Commitment to the melanocyte lineage is characterized by the onset of microphthalmia-associated transcription factor (Mitf) expression. Mitf plays a fundamental role in melanocyte development, with mice lacking Mitf being entirely devoid of pigment cells. In the absence of functional Mitf protein, melanoblasts expressing Mitf mRNA disappear around 2 days after their first appearance either by apoptosis or by losing their identity and adopting an alternative cell fate. The role of Mitf must therefore be to regulate genes required for melanoblast survival, proliferation, or the maintenance of melanoblast identity. Yet to date, Mitf has been shown to regulate genes such as Tyrosinase, Tyrp-1, and Dct, which are required for pigmentation, a differentiation-specific process. Because expression of these genes cannot account for the complete absence of pigment cells in Mitf-negative mice, Mitf must regulate the expression of other as yet uncharacterized genes. Here we provide several lines of evidence to suggest that Mitf may regulate the expression of the Tbx2 transcription factor, a member of the T-box family of proteins implicated in the maintenance of cell identity. First, isolation and sequencing of the entire murine Tbx2 gene revealed that the Tbx2 promoter contains a full consensus Mitf recognition element; second, Mitf could bind the promoter in vitro and activate Tbx2 expression in vivo in an E box-dependent fashion; and third, Tbx2 is expressed in melanoma cell lines expressing Mitf, but not in a line in which Mitf expression was not detectable. Taken together, with the fact that Tbx2 is expressed in Mitf-positive melanoblasts and melanocytes, but not in Mitf-negative melanoblast precursor cells, the evidence suggests that the Tbx2 gene may represent one of the first known targets for Mitf that is not a gene involved directly in the manufacture of pigment.

Understanding how specific cell lineages are established and maintained lies at the heart of developmental biology. The melanocyte lineage arises in the neural crest as nonpigmented precursor cells, termed melanoblasts, which then migrate to their final destinations in the epidermis and hair follicles, where they differentiate into mature pigment-producing melanocytes. Little is known of the precise program of events leading from a multipotent neural crest cell to a melanoblast, but the switch from a melanoblast precursor cell to a melanoblast is characterized by the onset of expression of the basic-helix-loop-helix-leucine zipper (bHLH-LZ)1 microphthalmia-associated transcription factor, Mitf (1, 2). Mitf plays a critical but poorly understood role in melanocyte development. Mice lacking Mitf generate neural crest-derived melanoblasts, as measured by the expression of Mitf mRNA, but these cells are no longer detectable around 2 days after their first appearance (3), resulting in a mouse devoid of all pigment cells. It is likely that the loss of melanoblasts in Mitf-negative mice reflects either the failure of the melanoblasts to survive or an inability to maintain their identity. Although Mitf has been shown to regulate the expression of genes such as Tyrosinase and Tyrp-1 (4–8), which are involved in the manufacture of the pigment melanin, expression of the pigmentation genes is not required for the genesis of the melanocyte lineage. This implies that Mitf must regulate other genes required for the survival, proliferation of melanoblasts, or the maintenance of their cell identity. However, apart from the pigmentation genes, no targets for Mitf in the melanocyte lineage have been identified to date.

Over the past few years considerable excitement has been generated by the discovery, by Bollag et al., (9) of a novel family of transcription factors defined by the conservation of the T box, which corresponds approximately to the DNA binding domain (10, 11). The T-box family plays a critical role in embryonic development (for reviews see Refs. 12–14) and is highly evolutionarily conserved, with family members being found in a wide range of organisms, including human, mouse, chicken, Xenopus, zebra fish, and Drosophila, with at least 21 T-box proteins being identified by sequence homology on completion of the sequence of the Caenorhabditis elegans genome (15). Consistent with the high degree of conservation within the T-box family, mutations within the family have a dramatic affect on development. For example, the prototype for the family, the T gene (16), encodes the Brachyury transcription factor (17), which is essential for mesoderm induction, whereas mutations in the human Tbx3 and Tbx5 genes result in the autosomal dominant ulnar-mammary (18) and Holt-Oram (19) syndromes, respectively. Both syndromes are characterized by developmental defects; in ulnar-mammary syndrome the limb

