lies
distal to *brown*). End fragments have also been isolated from other YACs
containing the *brown* gene and an indication of their relative orientation can
be established by determining which YACs overlap in these regions. End
fragments from YACs extending in the opposite direction to YAC 56E7, i.e. proximal to be brown gene, will be probed to the compound deletion DNAs, with the aim of defining the proximal boundary of the region which contains baf. If the end fragments of these YACs are not present in the compound deletions, they can be used to isolate overlapping YACs from a YAC library. This type of analysis will enable the region which contains the baf gene to be defined and physically isolated on YACs.

Once the baf gene has been localised on potentially as little as two YACs, its position can be further refined. The YACs can be analysed by restriction mapping using rare cutting restriction enzymes and PFGE. The restriction fragments can be used to analyse the compound and Harwell deletions. Fragments of the YACs present in the viable deletions b61H and b62H, but absent from the compound deletions, will be examined for the presence of protein coding sequences.

Various methods have been developed to identify and recover transcribed sequences from cloned DNA. CpG islands are known to be associated with the 5' end of genes (Bird, 1987). These unmethylated sequences are present in all the housekeeping genes analysed to date and in an estimated 40% of tissue specific genes (Antequera and Bird, 1993). They can be detected by the presence of a cluster of restriction sites for rare cutting enzymes with GC rich recognition sequences. This technique would be useful in identifying the position of potential genes on the YACs. However not all genes contain CpG islands and not all CpG islands are associated with genes.

Other techniques can also be used to identify and isolate genes. YACs have been used to screen cDNA libraries and isolate cDNA clones (Rommens et al., 1990). However, the appropriate cDNA library has to be screened for this technique to be used successfully. It is not known at what stage in development the baf gene is expressed or its pattern of expression. These factors would have to be determined before this technique could be employed. The detection of sequence homology between species ("zoo blots") is also an indicator of a protein coding sequence, due to evolutionary conservation (Monaco et al., 1986). The YACs could also be directly sequenced in search of open reading frames, however to sequence this length of DNA is rather time consuming and impractical. Other approaches,
such as exon amplification (Duyk et al., 1990; Buckler et al., 1991), would be more appropriate in this case. This technique involves the isolation of expressed sequences by the functional identification of sequences which are involved in RNA splicing. Recent adaptations to the technique of exon amplification have increased its efficiency (Church et al., 1994).

Once cDNA sequences have been isolated, they can be used to determine the time and pattern of expression of potential genes in the developing embryo. This information can be compared with that gained from studying the 1FCHLc deletion homozygotes. This should enable a candidate gene to be isolated, which would then be used, ultimately, to rescue the baf phenotype in these mice.

As a number of DNA markers have now been mapped to the brown locus, they will be used to isolate YACs from this 9cM region. Overlapping YACs can be detected by the methods previously described. Gaps in the contig can be filled by using end fragments to isolate overlapping YACs from YAC libraries. Similar strategies as those outlined above, can be used to localise and isolate genes from these YACs. Three lethal genes and dep have been genetically mapped to this region and it is likely that others are also present, that have not been detected by analysis of the b locus deletions.

Deletion analysis at the seven loci involved in the SLT has enabled these regions to be extensively characterised. Regions of the genome where such in depth analysis is possible, are limited. The knowledge gained from analysis of these loci, may be useful when designing future mapping strategies. As these loci are likely to be representative of other regions, they are an example of the organisation and structure of regions of the mammalian genome.


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