Distinct distal regulatory elements control tyrosinase expression in melanocytes and the retinal pigment epithelium

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Abstract

Pigment cells of mammals are characterized by two different developmental origins: cells of the retinal pigment epithelium (RPE) originate from the optic cup of the developing forebrain, whereas melanocytes arise from the neural crest. The pigmentation gene tyrosinase is expressed in all pigment cells but differentially regulated in melanocytes and RPE. The tyrosinase promoter does not confer strong expression in pigment cells in vivo, while inclusion of a distal regulatory element at position −15 kb is necessary and sufficient to provide strong expression in melanocytes. Nevertheless, the regulatory elements responsible for correct spatial and temporal tyrosinase expression in the RPE remained unidentified so far. In this report, we show that a 186 kb BAC containing the tyrosinase gene provides transgene expression in both RPE and melanocytes indicating the presence of regulatory sequences required for expression in the RPE. A deletion analysis of the BAC was performed demonstrating that a RPE-regulatory element resides between −17 and −75 kb. Using multi-species comparative genomic analysis we identified three conserved sequences within this region. When tested in transgenic mice one of these sequences located at −47 kb targeted expression to the RPE. In addition, deletion of this regulatory element within a tyrosinase∷lacZ BAC provided evidence that this sequence is not only sufficient but also required for correct spatial and temporal expression in the RPE. The identification of this novel element demonstrates that tyrosinase gene expression is controlled by separate distal regulatory sequences in melanocytes and RPE.

Keywords: Tyrosinase; Enhancer; Melanocyte; RPE; BAC; Albino; Eye; Pigment; Transgenic; lacZ

Introduction

Albinism is associated with a lack of eye, skin, and hair pigmentation and was one of the first genetic disorders studied (Castle and Allen, 1903). Different forms of albinism exist in human with the disorder corresponding to “albino” mice referred to oculocutaneous albinism type 1 (OCA1) (reviewed in Oetting et al., 2003). The gene mutated in OCA1 is tyrosinase (Tyr), which is located on chromosome 11 in human and 7 in mouse (Barton et al., 1988; Kwon et al., 1989; Ruppert et al., 1988). The mouse Tyr gene encodes the tyrosinase protein and is composed of 5 exons and 4 introns spanning 66 kb (Mouse Genome Database, 2006; Ruppert et al., 1988). Tyrosinase (EC 1.14.18.1) is a key enzyme in the melanin synthesis pathway and catalyzes the transformation of tyrosine into dopaquinone (Beermann et al., 2004; del Marmol and Beermann, 1996; Hearing and Tsukamoto, 1991; Tripathi et al., 1992).

More than 100 phenotypic alleles that result in albinism or hypopigmentation have been identified at the Tyr locus (Beermann et al., 2004; Mouse Genome Database, 2006; Oetting and Bennett, 2006; Oetting et al., 2003). These alleles encompass deletions, missense, and nonsense mutations but also regulatory mutations such as Tyr e−1R or Tyr e−m (chinchilla-mottled) (reviewed in Beermann et al., 2004). The Tyr e−1R allele is characterized by coat-color mottling in homozygous mutant mice and is caused by the insertion of an intracisternal A particle at −225 bp from the Tyr transcription start site (Wu et al., 1997). The Tyr e−m mouse also displays a coat-color mottling which was shown to be the consequence of a large rearrangement of the tyrosinase upstream sequence starting from −5 kb up to at least −30 kb from the transcription initiation site (Porter et al., 1991).