1 The abbreviations used are: bHLH-LZ, basic helix-loop-helix-leucine zipper; bp, base pair(s); FCS, fetal calf serum; PCR, polymerase chain reaction; RT, reverse transcription; BAC, bacterial artificial chromosome; ITT, in vitro transcribed/translated; Mitf, microphthalmia-associated transcription factor; USF1, upstream stimulatory factor 1; MSEu, upstream melanocyte-specific element; MSEi, initiator melanocyte-specific element; PBS, phosphate-buffered saline; Tyrp-1, Tyrosinase-related protein-1; Dct, dopachrome tautomerase.
and apocrine glands are affected, whereas Holt-Oram syndrome is characterized by abnormalities in the cardiac septum and forelimbs. In addition to these naturally occurring mutations, targeted disruption of the mouse Tbx6 gene results in an embryo in which the somites are transformed into neural tubes (20), whereas the Tbx3 and Tbx5 genes regulate limb identity (21, 22). Together the evidence suggests that T-box family members may play a crucial role in the maintenance of cell identity and that at least one family member may be expressed in all cell types.

We have shown previously that, in the melanocyte lineage, a single T-box factor gene Tbx2, is expressed in melanoblasts and melanocytes but not in neural crest-derived melanoblast precursor cells (23), that is, Tbx2 expression appears to occur after commitment to the melanocyte lineage. Given the critical role of the T-box family in the maintenance of cell identity in development, understanding the controls operating on Tbx2 expression should provide an important insight into the regulatory mechanisms operating during the genesis of the melanocyte lineage. To this end, we have isolated and sequenced the entire murine Tbx2 gene, characterized the intron/exon boundaries, and mapped the transcription start site. We demonstrate that the Tbx2 promoter functions in a cell type-specific fashion and can be regulated by Mitf through a specific Mitf-consensus binding site. Thus Tbx2 may represent one of the first known targets for Mitf in the melanocyte lineage other than those genes directly involved in the manufacture of pigment.

EXPERIMENTAL PROCEDURES

Isolation and Mapping of the Mouse Tbx2 Gene—Two primers corresponding to the 3' end of the mouse Tbx2 cDNA clone were synthesized: 5'-CTACGCAAAGGCGGC-3' and 5'-CCCTTCTGTGCTGTA-3'. These were used to amplify by polymerase chain reaction (PCR) a 192-base pair (bp) Tbx2 DNA fragment. This fragment was used as a probe to screen a mouse ES genomic library. A genomic clone of approximately 100 kilobases in a P1 vector was obtained, digested by BamHI or XhoI and SacI, and subcloned into either the BamHI or SacI sites of pBluescript-SK+.

The locations of the intron/exon boundaries were determined by sequencing either genomic PCR products isolated from subclones, and the transcription start site was determined by primer extension analysis. Primer extension was performed with a primer (5'-TCTCCGGGACATCCGGCGGTCCTCAGG-3') derived from the previously published Tbx2 cDNA sequence (9).

RNA Extraction and RT-PCR—Isolation of RNA and blotting procedures were described previously (24). For the reverse transcription (RT) PCR, RNA isolated from melanocytes and melanoma cell lines was reverse transcribed using different preparations of DNA. For each transfection used to obtain the results presented (see Fig. 2), we compared the values obtained for the lacZ reporter in the two cell lines and, assuming that any difference (which was around 20%) represented a difference in transfection efficiency, we used this value to adjust the relative levels of luciferase activity obtained using the Tbx2 promoter-reporter. By giving the luciferase activity from the Tbx2-luciferase reporter a value of 100% in B16 cells, we arrived for each transfection experiment at a value for the activity of the same reporter in HeLa cells as a percentage of that observed in B16 melanoma cells. We then took an average of the HeLa cells results and compared that with the result from B16 cells presented as 100%. The lacZ reporter was also used in other transfection experiments as a control for transfection efficiency, and the values obtained using the various Tbx2-luciferase reporters were adjusted accordingly.

