Characterization of TRP-1 mRNA levels in dominant and recessive mutations at the mouse brown (b) locus

Citation for published version:
Jackson, I, Chambers, D, Rinchik, EM & Bennett, DC 1990, 'Characterization of TRP-1 mRNA levels in dominant and recessive mutations at the mouse brown (b) locus' Genetics, vol. 126, no. 2, pp. 451-9.

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Genetics

Publisher Rights Statement:
Copyright 1990 by the Genetics Society of America

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Characterization of TRP-1 mRNA Levels in Dominant and Recessive Mutations at the Mouse brown (b) Locus

Ian J. Jackson,* Doreen Chambers,* Eugene M. Rinchik† and Dot C. Bennett‡

*MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland, †Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077, and ‡Department of Anatomy, St. George’s Hospital Medical School, London SW17 ORE, England

Manuscript received March 22, 1990
Accepted for publication June 20, 1990

ABSTRACT

The mouse brown locus encodes a putative membrane-bound metalloenzyme, tyrosinase-related protein-1 (TRP-1). We have examined the effect on mRNA expression of the locus of a number of mutant alleles. The common null mutant allele, brown, produces wild-type levels of TRP-1 mRNA, which is nonfunctional. Another recessive allele, cordovan-Harwell, has an intermediate, dark-brown phenotype and produces only very low levels of presumably normal TRP-1 mRNA. Two dominant alleles appear to act by killing the melanocyte in which they are expressed. One of them, Light, has normal size and amounts of TRP-1 mRNA. The other, White-based brown, produces no detectable TRP-1 mRNA. It has a gross DNA rearrangement at the 5' end, and we speculate that this results in activation of transcription of sequences not usually seen in melanocytes, and that this is toxic to the cell. The relationship between phenotype and molecular structure at the locus is discussed, and we draw some general principles applicable to other developmental genes.

THE formation of the mouse coat color is a developmental system particularly amenable to molecular genetic study (SILVERS 1979; JACKSON 1985). Pigmentation is not essential to the viability of the laboratory mouse, and new mutations will therefore generally survive. Furthermore, coat color is a particularly striking characteristic, thus new mutations are readily identified. A large number of mutations at many loci have been found to affect coat color. Six loci in particular [non-agouti (a), brown (b), albino (c), dilute (d), pink-eyed dilution (p) and piebald (s)] have been used for many years in specific-locus mutation experiments, which have produced many new alleles at these loci (RUSSELL 1951; SEARLE 1974). Some mutations affect only pigment or pigment-related functions, while others have marked developmental effects, usually due to deletions of DNA associated with the locus (RUSSELL 1971; RUSSELL, MONTGOMERY and RAYMER 1982).

The cDNA corresponding to the product of the murine chromosome 7 c locus, tyrosinase, has been cloned (KWON et al. 1987; YAMAMOTO et al. 1987; MULLER et al. 1988). We have recently shown that a different cDNA clone, known as pMT4 (SHIHABARA et al. 1986), encodes another protein, tyrosinase-related protein-1 (TRP-1), which shares approximately 40% amino acid identity with tyrosinase, and maps to the b locus on chromosome 4 (JACKSON 1988). Another tyrosinase-related protein (TRP-2) has been identified (called clone 5A), but not as yet mapped (JACKSON 1988; I. J. JACKSON, unpublished results).

These three proteins have several common features. All have a hydrophobic residue near to the C terminus, which is probably a transmembrane domain (it is known that tyrosinase is a membrane-bound enzyme) [see HEARING and JIMENEZ (1989) for example]. All three also have striking similarity to tyrosinases of lower eukaryotes (Neurospora crassa) and of prokaryotes (Streptomyces species) in two particular domains (LERCH, LONCONI and JORDI 1982, HUBER, HINTERMAN and LERCH 1985; MULLER et al. 1988). One of these regions is also remarkably similar to the copper-binding domain of hemocyanins, particularly in conservation of the histidine residues (GAYKAMA et al. 1984; HUBER, HINTERMAN and LERCH 1985). Neurospora and Streptomyces tyrosinases are known to contain two copper atoms per molecule (SOLOMON 1981; HUBER, HINTERMAN and LERCH 1985). In the mouse it would appear that there are at least three membrane-bound, copper-containing enzymes, which are most likely localized on the inner membrane faces of the melanosomes, the site of pigment synthesis within the melanocyte.

