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A conserved transcriptional enhancer that specifies *Tyrp1* expression to melanocytes

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Abstract

Pigment cells of mammals originate from two different lineages: melanocytes arise from the neural crest, whereas cells of the retinal pigment epithelium (RPE) originate from the optic cup of the developing forebrain. Previous studies have suggested that pigmentation genes are controlled by different regulatory networks in melanocytes and RPE. The promoter of the tyrosinase-related family gene *Tyrp1* has been shown to drive detectable transgene expression only to the RPE, even though the gene is also expressed in melanocytes as evident from *Tyrp1*-mutant mice. This indicates that the regulatory elements responsible for *Tyrp1* gene expression in the RPE are not sufficient for expression in melanocytes. We thus searched for a putative melanocyte-specific regulatory sequence and demonstrate that a bacterial artificial chromosome (BAC) containing the *Tyrp1* gene and surrounding sequences is able to target transgenic expression to melanocytes and to rescue the $Tyrp1^b$ (brown) phenotype. This BAC contains several highly conserved non-coding sequences that might represent novel regulatory elements. We further focused on a sequence located at −15 kb, which we identified as a melanocyte-specific enhancer as shown by cell culture and transgenic mice experiments. In addition, we show that the transcription factor Sox10 can activate this conserved enhancer. The presence of a distal *Tyrp1* regulatory element, which specifies melanocyte-specific expression, supports the idea that separate regulatory sequences can mediate differential gene expression in melanocytes and RPE.

Keywords: BAC; lacZ; Melanocyte; Neural crest; Pigmentation; RPE; Transgenic; Tyrosinase; Tyrp1

Introduction

The pigment melanin is produced by neural crest-derived melanocytes and by the cells of the retinal pigment epithelium (RPE), which originate from the neural tube (Martinez-Morales et al., 2004; Mayer, 1973). The emergence of RPE and melanocytes during evolution is still poorly understood, and it has been hypothesized that they have evolved by either divergence from a primitive pigment cell or by recruitment of the pigment production machinery to a neural crest or a neural tube derivative (Arnheiter, 1998; Martinez-Morales et al., 2004; Sato et al., 1999). Among diverse approaches, comparison of the regulatory networks controlling pigment gene expression in melanocytes and RPE might shed a new light on the evolution of pigment cells. A large set of genes are involved in pigmentation, including the members of the tyrosinase gene family, namely tyrosinase, *Tyrp1* and *Dct*. Pigment production is impaired in the absence of functional tyrosinase, which leads to the classical albino phenotype (Beermann et al., 2004). Mutations in *Tyrp1* and *Dct* alter the type of pigment produced. A brown pigment is produced in *Tyrp1*-mutant mice (Bennett et al., 1990; Hertwig, 1942; Zdarsky et al., 1990) and absence of *Dct* leads to a dark-grey coat color (Guyonneau et al., 2004).

The mouse *Tyrp1* gene (17.5 kb) is located on chromosome 4 and composed of eight exons (Bell et al., 1995; Jackson, 1988; Jackson et al., 1991). Its promoter contains several putative binding sites for melanocyte- or RPE-specific transcription factors (Murisier and Beermann, 2006), including the major transcriptional regulator of pigmentation genes Mitf (microphthalmia transcription factor) (Aksan and Goding, 1998; Bertolotto et al., 1998; Jackson et al., 1991; Lowings et al., 1992; Yasumoto et al., 1995, 1997). Mitf is expressed in both types of pigment cells but displays cell type-specific isoforms