The luciferase assays were performed as instructed by the manufacturer of the luciferase assay reagent (Promega) and were quantitated with a Berthold MicroLumat LB 960 plate luminometer. The origin and character of the B16, melan-a, and melan-c cell lines has been described previously (28–31) with the MM86 and K1735 cell lines being provided Dr. Dot Bennett.

RESULTS

Characterization of the Tbx2 Gene—To gain an insight into the regulation of Tbx2 gene expression in the melanocyte lineage, it was necessary to isolate the sequences controlling its expression. To this end a mouse BAC library was screened for clones containing the Tbx2 gene. A single BAC clone was isolated, which hybridized to probes derived from the 5'- and 3'-ends of the mouse Tbx2 cDNA. The BAC DNA was initially digested with BamHI and subcloned into pUC19. One subclone, containing approximately 9 kilobases of DNA, hybridized to probes derived from the 5'- and 3'-ends of the Tbx2 cDNA and was therefore likely to contain the entire Tbx2 gene. Sequence analysis confirmed that this was the case, and eventually the sequence of the whole Tbx2 gene was obtained.

The mouse Tbx2 gene was found to comprise seven exons, the relative locations of which are depicted in Fig. 1A. Previous work (32) had identified the intron/exon boundaries of the human gene, which are aligned with those deduced for the mouse gene in Fig. 1B, and the contribution of each exon to the Tbx2 protein is depicted in Fig. 1C. The overall structure of the
The murine Tbx2 gene is remarkably conserved in terms of number and position of exons when compared with that of the human gene. The evolutionary implications of the conservation of gene structure within the T-box family has been discussed previously (33, 34).

The Tbx2 Promoter—Although the structure of the T-box genes is of considerable interest to evolutionary biologists, our primary concern here was to isolate the Tbx2 promoter and to gain some clues as to its regulation. Sequencing revealed that the initial BamHI fragment containing the Tbx2 gene extended only some 350 bp 5′ from the ATG initiation codon, and it was not clear whether this 5′ sequence contained a significant region of the Tbx2 promoter. To obtain additional Tbx2 promoter sequence, the original BAC DNA was restricted with both XhoI and SalI and subcloned into pUC19. The clones isolated were screened using a probe derived from the 5′ end of the Tbx2 cDNA and positive clones sequenced. The results obtained enabled us to determine the sequence of an additional 829 bp of the Tbx2 promoter. The full sequence of this region is shown in Fig. 2 and contains potential binding sites for several transcription factors, including AP1, AP2, SP1, SIF-1, GATA, HIF-1, and EGR-2 (not shown). However, although such an analysis might give some indication as to the potential of the promoter to be regulated, an extensive mutational analysis would be required to determine whether any of these binding sites might be functional. Moreover, because all of these factors are known to be expressed in a wide range of tissues, they were not of immediate interest in terms of the regulation of Tbx2 expression in the melanocyte lineage.

In contrast, an E-box element, CATGTG, located between 332 and 327 bp 5′ to the initiator ATG, attracted our attention. E boxes are recognized by members of the bHLH and bHLH-LZ families of transcription factors. In the melanocyte lineage, the bHLH-LZ transcription factor Mitf plays a critical role; mice devoid of a functional Mitf lack all pigment cells. Mitf has been shown previously to regulate a number of genes, including Tyrosinase, Tyrp-1, and Dct involved in the manufacture of the pigment melanin, a process specific to melanocytes and the retinal pigment epithelium. The sequences recognized in all these Mitf target promoters are E boxes with the sequence CATGTG, including the highly conserved M-box motif (35). Although such E-box elements are present in many genes, we recently established that Mitf can only recognize a subset of E-box motifs in vitro and in vivo (27). Thus Mitf will recognize a CATGTG E box only if it is flanked by a specific 5′ T residue. In other words, Mitf recognizes TCATGTG, CATGTGA, or TCATGTGA. In addition, these elements may also be targeted by a second bHLH-LZ factor, USF1, which is ubiquitously expressed. As we discuss below, whether Mitf or USF1 will recognize these elements in vivo will depend on a number of
factors related to the relative abundance of DNA-binding competent USF1 and Mitf in the cell at a given time.

