A distal tyrosinase upstream element stimulates gene expression in neural-crest-derived melanocytes of transgenic mice: position-independent and mosaic expression

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SUMMARY

We have assessed the importance of a melanocyte-specific DNase I hypersensitive site and matrix attachment region situated 15 kb upstream of the mouse tyrosinase gene by analysis in transgenic mice. Transgenes containing all, part, or none of this region linked to the tyrosinase promoter and human tyrosinase cDNA were introduced into genetically albino mice, and pigmentation and transgene message levels were analyzed in the resulting transgenic lines. The effect of the upstream region was to enhance significantly gene expression in melanocytes, and to provide position-independent expression of the transgene. Two exceptions to complete position independence were seen; these lines displayed a mosaic expression pattern in which the transgene was expressed fully in some melanocyte clones but less so in others, resulting in transverse stripes of colours ranging from near white to dark grey. Unexpectedly, pigmentation in the eye of all transgenic lines containing the upstream region was non-uniform, in that the neural-crest-derived melanocytes of the choroid and anterior iris contained significantly more pigment than those derived from the optic cup (retinal pigment epithelium and posterior iris). Transgenes containing a small part or none of the upstream region were expressed poorly and in a position-dependent manner; of those lines that were visibly pigmented, expression was equal in the neural crest and optic-cup-derived cells of the eye. Mice with transgenes containing DNA sequences encompassing the hypersensitive site but lacking most of the matrix attachment region were, on average, poorer expressors than those containing the entire upstream region; the highest expressing line of this series, however, had a pigmentation level similar to the lines with the full upstream region. Thus, full transcriptional enhancement activity may lie within the segment containing the hypersensitive site, but position-independent expression may require the flanking matrix attachment region containing sequences.

Key words: melanocytes, tyrosinase, transgenic mice, mosaic, neural crest

INTRODUCTION

Tyrosinase is the key enzyme of melanin biosynthesis. Its gene is expressed in all pigmented melanocytes, including those of neural crest origin (residing in the skin, choroid and internal organs) and the optic-cup-derived melanocytes of the retinal pigment epithelium (RPE). We are interested in defining the elements required for the establishment of an active tyrosinase chromosomal domain in melanocytes, to understand better the mechanisms involved in this aspect of gene regulation, as well as to help in the identification of trans-acting factors important in melanocyte-specific gene expression and differentiation.

The identification of cis-acting sequences important in higher order gene control has been approached primarily by the functional analysis of candidate sequences in transgenes randomly integrated into the genome of mice or cultured cells. High level expression of the genes, which is proportional to the number of copies integrated, is considered to indicate that sequences required for autonomous establishment of an open chromatin structure and for insulation of the transgene from neighbouring chromatin or regulatory elements, are included in the construct. The first of such elements isolated was the locus control region (LCR) of the β-globin gene cluster (Grosveld et al., 1987), and elements with similar properties from a number of other genes have been identified (Greaves et al., 1989; Abe and Oshima, 1990; Bonifer et al., 1990; Greer et al., 1990; Chamberlain et al., 1991; Aronow et al., 1992).

Studies of the tyrosinase gene regulatory elements using transgenic mice have shown that 270 bp of the promoter are sufficient for expression in skin and eye melanocytes (Beermann et al., 1992) and that the expression of the transgene, although lower than that of the endogenous gene, roughly recapitulates its developmental timing. Position independence of expression in this and in other studies using longer tyrosinase promoters (up to 6 kb) with the tyrosinase or heterologous cDNAs (Tanaka et al., 1990; Yokoyama et al., 1990;
Bradl et al., 1991a; Klüppel et al., 1991) was not specifically addressed; however, visual analysis of coat color suggested that expression levels often did not correspond to copy number. In addition, some transgenic lines (non-founders) displayed a mosaic expression in which melanocyte clones in the coat were pigmented to different degrees (Bradl et al., 1991b; Takeuchi et al., 1993), clearly a variegating type position effect. The three analyzed transgenic lines derived from microinjection of a yeast artificial chromosome (YAC) encompassing 235 kb of the tyrosinase gene (80 kb of the coding region and 155 kb of upstream sequences) showed position-independent transgene expression at levels comparable to that of the endogenous gene (Schedl et al., 1993), suggesting that all elements necessary for higher order gene control are located within these limits.

