The EMBO Journal vol.9 no.9 pp.2819 – 2826, 1990

Rescue of the albino phenotype by introduction of a functional tyrosinase gene into mice

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Communicated by G.Schütz

The *c*-locus of the mouse is thought to encode tyrosinase, the key enzyme for melanin synthesis in melanocytes of the skin and the eye. Recently, a mouse cDNA was isolated and shown to confer tyrosine activity on a cell line which expressed no specialized functions for melanin synthesis. To verify that the isolated tyrosinase gene is encoded at the genetically well characterized *c*-locus, a minigene was assembled from tyrosinase cDNA and tyrosinase genomic DNA and used for generation of transgenic mice. Following microinjection of this construct into fertilized eggs of an albino mouse strain, transgenic mice were obtained which showed pigmentation in skin and eyes. By *in situ* hybridization, we show expression of the transgene in melanocytes of the hair-bulb and in the pigmented cell layers of the eye. We conclude that we have rescued the albino mutation (*c/c*) by introduction and expression of a functional tyrosinase gene.

Key words: *c*-locus/pigmentation/transgenic mice/tyrosinase

Introduction

Genes affecting coat colour and pigmentation were among the first to be studied for Mendelian inheritance in mice. More than 130 mutations at more than 50 loci have been described in the house mouse (*Mus musculus* L.), e.g. *A* (agouti), *b* (brown), *c* (albino), *d* (dilute) and *e* (extension; Silvers, 1979; Hogan *et al.*, 1986).

The albino or *c*-series of alleles is characterized by deficiency or alteration in the structure of tyrosinase. This copper-containing enzyme catalyses two reactions, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (Dopa) and the oxidation of Dopa to dopaquinone (Hearing, 1987; Hearing and Jiménez, 1989). Dopaquinone is then further metabolized to either yellow pigment (phaeomelanin) or black and brown pigment (eumelanin). The main pigment-synthesizing cells are the melanocytes, which are derived from melanocyte precursor cells (melanoblasts) that migrate from the neural crest into hair follicles in the skin and the uveal layer of the eye (Silvers, 1979; Hogan *et al.*, 1986). The second pigmented cell layer in the eye, the pigmented retina, is derived from the ciliary margin of the optic vesicle (Morris-Kay and Tan, 1987; McKay, 1989). By genetic analysis, it has been established unambiguously that the same gene is responsible for pigment formation in skin and eyes (Silvers, 1979).

At the *c*-locus on chromosome 7, the wild-type allele (*C*) is dominant over all other alleles and provides full tyrosinase activity. In contrast, albino mice (*c/c*) have no pigment at all, either in the skin or in the eyes, regardless of other coat colour determinants (e.g. agouti or brown) which are then masked. Nevertheless, albino mice (*c/c*) possess a full complement of pigment cells, so-called amelanotic melanocytes, which show no tyrosinase activity. Other *c*-locus alleles like chinchilla (c^{ch}) show reduced pigmentation due to altered tyrosinase activity (Silvers, 1979; Hogan *et al.*, 1986). Until recently, it was not clear whether the *c*-locus encoded tyrosinase or a *trans*-regulatory protein, which inhibits or activates tyrosinase activity (Hearing, 1973; Pomerantz and Li, 1974).

Recently, several independent cDNA clones encoding tyrosinase with different sequence content were isolated (Kwon *et al.*, 1987; Yamamoto *et al.*, 1987; Ruppert *et al.*, 1988). Comparison of the single copy structural gene and various cDNAs provided strong evidence that the different tyrosinase cDNA clones were generated by alternative processing of a single transcript (Ruppert *et al.*, 1988). Only a construct containing the full complement of exons was able to confer tyrosinase enzyme activity to tyrosinase-negative cells upon transient expression (Müller *et al.*, 1988). This occurred irrespectively of whether the cells were derived from melanocytes or not. This result has been corroborated recently. By transfection with a mouse tyrosinase cDNA, cultured albino melanocytes derived from BALB/c mice produced melanin (Yamamoto *et al.*, 1989). When amelanotic melanoma cells were transfected with the tyrosinase cDNA the cells showed both tyrosine hydroxylase and Dopa oxidase activity (Takeda *et al.*, 1989).

Strong evidence that the isolated gene maps at the *c*-locus comes from localization of the cloned DNA to deletions that accompany an albino phenotype (Kwon *et al.*, 1987; Ruppert *et al.*, 1988; Ruppert, 1988). However, the ultimate proof that a functional tyrosinase gene is encoded at the *c*-locus is to rescue the albino phenotype in transgenic mice. We therefore constructed a tyrosinase minigene which was injected into fertilized eggs of the albino mouse strain NMRI. Several transgenic mice were obtained which showed pigmentation in both skin and eyes.