The analysis of the Tyr e−1R and the Tyr e−m mutant mice indicated that important regulatory elements are present within
the tyrosinase upstream sequence. Further analysis of the \textit{Tyr} locus revealed the presence of a melanocyte-specific DNase I hypersensitive site (HS) at position −15 kb from the transcription start site (Ganss et al., 1994a; Porter and Meyer, 1994; Porter et al., 1991). When a 3.7 kb element encompassing this DNase I HS site was combined with the tyrosinase promoter and used to drive a tyrosinase minigene in albino rescue experiments, the resulting transgenic mice showed strong transgene expression with grey to black hair pigmentation (Ganss et al., 1994a; Porter and Meyer, 1994). On the contrary, when only the tyrosinase promoter was used in front of the tyrosinase minigene, the albino phenotype was only partially rescued leading to grey or mottled pigmentation (Beermann et al., 1990; Ganss et al., 1994a; Klüppel et al., 1991; Yokoyama et al., 1990). This distal regulatory element (DRE) located at −15 kb was shown to be required for proper tyrosinase expression in melanocytes and was proposed to act as a locus control region (LCR) (Gimenez et al., 2001; Montoliu et al., 1996). This element was also identified in the human \textit{Tyr} locus and was shown to transactivate gene expression specifically in melanocyte cell lines (Fryer et al., 2003; Regales et al., 2003). Interestingly, the DRE behaves differently in the two pigment cell lineages. The enhancer present within the DRE was shown to be mainly active in melanocytes, which are of neural crest developmental origin, and not active or weakly active in the retinal pigment epithelium (RPE), which originates from the neural tube (Porter and Meyer, 1994; Porter et al., 1999). When the activity of the tyrosinase DRE was assayed in transgenic mice using a \textit{lacZ} reporter combined to the tyrosinase promoter, it was observed that the 3.7 kb DRE acts as a strong enhancer in melanocytes but not in the RPE. The DRE was required to confer detectable \textit{lacZ} expression to melanocytes, whereas the tyrosinase promoter was sufficient for weak but detectable expression in the RPE. Strikingly, the DRE not only failed to transactivate expression in the RPE but rather repressed transgene expression in this cell type (Camacho-Hübner and Beermann, 2001). These results suggested that the control of tyrosinase expression is mediated by different regulatory elements in melanocytes and RPE (reviewed in Murisier and Beermann, 2006).

In this report, we analysed the involvement of distal regulatory elements in controlling differential tyrosinase gene expression in melanocytes and RPE. We performed a global analysis of the \textit{Tyr} locus using comparative sequence analysis and bacterial artificial chromosome (BAC) transgenesis. Using this approach, we identified and characterized a novel distal regulatory element that is required and sufficient for correct spatial and temporal expression in the RPE.

**Methods**

**BAC constructs**

The BAC RP24-459G24 (NCBI reference AC122517), which is derived from the RPCI C57BL/6J mouse library (Osoegawa et al., 2000), was obtained from the BACPAC Resource Center (Children’s Hospital Oakland Research Institute, Oakland, California). This BAC was used for both transgenic rescue experiments and \textit{Tyr}:\textit{lacZ} BAC experiments. Homologous recombination in bacteria (Lee et al., 2001) was used to insert a \textit{lacZ} reporter gene at the position of the \textit{Tyr} first exon in the BAC. The targeting vector was generated as follows: the 3′ homologous regions consisted of a 266 bp \textit{XhoI}–\textit{ApaI} fragment generated from the tyrosinase first exon (position from the transcription start site: +124 to +390). The 5′ homologous region consisting of the 270 bp \textit{Tyr} promoter and the \textit{lacZ} transgene was derived from the tyr(0.27):\textit{lacZ} construct (Camacho-Hübner and Beermann, 2001). An ampicillin cassette flanked by two FRT sites (provided by M. Foretz, Lausanne) was cloned between the \textit{lacZ} and the 3′ homologous region. Recombination and ampicillin cassette removal by flipase was performed as described (Lee et al., 2001).

A second modification by homologous recombination resulting in the deletion of the sequence located between −17.6 and −74.4 kb was then performed on the BAC \textit{Tyr}:\textit{lacZ} to generate the BAC \textit{Tyr}:\textit{lacZ-del-5′}. For that purpose, the targeting cassette was generated as follows: The 3′ homologous region consisted of the 3.7 kb tyrosinase DRE (Camacho-Hübner and Beermann, 2001). The 5′ homologous region was cloned by PCR from the BAC-\textit{Tyr} using the following primers: 5′-\textit{GTTGCGATGACCCCGTGT}-3′ and 5′-\textit{TGTGCGAGGAGCCAAATG}-3′. This 479 bp fragment is located at positions −74.9 kb to −74.4 kb from the \textit{Tyr} transcription start site. To avoid recombination with the FRT site already inserted in the BAC during the first homologous recombination, we used a mutated FRT site (M3) which does not recombine with wild-type FRT sites (Schlake and Bode, 1994). We flanked a M3 site at each end of an ampicillin cassette, which was itself amplified by PCR of the pHblueScript II KS plasmid (Stratagene) with the following primers: 5′-\textit{AAAGTATATGTAGTAAACTTTGGTCTC}-3′ and 5′-\textit{GCGGAAACCCT-TATTTGTAATG}-3′. The resulting M3-Amp-M3 cassette was cloned between the two homologous regions described above. Recombination and ampicillin cassette removal by flipase was performed as described (Lee et al., 2001).

