Phenotypic rescue of mutant brown melanocytes by a retrovirus carrying a wild-type tyrosinase-related protein gene

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Summary

A mouse cDNA for the developmentally controlled, melanocyte-specific protein, tyrosinase-related protein 1 (TRP-1), was previously cloned and reported to show genetic linkage with the coat-colour locus brown (b) on mouse chromosome 4. The cDNA has been inserted into a retroviral vector derived from Moloney murine leukaemia virus, under the control of the human histone H4 promoter. This vector was used to infect melanocytes of the immortal line melan-b, which are homozygous for the b mutation and which display light brown pigmentation in culture. Infected cultures containing between 0.2 and 2 copies of provirus per cell displayed an altered phenotype: 20–50% of cells now had the black to dark brown colour characteristic of cultured wild-type (Black, B/B) mouse melanocytes. Thus the TRP-1 gene complements the brown mutation. We conclude that TRP-1 is the product of the wild-type b-locus.

Key words: mouse, genetics, brown, melanocyte, culture, TRP-1, retrovirus, vector.

Introduction

The generation of pigmentation and coat colour in the mouse by melanocytes is a developmental process which is particularly amenable to molecular-genetic analysis (Jackson, 1985). As pigmentation is a readily observed phenotype, which is not essential for viability, mutations are easily obtained and many spontaneous and induced mutations at numerous loci have been isolated (Silvers, 1979). The process also lends itself to study in vitro; both melanocytes and their embryonic progenitors, neural crest cells and melanoblasts, can be grown and manipulated in culture (Ito and Takeuchi, 1984; Jaenisch, 1985; Sato et al. 1985; Tamura et al. 1987; Bennett et al. 1987, 1989).

The key enzyme in the synthesis of melanin pigment is tyrosinase, a copper-containing metalloprotein which is the product of the albino (c)-locus (Kwon et al. 1987; Müller et al. 1988). There are at least two other proteins whose mRNAs are developmentally controlled, being expressed specifically in melanocytes, and which show evolutionary relationship to tyrosinase. In particular both proteins contain, like tyrosinase, a transmembrane domain towards the C-terminus, and two potential copper-binding sites (Müller et al. 1988; Hearing and Jiménez, 1989; I.J.J., unpublished data). These proteins, tyrosinase-related proteins 1 and 2 (TRP-1 and -2), are encoded by the cDNAs pMT4 and 5A (Shibahara et al. 1986; Jackson, 1988). They are presumably localized, like tyrosinase, on the inner face of the melanosome, the intracellular site of pigment production, perhaps within a multi-enzyme complex for the synthesis of melanin.

We have shown that the gene for TRP-1 maps at or close to the mouse brown (b)-locus (Jackson, 1988). The wild-type product of this locus is not essential for pigmentation, but is necessary for the formation of black melanin (eumelanin) rather than brown.

Immortal lines of melanocytes have been derived from mice wild-type or mutant at the b-locus; these cells retain their black or brown phenotype in culture (Bennett et al. 1989), and may provide a clue to the role of the b-locus product. Although the black and brown lines melan-a and melan-b are visibly different in culture, this difference becomes more accentuated with time after fixation in formalin. The black pigment is stable, and fixed melan-a cells retain their dark pigmentation over time, whilst the pigment of melan-b cells becomes progressively lighter brown over a period of weeks to months, eventually approaching white...
(D.C.B., unpublished data). The b-locus product is thus responsible both for the different quality or colour of pigment and for its long-term stability (properties which might be interrelated).

To study further the function of the b-locus product, and to examine the numerous mutations at the locus, the identity of the encoded mRNA and protein needs to be determined. The genetic linkage observed between TRP-1 and the b-locus places the two genes within 2.8 cMorgan, at 95% confidence limits (Jackson, 1988). This, coupled with the melanocyte-specific expression of the TRP-1 mRNA, suggests that TRP-1 is the product of the b-locus. To test this relationship we have constructed a retroviral vector bearing the wild-type TRP-1 cDNA, under the control of a heterologous promoter. The retrovirus rescues the brown phenotype of melan-b cells, converting their colour to dark brown or black.

**Materials and methods**

**Cell stocks**

Melan-a, melan-b cells and derivatives were grown in a medium containing 200 nm tetradecanoyl phorbol acetate (TPA) and other supplements as described (Bennett et al. 1989), but without mercaptoethanol (melanocyte medium).

Psi-2 producer cells and NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum or foetal calf serum. All cultures were gassed with 10% CO₂ in air.

**Retroviral vector production**

The vector pHS-TRP1 was constructed by replacing the neo gene of the retroviral vector pHS-neo (Gilbert et al. 1984), as indicated by the designation En~. The hatched box represents the pMT4 cDNA, encoding TRP-1. The retrovirus contains the cDNA encoding mouse TRP-1 (Shibahara et al. 1986; Jackson, 1988), under the control of the human histone H4 promoter (Hanly et al. 1985; Guild et al. 1988). A diagram of the provirus is shown in Fig. 1.