Results

Rescue of the albino phenotype by production of transgenic mice carrying a tyrosinase minigene

The minigene construct ptrTyr4 used for injection is illustrated in Figure 1. In order to facilitate transcription of the microinjected gene, the first intron was included (Brinster *et al.*, 1988). To construct this hybrid minigene ptrTyr4 we

used genomic DNA derived from chinchilla mice (c^{ch}; Ruppert *et al.*, 1988) and cDNA sequences (Müller *et al.*, 1988; Ruppert *et al.*, 1988) derived from B16 melanoma cells (*C*; Pasztor *et al.*, 1976). This construct was sequenced according to the strategy shown in Figure 1. We did not observe any deviation from the tyrosinase sequences published by Terao *et al.* (1989) and Yamamoto *et al.* (1989). However, comparing the genomic sequences used here with the sequence of the cDNA clone described (Müller *et al.*, 1988) we noticed an Ile (ATC) to Ser (AGC) exchange at position 246 (Müller *et al.*, 1988), possibly due to microheterogeneity of reverse transcriptase generated cDNA clones. We also sequenced part of the chinchilla upstream region up to position −265 (data not shown) putatively including regulatory promoter elements and found no sequence difference to the published wild-type sequence (Yamamoto *et al.*, 1989). Thus we conclude that the introduced minigene encodes the information for wild-type (*C*) tyrosinase.

The construct was injected into fertilized eggs from the outbred albino strain NMRI. 297 injected embryos were transferred to both oviducts of seven pseudopregnant females; three of these became pregnant and gave rise to 21 offspring. At birth, five out of these had pigmented eyes. By Southern blot analysis of tail biopsies, six out of the 21 mice were shown to be transgenic (not shown). Whereas the five pigmented mice showed the expected 3.1 kb *Hin*dIII fragment on Southern blots, which is diagnostic for the transgene (see Figure 1), one unpigmented female (no. 15) showed an aberrant fragment pattern (not shown and was not analysed further.

One male (no. 37), which had very faint pigmentation in the skin, but also strong pigmentation in the eye, showed germline-soma mosaicism of the transgene. None of the 52 offspring of this mouse was transgenic. The other four pigmented mice all transmitted the transgene to their offspring (no. 18: 19 transgenic offspring out of 38 born; no. 19: 5 out of 8; no. 34: 13 out of 25; no. 35: 11 out of 35). About 10 copies had integrated in line no. 18, whereas ~5 copies were present in line no. 34 and one copy in line no. 35 (not shown). In founder female no. 19, however, at least two independent integration sites of the transgene were present. This was obvious from both Southern blot analysis and variations of coat colour among the offspring (not shown).

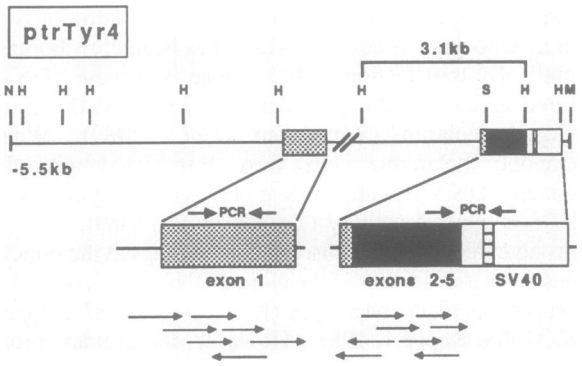

Fig. 1. Scheme of the tyrosinase minigene used for injection. The components of ptrTyr4 are as follows: thin lines, 5′ upstream sequences and first intron of the tyrosinase gene derived from genomic DNA of chinchilla mice (c^{ch}); stippled box, exon 1 and 60 bp of exon 2 of the tyrosinase coding sequence derived from chinchilla DNA (c^{ch}); black box, part of exon 2 and exons 3−5 of the tyrosinase cDNA isolated from B16 melanoma cells (*C*); open box, SV40 splice and polyadenylation site; striped box, 66 bp small T intron of SV40. The 3.1 kb *Hin*dIII fragment diagnostic for the transgene on Southern blots is indicated. The approximate location and orientation of the oligonucleotide primers used for PCR analysis of the tyrosinase gene (exon 1) and of the transgene-specific transcript (exon 5/SV40) are indicated. The sequencing strategy to determine the coding region in the minigene construct is shown below the indicated exons. Arrows depict the direction and extent of dideoxy sequencing reactions. H = *Hin*dIII; M = *Mlu*I; N = *Not*I; S = *Sau*I. The *Sau*I site (+959) was used for fusion of the genomic DNA and the cDNA and is not a unique site.

The coat colour of all these transgenic animals resembled a chinchilla-like phenotype (see below) on an agouti (*A*), black (*B*) genetic background (Figure 2) as was expected from the genetic status of the outbred albino strain NMRI at both the agouti locus and the *b*-locus (see Materials and methods). The various transgenic lines differed only with respect to the intensity of pigmentation. The most intense pigmentation was seen in line no. 18. Whereas animals of line no. 35 showed light pigmentation, transgenic offspring of line no. 34 were only slightly less pigmented than those of line no. 18. The following analyses of expression of the transgene were performed only on line no. 18.

Expression of the tyrosinase gene and the transgene in skin and eye

Very few melanocytes are present in skin; they each contain low amounts of tyrosinase (Hearing and Jimenez, 1989). The enzymatic activity of tyrosinase increases between 4 and 6 days after birth and declines thereafter (Movaghar, 1989). Therefore, we monitored expression of both the endogenous tyrosinase gene and the transgene in the eyes and skin of nontransgenic albino mice (NMRI) and the transgenic mouse line no. 18 by *in situ* hybridization on frozen sections of 4 day old mice. Expression of the transgene was further analysed with the polymerase chain reaction (PCR) of cDNA derived from several organs.