To facilitate cloning of PCR products, nucleotides containing restriction sites were added at the 5′ end of certain PCR primers (indicated by small letters). To generate the BAC \textit{lacZ-del-3′} the sequence located between +0.4 and +9.5 kb was deleted by homologous recombination on the BAC \textit{Tyr}:\textit{lacZ}. The targeting cassette was generated as follows: The 5′ homologous region consisted of a 740 bp fragment corresponding to a large fraction of the first exon of tyrosinase and cloned by PCR from the BAC-\textit{Tyr} using the primers 5′-\textit{ecggattg-cegctggcccCTCGAGATGACCCCGTGT}-3′ and 5′-\textit{ecgctggccCGATGCGTGGCTTAAATG}-3′. The 3′ homologous region consisted of a 835 bp fragment corresponding to a large fraction of the last exon of \textit{Nos} and cloned by PCR from the BAC-\textit{Tyr} using the primers 5′-\textit{ecgctggccAGGGAGCA-GATTTCTCTA-3′} and 5′-\textit{ecgctggccGGCTTGGTCTGGTCTGC}-3′. The M3-Amp-M3 cassette described above was cloned between these two homologous regions. Recombination and ampicillin cassette removal by flipase was performed as described (Lee et al., 2001).

The BAC \textit{Tyr}:\textit{lacZ-del-CNS2} was generated by homologous recombination on the BAC \textit{Tyr}:\textit{lacZ}. The targeting cassette was generated as follows: The 5′ homologous region consisted of a 750 bp fragment located 30 kb immediately upstream of the CNS-2 element and cloned by PCR from the BAC-\textit{Tyr} using the primers 5′-\textit{ecgctggccAATGGGCGATCCACATGAG}-3′ and 5′-\textit{ecgctggccGCAACAACACACACACACACACACATGAG}-3′. The 3′ homologous region consisted of a 848 bp fragment located immediately downstream of the CNS-2 element and cloned by PCR from the BAC-\textit{Tyr} using the primers 5′-\textit{ecgctggccGCAACAACACACACACACACACACATGAG}-3′ and 5′-\textit{ecgctggccCGATGCGTGGCTTAAATG}-3′. The M3-Amp-M3 cassette described above was cloned between these two homologous regions. Recombination and ampicillin cassette removal by flipase was performed as described (Lee et al., 2001).

**Plasmid constructs**

The large-\textit{Tyr}:\textit{lacZ} was generated by cloning 18 kb of tyrosinase upstream sequence by homologous recombination from the BAC \textit{Tyr}:\textit{lacZ} (Lee et al., 2001). The targeting vector consisted of the plasmid \textit{tiyt}(hs3.6/6.1)–\textit{lacZ} (Camacho-Hübner and Beermann, 2001) linearized with \textit{XhoI}. Similarly, the Super-large-\textit{Tyr}:\textit{lacZ} was cloned by generating 30 kb of tyrosinase upstream sequence and a \textit{lacZ} reporter gene in the plasmid pHBr322 (Fermentas Life Sciences). Here, the 5′ homologous regions consisted of a 3.6 kb fragment corresponding to the 5′ end of the CNS-1 and subcloned from CNS-1-\textit{hsp68}:\textit{lacZ} (see below) using \textit{Sal}I and \textit{Hind}III restriction enzymes. The 3′
homologous region consisted of the polyA cloned from tyr(0.27)-lacZ (Camacho-Hübner and Beermann, 2001). Both homologous regions were cloned in pBR322. The resulting plasmid was then linearized by digestion with HindIII, which cuts between the two homologous regions, and used to rescue the 35 kb sequence from the BAC Tyr∷lacZ by homologous recombination in bacteria (Lee et al., 2001). The CNS sequences cloned in front of the hsp68 and thus indicating rescue of the albino phenotype. (C, D) Tyr∷lacZ reporter plasmid (Kothary et al., 1989) were isolated using the following primers: CNS-1 (4.2 kb), 5′-GAGGTTTGGTGATGCGAGGA-3′ and 5′-ACAGGATGAGAGGTGTCGAGA-3′; CNS-2 (4.7 kb), 5′-GCTTCTTTGCCCTTTCCCT-3′ and 5′-TCCCATATGTTGCAGTATGGA-3′; CNS-3 (5 kb), 5′-GTCCTGCTGAGGCTAAGGTG-3′.