High titres of the retroviral vector were obtained from psi-2 packaging cells containing the provirus. As assessed by Southern blot hybridization of infected cells, infection of 6×10⁵ NIH-3T3 cells resulted in an average of 1 proviral copy per cell (D.H., data not shown).

**Results**

**The retroviral vector**

The pHS-TRP1 retrovirus contains the cDNA encoding mouse TRP-1 (Shibahara et al. 1986; Jackson, 1988), under the control of the human histone H4 promoter (Hanly et al. 1985; Guild et al. 1988). A diagram of the provirus is shown in Fig. 1.

**RNA analysis**

RNA was extracted from cells as described in detail elsewhere (Robson et al. 1985). Total cellular RNA (15 µg) was denatured with glyoxal, electrophoresed through a 1.1% agarose gel and transferred onto nitrocellulose (Schleicher and Schuell). Filters were hybridized with ³²P-labelled pMT4 cDNA by the method of Thomas (1980).

**Retroviral infection of melanocytes**

Nearly-confluent psi-2 producer cells were incubated for at least 6 h in melanocyte medium without TPA. The medium containing virus was harvested, passed through a washed 0.22 µm filter to remove cells, supplemented with TPA and 8 µg ml⁻¹ polybrene, and placed on melan-b cultures plated the previous day at 2×10⁴ cells ml⁻¹. The medium was replaced after at least 6 h with fresh melanocyte medium or, for multiple infections, with medium freshly removed from psi-2 cells and supplemented as above. Control cultures were mock-infected with medium not exposed to psi-2 cells but containing polybrene.

**Colony assay of phenotypic rescue**

Cells were resuspended and plated at 500 cells per 85 mm dish in Ham's F10 medium with 5% FCS and 200 nM TPA. Colony-forming efficiencies varied between 45% and 75%. Cultures were fixed in formalin after 17 days, washed in distilled water, dried, coded and all colonies on each dish were counted in the categories 'pale' and 'dark' by an observer unaware of the nature of the experiment.

**DNA analysis**

DNA was made from frozen cell pellets by resuspending the cells in 50 mM Tris pH 8.5, 10 mM EDTA, 100 mM NaCl, followed by addition of 100 µg ml⁻¹ proteinase K and 1% SDS. After incubation at 37° C for several hours the DNA was extracted twice with phenol, twice with chloroform and precipitated with ethanol. The DNA was dissolved in 10 mM Tris, pH 7.5, with 1 mM EDTA.

Restriction endonuclease digestion was performed according to manufacturers' recommendations. DNA concentrations were determined by absorption at 260 nm, and also by measurement of fluorescence in Hoechst 33258 with a Hoefer fluorimeter. After electrophoresis the gel was soaked in 0.5 M NaOH, 1.5 M NaCl and transferred without wick to Hybond N (Amersham).

Hybridization was carried out according to Church and Gilbert (1984), and the probe labelled with ³²P-CTP by the random priming method (Feinberg and Vogelstein, 1983).

![Fig. 1. Schematic diagram of the pHS-TRP1 provirus. The horizontal lines represent Moloney murine leukaemia virus (MoMLV) sequences and the open boxes indicate the MoMLV long terminal repeats (LTRs). Viral enhancer sequences have been deleted from the 3' LTR (Guild et al. 1988), as indicated by the designation En~. The hatched box represents the pMT4 cDNA, encoding TRP-1. Transcriptional orientation is indicated by the arrow.](image-url)
Cloned gene complements brown mutation

Fig. 2. Northern blot analysis of pH5-TRPI-infected NIH-3T3 cells. Total cellular RNA (15 μg) from pH5-TRPI-infected (lane 1) and uninfected (lane 2) 3T3 cells was hybridized with 32P-labelled pMT4 cDNA.

RNA hybridized to the pMT4 cDNA. As shown in Fig. 2, lane 1, pH5-TRPI-infected cells contain one major RNA species of 2.7 kb, corresponding in size to a transcript initiated at the H4 promoter and terminated in the viral 3' long terminal repeat (LTR). The very low level of transcription observed from the retroviral promoter in the 5' LTR (indicated by the faint band at 3.9 kb) results from deletion of viral enhancer sequences from the 5' LTR of the integrated provirus. This loss is a consequence of retroviral replication, whereby the region of the 3' LTR containing an engineered enhancer deletion (see Fig. 1) serves as template for both the 5' and 3' LTRs during reverse transcription (Varmus and Swanstrom, 1982).

Infection of melan-b cells in culture

The melan-b cell line is derived from a homozygous brown mouse embryo of the outbred Q stock maintained at the MRC Mammalian Development Unit (Bennett et al. 1989). The cells maintain their brown phenotype in culture. Two infected populations of melan-b cells were generated, 'V1' which was exposed to virus-containing medium for 6 h, and 'V3' which received three exposures to the virus over 42 h. The following analyses were all done with cultures from the same experiment, although a second experiment was performed with similar results.