As shown in Figure 3A, tyrosinase mRNA is present in skin melanocytes of nontransgenic NMRI (i.e. albino) mice. Tyrosinase mRNA is only detected in the lower parts of the hairbulbs, where the melanocytes are located (Silvers, 1979). The finding that the tyrosinase gene in NMRI mice is transcribed is in agreement with recent experiments describing tyrosinase RNA in another albino mouse strain, suggesting that the albino mutation does not affect levels of tyrosinase mRNA (Tamate *et al.*, 1989). As expected, no signals were detected using a transgene specific probe (pSV-H) prepared from the SV40 splice and polyadenylation signal (not shown). This probe was proven to be transgene-specific by hybridization to Southern blots of DNA from transgenic and nontransgenic animals (not shown).

The same specific grain distribution seen in the albino mouse was observed when successive skin sections of a 4 day old transgenic mouse (line no. 18) were hybridized with both the transgene-specific probe (Figure 3C) and the tyrosinase probe (Figure 3D). As expected, transcription of the transgene was restricted to the interior of the root sheath at the lower part of the hairbulb. This represents the prospective location of melanocytes. The perfect concordance between sites of expression revealed by the transgene-specific (Figure 3C) and tyrosinase probes (Figure 3B,D) in transgenic mice and the tyrosinase probe in control

mice (Figure 3A) indicates correct expression of the transgene.

The second location of pigmented cells is the eye. Pigmentation is found in two cell types, the pigmented epithelial cells derived from the optic cup and the melanocytes of the uvea derived from the neural crest (McKay, 1989; Morris-Kay and Tan, 1987; Wheater *et al.*, 1987). As shown by hybridization with the tyrosinase probe, tyrosinase mRNA is restricted to the same cell layers in both the albino mouse (Figure 4A) and the transgenic mouse (Figure 4B). When sections of the transgenic mouse were hybridized to the transgene specific probe, transcripts were only detected in those cell layers that also showed expression of the endogenous tyrosinase gene (Figure 4D). In the non-transgenic mouse, no specific signals were observed with this probe (Figure 4C). Moreover, expression from the transgene has resulted in the production of pigment (Figure 4B and D) in the proper location. Thus, the expression of the transgene in hairbulbs and the pigmented cell layers of the eye suggests that the minigene (Figure 1) contains sufficient information (*cis*-elements) for cell type-specific expression.

To investigate the expression of the transgene in various tissues, we used *in vitro* amplification of cDNA by the polymerase chain reaction (Chelly *et al.*, 1989; Schlissel and Baltimore, 1989). cDNA was synthesized from total RNA via reverse transcription using oligo(dT) as a primer. For amplification, two primers were chosen, one in exon 5 of the tyrosinase gene and one in the SV40 polyadenylation site 3′ of the SV40 intron (Figure 1; see Materials and methods), which enabled us to distinguish between amplified products of DNA (including the 66 bp small T intron; 515 bp; Figure 5) and amplified cDNA (without the 66 bp intron; 449 bp; Figure 5). The products were analysed on an agarose gel and visualized by staining with ethidium bromide. To confirm the transgene-specificity of the PCR product, the gel was analysed by Southern blot hybridization to the transgene-specific probe pSV-H.

The results shown in Figure 5 were obtained with 24 cycles of PCR. The highest levels of amplified transcripts were observed in samples of eye and skin (Figure 5A). Also, transgene specific transcripts were found in brain, and at a much lower level in several tissues analysed (Figure 5B). In samples from organs of nontransgenic NMRI mice, run in parallel, no amplified products were observed (Figure 5), even following 30 cycles of amplification (not shown). Since only a minor part of the cells in the skin and the eye are melanocytes, transcription of the transgene in other organs is orders of magnitudes lower.

Although the introduced minigene construct ptrTyr4 (Figure 1) codes for wild-type (*C*) tyrosinase as evidenced by DNA sequence analysis, this construct, when introduced into an albino mouse strain, rescued the albino phenotype by producing a chinchilla-like coat colour (Figure 2). This coat colour could be due to a low enzyme activity caused by an altered expression of the transgene. Therefore, we analysed the expression of both the transgene and the endogenous tyrosinase gene in skin and eye by quantitative PCR. We used those primers shown in Figure 1 that detect only the transgene specific transcript (449 bp), and a pair of primers located in the first exon which detect transcripts of both the transgene and the endogenous tyrosinase gene (310 bp; Figure 1 and Materials and methods). As shown in Figure 6, expression of the transgene in both skin and eye is similar to the expression of the endogenous tyrosinase gene. These results suggests that either the presumptive lowered expression is in a range not detectable by the PCR technique or that lowered enzyme activity has to be attributed to a reduced translation efficiency e.g. due to the incorporation of SV40 derived sequences. That intensity of coat colour is indeed correlated with amount of enzyme is demonstrated by comparing mice homozygous and hemizygous for the transgene (Figure 7).