Before undertaking an analysis of the potential role of this E-box element in the Tbx2 promoter function, we first identified the transcription initiation site by primer extension analysis using a primer corresponding to sequences located between 24 and 52 bp 5′ to the ATG translation initiation codon and mRNA derived from a B16 melanoma cell line. The primer location was chosen because previously isolated Tbx2 cDNAs extended up to a maximum of 57 bp 5′ from the initiator ATG. The results (Fig. 2B) revealed the presence of two bands corresponding to cDNAs extending some 117 bp 5′ to the initiator ATG and 60 bp 5′ to the end of the longest previously published cDNAs. Because no cDNAs extending beyond these positions were obtained using other primers located within the putative Tbx2 leader sequence (not shown), we believe that the 5′ ends of the cDNAs derived by primer extension most likely correspond to the location of the transcription initiation site. These are indicated in Fig. 2A.

**Tbx2 Expression in Melanoma Cell Lines**—Prior to examining the requirements for Tbx2 expression in melanocytes, we asked whether the gene was expressed in a number of cell lines that are regularly used for the analysis of melanocyte-specific gene expression. We had previously shown that Tbx2 was expressed in both melanoblasts and all melanocyte cell lines tested but was not expressed in a melanoblast precursor cell line (23). However, we had not determined whether Tbx2 was also present in melanoma cell lines. We therefore asked whether Tbx2 was expressed in two Mitf-positive melanoma cell lines, B16 and MM96, one Mitf-negative melanoma line, K1735, and the melanocyte cell line melan-c, whereas melan-a melanocytes were used as a positive control. The results, shown in Fig. 3, reveal that Tbx2 was expressed in all cell lines that also expressed Mitf, that is, melan-a, melan-c, and the two melanoma lines B16 and MM96. However, intriguingly, given the presence of the potential Mitf binding site in the Tbx2 promoter, no expression was detected in the Mitf-negative cell line K1735. These results were confirmed independently by Northern blotting.2

**The Tbx2 Promoter Contains a Functional E-box Motif**—To determine whether the Tbx2 promoter isolated was sufficient to direct cell type-specific expression, sequences between −859 and +121, relative to the transcription start site, were cloned upstream from a luciferase reporter (Fig. 4A), and promoter activity was determined following transfection into B16 melanoma cells. As a control, the Tbx2 promoter-luciferase reporter was also transfected into HeLa cells, which we have shown previously do not express Tbx2 (23), and the relative promoter strength was determined by comparison to the activity of a cotransfected SV40-lacZ reporter. The results shown in Fig. 4B demonstrate that the Tbx2 promoter used was sufficient to direct expression specifically in the melanoma cell line; relative to the activity of the lacZ reporter, the Tbx2 promoter was at least 30-fold better expressed in B16 cells than in HeLa cells.

We next determined whether the minimal promoter, which still retained the E-box motif, was functional using promoter deletion mutants (Fig. 5A). Deletion of the Tbx2 promoter to −230 resulted in no more than around a 3-fold decrease in promoter activity relative to the promoter extending to −859 (Fig. 5B). However, mutating the CATGTG E box motif to CCTTG in the context of the −230 deletion resulted in a further 5-fold decrease in promoter activity, suggesting that this element plays a significant role in Tbx2 promoter function. The importance of this element was highlighted by the fact that the activity of the mutated promoter extending to position −230 was no more than 2-fold greater than when the promoter was almost entirely deleted to −11.