A sequence at 15 kb upstream from the first exon of the tyrosinase gene is hypersensitive to DNase I in melanocytes and has in vitro nuclear matrix attachment activity (Porter et al., 1991). It is located within a region removed by rearrangement from the rest of the tyrosinase gene in the variegating mutation of the tyrosinase locus, chinchilla-mottled (c<sup>m</sup>) (Porter et al., 1991). This autosomal, somatically stable mutation results in light and dark grey stripes on the coat of homozygous mice, which are accounted for by altered tyrosinase mRNA levels (a roughly 10-fold difference between the light and darkly pigmented cells, and between the dark c<sup>m</sup>c<sup>m</sup> and wild-type melanocytes). The difference in gene expression between the light and dark cells could be attributed to alternate chromatin conformations of the tyrosinase locus. Thus, the rearrangement of the tyrosinase gene causes aberrant chromatin formation in some clones and reduced expression in all clones (even those with apparently normal chromatin structure). The –15 kb region was hypothesized to encompass a potential regulatory element important in transcriptional enhancement of the tyrosinase gene and/or in the establishment of open chromatin, as it was the only DNase I hypersensitive site within 20 kb of the promoter that was separated from the gene in the c<sup>m</sup> mutation. That all sequences required for full expression and open chromatin conformation were not present in the immediate vicinity of the coding sequence was concluded independently from the transgenic experiments discussed above.

To address the hypothesis that this region is important in tyrosinase gene control in melanocytes, we generated transgenic mice containing the tyrosinase promoter, human tyrosinase cDNA, and part or all of this –15 kb region. We found that the region imparted high level, position-independent expression in all lines except two, which showed variegating position effects. In addition, we found the region to enhance tyrosinase expression in neural-crest-derived melanocytes, but not in optic-cup-derived melanocytes, indicating the existence of distinct tyrosinase regulatory elements for these two developmental lineages. The sequence was also shown to be hypersensitive in melanoma cells, but not in neuroblastoma, liver or brain cells.

**MATERIALS AND METHODS**

**DNase I hypersensitivity analyses**

Cell cultures of Neuro-2a and Cloudman S91 (clone M-3) melanoma (both from ATCC) were propagated as recommended by the ATCC. Near confluent cultures were harvested by scraping, and nuclei were isolated and subjected to incremental DNase I (Gibco BRL) digestions as previously described (Porter et al., 1991).

Brain and liver were obtained from BALB/c mice. The tissues were minced, washed 3 times in ice-cold SSC/10 mM Tris-HCl pH 7.5 and homogenized in a dounce homogenizer. Nuclei were isolated (after passage through gauge) and DNase I digestions were carried out as above.

For Southern analysis, DNA was digested with EcoRI, separated by gel electrophoresis, blotted to Nytran (Schleicher and Schuell) and probed according to the manufacturer’s directions.