The chinchilla tyrosinase differs by only one amino acid substitution from the wild-type tyrosinase

We were surprised that we had obtained a chinchilla-like phenotype with the normal tyrosinase coding region. Since it had previously been documented that the chinchilla phenotype is not due to altered expression of the tyrosinase gene and since furthermore an increased susceptibility to proteolytic degradation of the chinchilla protein had been observed, Halaban *et al.* (1988) suggested that this phenotype is caused by a structural gene mutation. In order to test this hypothesis, we sequenced the coding sequences of the entire chinchilla allele and found an amino acid exchange at position 464 (Müller *et al.*, 1988) as shown:

wild-type:	GGG	GCA	GCA	CTG	GTG
	Gly –	Ala –	Ala –	Leu –	Val
chinchilla:	GGG	GCA	ACA	CTG	GTG
	Gly –	Ala –	**Thr** –	Leu –	Val

Since no other change in the entire coding region (or the putative promoter region) has been detected and since Halaban *et al.* (1988) had detected an increased susceptibility of the chinchilla tyrosinase towards proteolytic degradation the conclusion is compelling that this substitution is the basis for the chinchilla mutation.

Discussion

A functional tyrosinase gene rescues the albino mutation (c/c)

In the past, several cDNA clones have been isolated as candidates for human and mouse tyrosinase (Shibahara *et al.*, 1986; Yamamoto *et al.*, 1987; Kwon *et al.*, 1987, 1988; Ruppert *et al.*, 1988; Terao *et al.*, 1989). These clones differed from one another in structure or sequence. One of them, pMT4 (Shibahara *et al.*, 1986), encodes a tyrosinase-related protein (Müller *et al.*, 1988) and maps to the brown locus (Jackson, 1988). The clone pmcTyr1 (Ruppert *et al.*, 1988), however, was able to confer tyrosinase enzymatic activity to tyrosinase-negative cell lines, when tested by transient transfection (Müller *et al.*, 1988) and, furthermore, maps to the *c*-locus (see below).

The *c*-locus on mouse chromosome 7 was for a long time thought to affect pigmentation reactions by controlling the activity of tyrosinase (Russell and Russell, 1948; Coleman, 1962). It was not clear whether the albino locus encoded a regulator for tyrosinase (Hearing, 1973; Pomerantz and Li, 1974) or the structural gene for tyrosinase (Silvers, 1979). Several recent findings are relevant: firstly, the mouse tyrosinase cDNA clone pmcTyr1, which encodes a protein with sequence homology to other tyrosinase proteins, confers

Fig. 2. Coat colour of transgenic mice. The photograph shows a nontransgenic albino mouse (NMRI) and a transgenic mouse (line no. 18). The introduction of a functional tyrosinase gene rescues the albino mutation and results in a chinchilla-like coat colour on an agouti, black genetic background.

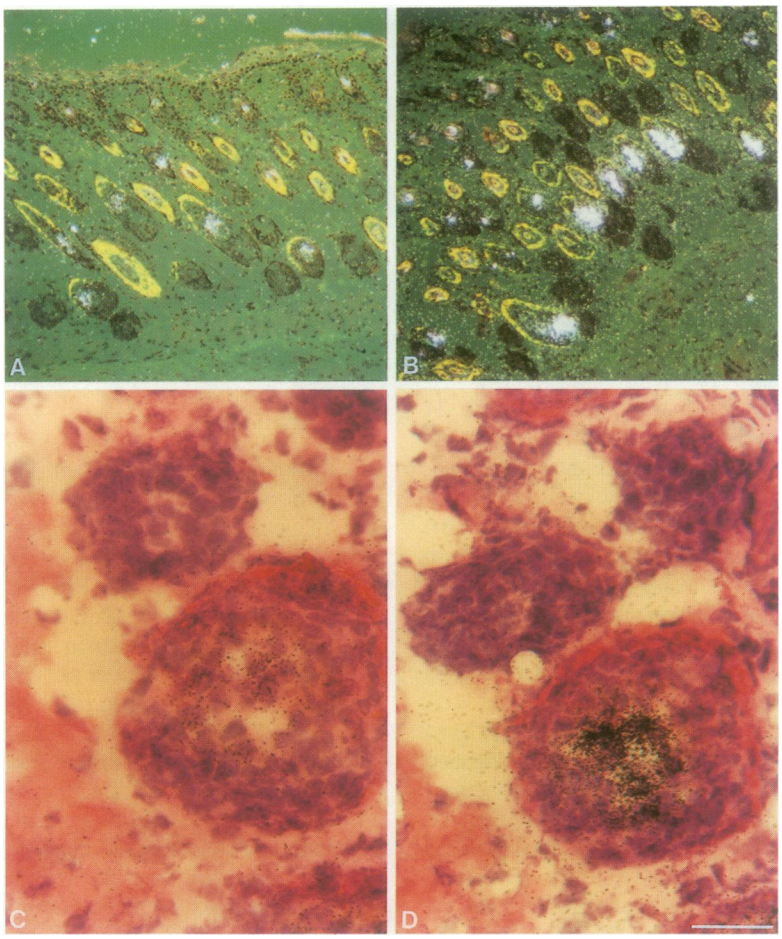

Fig. 3. The transgene is specifically expressed in melanocytes of the skin. *In situ* hybridization was performed on sections from skin of 4-day-old transgenic and nontransgenic mice. (**A**) Tyrosinase gene transcripts detected in hairbulbs of a nontransgenic albino NMRI mouse (dark field photograph). (**B**) Tyrosinase gene and transgene transcripts in hairbulbs of a transgenic mouse (line no. 18). Note that localization of mRNA is restricted to the interior of the root sheath (yellow staining) at the lower part of the hairbulb (dark field photograph). (**C,D**) Bright field photographs in higher magnification show specific hybridization of successive sections from hairbulbs of the transgenic mouse to a transgene-specific-probe (C) and a tyrosinase gene-specific probe (D). Note that the tyrosinase gene-specific probe detects both endogenous and transgene transcripts. The bar represents 120 μm (A,B) or 20 μm (C,D).