**USF and Mitf Bind the Tbx2 Promoter**—The results obtained so far suggest that the Tbx2 promoter is cell type-specific and contains a functional E-box motif that contributes substantially to promoter function. In the melanocyte-specific Tyrosinase Tyrp-1 and Dct2 promoters, the specific E-box elements that are essential for their expression have been shown to bind both the bHLH-LZ transcription factors USF1 and Mitf (4, 6, 7, 26, 35, 36). To determine whether USF1 could recognize the E box present in the Tbx2 promoter, we performed a DNA binding band shift assay using a radiolabeled oligonucleotide probe spanning the E box and in vitro transcribed/translated (ITT) USF1. The sequence of the probe is shown in Fig. 6A, and the results of the DNA binding assay are shown in Fig. 6B. The ability of USF1 to recognize the Tbx2 E box was demonstrated by the presence of a band that was absent when unpro-

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2 D. Bennett, personal communication.
The E box is required for Tbx2 promoter activity. A, Tbx2 promoter fragments extending between −859, −230, or −11 and +121 were fused to a luciferase reporter. The black bar indicates the relative position of the Tbx2 E box. B, the indicated Tbx2 promoter–luciferase reporters were transfected into B16 melanoma cells and luciferase activity determined 48 h post-transfection. The sequence of the mutation introduced into the E box in the mutant E (mE) box reporter is also indicated. The results presented are an average of at least three experiments.

programmed reticulocyte lysate was used. This band corresponded to USF1, because it was supershifted in the presence of a specific anti-USF1 antibody. Consistent with this, the USF1 complex was competed by an oligonucleotide containing a known USF1 binding site, the M box from the Tyrosinase promoter (27, 35), as well as by the Tbx2 E box, whereas no binding was observed to a mutated Tbx2 E box in which the CATGTA motif had been changed to CTTTTG. The complex present in unprogrammed lysate almost certainly corresponds to rabbit USF1 and would not be recognized by the anti-USF1 antibody used. USF1 binding could also be detected using B16 melanoma cell nuclear extract (Fig. 6A) with the USF1 complex being abolished using the anti-USF1 antibody.

Although USF1 can bind the highly conserved E-box elements present in the Tyrosinase, Trypt-1, and Dct2 promoters, targeted disruption of the Usf1 gene results in normally pigmented mice. The evidence available would therefore indicate that, although the Tyrosinase, Trypt-1, and Dct2 promoters can bind USF1 in vitro, in vivo their expression is regulated by Mitf, which can recognize the same E-box motifs with a similar specificity to USF1 (27). Given that the E box within the Tbx2 promoter fits the full consensus for Mitf binding (27), it seemed likely that the Tbx2 promoter might also be regulated by Mitf. To investigate this possibility, we used ITT Mitf to compare the relative binding affinity of Mitf for the Tbx2 E box to that of a known Mitf target, the M box in a band shift assay using the M-box or Tbx2 E-box competitor oligonucleotides (Fig. 7A). The results (Fig. 7B) revealed that Mitf binding to the M box was competed by both the M box and Tbx2 E box with a similar efficiency. To confirm that Mitf derived from cells was also competent to bind the Tbx2 E box, we transfected the Mitf-negative COS cell line with a vector expressing SV5 epitope-tagged Mitf and used nuclear extracts from the transfected cells to assay for Mitf binding to a radiolabeled Tbx2 E box. The binding conditions used in the band shift assay have been described previously (37, 38) and enable detection of Mitf binding while eliminating binding by USF1. The result (Fig. 7C) shows that the complex formed using the transfected cell extract can be partially supershifted using an antibody directed against a peptide derived from the C terminus of Mitf and completely abolished using the anti-SV5 epitope antibody. We also verified that Mitf derived from B16 melanoma cells could bind the Tbx2 E box. In this experiment (Fig. 7D), the complexes formed using extracts from B16 cells together with the Tbx2 E box probe were supershifted using either the anti-Mitf peptide antibody or a second antibody directed against the N-terminal 70 amino acids of Mitf. Note that the anti-Mitf antibody alone does not bind the probe. Taken together, the evidence provided indicates that Mitf can bind the E-box element present in the Tbx2 promoter.