**Comparative sequence analysis**

Genomic sequences were obtained from the Ensembl genome browser (Ensembl, 2006) and analysed with the zPicture software (Ovcharenko et al., 2004). The zPicture parameters were the following: sequences masked for repetitive elements, ECR length: 100 bp, ECR similarity: 70%, and bottom cut-off: 50%. The mouse sequence from the BAC was used as template. (NCBI accession number AC122517) and compared to rat (RGSC 3.4 assembly, Chr. 1, position 14344435–14394434), human (NCBI 36 assembly, Chr. 11, position 88359371–88859370), macaque (MMUL 0.1 assembly, Gene Scaffold 574, position 1–447438), and chimpanzee (PanTro 1.0 assembly, Chr. 11, position 90198593–90698592) sequences.

**Generation and analysis of transgenic mice**

Transgenic mice were generated and analysed by X-gal staining of embryos essentially as described previously (Murisier et al., 2006; Porret et al., 2006; Schmidt et al., 1998). Large-Tyr∷lacZ, Super-large-Tyr∷lacZ, CNS-1-hsp68∷lacZ, CNS-2-hsp68∷lacZ and CNS-3-hsp68∷lacZ were deliberated from vector sequences prior to injection in oocytes derived from mating of FVB or B6D2F1 mice. BACs were purified from bacterial cultures using the “Large construct kit” (Qiagen) and injected in circular form. The BAC-Tyr was injected into fertilized oocytes derived from mating of FVB mice. LacZ transgenic mice and embryos were identified by PCR using the primers 5′-GTGATATCGGGAAGGAAAA-3′ and 5′-TTATGCGCCCTGAAGAGGATG-3′.

**Results**

To address the localization of the tyrosinase regulatory elements, we asked whether a bacterial artificial chromosome (BAC) containing the Tyr gene and large amount of surrounding sequences would be able to rescue the Tyr⁺ albino phenotype in mice. The Tyr⁺ mouse is characterized by an absence of pigmentation of the coat and eyes (Fig. 1B). This phenotype is due to a single base mutation in the Tyr coding sequence which leads to substitution of a cysteine to a serine at amino acid 103, and which is sufficient to abrogate the catalytic activity of the tyrosinase protein (Yokoyama et al., 1990). We selected a BAC from a C57BL/6J mouse library (Osogawa et al., 2000) which contains the wild-type Tyr gene (66 kb), 86 kb of 5′ upstream sequence, and 34 kb of 3′ downstream sequence (Fig. 1A). We then generated one transgenic mouse line with this 186 kb construct on a homozygous albino background (Fig. 1B). These mice showed an agouti black coat color and pigmented black eyes. Visually, the pigmentation levels were similar to those

Fig. 1. Rescue of the albino (Tyr⁺) phenotype by introduction of a functional Tyr gene into mice. (A) Schematic view of the BAC-Tyr and the modified BAC Tyr∷lacZ. The Tyr gene is composed of 5 exons spanning 66 kb. In the BAC the Tyr gene is surrounded by ~86 kb of 5′ sequence and ~34 kb of 3′ sequence. The last exon of the Nox4 gene is present on the BAC. (B) A BAC-Tyr transgenic mouse (right) generated on a Tyr⁺/Tyr⁺ mutant background (left) showing an agouti black coat color and thus indicating rescue of the albino phenotype. (C, D) LacZ expression in melanocytes (black arrows) and RPE (white arrows) in E13.5 transgenic embryos generated with the BAC Tyr∷lacZ construct.
observed in mice carrying the wild-type Tyr allele. The rescue of the albino phenotype mediated by the BAC-Tyr thus demonstrates that the BAC contains a functional Tyr gene but also the regulatory sequences required for tyrosinase gene expression in melanocytes and RPE.