tyrosinase activity to a breast cancer cell line with no specialized functions for melanin synthesis (Müller *et al.*, 1988). Secondly, the genomic DNA sequences hybridizing to a human tyrosinase cDNA clone and to pmcTyr1 are absent in mice carrying radiation-induced deletions at the *c*-locus (e.g. c^{14Cos}/c^{14Cos}; Kwon *et al.*, 1987; Ruppert *et al.*, 1988) including the balanced heterozygote c^{6H}/c^{14CoS} which shows no phenotype beyond albinism (Ruppert, 1988). However, BALB/c mice indicate no

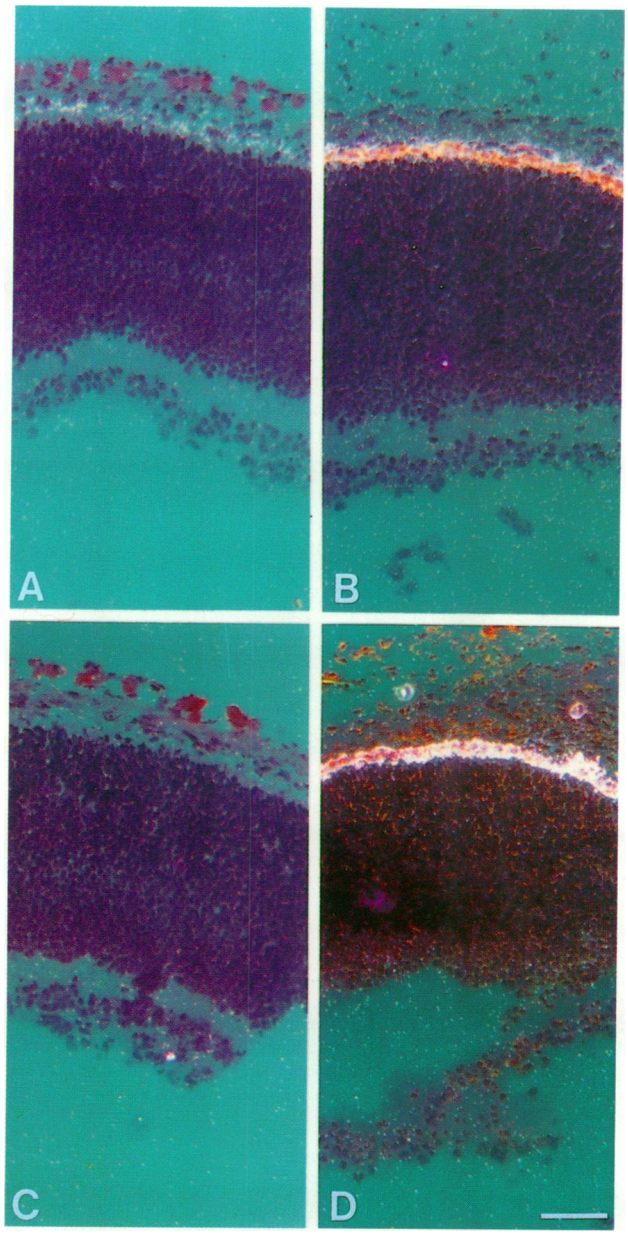

Fig. 4. Localization of tyrosinase mRNA and transgene mRNA in mouse eyes. Sections from a nontransgenic NMRI (albino) mouse (**A,C**) and from a transgenic mouse of line no. 18 (**B,D**) were hybridized to ^{32}P-labelled riboprobes as described in Materials and methods. Due to the presence of pigment in B and D, the dark field photographs give rise to non-specific illumination which can, however, be discriminated from the specific silver grain signal using colour photography. (**A,B**) Detection of tyrosinase transcripts in the NMRI (A) and the transgenic (B) mouse. (C) In the eye of the NMRI mouse, no hybridization was obtained using the transgene-specific probe (pSV-H). (D) In the eye of the transgenic mouse, transgene-specific transcripts were detected in the cell layers that are pigmented. The bar represents 50 μm.

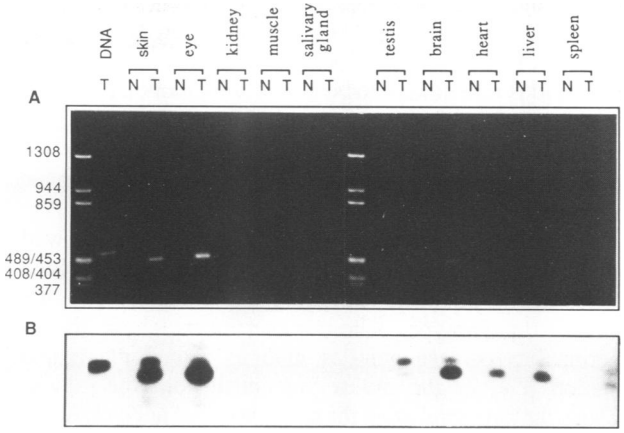

Fig. 5. Detection of transgene-specific transcripts. Several tissues of mice from transgenic line no. 18 were analysed for expression by the polymerase chain reaction of cDNA synthesized from total RNA. Amplified products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. Compared to genomic DNA (515 bp), amplified cDNA results in a shorter fragment (449 bp) due to the splicing of the 66 bp intron of SV40 (see Figure 1 and Materials and methods). The size of the marker (pTATCAT digested with *Hpa*II; Jantzen *et al.*, 1987) is indicated in bp. N = nontransgenic NMRI mouse; T = transgenic mouse (line no. 18).