The tight linkage we have reported of the TRP-1 probe to brown merely suggests, but does not prove, allelism. Recently we have shown sequence differences in the TRP-1 mRNA which are causative of the brown phenotype (ZDARSKY, FAVOR and JACKSON 1990). In this study, we find changes in TRP-1 mRNA expression and in TRP-1 gene structure in certain b-locus mutants, which provide additional evidence of allelism and allow an explanation of the phenotypes.
Mice doubly heterozygous for certain radiation-induced brown mutations are fully viable but have complete deletions of the TRP-1 locus and have a brown phenotype indistinguishable from the classical brown (E. M. Rinchik unpublished results). Brown is therefore the null phenotype and results in melanocytes containing brown eumelanin instead of the wildtype black. Previously, examination of brown melanocytes led to the suggestion that its gene product played a role in melanosome morphology (Rittenhouse 1968). The sequence of TRP-1, however, would lead us now to propose that it is a membrane-bound metalloprotein that has some enzymatic function and shares a common ancestry with tyrosinase. While null mutations of tyrosinase (the e locus) cause complete absence of pigment, this is not the case at the b locus. The function of wild-type TRP-1 is therefore one that is not essential for pigment synthesis, but is necessary for black rather than brown pigment to be formed. The enzymology of melanin synthesis is not well understood, but there are a number of candidate enzymes which could be represented by TRP-1 (Körper and Pawalek 1980, 1982; Barber et al. 1984).

In this work we have examined expression of TRP-1 mRNA from a number of alleles at the b locus. This locus is unusual among pigmentation genes as it has mutant alleles both recessive and dominant to wild type (Black). The recessive alleles result in production of either brown or dark-brown eumelanin. The two dominant alleles, Light (B") and White-based brown (B"), result in the tips of the hairs being pigmented, but the bases being much paler (Macdowell 1950; Hunsicker 1969). Both are expressed when heterozygous with wild type (Black) but are more extreme in phenotype when homozygous. The phenotype appears to be due to a "suicide activation" of the b locus, resulting in death, or failure to remain in the hair bulb, late in the hair cycle, of those melanocytes which are undertaking pigment synthesis. In Light mice, at each hair growth cycle, pigment is made for a time, before the cells begin to die and are incorporated into the hair shaft. By the end of the growth cycle there are no melanocytes visible in the hair bulb (Quevedo and Chase 1958; Sweet and Quevedo 1968; Quevedo, Fleischmann and Dyckman 1981). New growths of hair, following molting, have pigmentation restored, but successive rounds of hair growth have less pigment at the hair tip, possibly reflecting a decreasing pool of melanocyte precursor cells on which to draw or, less likely, a more rapid suicide of newly recruited melanocytes (perhaps due to their having a low level of b locus transcription before recruitment).

Table 1 summarizes the mutants examined in this study, their origin and their phenotypes. We report the characterization of expression of the alleles and present the basis for detailed further molecular analysis of the relationship between genotype and phenotype at the b locus.

**MATERIALS AND METHODS**

**Mice and cell lines:** C57BL/6J, cordovan-Harwell and Light mice are maintained at the Animal Unit of the Western General Hospital. White-based brown mice are maintained at the Biology Division of Oak Ridge National Laboratory.

The B16C3 melanoma cells were obtained originally from J. Kreider and grown as described previously (Bennett 1983). Melan-a and melan-b lines were described by Bennett, Cooper and Hart (1987) and Bennett et al. (1989) and grown as described.