Regulation of the Tbx2 Promoter by USF and Mitf—Although the Tbx2 promoter E box made a significant contribution to Tbx2 promoter function and could bind Mitf and USF1 in vitro, it was necessary to determine whether the Tbx2 promoter, and the E box in particular, was a target for USF1 or Mitf in vivo. To this end, the ability of the Tbx2 promoter extending to −859 to be up-regulated by cotransfection with an Mitf or USF1 expression vector was assessed. The results obtained following transfection into the melanocyte cell line melan-c (Fig. 8A) reveal that, although the Tbx2 promoter could be activated around 4-fold by Mitf, no significant activation was observed using the USF1 expression vector. Similar results were obtained using the tyrosinase promoter that is a known Mitf target (Fig. 8B). Therefore, in the melan-c cells Mitf but not USF1 was able to activate transcription. In contrast, when the assay was repeated in B16 melanoma cells (Fig. 8C), Mitf was able to activate the Tbx2 promoter around 6-fold, but USF1 was also able to induce promoter activity, by around 3-fold. Similar
results in an increase in the total amount of Mitf bound to the probe. melanocyte development being underscored by the fact that Tbx2 results were obtained using the Tbx2 promoter extending to −230, where Mitf and USF1 could both activate around 5- to 6-fold and 7-fold, respectively, in transfected B16 cells (A). The activation of transcription by both Mitf and USF1 was dependent on the presence of an intact E box, with no significant activation by Mitf alone. The characterization of the Tbx2 promoter revealed that it contained an E box with the sequence ACATGTG, which fits the consensus for binding by Mitf, which will only recognize CATCTG T or 3′ A residues (27). Such (T)CATGAT(A) motifs are found at all known Mitf target promoters, including those of the Tyrosinase, Tprp-1, Dct, and Tyrp genes, and Mitf cannot bind a related CATCTG element in the P-gene promoter, which lacks the required flanking bases (27). Second, the E box in the Tbx2 promoter was able to bind Mitf derived either from transfected cells or from B16 melanoma cell nuclear extract and was essential for the efficient expression of the Tbx2 promoter. Thus, the Tbx2 promoter was activated by cotransfection of an Mitf

DISCUSSION

Commitment to the melanocyte lineage is characterized by the onset of Mitf expression, the fundamental role of Mitf in melanocyte development being underscored by the fact that mice lacking Mitf are entirely devoid of pigment cells. In the absence of functional Mitf protein, melanoblasts expressing Mitf mRNA appear in the embryo but disappear after around 2 days (3), either by apoptosis or by losing their identity and adopting an alternative cell fate. The role of Mitf must therefore be to regulate genes required for melanoblast survival, proliferation, or the maintenance of melanoblast identity. Yet to date, Mitf has been shown to regulate the Tyrosinase, Tprp-1, and Dct genes, which are required for pigmentation, a differentiation-specific process. Because expression of these genes cannot account for the complete absence of pigment cells in Mitf-negative mice, Mitf must regulate the expression of other as yet uncharacterized genes. Here we provide several lines of evidence to suggest that Mitf may also regulate the Tbx2 promoter-luciferase reporter that was used as a control for transfection efficiency, and the results represent an average of three experiments.