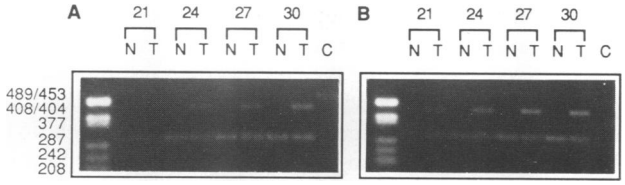

Fig. 6. Detection of tyrosinase and transgene specific transcripts by PCR. Expression of both tyrosinase and transgene specific transcripts in skin (**A**) and eye (**B**) was analysed in parallel by PCR on cDNA using two pairs of primers. Primer pair I detects transgene specific transcripts (449 bp; see Figure 5 and Materials and methods), primer pair II detects endogenous and transgenic tyrosinase transcripts (310 bp; see Materials and methods). PCR products were analysed after 21, 24, 27 and 30 cycles on an agarose gel. The size of the marker (pTATCAT digested with *Hpa*II; Jantzen *et al.*, 1987) is indicated in bp. N = nontransgenic NMRI mouse; T = transgenic mouse (line no. 18); C = transgenic genomic DNA amplified using the transgene specific primers (515 bp; see Figure 5 and Materials and methods).

Fig. 7. A homozygous transgenic mouse shows a darker coat colour than its hemizygous transgenic littermate (line no. 18).

rearrangements of the tyrosinase gene in genomic digests (Ruppert, 1988), and tyrosinase mRNA is detected in other *c/c* mice (Tamate *et al.*, 1989). Therefore, the classical *c*-mutation is most likely to be a point mutation. To test the hypothesis that the *c*-locus encodes the structural gene for tyrosinase, the *c*-mutation was rescued by generating transgenic mice carrying a functional tyrosinase minigene (Figure 1).

Five transgenic mice were obtained which showed pigmentation in skin and eyes. Four of these mice transmitted the transgene to their offspring, which inherited the pigmented phenotype. These experiments show that a functional tyrosinase gene is able to rescue the albino mutation (*c/c*) of the mouse. In conclusion, the *c*-locus encodes the structural gene for tyrosinase and not a regulator for tyrosinase expression.

The coat colour of the transgenic animals resembles that of the *c*-locus allele chinchilla on an agouti, black genetic background. The c^{ch} mutation reduces the production of phaeomelanin (yellow pigment) and slightly reduces that of eumelanin in the hair (black pigment; Silvers, 1979). However, the basic defect for this mutation is not known. Since recent investigations showed that the levels of tyrosinase mRNA in chinchilla mice and even in albino mice (*c/c*) are equal to that of wildtype (*C/C*) mice (Halaban *et al.*, 1988; Tamate *et al.*, 1989), it was suggested that the reduced pigmentation in chinchilla mice (c^{ch}/c or c^{ch}/c^{ch}) is due to a weaker activity of a mutated protein (Halaban *et al.*, 1988). This hypothesis is strongly supported by the observation that the enzyme isolated from chinchilla mice is more susceptible to proteolysis. Sequencing the entire coding information of the genomic tyrosinase gene derived from the chinchilla allele we detected a single amino acid exchange at position 464 (Müller *et al.*, 1988) changing Ala (GCA) in the wildtype sequence (Müller *et al.*, 1988; Terao *et al.*, 1989; Yamamoto *et al.*, 1989) to Thr (ACA). We therefore wish to suggest that this amino acid exchange is the nature of the chinchilla mutation leading to an altered enzymatic activity. Since we also sequenced the entire coding information of the transgene (Figure 1) without detecting this alteration or any other, we conclude that two different mechanisms may cause a chinchilla phenotype: a mutant protein with lowered enzyme activity but normal expression as in the chinchilla mutation, or a diminished level of normal enzyme as in our transgenic mice. This conclusion is corroborated by the sequence analysis of the putative promoter of the chinchilla allele. We did not find any sequence difference between this and the promoter of the wildtype allele, thus ruling out the possibility that the classical chinchilla mutation is a promoter down-mutation.

The different alleles at the *c*-locus (e.g. chinchilla) lead to lower tyrosinase activity and to lighter pigmentation in heterozygotes with the albino allele (e.g. c^{ch}/c) when compared to the respective homozygous situation (e.g. c^{ch}/c^{ch}) (Silvers, 1979). This characteristic of the chinchilla mutation is also reflected by the transgene. When homozygous transgenic animals were produced, the coat colour was much darker than that of their hemizygous transgenic littermates (Figure 7).