The regulatory sequences present on the BAC-Tyr are sufficient to drive lacZ expression to endogenous tyrosinase expression sites

We further characterized the activity of the tyrosinase regulatory elements present on the BAC-Tyr by inserting a lacZ reporter at the position of the first exon (BAC Tyr∷lacZ) (Fig. 1A). 5 transient transgenic and 2 stable transgenic lines were generated with this construct. At embryonic day (E) 13.5, five of them showed expression in both melanocytes and RPE, one depicted expression only in RPE and one only in melanocytes (Figs. 1C, D and 2B–D). This indicates that the regulatory sequences included in the BAC are sufficient to drive detectable lacZ expression to both pigment cell lineages, the melanocytes of neural crest origin and the RPE of neural tube origin. This contrasts markedly with the results obtained with either the tyrosinase promoter alone or combined with the DRE since these mice depicted lacZ expression only weakly in RPE or only in melanocytes, respectively (Camacho-Hübner and Beermann, 2001; Tief et al., 1996).

Fig. 2. The 5′ Tyr upstream sequence is required for expression in melanocytes and RPE. (A) Scheme of the BAC Tyr∷lacZ, large-Tyr∷lacZ, BAC Tyr∷lacZ-del-5′, and BAC Tyr∷lacZ-del-3′ constructs. The large-Tyr∷lacZ comprises 18 kb of Tyr 5′ sequence, encompassing the promoter and the DRE, and fused to a lacZ reporter gene. A deletion spanning from −17.6 kb to −74.4 kb was generated in the BAC Tyr∷lacZ to generate the BAC Tyr∷lacZ-del-5′ whereas the sequence located between +0.4 kb and +95.8 kb was deleted in the BAC Tyr∷lacZ-del-3′. (B) Table recapitulating transgenic lacZ expression in RPE and melanocytes. (C, D) E13.5 embryo generated with the BAC Tyr∷lacZ and depicting lacZ expression in both melanocytes (black arrow) and RPE (white arrow) (see also Figs. 1C, D). (E, F) E13.5 embryo generated with the large-Tyr∷lacZ and depicting lacZ expression in melanocytes. Expression in the RPE was not observed. The ectopic expression in the head had already been observed previously (Camacho-Hübner and Beermann, 2001) and was not followed further. (G, H) In E13.5 embryos generated with the BAC Tyr∷lacZ-del-5′ lacZ expression was observed in melanocytes but not in the RPE. (I, J) E13.5 transient transgenic embryo generated with the BAC Tyr∷lacZ-del-3′ and depicting expression in both melanocytes and RPE.
The 5′ upstream region is required for expression in melanocytes and RPE

In order to localize more precisely the regulatory elements that are mediating pigment cell-specific expression, additional transgenic lacZ experiments were performed. Since the DRE located at −15 kb was shown to be essential for expression in melanocytes (Camacho-Hübner and Beermann, 2001; Gimenez et al., 2001), we asked whether 18 kb of 5′ upstream sequence containing the Tyr promoter and the DRE would be sufficient to provide lacZ expression to both pigment cell types (large-Tyr∷lacZ construct, Fig. 2A). 3 transient transgenic (E13.5) embryos were generated and all of them showed lacZ expression in melanocytes whereas expression in the RPE was never detected (Figs. 2B, E, F). This indicates that the regulatory elements required for expression in the RPE are either located upstream of the −15 kb DRE or downstream of the Tyr promoter. In order to discriminate between these two possibilities, we addressed whether the deletion of the sequence located upstream of the DRE would affect the pattern of expression mediated by the BAC Tyr∷lacZ. For this purpose, a 56.8 kb sequence located between −17.6 kb and −74.4 kb was deleted from the BAC Tyr∷lacZ (BAC Tyr∷lacZ-del-5′ construct, Fig. 2A). 7 transient transgenic embryos (E13.5) were obtained and 6 of them displayed lacZ expression in melanocytes, whereas none depicted expression in the RPE (Figs. 2B, G, H). Since the embryos generated with the entire BAC Tyr∷lacZ showed lacZ expression in both melanocytes and RPE (Figs. 1C, D and 2C, D), this result suggests that the regulatory elements required for expression in the RPE are located somewhere between −17.6 kb and −74.4 kb. It might nevertheless be feasible that important regulatory elements are also located outside of this region. As a control experiment we therefore deleted most of the 3′ region (+0.4 kb to +95.8) in the BAC Tyr∷lacZ (BAC Tyr∷lacZ-del-3′ construct, Fig. 2A). The transient transgenic E13.5 embryo generated with this construct showed expression in both melanocytes and RPE (Figs. 2B, I, J) indicating that the region located downstream of the first exon is not required for expression in melanocytes and RPE, and that all major regulatory elements are located in the 5′ region.