FIG. 7. Mitf binds the Tbx2 E box. A, sequence of the WT and mutated Tbx2 E-box and M-box oligonucleotides used in the DNA binding assays. The E-box elements are overlaid and mutated bases indicated in lowercase and underline. The location of the sequences shown relative to the Tbx2 transcription start site is also indicated. B, ITT Mitf used in a band shift assay together with a radiolabeled E-box probe and competition by the M-box or Tbx2 oligonucleotides. Competitor DNA was used at 10, 50, and 250 ng. Only the bound DNA is shown. C, ectopically expressed Mitf binds the Tbx2 E box. Nuclear extracts from COS cells transfected with a vector expressing SV5 epitope-tagged Mitf were used together with a radiolabeled Tbx2 E-box probe in a band shift assay, either in the presence or absence of anti-SV5 or anti-Mitf peptide antibody as indicated. D, B16 cell nuclear extract was used together with a radiolabeled Tbx2 E-box probe in a band shift assay, either in the presence or absence of anti-Mitf N terminus or anti-Mitf peptide antibody as indicated. A control in which anti-Mitf antibody was incubated with the probe alone is also shown. Note that in our hands one of the characteristics of anti-Mitf antibodies is that the addition of antibody, even to bacterially expressed Mitf, tends to increase the total amount of Mitf available to bind DNA if the antibody is not in excess of the Mitf protein present. We believe that the most likely reason for this is that the majority of Mitf is in an inactive conformation that is altered when bound by antibody such that the antibody-Mitf complex binds DNA better than does Mitf alone. The ability of antibody to induce a conformational change in target proteins is well known. Thus, the addition of anti-Mitf antibody to cell extracts in A–D results in an increase in the total amount of Mitf bound to the probe.

FIG. 8. Regulation of the Tbx2 promoter by Mitf and USF1. The Tbx2 promoter-luciferase reporter was transfected into melan-c cells (A) or B16 cells (C) in the presence or absence of a cotransfected Mitf or USF1 expression vector as indicated, and luciferase activity was determined 48 h post-transfection. B, a tyrosinase promoter-luciferase reporter was transfected into melan-c cells in the presence or absence of a cotransfected Mitf or USF1 expression vector as indicated, and luciferase activity was determined 48 h post-transfection. The tyrosinase promoter used has been described previously (6) and extends between −300 and +80 with respect to the transcription start site. D, a WT or E-box-mutated Tbx2 promoter extending to −230 was fused to a luciferase reporter and cotransfected into B16 melanoma cells in the presence of either cotransfected Mitf or USF1 expression vectors as indicated. The sequence of the E-box mutation is indicated. Luciferase activity was determined 48 h post-transfection. The values presented in A–D have been adjusted relative to the activity of a cotransfected SV40-lacZ reporter that was used as a control for transfection efficiency, and the results represent an average of three experiments.
expression vector both in the melan-c melanocytes and in the B16 melanoma cell line. Fourth, Tbx2 was expressed in both the B16 and MM96 melanoma cell lines, which express Mitf, but not in the K1735 melanoma cells, which are Mitf-negative, and our previous work (23) has demonstrated that Tbx2 is present in Mitf-positive melanoblasts as well as melanocytes but not in melanoblast precursor cells that do not express Mitf. Although the pattern of Tbx2 expression in the melanocyte lineage in the developing embryo has yet to be determined, the evidence presented here provides a strong indication that the Tbx2 gene is a candidate for regulation by Mitf.

If the Tbx2 gene is regulated by Mitf, this then raises the important question as to what function is likely to be performed by Tbx2 in the melanocyte lineage. Tbx2 is a member of the T-box family of transcription factors, which plays a critical role in development. In particular, the evidence available implicates T-box proteins in the maintenance of cell identity. For example, the loss of Tbx6 results in a somite to neural tube transition, resulting in an embryo with three neural tubes (20). In addition, the Tbx4 and Tbx5 genes have been implicated in limb identity; missexpression of Tbx5 in the hindlimb bud in chick embryos results in a leg to wing transition, whereas ectopic Tbx4 expression in the forelimb bud converts the wing into a leg (21, 22). Our view is that a similar role may be played by Tbx2 in the melanocyte lineage, and a working model might be that the onset of Mitf expression results in the specification of a melanoblast but that the continued identity of an Mitf-positive cell may require the Mitf-dependent expression of Tbx2. Whether such a model bears any resemblance to reality will await the detailed characterization of the expression pattern of Tbx2 in the melanocyte lineage in WT and Mitf-negative embryos and results from the targeted disruption of the Tbx2 gene.