Fig. 3 shows a Southern blot hybridization of DNA from uninfected melan-b cells and from V1 and V3 cells, cut with SacI and hybridized with the 1.6 kb HindIII fragment of pMT4. As a quantitative comparison, SacI-digested mouse DNA, doped with pH5-TRPI plasmid to provide 0, 0.2, 0.5, 1, 2 and 5 proviral copies, was also run. The pMT4 probe detected the 2 expected proviral SacI fragments of 2.56 kb and 0.7 kb in infected cells (see Fig. 1), in addition to the endogenous genomic fragments, which served as internal controls. Comparison of the hybridization signals showed that the V1 population contained, on average, about 0.2 to 0.5 copies per cell, whilst the V3 population averaged 1 to 2 copies per cell.

Phenotype rescue

The cell populations which had been infected with retrovirus had darker pigmentation than the mock-infected cells. Their macroscopic appearance is shown in Fig. 4 (monochrome). Both V1 and V3 appeared darker than the control cells, but the V3 population, with more copies on average, showed a greater darkening.
Fig. 4. Macroscopic effects of infection with phs-TRP1 virus. Mock-infected and infected cultures were plated in 3 cm dishes at the second passage after infection and grown to saturation density. They were fixed with formalin and stored dry until photographed. Left, mock-infected; centre, V1 cells; right, V3 cells.

Fig. 5 shows colour light micrographs of the same cultures, compared also with melan-a black (a/a, B/B) melanocytes. The pale brown colour of mock-infected melan-b cells was barely visible and clearly different from the black to dark brown granularity of melan-a cells. Patches (probably clones) of black cells were evident in the V3 cultures, as well as patches of unusually dark-brown cells. The V1 cultures contained fewer patches of both. Some of the variation in colour among the dark cells may have reflected differences in the level of TRP-1 expression among different clones; however, there were also some dark brown cells even among the homoyzgous black melan-a cells. (One possible explanation is production of yellow pheomelanin, as reported for another line of black melanocytes by Sato et al. (1985)). The cultures had been fixed for over a year at the time of photography, showing that the dark pigment in V1 and V3 cultures did not fade.

More dark cells were observed in V3 than in V1 cultures. To quantify this, dark and light cells were counted by haemocytometer at the second passage after infection. The percentages of dark cells seen in control, V1 and V3 cultures were 2.6±0.6, 29.6±0.5 and 56.1±1.7, respectively (means and standard errors of 4 counts each). As an independent test, each line was plated sparsely at passage 1 and allowed to form clonal colonies, after which the proportion of visibly dark colonies on each was counted (see Materials and methods). The percentages of dark colonies for control, V1 and V3 cultures were, respectively, 5.4±0.7, 23.7±1.9 and 33.3±2.2 (means and s.e. of 4 counts each). Interestingly, a high proportion of colonies scored macroscopically as dark had a rim of pale cells around the edges, only the colony centre being dark. In addition, attempts to purify black cells by cloning had to be discontinued because initially dark clones soon produced mixtures of light and dark cells, suggesting an instability of vector expression. The proportion of dark cells in a clone seemed to drop progressively.

Discussion

These experiments demonstrate clearly that melan-b melanocytes, homozygous for the brown mutation, can be rescued to give a wild-type colour by infection with a retroviral vector bearing a wild-type cDNA of the TRP-1 gene. It is therefore highly probable that TRP-1 is the product of the b-locus. The way is now open for examination of the molecular nature of mutant alleles at the locus.

Comparison of the average number of proviruses per cell with the proportion of visibly dark cells indicates that 1 proviral copy may be sufficient to produce a dark phenotype; 0.2 to 0.5 proviral copies per cell in the V1 population resulted in 20–30 % dark cells, while 1–2 copies per cell gave 30–50 % dark cells. The small number of dark cells counted in uninfected populations may be due to some natural variation in the colour of the cells; on re-examination these cells were judged to be dark brown rather than black. The black phenotype of infected melan-b cultures was gradually lost during growth of clonal cultures. Since retroviral integration into chromosomal DNA is stable (Varmus and Swanson, 1982), this is probably due to an inhibition of TRP-1 expression by epigenetic mechanisms. Mosaic expression of the enzyme β-galactosidase has also been observed by ourselves (unpublished observations) and others (Price et al. 1987) in clones of cells infected with retroviral vectors bearing this enzyme.

Both functions of the b-locus product are supplied by the TRP-1-bearing provirus. The rescued cells not only have melanin which is darker than that of the melan-b cells, but the pigment is also stable with time after fixation, unlike that of the parental cells.

Introduction and expression of the TRP-1 gene in non-pigment cells will now provide a valuable route for the elucidation of the enzymatic function of TRP-1. It should also soon be possible in future to study the developmental control of this gene by analysis of genomic sequences and the requirements for their melanocyte-specific expression.

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References


Fig. 5. Colour photomicrographs of infected and uninfected cells. The same cultures as in Fig. 4 were used, as well as a confluent dish of melan-a black (a/a, B/B) melanocytes (Bennett et al. 1987), for comparison. Water was added to the dishes before photography. High-voltage, bright-field illumination was used throughout, and all photographic settings and procedures were identical. (A) Mock-infected melan-b cells; (B) melan-a cells; (C) V1 cells; (D) V3 cells. Scale bar: 200 μm.


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