**Hybridization probes:** Most hybridizations used the 1.6-kb internal HindIII fragment of pMT4, recloned into Bluescribe (Stratagene) (Jackson 1988; Shibahara et al. 1986). This fragment detects the diagnostic D- and B-haplotypic fragments, in addition to several others. To examine the 5' end of TRP-1 an 800-bp EcoRI to PstI fragment from a genomic clone of the TRP gene was used. The TRP-2 probe was the 1200-bp EcoRI fragment of clone 5A (Jackson 1988), subcloned into Bluescribe. Actin mRNA was detected using the cDNA from clone pAM91 (Minty et al. 1981). The fragments were isolated from low gelling temperature agarose, diluted with 3 volumes of water and labeled with 32P using the method of Feinberg and Vogelstein (1983).

**DNA methods:** DNA was made by homogenization of organs in STE [100 mM NaCl, 50 mM Tris-HCl (pH 8), and 10 mM EDTA], followed by treatment with proteinase K (100 µg/ml) in 0.5% sodium dodecyl sulfate (SDS) for several hours. After phenol extraction and chloroform extraction the DNA was precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Restriction digestions were performed using manufacturers' recommended buffers.

DNA was electrophoresed through 0.8% agarose in TAE [40 mM Tris-acetate (pH 7.5) and 1 mM EDTA] and transferred to Hybond-N (Amersham) nylon filters according to manufacturer's instructions.

Southern blot hybridizations were carried out in 3 × SSC, 10 × Denhardt's solution supplemented with 100 µg/ml denatured salmon sperm DNA, 0.1% SDS and 0.1% sodium pyrophosphate (Jeffreys and Flavell 1977) [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate (pH 7) and 1 × Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% and polyvinylpyrrolidone] or in 0.5 M sodium phosphate (pH 7.2), 7% SDS (Church and Gilbert 1984) and washed at 68° down to 0.1 × SSC and 0.1% SDS before autoradiography.

**RNA methods:** RNA was made from fresh or frozen melanoma or melanocyte cell pellets, and from fresh or frozen dorsal skin of 1–4-day-old mice. Cells or skin were homogenized in 3 M LiCl, 6 M urea (Lovell-Badge 1987). The RNA precipitated overnight, centrifuged and washed in homogenization buffer. After resuspension in 10 mM Tris-HCl (pH 7.5) and 0.1% SDS, the RNA was digested with proteinase K at 100 µg/ml at 65° for several hours, phenol extracted, chloroform extracted, and ethanol precipitated.

The RNA was electrophoresed through 1% agarose gels containing 2.2 M formaldehyde in 40 mM MOPS (pH 7.5) 40% sodium acetate and 1 mM EDTA (Lehrach et al. 1977) before blotting in 10 × SSC to nitrocellulose filters.

Northern-biot hybridizations were carried out in 50% formamide, 4 × SSC, 10 × Denhardt’s solution, 50 mM sodium phosphate, 100 µg/ml salmon sperm DNA and 0.1% SDS overnight at 48° and washed down to 0.3 × SSC at 68° before autoradiography.
Expression of brown Locus Alleles

TABLE 1
Alleles at the brown locus

<table>
<thead>
<tr>
<th>Allele</th>
<th>Symbol</th>
<th>Phenotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>B</td>
<td>Black eumelanin</td>
<td>Wild type</td>
</tr>
<tr>
<td>brown</td>
<td>b</td>
<td>Brown eumelanin</td>
<td>Mouse fancy</td>
</tr>
<tr>
<td>cordovan-Harwell</td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Dark-brown eumelanin</td>
<td>Radiation mutagenesis</td>
</tr>
<tr>
<td>Light</td>
<td>B&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Dominant; hair tip more pigmented than base, and is black or dark-brown</td>
<td>Spontaneous</td>
</tr>
<tr>
<td>White-based brown</td>
<td>B&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Dominant, similar to Light but more extreme, and pigment is brown</td>
<td>Radiation mutagenesis</td>
</tr>
</tbody>
</table>

Reverse transcription and the polymerase chain reaction: Double-stranded cDNA was prepared using a kit supplied by Boehringer Mannheim Ltd., according to the manufacturer's instructions, except that oligo(dT) primer was replaced with 36 μg/ml random hexanucleotides (dN<sub>6</sub>) (Pharmacia). The oligonucleotide primers used for the polymerase chain reaction (PCR) were: TCCGAAATTCAAGGGCTGATGACCG (bases 293 to 312 plus a terminal EcoRI site) and GACACATGATATGCATCA (-175 to -157) and GGAAGGGTTTCTCTGCTGA (-97 to -114) from the 5' untranslated region of TRP-1 cDNA; and GCTGAGAGGCCCTCTTCTTC (714 to 752) and GAGGCCTGCGACTGCTGGTCT (961 to 979) from the middle of TRP-1 cDNA (Shibahara et al. 1986).