**Transgenic mice**

All DNA constructs used to generate transgenic mice contain the mouse tyrosinase promoter and human tyrosinase cDNA, and some contain sequences from the –15 kb region of the mouse tyrosinase gene. The promoter was derived from AgTYR101, and the –15 kb region from AgTYR14 (Ruppert et al., 1988). These genomic fragments were derived from the chinchilla allele, which contains a point change in the coding region (Beermann et al., 1992) and exhibits no apparent alteration in the transcript size or level (Halaban et al., 1988). The human cDNA, which includes a polyadenylation signal, was derived from BBTY-1 (Bouchard et al., 1989). The promoter and cDNA in all constructs are identical and consist of a 2.5 kb EcoRI-Sau3A3 segment of the immediate upstream region of the tyrosinase gene (the Sau3A site is situated 65 bp downstream of the major transcription initiation site; Ruppert et al., 1988; Yamamoto et al., 1989) fused through a 21 bp polynucleotide to the EcoRI fragment of BBTY-1. For UPT, a 3.6 kb EcoRI-HindIII fragment encompassing the –15 kb hypersensitive site was ligated to the EcoRI site (blunt-ended) of the promoter, and for 3PT, a 1 kb AvaII-SstI subfragment of the 3.6 kb fragment was blunt-end ligated to the promoter. 2PT was derived directly from UPT by cutting the final construct at the 5′ end with SstI (which removes all sequences from the 3.6 kb fragment except the 3′ terminal 300 bp). Constructs were digested with XhoI to release the 3′ end of the construct from the vector, and either Smal (UPT), BamHI (PT), or Srl (3PT and 2PT) to release the 5′ end. The DNA was gel-purified by Geneclean (Bio 101) for injection.

Fertilized mouse oocytes were obtained from superovulated BALB/c females crossed with BALB/c males (for TgUPT5, 6 and 7) or CD-1 males (all remaining lines), and were cultured, injected and implanted using standard methods (Hogan et al., 1986). Positive founders were initially identified by PCR analysis of tail DNA samples from all mice developed to term.

**Copy number determination**

Tail DNA samples from the progeny of UPT founder mice were digested with EcoRI, and analyzed by Southern blotting using the 3.6 kb EcoRI-HindIII fragment as probe. This gives a 6.1 kb transgene-specific band and a 3.6 kb band from the endogenous sequence (corresponding to two copies). Additional bands were apparent in most samples, presumably corresponding to partial truncations. DNA from the other lines was digested with PstI (which cuts once in the construct) and probed with the tyrosinase promoter sequence, which recognizes a 12 kb endogenous fragment, a band corresponding to the full length of each construct representing head-to-tail tandem integrations, and at least one other band representing the terminal transgene copy and separately integrated copies. DNA was blotted onto Nytran and hybridized with random primer 32P-labelled DNA probes. Blots were exposed to either preflashed X-ray film at –70°C with intensifying screens, or to non-flashed film without screens. The autoradiograms were quantitated using a Zeineh hand-held scanning densitometer (AAB).

**Quantitation of transgene expression**

RNA was isolated from the dorsal skin of 7-8 day transgenic offspring of founders crossed with BALB/c mice by the method of Chom-
czynski and Sacchi (1987), and 50 μg of each sample were used for analysis by RNase protection. RNA probes used for the assays were radiolabelled with [32P]CTP (using an RNA transcription kit, Stratagene), and corresponded to the antisense strand encompassing the 3′ terminal 275 bp (HaeIII site to terminus) of the human tyrosinase cDNA (BBTY-1) including 65 bp of vector sequence (39 of which are also present at the 3′ end of the transgene) and, as an internal control, the antisense 3′ terminal 188 bp (TaqI site to terminus) of the mouse tyrosinase cDNA (Terao et al., 1989).

Assays were carried out by standard methods (Ausubel et al., 1993), using approximately 10,000 cts/minute of each probe simultaneously. Products of the RNase digestions were separated by denaturing gel electrophoresis (6% polyacrylamide/7 M urea), and autoradiograms were prepared and quantitated as described for the copy number determination analyses. An average of the ratio of the human tyrosinase protected fragment (transgene) to that of the mouse (endogenous) was taken of two separate assays and normalized to line UPT3 (1 transgene copy).

Histology
All tissue samples were fixed in 10% buffered formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin for microscopic examination.

RESULTS

Cell type specificity of the −15 kb hypersensitive site
The −15 kb region of the tyrosinase gene was examined in a number of cell types for hypersensitivity to DNase I. As seen in Fig. 1, neither liver, nor brain nor the neuroblastoma cell line Neuro-2a was hypersensitive at −15 kb. The pigmented melanoma cell line Cloudman S91 (clone M-3) was hypersensitive at this point, as expected.