Cell type-specific expression of the tyrosinase transgene

In order to study cell type-specific expression of the transgene, we performed *in situ* hybridization on tissue sections of skin and eyes from both transgenic (line no. 18) and nontransgenic NMRI mice. These *in situ* hybridizations showed that the introduced tyrosinase minigene is expressed in a cell type-specific manner that precisely overlaps with the expression of the endogenous gene. This is confirmed by the analysis of cDNA-PCR amplified transgenic transcripts, where expression in the pigmented cells of skin and eyes is orders of magnitude higher than in other tissues.

All elements necessary to ensure correct transcription in pigmented cells appear to be contained in the introduced construct. The finding that expression of tyrosinase in skin and eyes is restricted to melanocytes suggests the possibility of using a tyrosinase construct as a neural crest cell marker (Anderson, 1989). Because expression of the tyrosinase gene is easily detectable by the pigmented phenotype, this construct might also be used as a coinjected marker for production of transgenic animals thereby circumventing DNA blot analyses. In development, pigmentation of the eye starts much earlier (e.g. at day 12 of gestation in the rat; Rothman *et al.*, 1980) than in the skin. This developmentally regulated gene expression might therefore require different elements or different *trans*-acting proteins. Since the construct (Figure 1) is expressed in both eyes and skin, we can now start to analyse the developmental onset of expression from this construct and then define regulatory elements for the cell type-specific expression.

Materials and methods

Mice

NMRI mice were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, FRG) or from Charles River Wiga (Sulzfeld, FRG). They were then bred and maintained on a constant light–dark cycle (with darkness lasting from 7 p.m. to 5 a.m.) at the animal house of the German Cancer Research Center, where the genetic manipulation of mice was performed.

Since the experiments were done in an outbred strain, we determined the genetic status of this strain at the agouti locus and *b*-locus to exclude any *trans*-acting effect of the transgene on these loci. The genotype for the agouti locus was determined for offspring of line no. 18 by breeding to C57BL/6J mice (*a/a; B/B; C/C*). Analysis of the coat colour (agouti or non-agouti) in offspring demonstrated that mice of line no. 18 are agouti (*A/a* or *A/A*), but not non-agouti (*a/a*). This was then confirmed by use of the molecular probe p15.4 which reveals a diagnostic restriction fragment length polymorphism on Southern blots of genomic DNA digested with *Hin*dIII (Siracusa *et al.*, 1987). At the *b*-locus, NMRI mice had the wildtype allele (*B/B*), as was evident by a diagnostic *Taq*I polymorphism specific for the *b*-locus (Jackson, 1988). Hybridizing the plasmid pMT4 (Shibahara *et al.*, 1986), which maps to the *b*-locus (Jackson, 1988), to *Taq*I digested DNA, we observed the 1.2 kb and 3.7 kb fragments characteristic for the *B*-genotype.

Construction of plasmids

To construct ptrTyr4, 5′ upstream sequences, exon 1, intron 1 and 60 bp of exon 2 were derived from genomic DNA derived from chinchilla mice and isolated by different cloning steps from λgTYR101 (Ruppert *et al.*, 1988). They were inserted into ptrTyr4 as a 14 kb *Sal*I/*Sau*I fragment and fused to part of exon 2 and exons 3–5 isolated from the tyrosinase cDNA as a *Sau*I/*Spe*I (+959 to +1760) fragment of pHDmcTyr1 (Müller *et al.*, 1988). Polylinker sequences (54 bp; Bluescript, Stratagene) were added to the *Spe*I site; the SV40 splice and polyadenylation sequences were then fused to the *Acc*I site in the polylinker as an *Hpa*II/*Eco*RI (0.85 kb) fragment of pHDmcTyr1. The minigene was cloned in a modified Bluescript vector (Stratagene) containing rare cutting sites and was recovered as a 15.6 kb *Not*I/*Mlu*I fragment. Further details of the construction are available on request.

Plasmids used as probes were as follows: mouse tyrosinase cDNA clones pmcTyr54 and pmcTyr63 (Ruppert *et al.*, 1988). pSV-H was made by subcloning the 0.85 kb *Hin*dIII fragment containing the SV40 splice and

polyadenylation site of ptrTyr4 in Bluescript (Stratagene) and was used to generate a transgene-specific riboprobe. The plasmid p15.4 was used to discern the alleles at the agouti locus (Siracusa *et al.*, 1987). The insert of pMT4 (Shibahara *et al.*, 1986) was used to genotype NMRI mice at the *b*-locus (Jackson, 1988).

DNA sequencing

The tyrosinase encoding sequences of ptrTyr4 were sequenced by using the dideoxy chain termination method (Sanger *et al.*, 1977) with modifications made for sequencing double-stranded plasmid DNA (Chen and Seeburg, 1985). Tyrosinase specific primers deduced from the cDNA sequence by Müller *et al.*, (1988) were used for sequencing with sequenase (United States Biochemical Corporation).

Microinjection

The plasmid ptrTyr4 was digested with *Not*I and *Mlu*I and the fragment containing the minigene was isolated from an agarose gel. It was further purified on an ElutipD column (Schleicher and Schuell) followed by dialysis against injection buffer (10 mM Tris, pH 7.5, 0.25 mM EDTA) on dialysis filters (Millipore). Procedures for generation of transgenic mice followed methods described previously (Beermann *et al.*, 1988; Beermann, 1989; Hummler, 1989). The DNA (1–2 ng/μl) was injected into one of the pronuclei of fertilized eggs. After injection, embryos were surgically transferred to both oviducts of pseudopregnant NMRI females which carried them to term.