The reactions were performed essentially as in SaiKI et al. (1988). Each reaction contained 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 0.01% NP-40, all six oligonucleotide primers at 3 μg/ml, all four deoxynucleotide triphosphates at 0.1 mM and 2.5 units Taq DNA polymerase (Amersham) in a volume of 50 μl. Some reaction mixes were irradiated with UV by placing on a UV Products transilluminator for 10 min before addition of enzyme and substrate (Sarkar and Sommer 1990). Each CDNA amplification used the product of reverse transcription of approximately 0.1 μg skin total RNA. The reaction was cycled 25 times through 90 sec at 92°, 90 sec at 55° and 120 sec at 72°. Thirty percent of the reaction was analyzed by electrophoresis on gels of 4% NuSieve GTG agarose (FMC Bioproducts) for ethidium bromide staining, or of 1.5% agarose (Sigma) for Southern blotting.

RESULTS AND DISCUSSION

Both TRP-1 and TRP-2 are melanocyte-specific, highly expressed mRNAs. Northern blot analysis on a range of tissues reveals no evidence of expression in cells other than melanocytes and melanoma. In situ hybridization to sections of neonatal mouse skin shows that TRP-1 is expressed exclusively in the melanocytes of the hair follicles (K. Steel, D. R. Davidson and I. J. Jackson, unpublished results).

Shibahara et al. (1986) reported two discrete TRP-1 mRNA species visible on Northern blots. Although the existence of two species is demonstrated by cDNA clones differing at their 3' ends (Shibahara et al. 1986), when blots are washed at high stringency we see only a single species (see Figures 1, 2 and 3, for example), although additional bands can be seen at lower stringency. The high abundance of TRP-1 mRNA in melanocytes permits its detection in neonatal skin samples, where melanocytes comprise only a small minority of cells. Approximately the same signal is obtained on a Northern blot of 10 μg of 2-day-old skin total RNA, as from 100 ng of melanoma total RNA. Hybridization of TRP-2 cDNA to Northern blots reveals a slightly smaller mRNA, which, in this study, we use as a control, not only for the integrity and loading of RNA, but also to confirm the presence of melanocytes in the skin samples examined.

The brown (b) mutation: The common, recessive brown (b) mutation is an old mutation of the mouse fancy. We have shown (Jackson 1988) that the mutation present in a number of different laboratory inbred and outbred stocks is associated with a 4.9-kb TaqI TRP-1 fragment, the D haplotype. All other mice have the B haplotype comprising a 3.7- and 1.2-kb TaqI fragment.

Figure 1 shows autoradiography of a Northern blot, probing total RNA made from melanoma cells and melanocytes grown in vitro. B16 melanoma cells and melan-a cells (Bennett, Cooper and Hart 1987) are both derived from C57BL/6 inbred mice, which are wild type (Black) at the b locus. Melan-b cells (Bennett et al. 1989) are derived from homozygous brown mice of the outbred Q stock. After washing to remove the first probe, the filter was rehybridized with TRP-2 to check for loading. There is no significant difference in the abundance or size of TRP-1 mRNA between Black and brown melanocytes, or between melanoma and cultured melanocytes. The Q-stock brown mutation is associated with the D haplotype (Jackson 1988), and this result is therefore most likely applicable to the common brown mutation seen in all laboratory mice.

The defect which results in the brown phenotype is not one of transcription or processing, but must be a small change, probably a point mutation, which renders the TRP-1 mRNA or its protein product inactive. We have recent sequencing evidence that there are two amino acid differences between the TRP-1 product of Black and brown mice, and can show that only one of these, Cys-86 to tyrosine, results in the mutant
phenotype (Zdarsky, Favor and Jackson 1990).