Effect of the −15 kb region on expression patterns in transgenic mice
The constructs used to generate transgenic mice are illustrated schematically in Fig. 2. Each transgene contains the identical promoter fragment (2.5 kb upstream from the transcription initiation point of the mouse tyrosinase gene) and human tyrosinase cDNA/polyadenylation signal. The human tyrosinase cDNA was used as a reporter gene to allow the level and uniformity of transgene expression to be determined by visual analysis of the pigmentation pattern; quantitative comparison of expression levels was also possible as the transgene message was readily distinguished from that of the endogenous tyrosinase gene by RNase protection analysis. The constructs contain the following additional sequences: (1) UPT contains the 3.6 kb EcoRI-HindIII fragment encompassing the hypersensitive site and matrix attachment region (MAR) (Porter et al., 1991), (2) 3PT contains the 1 kb AvaII-SstI fragment, which includes the hypersensitive site but lacks most of the MAR, (3) 2PT contains only the 3′ terminal 300 bp of this fragment, and (4) PT contains none of the −15 kb region.

A representative mouse of each transgenic line generated with the above constructs is shown in Fig. 3. Each of the pigmented mice shown here are genotypically black (an allele of the brown locus) and agouti. None of the mice containing the PT transgene (Fig. 3B) have visibly pigmented coats; however, two (TgPT2 and TgPT4) have ruby-colored eyes and pigmented tail, ears and scrotum. One line out of four generated with the 2PT construct (Fig. 3C), Tg2PT1, is pigmented in all normally pigmented structures, but the level is very low. Lines generated from 3PT (Fig. 3D) are either light grey, non-pigmented or pigmented in a mosaic pattern. The eyes of the two pigmented lines (Tg3PT2 and Tg3PT4) have ruby-colored eyes and pigmented tail, ears and scrotum. One line out of four generated with the 2PT construct containing the entire 3.6 kb upstream fragment are pigmented (Fig. 3A), with colors ranging from tan (TgUPT3) to black (TgUPT5, 6 and 8). The eyes of each line are black. An additional founder animal that died at birth was also pigmented. Two of the TgUPT lines (1 and 9) have a mosaic coat pattern of stripes. Mice of line TgUPT1 are more highly pigmented overall than those of line TgUPT9, and although some TgUPT1 mice have a distinctive dark grey and
white pattern, most have stripes of varying shades of grey. The number of pigmented clones in line TgUPT9 mice is generally much smaller, although this is extremely variable within the line. In addition, the ears of some mice of this line are darkly pigmented, whereas others have no visible ear pigmentation.

The eyes of all pigmented lines were examined histologically to assess the amount and distribution of pigment (Fig. 4). The eyes of the pigmented TgPT and Tg2PT lines, whose transgenes contain little or none of the upstream region, had only a small amount of pigment in the eye, and it was distributed evenly between the choroid, iris and RPE (Fig. 4D,H). The distribution of ocular pigment in every TgUPT line and in the two pigmented Tg3PT lines, however, was very different. Whereas every TgUPT line had significant (typically wild-type) levels of pigmentation in the choroid and anterior half of the iris (which is continuous with the choroid), most lines had no detectable pigmentation of the RPE or posterior iris (which is derived from the retina) (Fig. 4D,F). Line TgUPT1 had an occasional cluster of lightly pigmented cells in the RPE (0-3 clusters of 2-3 cells each, per section), and lines TgUPT6 and 8, with the highest transgene copy numbers, had a moderate but subnormal amount of RPE and posterior iris pigmentation (Fig. 4C,G). The eyes of the pigmented Tg3PT lines were less pigmented than those of any TgUPT line, but the distribution of melanin granules (only in the choroid and anterior iris) was the same (data not shown).