Identification of a second distal regulatory element at the Tyr locus

Comparative sequence analysis represents a powerful approach to detect putative functional elements in noncoding DNA sequences (Hardison, 2000; Margulies et al., 2003; Pennacchio, 2003). We thus searched for conserved noncoding sequences (CNS) that might represent putative novel regulatory elements of the Tyr gene. The mouse sequence from the BAC RP24-459G24 was compared to several vertebrate genomes (Ensembl, 2006) using the zPicture software (Ovcharenko et al., 2004). No obvious CNS was identified when the mouse sequence was compared with non-mammalian sequences such as those of zebrafish, fugu, or chicken (not shown). In contrast, several CNS were detected between mammalian sequences such as those from mouse, rat, human, chimpanzee and macaque (Fig. 3). According to the results obtained with the BAC Tyr∷lacZ-del-5′, the BAC Tyr∷lacZ-del-3′ and the large-Tyr∷lacZ constructs, the regulatory elements required for expression in the RPE are located between −17.6 kb and −74.4 kb (see arrows in Fig. 3). We thus focussed our analysis on the 3 major CNS that are located within this interval at positions −28 kb (CNS-1), −47 kb (CNS-2), and −65 kb (CNS-3) (Fig. 3). In addition, two CNS are found in the region located between the exon 1 and the CNS-1 and correspond to the DRE and the Tyr promoter (Fig. 3).

Since no appropriate RPE cell culture system is available to functionally address the activity of these CNS, we functionally tested the activity of these elements in lacZ transgenic mice. As
a first experiment, the genomic region spanning from the Tyr promoter, the DRE, and the CNS-1 was cloned in a lacZ reporter construct (Super-large-Tyr::lacZ construct, Fig. 4A). 2 transient transgenic E13.5 embryos and one stable line were obtained which depicted strong expression in melanocytes and no expression in the RPE (Figs. 4B, C, G). This result indicates that the 30 kb upstream sequence is not sufficient to provide expression to both pigment cell types. The 3 CNS were then individually cloned (CNS-1, 4.2 kb; CNS-2, 4.7 kb; CNS-3, 5 kb) upstream of a hsp68::lacZ reporter plasmid (Fig. 4A) (Beermann et al., 2006; Kothary et al., 1989) and tested in transient transgenic experiments for their ability to target RPE-specific lacZ expression in mouse embryos. The tyrosinase promoter itself is capable to provide low levels of transgenic lacZ expression to the developing RPE (Camacho-Hübner and Beermann, 2001; Tief et al., 1996), and was thus considered to be not suitable for these experiments. Instead, we chose the hsp68 promoter, which has been widely used to identify enhancer activity of conserved noncoding sequences in transgenic experiments (Beermann et al., 2006; Nobrega et al., 2003; Sumiyama and Ruddle, 2003). 7 transient transgenic E13.5 embryos were obtained for the CNS-1 construct (CNS-1-hsp68::lacZ) and 5 were obtained for the CNS-3 construct (CNS-3-hsp68::lacZ). All of them showed weak and unspecific lacZ expression (Figs. 4D, F, G). On the contrary, the 7 transient transgenic E13.5 embryos generated with the CNS-2 construct (CNS-2-hsp68::lacZ) depicted strong lacZ expression in the RPE (Figs. 4E, G) indicating that the CNS-2 is a promising candidate for a distal regulatory element directing expression to the RPE.

The CNS-2 is required for expression in the RPE

The transgenic embryos generated with the CNS-2-hsp68::lacZ construct depicted strong lacZ expression in the RPE indicating that the CNS-2 represents a novel tissue-specific tyrosinase enhancer. In order to confirm the functional relevance of this element, the CNS-2 was deleted form the BAC Tyr::lacZ (Fig. 5A). The resulting BAC Tyr::lacZ-del-CNS-2 was used to generate 3 transient transgenic E13.5 embryos which depicted lacZ expression in melanocytes but not in the RPE (Figs. 5B–E). In comparison to the results obtained with the entire BAC Tyr::lacZ conferring strong lacZ expression in both melanocytes and RPE (Figs. 1C, D and 2C, D), this result indicates that the CNS-2 located at ~47 kb represents a novel distal regulatory element which is required to provide correct spatial and temporal tyrosinase gene expression to the RPE.