The cordovan-Harwell (b") mutation: A number of alleles of brown have been described that are intermediate in phenotype between black and brown. They have been variously called dark-brown or cordovan. The first intermediate allele to be described, called cordovan (F), arose spontaneously (Miller and Potas 1955). We have examined transcription of another intermediate allele, cordovan-Hamell (b") which arose in a γ-irradiation mutagenesis experiment in a (C3H/HeH x 101/H)F1 male receiving approximately 600 rad (6 Gy) over a period of about 12 weeks (Batchelor, Phillips and Searle 1966).

We have examined mRNA levels of the b" allele in RNA prepared from neonatal skin of litters segregating b" and b. It is not possible to distinguish mice homozygous for cordovan-Harwell from those heterozygous with brown (i.e., the mutation is fully dominant over brown). Furthermore it is difficult to distinguish these mice from homozygous brown animals when they are only a few days old. However, as the b" mutation arose on mice carrying the B haplotype, Southern blots of DNA from these neonates allow distinction to be made between animals homozygous for either B or D haplotypes (b" or b, respectively), or compound heterozygotes (b/B). We prepared RNA from the skin and DNA from the kidney of each member of the litter. Figure 2a is an autoradiograph of a Northern blot of the skin RNAs probed with pMT4. TRP-2 was hybridized subsequently to confirm the RNAs were undegraded and equally loaded (Figure 2b). Animal 5 is the only b/b animal of the litter. As the mRNA abundance is the same in Black (+/+ or brown (b/b) melanocytes (see above), this animal serves as an indicator of wild-type TRP-1 mRNA level. Animals 2 and 3 are both homozygous b"/b", and the rest are b/b". Comparison of the hybridization signals, within the litter and with the control hybridization to TRP-2, shows that the homozygous cordovan-Harwell mice have very low levels of TRP-1 mRNA. The heterozygotes have about 50% wild-type amount of mRNA, largely due to the brown (b) allele. Hybridization to TRP-1 in the b"/b" skin is detectable in lanes 2 and 3, but we estimate from scanning the autoradiograph that it is present at approximately 1% of the abundance of b/b or +/+ mice.

As the phenotype of cordovan-Harwell is not brown, but is somewhat darker, we would expect the melanocytes to have reduced, but not absent, TRP-1 activity. It therefore is likely that the low level of TRP-1 seen represents normal mRNA, and that the phenotype is due to a greatly reduced level of its (normal) protein product.

The effect of dosage of TRP-1 on pigmentation is put into an interesting light by the mutation, and is summarized in Table 2. If we make the reasonable assumption that relative protein levels correspond to the observed mRNA levels, we can propose that al-
though complete lack of active protein (b/b) leads to a brown phenotype, half normal levels (B/b) results in a phenotype indistinguishable from wild type (B is dominant over b). A reduction to about 1% wild-type levels of TRP-1 in bH/bH mice leads to an clear change in pigmentation from black toward brown, but further reduction to about 0.5% (bH/b) has no further effect (bH is dominant over b). Two explanations present themselves. First the effect of TRP-1 on pigment synthesis might show a threshold or quantized effect, meaning that there exist discrete forms of pigment, governed by a particular dose of TRP-1, rather than a continuum, but we cannot perceive the difference between more than a limited number of states. A different situation occurs at the albino (c) locus, at which many intermediate alleles [such as chinchilla (cH)], are recessive to wild type but only semidominant over albino (so, for example, cH/c is darker than c/c) (SILVERS 1979). The difference between the b and c loci might be due to the function of their products. Tyrosinase, from the c locus regulates the amount of pigment produced while the b-locus product, TRP-1, governs the quality (or possibly stability) of the pigment color.

The molecular basis of the reduced TRP-1 mRNA abundance is currently under investigation. The nature of the change will be informative as to the regulation of mRNA levels in general and in melanocytes in particular. The reduction may be due to decreased transcription or increased degradation of the RNA or its precursor. It should be noted that we have not been able to detect by restriction fragment analysis any difference between this mutant TRP-1 gene and the wild-type allele, even though the mutation arose following γ-irradiation.