The approximate level of pigmentation in the ear and scrotum (where the majority of pigmentation results from dermal rather than epidermal melanocytes; Billingham and Silvers, 1960), Harderian gland (a pigmented lacrimal gland), eye structures and coat for all transgenic lines is summarized in Table 1. Harderian gland pigmentation was evaluated histologically, but as present or absent. Pigment cells in various locations were categorized as being derived from the optic cup (RPE and posterior iris), or from the neural crest (probably all other melanocytes; Billingham and Silvers, 1960). It is immediately evident from these results that a direct correlation exists between the presence of the −15 kb hypersensitive site in the transgene and increased expression of the gene in neural-crest-derived melanocytes. There appears to be little or no effect of the region on optic-cup-derived pigment cell expression, as the highest level of pigmentation seen in either the TgUPT or Tg3PT mice (lines TgUPT6 and 8, with 17 and 19 transgene copies, respectively) is not significantly higher than that seen in the strongest expressing line of the TgPT series (TgPT4, with 23 transgene copies).

Another conclusion that can be drawn from these data is that the expression of the UPT transgene in neural-crest-derived pigment cells in various locations is apparently proportionately...
normal, whereas that of the TgPT lines is not (in which expression in the ocular and dermal melanocytes is much higher than that in the follicular melanocytes). Melanocytes of the only expressing Tg2PT line are pigmented in apparently correctly proportional amounts and the Tg3PT lines, although underpigmented in the eye, have otherwise proportionate pigmentation. Three TgUPT lines with the highest transgene copy numbers (5, 6 and 8) were bred with strains containing different genetic coat color determinants that act downstream of tyrosinase in the synthesis of pigment. In each case, the transgenes behaved as true phenotypic revertants of albinism. That is, the brown agouti, black agouti and black non-agouti (carried out only on line 8) phenotypes were all comparable to those of wild-type (non-albino) animals with these genetic determinants.

**Quantitative analysis of transgene expression**

To address the question of position independence (copy number dependence) of transgene expression, and to estimate quantitatively the influence of the upstream region, RNase protection analyses were carried out on RNA from the dorsal skin of 7-8 day offspring of founder mice using both human (transgene) and mouse (endogenous)-specific probes (Fig. 5). An average of the ratio of human:mouse tyrosinase RNA, normalized to line TgUPT3, was taken of two experiments and expressed as a function of transgene copy number (Fig. 6). The results are consistent with a visual analysis of coat color in that expression of the transgene appears to be directly proportional to the transgene copy number for all lines except those exhibiting a mosaic coat color pattern. Analysis of transgene expression levels in some of the TgPT and Tg2PT lines was attempted (including Tg2PT1, the only one with a pigmented coat), but the level was too low to be quantitated. The Tg3PT series was not quantitatively analyzed. The pigmentation levels of the TgPT, Tg2PT and Tg3PT series mice show, however, that transgene expression does not correlate with copy number (see Table 1).

A comparison of coat colors of mice from different series can also give an indication of relative transgene expression efficiency, and thus of the quantitative effect of the upstream region. TgUPT3 mice have a single transgene copy which is expressed at a level (per copy number) comparable to those of the rest of the series (Fig. 6). Tg2PT1 homozygotes, which have 12 transgene copies, have coat colors which are indistinguishable from those of TgUPT3 hemizygotes, implying that the difference in gene expression of the highest expressing Tg2PT line and the average TgUPT line is approximately 12 fold. Likewise, one can conclude that the relative expression level of the highest expressing Tg3PT line (Tg3PT3; 2 copies) is similar to that of the average TgUPT line, as a Tg3PT3 hemizygous mouse has a coat color intermediate between that of a TgUPT3 (1 copy) and TgUPT7 hemizygote (2 copies, with slightly higher than average expression).