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such as Mitf-M in melanocytes or Mitf-A in the RPE (Amae et al., 1998; Hershey and Fisher, 2005; Yajima et al., 1999). In addition, the paired domain transcription factor Pax3 transactivates the *Tyrp1* promoter only in melanocytes (Galibert et al., 1999), whereas the homeobox factor Otx2 is involved in RPE-specific control of *Tyrp1* expression (Martinez-Morales et al., 2003). Taken together, this suggests that *Tyrp1* expression is controlled by different regulatory networks in melanocytes and RPE (Murisier and Beermann, 2006). In transgenic mice, the difference between the two pigment cell types is even more obvious because the *Tyrp1* promoter was shown to confer transgene expression only to the RPE (Mori et al., 2002; Penna et al., 1998; Raymond and Jackson, 1995; Rousseau et al., 2000; Schmidt et al., 1999). In contrast, with exception of a single transgenic mouse (Raymond and Jackson, 1995), the *Tyrp1* promoter was not sufficient to provide detectable transgene expression to melanocytes. Nevertheless, the *Tyrp1* gene is expressed in melanocytes (Steel et al., 1992) as evident from *Tyrp1*-mutant mice (*Tyrp1*^{b}) which display a brown coat color phenotype (Zdarsky et al., 1990). We thus assumed that genetic elements responsible for *Tyrp1* expression in melanocytes are located outside of the promoter such as in introns or in more distal regions.

Previous reports have indicated that the control of pigmentation gene expression is achieved by distinct *cis*-regulatory elements in melanocytes and RPE (for a review, see Murisier and Beermann, 2006). In this report, we have used comparative sequence analysis as a tool to identify putative distal regulatory elements at the *Tyrp1* locus. We show that a conserved region located at −15 kb acts as a melanocyte-specific enhancer and positively regulates reporter gene expression in cell culture and transgenic mice. In addition, further functional analysis identified the transcription factor Sox10 as being able to transactivate this distal enhancer. It has been proposed that the genetic network controlling pigment production has evolved initially in the context of primitive eye function and then was adapted by the neural crest to form melanocytes (Arnheiter, 1998; Martinez-Morales et al., 2004). Following this hypothesis, the existence of separate regulatory elements controlling *Tyrp1* expression in melanocytes and RPE might be consecutive to this adaptive evolutionary process. In that way, the *Tyrp1* enhancer might have contributed to recruit gene expression to melanocytes.