The dominant Light (B") mutation: Light (B") is the better studied of the dominant alleles of brown (QUEVEDO and CHASE 1958; SWEET and QUEVEDO 1968; QUEVEDO, FLEISCHMANN and DYCKMAN 1981). It arose spontaneously on a C58 background, and Southern blot examination of DNA from homozygous Light mice shows no evidence of alteration of gene structure. We have examined the TRP-1 mRNA level in the skin of homozygous B" neonates (whose genetic status was confirmed by haplotype analysis) compared with age-matched C57BL/6 (wild-type) animals. At this early stage of the hair cycle we would not yet expect to see a depletion in melanocyte numbers. Figure 3 shows a Northern blot of these RNAs probed with the pMT4 plasmid, which indicates that there is no difference in TRP-1 mRNA size or abundance.

We conclude that the Light mutation most likely exerts its effect through a mutant protein which causes the observed death of the melanocytes. This protein probably has some residual, normal TRP-1 activity, as the pigment produced by homozygous mutant melanocytes is dark brown rather than brown, but, in addition the protein must be toxic to the cell. Either it is itself toxic (through mislocalization in the melanocyte, for example), or its enzymatic function has been altered so that a toxic product accumulates in the cell.

The dominant White-based brown (B") mutation: The phenotype of White-based brown (B") mice is very similar to that of Light, although it has not been studied in the same detail. We propose that the product of the B" allele, like that of B", causes melanocyte death, but, unlike B", homozygous B"/B" mice, or compound heterozygotes with brown (B"/b), have brown pigment at the hair tips. Thus the mutation results in a recessive null phenotype (brown) in addition to the dominant (white-base) phenotype. The mutation arose in a spermatogonium of a (101/R1 x C3H/RI)F1 male, exposed to 600 rad (6 Gy) of γ-radiation over a period of several weeks (HUNSICKER 1969).
There is an intervening sequence beginning after base 31 about 100 bp upstream of the 111(111. SIIIIC. liltel. Idlvl)ritli/ctl with rllr I.fi-kb the 800-l)p /:'roKI to 10(. right-hand track intlic;~tctl above. t11c lcft-l~and r~.;~c-k 1nicc. 111

an 800-bp EroRI-Pstl fragment. The EcoRl site is compared DNA restriction fragments from homozv-

gous which has two different chromosomes 4, but we do rearrangement detected by TRP-1
tiallv identical fragments M.llen compared with 10 1 nomic probes. The mutation  arose in a hybrid mouse,

not kno~v on which chromosome it occurred. We have ever, when probes toward the

encompassing most of the coding region of the TRP-

fragment cross-hybridizing with the 456 DNA (with the exception of variants in an unlinked

gene

Figure 4 shows this allele is associated with a DNA rearrangement detected by TRP-1 cDNA and gen-

probes. The mutation arose in a hybrid mouse, which has two different chromosomes 4, but we do not know on which chromosome it occurred. We have compared DNA restriction fragments from homozygous White-based brown (B'/B') mice. In each pair of tracks, digested with the restriction enzyme indicated above, the left-hand track is White-based brown DNA and the right-hand track is C3H/He DNA. Panel a was hybridized with the 800-bp EcoRl to Pstl genomic clone fragment and panel b is the same filter rehybridized with the 1.6-kb HindIII cDNA fragment.

Figure 4 shows this allele is associated with a DNA rearrangement detected by TRP-1 cDNA and genomic probes. The mutation arose in a hybrid mouse, which has two different chromosomes 4, but we do not know on which chromosome it occurred. We have compared DNA restriction fragments from homozygous White-based brown (B'/B') mice with C3H DNA, but we observe essentially identical fragments when compared with 101 DNA (with the exception of variants in an unlinked fragment cross-hybridizing with the 3' end). Probes encompassing most of the coding region of the TRP-1 gene do not detect any differences between the B' allele and the C3H wild-type gene (Figure 4b). However, when probes toward the 5' end of TRP-1 are used, differences are found between the two genes.