**DISCUSSION**

Combined evidence from a number of transgenic mouse studies and from a molecular analysis of the chinchilla-mottled mutation has led to the conclusion that regulatory elements necessary for full expression of the tyrosinase gene are present between 5.5 and 155 kb upstream from the first exon. A candidate region centered at −15 kb was tested in transgenic mice and was shown to encompass an element with strong enhancer activity in melanocytes of neural crest origin. This element was also able to insulate the transgene from all position effects except variegating ones.

**Enhancer activity of the −15 kb region**

None of the six transgenic lines generated with the PT construct (containing the mouse tyrosinase promoter and human tyrosinase cDNA) had pigmented coats, whereas all eight lines containing the same DNA fused to the −15 kb region fragment were pigmented, three at wild-type levels. Mice transgenic for the 2PT construct (with only 300 bp of the upstream fragment) were, like the TgPT animals, poor expressors, with only one line in four showing any pigmentation. These results point to a strong effect of this 3.6 kb upstream region in enhancing gene expression and/or insulating the
transgene from position effects. It was somewhat surprising to find such a low level of coat pigmentation in the TgPT series of mice, as the majority of other transgenic mice containing the same or similar promoter fragments fused to the mouse tyrosinase cDNA (Tanaka et al., 1990) or mini-gene (Yokoyama et al., 1990) were visibly pigmented. Possible reasons for this discrepancy include strain differences and/or the substitution of human sequences for those of the mouse.

It can be difficult to differentiate poor expression due to a lack of strong regulatory elements from that due to position effects on randomly integrated DNA, and it appears that both of these factors play a role in the reduced expression of the TgPT and Tg2PT series relative to the TgUPT series. The existence of position effects can be surmised from a comparison of phenotypes within the Tg2PT series of mice, where a line with 6 copies (Tg2PT1) is visibly pigmented, although all other lines, with equal or greatly increased numbers of transgene copies, are not. Cell-type-position effects are also likely to be prevalent in the TgPT series, as the two pigmented lines have an aberrant distribution of pigment, in which the eyes, ears, scrotum and Harderian gland (in one of them) are pigmented, but the coat is not. The presence of ear pigmentation in mice with little or no coat pigmentation has been observed in other transgenic lines containing the tyrosinase promoter (Yokoyama et al., 1990). The fact that even the highest expressing lines (Tg2PT1 for the coat, TgPT4 for the eye) are significantly less pigmented than any of the UPT lines indicates that, even in the absence of position effects, transgenes lacking the −15 kb region are expressed at a much lower level than those containing it.

The reduction of expression of transgenes lacking most or all of the −15 kb region compared to those containing the entire region, in lines presumably not showing position effects (using a coat color comparison of Tg2PT1 and TgUPT3, described in Table 1).
mosomal position effects (Stief et al., 1989; Phi-Van et al., 1990; McKnight et al., 1992); it also showed transcriptional activation activity in stably transformed cells (Stief et al., 1989), but did not appear significantly to activate linked genes in mice (McKnight et al., 1992). The mean level of gene activity in transgenic mice containing immunoglobulin κ gene constructs with an intact intronic MAR was twice as high as those containing transgenes with no MAR (Xu et al., 1989). As in our series, however, the highest expressing animal with no (in our case, little) transgenic MAR sequence had an expression level similar to the average expression of the MAR-containing mice. This suggests that, in the absence of a negative position effect, a MAR has little transcriptional activation activity in transgenic mice.

**Cell type specificity of enhancer**

Ocular pigment in the pigmented TgPT and Tg2PT mice was distributed evenly between the choroid, RPE and iris. An equivalent expression in these structures was also seen in transgenic mice with the *tyrosinase* mini-gene driven by the *tyrosinase* promoter (Klüppel et al., 1991). Interestingly, however, the TgUPT and Tg3PT transgenes were expressed consistently at much higher levels in the choroid and anterior iris than in the RPE and posterior iris. Thus, neural-crest-derived melanocytes had enhanced expression relative to optic-cup-derived melanocytes. What expression there is in the RPE, from the UPT construct, appears to be as independent of position as in the other melanocytes, with visible pigmentation only in the lines with highest transgene copy numbers.