DNA analysis

Transgenic offspring were identified by Southern blot analysis of DNA which was extracted from tails of 3- to 4-week-old mice (Hogan *et al.*, 1986). The DNA was digested with the respective restriction enzyme, electrophoresed in 0.9% agarose gels and transferred to nylon membranes. The DNA was hybridized to ^{32}P-labeled *in vitro* transcribed RNA probes (Melton *et al.*, 1984) or ^{32}P-labeled DNA probes generated by random priming (Feinberg and Vogelstein, 1983). Conditions for hybridization and washing were as described (Ruppert *et al.*, 1988).

In situ hybridization

Hybridization was performed (by F.X.B.) essentially as described (Leube *et al.*, 1986; Bosch *et al.*, 1989). Skin and eyes of both nontransgenic and transgenic littermates were isolated and immediately shock frozen in isopentane/liquid nitrogen. 5 μm sections were fixed in 4% paraformaldehyde, dehydrated, treated with proteinase K and acetylated. Prehybridization was done for 1–2 h at 50°C in 50% formamide and 2 × SSPE. The ^{32}P-labeled antisense RNA probe (generated from pmcTyr54 or pSV-H; see above) was partly hydrolysed by mild alkaline treatment (Cox *et al.*, 1984) and 2 × 10^6 d.p.m. (~1 ng RNA) per section were added to the hybridization solution (50% deionized formamide, 10% dextran sulphate, 2 × SSPE, 0.1% SDS, 1 × Denhardt's solution, 0.5 mg/ml tRNA and 0.1 mg/ml denatured salmon sperm DNA). Hybridizations were performed in moist chambers at 50°C for 5–7 h or overnight. First washing was performed in 50% formamide and 2 × SSPE (2 h at 37°C). The sections were then equilibrated in 2 × SSC and 0.1% SDS for 10 min and digested with 50 μg/ml RNase A for 30 min at 37°C. The final washing was done in 50% formamide and 0.1 × SSC for 30 min at 37°C. The sections were dehydrated in ascending ethanol concentrations in the presence of 0.3 M ammonium acetate and dipped in Kodak NTB 2 emulsion. After 1–2 weeks exposure, the sections were developed and stained with haematoxylin and eosin.

Analysis of expression by PCR of cDNA

RNA was extracted from tissues by homogenization in guanidinium isothiocyanate followed by centrifugation on a CsCl gradient (Davis *et al.*, 1986). Single-stranded cDNA was prepared from 10 μg of total RNA using reverse transcriptase (Boehringer) primed with oligo(dT) (Ruppert, 1988; Ruppert *et al.*, 1988). For the amplification of the transgene specific transcripts, the following oligonucleotides were used: one oligonucleotide (5′-GAGCCTTACTTGGAACAAGCC-3′) is located at position +1475 of the tyrosinase sequence (Müller *et al.*, 1988). The second oligonucleotide (5′-CTGCTCCCATTCATCAGTTCC-3′) is derived from the SV40 small T antigen and is located at position +4522 of the published sequence (from Buchman *et al.*, 1980). Since the small T intron is spliced out, a 449 bp fragment or a 515 bp fragment are generated, specific for RNA or DNA, respectively. To amplify transgenic and endogenous tyrosinase sequences, two oligonucleotides, 5′-CAGGCAGAGGTTCCTGCCAG-3′ (position +231 to +250; Müller *et al.*, 1988) and 5′-GTGGGGATGACATAG-ACTGA-3′ (position +520 to +540; Müller *et al.*, 1988) were used, giving rise to a 310 bp fragment. One tenth of the cDNA reaction or 2 μg of genomic DNA was subjected to PCR using 50 pmol of each oligomer. PCR assays were done in a final volume of 100 μl buffer consisting of 16.6 mM ammonium sulphate, 67 mM Tris (pH 8.8), 5.0 mM $MgCl_2$, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 1.7 mg/ml BSA, 10% (v/v) dimethyl sulphoxide and 0.5 mM dNTP (dATP, dCTP, dGTP and dTTP). 2 U of *Taq*I polymerase (Cetus) were added. Cycles, repeated up to 30 times, consisted of 1 min denaturation at 92°C, 1 min annealing at 55°C and 2.5 min polymerization at 72°C and a final 9.9 min incubation at 72°C to ensure complete elongation. After 15, 18, 21, 24, 27 and 30 cycles, 15 μl of each reaction were taken out. 8 μl of each reaction were electrophoresed through a 1.5% agarose gel stained with ethidium bromide. The gels were blotted to nylon membranes and hybridized to a labeled transgene-specific probe (generated from pSV-H).

Acknowledgements

We thank Dr Nancy Jenkins for providing the probe p15.4, Cilly Kuhn and Sabine Stumpp for cryostat sections, Drs Gavin Kelsey and Hans Weiher for their critical reading of the manuscript, Werner Fleischer and Dr Bruno Luckow for photographic work, Cornelia Schneider for help with the manuscript and Angelika Frenznik, Daniela Frey and Achim Hickel for animal care taking. The work was supported by the Deutsche Forschungsgemeinschaft through SFB 229 and the Leibniz Programm and the Fonds der Chemischen Industrie.

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Received on November 27, 1989; revised on May 17, 1990