The 5' end of the wild-type gene is enclosed within an 800-bp EcoRl-Pstl fragment. The EcoRl site is about 100 bp upstream of the 5' end of the mRNA. There is an intervening sequence beginning after base -87 of the cDNA sequence (i.e., 99 bp from the end of the published sequence) and the Pstl site is approximately 600 bp into this intron (see Figure 5a). This EcoRl-Pstl fragment, containing exon I, was used to probe Southern blots of DNA from homozygous White-based brown and wild-type animals and the results are shown in Figure 4a. When digested with Pstl, the B'/B' DNA has the same fragment size as wild type (4.5 kb), indicating that sequences 5' of the Pstl site in the first intron are unrearranged (which include a HindIII and an EcoRI site). However, the 6.5-kb EcoRl wild-type fragment is replaced in White-based brown DNA by one of 2.4 kb, and  the 2.15 kb HindIII fragment replaced by one of 1.3 kb. There is therefore a DNA rearrangement within the B' TRP-1 gene, downstream of the Pstl site in the first intron, but upstream of the HindIII and EcoRI sites, which are in the second and third introns, respectively. As the novel HindIII site lies only 0.2 to 0.3 kb downstream of the (unaffected) Pstl site, the rearrangement must have an end point in this few hundred base-pair interval. Other data (not shown) indicates the breakpoint lies close to the 3' end of the first intron.

The exact nature of the rearrangement has to be established, but it involves a juxtaposition of novel DNA sequences with the 5' end of the gene. The data are consistent with an insertion of DNA at this point, or an inversion of the region with one breakpoint mapping to end of the intron, and the other an unknown distance upstream. Figure 5 is a schematic representation of the first 3 introns of the wild-type TRP-1 gene, and both halves of the rearrangement in the B' mutation, and includes additional mapping data.

We are unable to detect transcription from the B' mutant allele. Figure 6 shows the results of probing a Northern blot of RNA made from 2-day-old wild-type (C57BL/6) skin (lanes 1, 3, 5 and 7) and from 2-day-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Southern blot hybridization to DNA prepared from C3H/He mice and from homozygous White-based brown (B'/B') mice. In each pair of tracks, digested with the restriction enzyme indicated above, the left-hand track is White-based brown DNA and the right-hand track is C3H/He DNA. Panel a was hybridized with the 800-bp EcoRl to Pstl genomic clone fragment and panel b is the same filter rehybridized with the 1.6-kb HindIII cDNA fragment.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Schematic representation of the 5' ends of the wild-type and B' mutant TRP-1 genes. (a) Wild-type gene, showing first 3 exons, numbered I, II and III. (b) B' mutant gene. Novel DNA sequences are represented by dashed lines. It is not known if these sequences are a single insertion into the locus, or are flanking a site of a large inversion. Both halves of the rearrangement are shown. P, Pstl; E, EcoRI; H, HindIII. Not all sites are shown.}
\end{figure}
old $B^e/B^e$ skin (lanes 2, 4, 6 and 8). When the blot is probed either with the 1600 bp cDNA fragment from downstream of the rearrangement (lanes 1 and 2) or with the EcoRI/PstI genomic fragment containing the first exon (upstream of the rearrangement) (lanes 3 and 4) there is no evidence of a TRP-1-containing transcript from the $B^e$ allele. Reprobing the blot with TRP-2 cDNA (lanes 5 and 6) results in hybridization to all tracks, indicating that the RNA from $B^e$ mice is intact, and that there are indeed melanocytes present in the skin. The hybridization to TRP-2 mRNA is somewhat weaker in the $B^e/B^e$ RNA than the wild type. This is not due to a lower RNA loading as reprobing with actin cDNA (lanes 7 and 8) shows that there is, in fact, more RNA in the mutant track. It is possible that we may be seeing here an early indication of the depletion of the melanocyte pool, which ultimately results in the phenotype.