Discussion

The control of gene expression is a tightly regulated and complex process which follows strict time and space constraints. Moreover, upon appropriate intra- or extra cellular stimuli, the level of gene expression is adaptable in order to perfectly fit with cellular and body requirements. These rules also apply to the genes that are involved in pigment production, such as tyrosinase. This gene is expressed in very specific cell types, namely the melanocytes which developmentally originate from the neural crest, and the cells of the RPE which arise from the neural tube. During the development of the mouse embryo, tyrosinase is expressed from E12.5 in melanocytes and from E10.5 in the RPE (Beermann et al., 1992). Tyrosinase is not only transcriptionally regulated in time and space but also by specific external stimuli such as those occurring during hair regeneration or following UV exposure (Gilchrest et al., 1996; Slominski et al., 1991). The genetic elements controlling tyrosinase expression have thus evolved in order to satisfy these multiple constraints. In this study, a global analysis of the Tyr locus was carried out in order to identify and characterize the genetic elements involved in temporal and spatial regulation of tyrosinase expression.

Previous reports have indicated that tyrosinase gene expression is controlled by a complex arrangement of regulatory elements. It was shown by albino rescue experiments that a 270 bp tyrosinase promoter is sufficient to drive expression to melanocytes and RPE (Klüppel et al., 1991). The levels of expression were however rather low even when larger fragments of the tyrosinase promoter were analysed (Beermann et al., 1990, 1992; Ganss et al., 1994b; Klüppel et al., 1991; Yokoyama et al., 1990). This indicates that, in addition to the promoter, correct spatial and temporal tyrosinase expression also requires other regulatory elements. Among them the tyrosinase distal regulatory element (DRE) located at ~15 kb was shown to be required for strong, copy number-dependent, and position-independent transgene expression (Ganss et al., 1994a; Gimenez et al., 2001; Montoliu et al., 1996; Porter and Meyer, 1994). Subsequent experiments have however indicated that, even if the tyrosinase promoter and the DRE are key elements in controlling gene expression, they are not entirely sufficient. For instance, transgenic mice generated with a construct containing the 3.7 kb DRE, the tyrosinase promoter, and a lacZ reporter displayed strong expression in melanocytes but no expression in the RPE (Camacho-Hübner and Beermann, 2001). This not only indicates that tyrosinase expression is differentially regulated in melanocytes and RPE but, moreover, that correct spatial and temporal expression in the RPE requires regulatory elements that are located outside of the DRE and promoter.

In order to address the localization of putative novel regulatory elements within the Tyr locus, we asked whether a BAC containing the Tyr gene and large parts of surrounding genomic sequences would be sufficient to rescue the albino phenotype in transgenic mice. The transgenic mouse obtained showed an agouti black coat color and pigmented eyes and was undistinguishable from wild-type agouti black mice. This indicates that the BAC contains the regulatory sequences required for expression in both melanocytes and RPE. However, such an albino rescue experiment does not allow precise analysis of the spatio-temporal pattern of expression mediated by the BAC, since the transgenic expression is only visible by the progressive accumulation of melanin. We thus used a lacZ reporter gene inserted in the BAC to monitor tyrosinase gene regulation in transgenic mice. At E12.5, which corresponds to
Identification of a RPE-specific enhancer at the *Tyr* locus. (A) Scheme of the *Tyr* upstream sequence depicting the position of the different elements used to generate the Super-large-*Tyr*:lacZ, the CNS-1-hsp68::lacZ, the CNS-2-hsp68::lacZ, and the CNS-3-hsp68::lacZ constructs. (B, C) E13.5 embryo with the Super-large-*Tyr*:lacZ construct and depicting strong lacZ expression in melanocytes as shown by higher magnification of the back skin (C, black arrow) and no X-Gal staining in the RPE. (D) E13.5 embryo with the CNS-1 cloned in front of the hsp68::lacZ reporter construct (Kothary et al., 1989) depicting weak and unspecific X-Gal staining. (E) E13.5 embryo with the CNS-2 reporter construct (CNS-2-hsp68::lacZ) depicting strong and RPE-specific lacZ expression. (F) E13.5 embryo with the CNS-3 (CNS-3-hsp68::lacZ) depicting almost no X-Gal staining. (G) Table recapitulating the pattern of lacZ expression in the different transgenic experiments.