It is possible that the discrepancy in gene expression is not a function of developmental lineage per se, but rather of differing environments. This seems unlikely for a number of reasons, including (1) all analyzed neural-crest-derived melanocytes throughout the body of the transgenic mice seemed to be pigmented in normally proportional amounts (dermal melanocytes of the ear and scrotum, choroidal melanocytes, Harderian gland melanocytes and hair follicle melanocytes) and (2) the posterior layer of the iris, which is derived from the retina, but which exists in a different environment from that of the RPE, displays the same level of pigmentation as the RPE in all lines.

There appears to little or no effect of the −15 kb region on expression in the RPE, even when the transgenes are apparently poised for transcription in this cell type (i.e., not subject to position effect). It is likely, therefore, that transcription factor(s) specific to neural crest melanocytes play an important role in *tyrosinase* gene expression in these cells. A number of nuclear proteins bind this region (data not shown), but their identity has not yet been determined. It also seems likely that there exists an optic-cup-specific enhancer outside this region, but difficulty in obtaining long-term cultures of melanocytes from the RPE has precluded preliminary investigation by molecular means.

The pigmented cells of the RPE of *cmt/cm* melanocytes throughout the body of the transgenic mice had enhanced expression relative to optic-cup-derived melanocytes. What expression there is in the RPE, from the UPT construct, appears to be as independent of position as in the other melanocytes, with visible pigmentation only in the lines with highest transgene copy numbers.

The two pigmented lines of the Tg3PT series have reduced

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**Fig. 6.** Relationship of TgUPT transgene expression to copy number. Transgene expression was quantitated by RNase protection analysis; each point represents an average from two assays of the ratio of the transgene-specific fragment to that of the endogenous *tyrosinase* gene for a hemizygous representative of each UPT line, normalized to line TgUPT3 (1 copy). Transgene copy number was determined by quantitative Southern blot. Open boxes represent mice with mosaic pigmentation (lines TgUPT1 and 9), the values of which were not used to plot the line of best fit.
pigmentation in the choroid relative to their coat color, as compared to the choroidal and coat pigmentation levels of mice of the TgUPT series. This may mean that sequences needed for choroid melanocyte expression are missing in this construct, or that the 3PT transgene in these cells is affected by neighbouring sequences only in this cell type.

**Position-independent expression of non-mosaic UPT animals**

Position effects can cause uniformly reduced (or enhanced) expression of randomly integrated DNA, altered patterns of cell-type-specific expression, or variegation. Position independence in terms of a direct correlation of expression level to transgene copy number is a criterion generally used in defining chromosomal domain control elements. A linear relationship of transgene expression to copy number was found for the six non-mosaic TgUPT mice, in dramatic contrast to the mice of all other series (TgPT, Tg2PT and Tg3PT), which displayed little correspondence of copy number and expression level. Elimination of most of the MAR containing sequences in the 3PT construct appeared to result in loss of position independence. Lines Tg3PT2 and Tg3PTS (2 and 1 transgene copy, respectively) are not detectably pigmented, whereas line Tg3PT3, with 2 transgene copies, is substantially so. This is consistent with the previously mentioned studies showing that MARs can contribute to insulation of genes against position effects. Many sequences with no MAR activity, however, have also been shown to contribute to position independence, and so it remains to be determined whether MAR activity itself is required for the effect of the UPT transgene, or whether it is another (or perhaps non-specific) attribute of the 3' sequences.