It is not surprising that the DNA rearrangement results in no transcription from the main part of the gene. However, as it appears from Southern blotting that the 5' flanking region is intact, it may be capable of driving transcription of the first exon, as well as sequences downstream including sequences within the rearrangement. Such transcripts may not be of discrete size, and may be hidden in the smear seen in track 4 of Figure 6. We therefore used the polymerase chain reaction (PCR) to examine homozygous $B^e$ RNA. We made double-stranded cDNA from both wild-type and mutant skin RNA [priming the first strand with random hexanucleotides to allow for a possible lack of poly(A) tail]. This was then used as a substrate for the PCR (Saito et al. 1988), primed by oligonucleotide pairs from downstream of the rearrangement, from upstream (within the first exon) and, as control, from the tyrosinase gene. Figure 7 (top) shows an ethidium bromide-stained gel of the amplified DNA. Amplification of wild-type cDNA gives rise to a 348-bp fragment deriving from the tyrosinase mRNA, and to 266- and 78-bp fragments from TRP-1 mRNA (track 2). $B^e$ cDNA gives rise only to the amplified tyrosinase fragment; TRP-1 transcripts from either side of the rearrangement are not visible (track 3). Figure 7 (bottom) is a Southern blot of a similar gel probed with exon 1, and shows at higher sensitivity that no exon 1 transcripts are present in $B^e/B^e$ skin. We conclude that the TRP-1 promoter associated with the $B^e$ allele most likely is nonfunctional due to disruption by the rearrangement.
White-based brown has a recessive brown phenotype which is obviously due to absence of any TRP-1 transcript. However, the mutation also has a true dominant effect, and therefore it is likely that a transcript derives from the region of the locus, and this transcript (or its translation product) causes melanocyte death. While it is possible that very low levels of a fusion transcript containing the first exon and other sequences might be the agent, this is perhaps unlikely. A better hypothesis is that the rearrangement has brought another gene (or genes), not normally expressed in melanocytes, into the proximity of the TRP-1 gene, which supplies it with enhancer (but not necessarily promoter) function. It might be the ectopic expression of this gene (either high levels of an aberrant RNA alone or the translation product of an ectopic mRNA) which results in the dominant phenotype. The nature of the DNA rearrangement in White-based brown mice, and the sequences encompassed by it are of great interest, and will be further characterized by DNA cloning.

GENERAL CONCLUSIONS

This study raises several issues that are generally applicable and should be borne in mind when other developmental mutations are considered. First, phenotype can be quantized in addition to the usually observed dominance of wild-type over null mutation. Second, simple mutations in highly expressed enzymes can lead to neomorph dominant functions which can have profound effects on cell function or survival. Furthermore it seems that a mutation involving only a small change (B'C) can have a very similar phenotypic effect to one resulting from a much more severe DNA disruption (B'C'). The mode of action of these dominant, cell-suicide, alleles is of general relevance to developmental genetics; cell-type specific, autonomic, cell-suicide, alleles is of general relevance to morphology. Finally, it should be noted that two mutations examined here arose in γ-irradiation experiments, and both received approximately the same dose (600 rad or 5 Gy) over approximately the same time period, but only in one have we detected a gross DNA rearrangement. However, both mutations were selected on the basis of being distinguishable from brown, and so are not representative of the large number of other brown alleles obtained by radiation mutagenesis.

We thank Nick Hastie and Ruth Johnson for useful discussion and encouragement, Eman Zdarsky for developing the RT-PCR methodology, Linda Devlin for skilled assistance and Nick Hastie, John Evans and Ruth Johnson for comments on the manuscript. Norman Davidson, Douglas Stuart and Sandy Bruce provided excellent photographic and art work. We also thank the Medical Research Council (MRC) and the Lister Institute for Preventive Medicine for financial support. The work has been funded in part by an MRC Project Grant to I.J.J., by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems Inc. (E.M.R.) and by a CRC grant to D.C.B. I.J.J. is a Lister Fellow.

LITERATURE CITED


Lovell-Badge, 1987 Introduction of DNA into embryonic stem
Expression of brown Locus Alleles


Communicating editor: R. E. GANSCHOW