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the onset of endogenous Tyr expression in melanocytes, the embryos generated with the BAC Tyr∷lacZ showed expression in both melanocytes and RPE. This specific pattern of lacZ expression, which is likely to correspond to endogenous Tyr expression, was never observed in embryos generated with the Tyr promoter combined to the DRE which showed lacZ expression in melanocytes only (Camacho-Hübner and Beer-mann, 2001). This indicates that the BAC not only contains the known melanocyte-specific regulatory elements, but furthermore additional regulatory elements required for correct spatial and temporal tyrosinase expression in the RPE.

These regulatory elements might be located everywhere in the BAC, and we thus tried to reduce the size of the BAC without compromising its specific transcriptional activity. In a first attempt, a construct generated with 18 kb of Tyr upstream sequence was insufficient to provide expression to both melanocytes and RPE (large-Tyr∷lacZ construct). The pattern of lacZ expression was similar to what was observed with embryos generated with the tyrosinase promoter combined with the DRE (Camacho-Hübner and Beermann, 2001). This indicates that no obvious regulatory elements are located between the promoter and the DRE and that the regulatory elements necessary for expression in the RPE are located either upstream of the DRE or downstream of the promoter. To discriminate between these two possibilities, the region located immediately upstream of the DRE was deleted in the BAC Tyr∷lacZ. This deletion abolished expression in the RPE indicating that the RPE-specific regulatory elements are located within this 57 kb interval. In addition, a transgenic embryo generated with a BAC Tyr∷lacZ containing a deletion of the sequence localized between the exon 1 and +95 kb showed strong expression in both melanocytes and RPE. This indicates that this region does not contain important regulatory elements required for expression in melanocytes or RPE and thus confirms the importance of the 5′ region. We then further focussed on the region located upstream of the DRE which, according to the results obtained with the truncated BACs, is likely to contain a RPE-specific regulatory element.

Since the deletion of the sequence located between −17.6 kb and −74.4 kb in the BAC resulted in the loss of lacZ expression in the RPE, we searched for putative RPE-specific elements within this interval. Regulatory elements are likely to be conserved during evolution and thus multi-species sequence comparison was used as a predictive tool (Beermann et al., 2006; Hardison, 2000; Pennacchio and Rubin, 2001). Comparison of the mouse Tyr locus with the corresponding sequences from chicken or fish did not reveal any obvious conserved pattern with exception of the tyrosinase coding sequence. When the analysis was restricted to mammalian sequences, several stretches of conserved noncoding sequences (CNS) were identified. Among them, the DRE located at −15 kb (Montoliu et al., 1996) was conserved in all mammalian species analysed thus confirming the predictive capacity of the method. We further focussed our analysis on the region deleted in the BAC Tyr∷lacZ-del-5′ and identified three major CNS located at positions −65, −47, and −28 kb.

To address whether these CNS represent functional regulatory elements, they were cloned in front of the hsp68∷lacZ reporter construct (Beermann et al., 2006; Kothary et al., 1989) and analysed in transgenic mice. Since the hsp68 promoter alone is not sufficient to provide strong expression, any specific pattern of lacZ expression indicates that the cloned CNS represents a putative enhancer. This experimental system allows to rapidly determine whether a given DNA fragment has
The transcriptional activity mediated by the tyrosinase promoter is not sufficient for strong expression in melanocytes and RPE and thus requires additional regulatory elements. The DRE located at −15 kb acts as a strong transcriptional enhancer in melanocytes. This enhancer is however not active in the RPE and can repress gene expression in this cell type, as evident from comparison of Tyr::lacZ constructs with or without the DRE (Camacho-Hübner and Beermann, 2001). The CNS-2 located at −47 kb is responsible and required for correct spatial and temporal tyrosinase expression in the RPE.

In conclusion, we propose a novel regulatory model in which tyrosinase gene expression is controlled by separate distal regulatory elements in melanocytes or RPE. The transcriptional activity mediated by the tyrosinase promoter is not sufficient for strong expression in melanocytes and RPE and thus requires additional regulatory elements. The DRE located at −15 kb acts as a strong transcriptional enhancer in melanocytes. This enhancer is however not active in the RPE and can repress gene expression in this cell type, as evident from comparison of Tyr::lacZ constructs with or without the DRE (Camacho-Hübner and Beermann, 2001). The CNS-2 located at −47 kb is responsible and required for correct spatial and temporal tyrosinase expression in the RPE.

References


847