**Variegating position effects**

Two of the eight TgUPT transgenic lines have a mosaic coat consisting of differently pigmented patches, usually transverse stripes of variable widths. This pattern reiterates that of the clonal developmental history of melanocytes (Mintz, 1967), although some of the transgene-induced stripes are narrower. The darkest color of each line is roughly equivalent to that of mice in the same series with similar copy numbers of transgenes. This suggests that the dark clones are fully expressing the transgenes, and that much poorer (but usually some) expression of the transgene occurs in the light clones. This is in contrast to position effect variegation in *Drosophila*, where most variegating genes are either completely on or completely repressed (Spofford, 1976). Mice of the TgUPT1 line generally have more clones that are visibly pigmented than do the mice of line TgUPT9, indicating either an earlier inactivation event (i.e. prior to melanoblast determination) in line TgUPT9, or increased probability of inactivation. As the mice are not inbred, there may also be different genetic modifiers that play a role in the modulation of gene activity. The uniformity of transgene expression of these mice in cell types other than follicular melanocytes is difficult to assess. Mosaicism of expression in the RPE of line TgUPT1 is discernable, but no RPE pigmentation is detectable in line TgUPT9.

Mosaic expression of other transgenes expressed in melanocytes (Bradl et al., 1991b; Mintz and Bradl, 1991; Takeuchi et al., 1993) and other cell types (Sweetser et al., 1988a,b; Katsuki et al., 1988; McGowan et al., 1989) has been observed, and as mosaicism of expression is rarely specifically addressed, it seems likely that it is not an unusual occurrence. Heterogeneity of (autosomal) gene expression between cellular clones of the same cell type can also occur naturally in vivo (Rubin et al., 1989; Michaelson, 1993), and may be of fundamental importance in normal developmental and physiological processes (Mintz, 1971). Little is known of the mechanisms contributing to mosaic expression of autosomal genes or transgenes in mice. Variability of chromatin structure may play a role, as it does in *chinchilla-mottled* mice and in position effect variegation in *Drosophila*; mosaicism in DNA methylation has also been found to be associated with mosaic transgene expression (McGowan et al., 1989). Neither the TgUPT1 nor TgUPT9 line phenotype is noticeably affected by the sex of the parent transmitting the transgene, making it unlikely to be a result of or affected by gamete-of-origin-specific imprinting (see Sapienza, 1990).

The mosaic pattern of pigmentation in the Tg3PT1 line is slightly unusual in that the melanocyte clones of the head are more often pigmented than those of the body. This may reflect a time-dependent (the anterior region of the neural crest being formed later than others) or environmentally affected gene activation event related to the chromosomal position of the transgene. If the variegating effects observed with the TgUPT lines have a similar molecular basis as those observed in *Drosophila*, it is not surprising that regulatory or structural elements (enhancers and MARs) fail to inhibit the inactivation of the gene in some clones. That is, gene inactivation in *Drosophila* can spread over 60 chromosome bands in variegating mutations (see Tartof et al., 1989), implying that normal domain boundaries and the regulatory elements contained within the domains do not insulate internal sequences from this particular inactivating phenomenon. This has in fact been shown experimentally by Kellum and Schedl (1991) who tested the activity of chromosomal domain boundary sequences in insulating against position effects in *Drosophila*. Although nine lines transformed with the *white* gene flanked by these elements fully expressed the gene, a tenth line had a variegated expression pattern, indicating that the boundary elements did not protect against this type of position effect.

An important advantage of studying genes affecting coat colour is the ease with which uniformity of expression can be addressed. Other studies using transgenic mice with putative LCR-containing transgenes have analyzed transgene expression in total tissue, and variability of expression between individual cells or clones has not been a focus of attention. Thus, variegating position effects have not been accounted for in these experiments, and mosaic expression may have occurred unnoticed as LCR containing transgenes can often be expressed, per copy number, within a 2- or 3-fold range of each other. It will be interesting to determine whether variegating position effects do occur in LCR-containing transgenic mice, and indeed what causes these effects. Until then, one cannot conclude with certainty that the UPT construct contains LCR activity comparable to other described LCR like elements. Little is known about gene regulation in melanocytes, and the identification and characterization of this genetic element will be of use in defining regulatory mechanisms in this cell type as well as in inducing high level, position-independent expression of heterologous genes in melanocytes of transgenic mice.
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