Of course, although we believe that Tbx2 is likely to play an important role in the development of the melanocyte lineage, this is not to say that Tbx2 will not regulate other genes more characteristic of melanocyte differentiation. A precedent for this is provided by both Mitf and Pax3, which are both essential for the genesis of pigment cells, but both of which also regulate the expression of pigmentation genes, Mitf acting via the M-box and other E-box elements in the Tyrosinase, Tyrp-1, and Dct promoters (4–8), whereas Pax3 binds the MSEu and MSEi elements in the Tyrp-1 promoter (39). Intriguingly, the Tyrp-1 MSEu and MSEi elements are also recognized by Tbx2 where binding appears to correlate with transcriptional repression (23), and it may be that one role of Tbx2 is to compete with Pax3 for binding to specific Pax3/Tbx2 recognition elements. This may be important given that mutations in Pax3 can give rise to Splotch mice (40) and human Waardenburg syndrome type 1 (41, 42), which is characterized by defects in the development of the melanocyte lineage resulting in pigmentation abnormalities. However, although the effects of Pax3 appear to be mediated at least in part by its ability to activate Mitf expression through a specific binding site in the Mitf promoter (43), we have not observed any Tbx2 binding to this site. Nevertheless, on some sites at least, competition between members of the T-box family and paired-homeo domain factors like Pax3 may be a key feature in development.

In addition to being recognized by Mitf, the Tbx2 E box is also a target for binding by USF1, and in B16 melanoma cells but not in melan-c cells, expression of USF1 was able to activate transcription from the Tbx2 promoter. Like Mitf, USF1 is able to recognize the E-box elements present in the Tyrosinase, Tyrp-1, and Dct promoters, and USF1 shares a very similar binding specificity to Mitf. However, mice lacking USF1 are pigmented normally (44, 45), indicating that in vivo, USF1 is unlikely to play a major role in regulating the basal level of expression of these genes. Moreover, USF1 is abundant in HeLa cells where, as we show here, the Tbx2 promoter is essentially inactive. However, this is not to say that USF1 will never act to regulate expression through the Mitf binding sites in these promoters. Whether a specific element will be targeted by USF1 or by Mitf will depend to a large extent on the relative abundance of each factor. In this respect it is significant that phosphorylation of Mitf on serine 73 by the mitogen-activated protein kinase ERK2 results in its destabilization (46, 47), while it is also known that USF1 DNA binding activity is regulated by phosphorylation, most likely by a stress-responsive kinase (48). Thus, it is likely that the activity of specific signal transduction pathways will dictate whether it is Mitf or USF1 that binds and regulates the expression from the promoters containing the specific E-box elements. Differential signaling might well explain why USF1 was unable to activate the Tbx2 promoter in melan-c melanocytes but could activate the promoter in the B16 melanoma cells.

Although in this paper we have focused on the potential regulation of Tbx2 expression in the melanocyte lineage, Tbx2 is also expressed in several other cell types. In mouse development, Tbx2 mRNA is not expressed at embryonic day 7.5 (E7.5), but by E9.5 was detected by in situ hybridization in the otic and optic vesicles and at later stages in a several other specific regions of the embryo, including the mesenchyme at the anterior and posterior margins of the limb buds (49). It has also been proposed that Tbx2, together with Tbx5, plays a critical role in limb morphogenesis. Northern blotting has also revealed mouse Tbx2 expression in adult lung, kidney, ovary, and heart (9). In these cells, the role of Mitf in expression of Tbx2 in melanocytes may be performed by other members of the bHLH-LZ transcription factor family, and it will be important to understand which elements control Tbx2 expression in these cells as well as in transgenic mice. Whatever regulates Tbx2 expression in other cell types, it seems likely that understanding the role of Tbx2 and its regulation by Mitf in the melanocyte lineage will provide a fascinating area for future research.

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