Methods

Plasmid constructs

The *Tyrp1∷luc* construct was generated by cloning the 1.4-kb *Tyrp1* promoter extending from −1434 bp to +107 bp (Jackson et al., 1991; Raymond and Jackson, 1995) in the pGL3-basic plasmid (Promega). The 1.8-kb *Tyrp1* enhancer was isolated by PCR (primers: 5′-CCCCTCACCTGGATCCTAA-3′ and 5′-GCACCTAACCATTGACCACA-3′) from the BAC RP23-372M15 obtained from the BACPAC Resource Center (Children's Hospital Oakland Research Institute, Oakland, CA) (Osoegawa et al., 2000) and cloned in pGEMT-easy (Promega). The 1.8-kb fragment was then cloned in *Tyrp1∷luc* to generate the 1.8 *Tyrp1∷luc* plasmid. This fragment was cut in two parts with *Pst*I to generate a 731-bp (SP) and a 1066-bp (KP) fragment, which were cloned into *Tyrp1∷luc* to generate *SP-Tyrp1∷luc* and *KP-Tyrp1∷luc*. Restriction digestion of the 1.8-kb fragment by *Hin*cII resulted in a 504-bp (EH) and 1293-bp (HE) fragment, which were used to generate *EH-Tyrp1∷luc* and *HE-Tyrp1∷luc*. An internal fragment of 227 bp (HP) was isolated by *Hin*cII and *Pst*I digestion and used to generate *HP-Tyrp1∷luc*. *SV40∷luc* corresponds to the pGL3-promoter plasmid (Promega). *EH-SV40∷luc* was generated by cloning the EH fragment from *EH-Tyrp1∷luc* into *SV40∷luc*. The 1.4-kb *Tyrp1* promoter (Jackson et al., 1991; Raymond and Jackson, 1995) was cloned in the 0.27 *Tyr∷lacZ* construct (Camacho-Hübner and Beermann, 2001) to generate the *Tyrp1∷lacZ* plasmid (with the *Tyr* [tyrosinase] promoter removed from the construct). *1.8-Tyrp1∷lacZ* was constructed by inserting the 1.8-kb enhancer in the *Tyrp1∷lacZ* plasmid. The EH fragment was reduced to 426 bp (EN fragment) by digestion with *Nsi*I to generate *EN-SV40∷luc*. Details on further deletion constructs are available on request. Briefly, the following sequences were deleted with respect to EN: EN1 (5′: 26 bp), EN2 (5′: 59 bp), EN3 (5′: 68 bp), EN4 (5′: 104 bp), EN5 (5′: 156 bp), EN6 (3′: 94 bp), EN7 (3′: 156 bp) and EN8 (5′: 59 bp and 3′: 84 bp). Point mutations were introduced by PCR into EN8: S1 was changed from TTTGAT to GAATTC, S2 was changed from ATTGAT to GAATTC, S3 was changed from AACAAA to GAATTC and E1 was changed from CAGTTG to CTGCAG. The constructs were checked by sequencing and restriction fragment analysis. The plasmids pCMV1/Brn2 (Kuhlbrodt et al., 1998a) and pCMV5-Pax 3 (Kuhlbrodt et al., 1998b) were kindly provided by M. Wegner. The plasmids CMV-Pax6 and pHCMV-Pax2 (Baumer et al., 2003) were kindly provided by P. Gruss. The plasmid pCMVSp2-Sox10 (Epstein et al., 1996) was kindly supplied by J.A. Epstein. The plasmids pCMV19a-Tbx2FL (Carreira et al., 1998), pCMV-Usf-1 (Galibert et al., 2001) and flag-mi were kindly provided by C.R. Goding. PCINEO-mOTX2 (Martinez-Morales et al., 2003) was kindly supplied by P. Bovolenta.

BAC constructs

The BAC RP23-100J5, 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, CA). This BAC was used for the transgenic rescue experiment and the generation of a *Tyrp1∷lacZ* BAC. Homologous recombination in bacteria (Lee et al., 2001) was used to replace the first *Tyrp1* coding exon by the lacZ reporter gene in the BAC. The targeting vector was generated as following: the 3′ homologous region consisted of a 2-kb fragment amplified by PCR from the BAC RP23-100J5 using the following primers: 5′-CCTGGCCTCTGAGGTTCTTT-3′ and 5′-TTCCAACATGCTATGCCTCT-3′. The 5′ homologous region consisted of the 1.4-kb *Tyrp1* promoter, the first non-coding exon and the first intron (Schmidt et al., 1999). The lacZ transgene was placed immediately after the ATG. An ampicillin cassette flanked by two FRT sites (a kind gift of M. Foretz) was cloned between the lacZ gene and the 3′ homologous region. Recombination and ampicillin cassette removal by flipase were performed as described (Lee et al., 2001).

Comparative sequence analysis

Genomic sequences were obtained from the Ensembl genome browser (Ensembl, 2005) and analyzed 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 cutoff: 50%. The mouse sequence extending from −76889 bp to +119318 bp (NCBI m34 assembly, chromosome 4, position 79756613-79952819) from the *Tyrp1* transcription start site was used as template and compared to rat (RGSC 3.4 assembly, chromosome 5, position 99368306-99686463), dog (CanFam 1.0 assembly, chromosome 11, position 34664792-34682086) and human (NCBI 35 assembly, chromosome 9, position 12683435-12700249) sequences extending from +150 kb to −150 kb. The sequence of the EN8 fragment was aligned to the corresponding sequences from rat, dog and human genomes (Ensembl, 2005) with the ClustalW software (Thompson et al., 1994).

Cell culture, transfection and luciferase assays

NIH3T3, 293T, B16F1 and B16F10 cells were maintained in low glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma).