The retinal pigment epithelium (RPE) is crucial for the function and survival of retinal photoreceptors. VMD2 encodes bestrophin, an oligomeric chloride channel that is preferentially expressed in the RPE and, when mutated, causes Best macular dystrophy. Previously, we defined the VMD2 upstream region from −253 to +38 bp as being sufficient to direct RPE-specific expression in the eye, and we suggested microphthalmia-associated transcription factor (MITF) as a possible positive regulator. Here we show that in transgenic mice the −154 to +38 bp region is sufficient for RPE expression, and mutation of two E-boxes, 1 and 2, within this region leads to loss of promoter activity. A yeast one-hybrid screen using bait containing E-box 1 identified clones encoding MITF, TFE3, and TFEB, and chromatin immunoprecipitation with antibodies against these proteins enriched the VMD2 proximal promoter. Analysis using in vivo electroporation with constructs containing mutation of each E-box indicated that expression in native RPE requires both E-boxes, yet in vitro DNA binding studies suggested that MITF binds well to E-box 1 but only minimally to E-box 2. MITF knockdown by small interfering RNA (siRNA) in cell culture revealed a strong correlation between MITF and VMD2 mRNA levels. Sequential transfection of a luciferase construct with expression vectors following MITF siRNA revealed that TFE3 and TFEB can also transactivate the VMD2 promoter. Taken together, we suggest that VMD2 is regulated by the MITF-TFE family through two E-boxes, with E-box 1 required for a direct interaction of MITF-TFE factors and E-box 2 for binding of the as yet unidentified factor(s).

The retinal pigment epithelium (RPE),5 a monolayer of neuroepithelium-derived cells located between the photoreceptor layer and choroid of the eye, is essential for supporting and maintaining the health and function of retinal photoreceptor cells (1, 2). Demonstrating the significance of the RPE to human disease, age-related macular degeneration, the leading cause of irreversible blindness in the elderly in the Western world, is thought to involve a primary or secondary defect in RPE function (3, 4). In addition, mutations in a growing number of genes that are specifically or preferentially expressed in the RPE, such as RPE65, RLBP1, RGR, TIMP3, RDHS, and VMD2, can cause a variety of human retinal dystrophies (5–16).

Despite the key role of the RPE in vision, relatively little is known about the mechanisms regulating RPE gene expression. Several groups have begun to define promoter regions capable of directing RPE-enriched or RPE-specific expression, and also to identify some of the regulatory elements involved (e.g. Rpe65 (17, 18), RPE65 (19), RLBP1 (20), tyrosinase (Tyr) (21–24), tyrosinase-related protein 1 (TTRP1) (25), and Oa1 (26)). Information about specific transcription factors regulating RPE expression is advancing, although still quite limited (27, 28). To analyze RPE gene regulation in the context of a gene important for retinal disease, we have been studying VMD2 regulation as a model system (29).

VMD2 is highly and preferentially expressed in the RPE, and its mutation causes Best disease (vitelliform macular dystrophy (VMD)), an autosomal dominant, juvenile-onset macular degeneration that is associated with an abnormal electrooculogram (15, 16). Histopathologically, Best disease is characterized by accumulation of lipofuscin-like material within and beneath the RPE, a finding similar to that seen in age-related macular degeneration (30–32). VMD2 encodes bestrophin, a multispan transmembrane protein that is localized at the basolateral plasma membrane (33, 34) and functions as an oligomeric chloride channel (35, 36). Mutations in VMD2 were also identified in families with nanophthalmos associated with autosomal...
dominant vitreoretinochoroidopathy, suggesting a role for VMD2 in ocular development (37).

In our previous analysis of the VMD2 promoter, transgenic mouse studies revealed that the human −253 to +38 bp promoter fragment is sufficient to direct RPE-specific expression in the eye (29). Using transient transfection assays with the D407 human RPE cell line, two positive regulatory regions were identified as follows: −585 to −514 bp for high level expression and −56 to −42 bp for low level expression. The −56 to −42 bp region contains a canonical E-box (designated E-box 1, −47 to −42 bp), and another E-box is located nearby (E-box 2, −69 to −64 bp). Mutation of E-box 1 greatly diminished luciferase expression in D407 cells and abolished the bands shifted with bovine RPE nuclear extract in electrophoretic mobility shift assays (EMSAs), although mutation of E-box 2 had only a moderate effect on luciferase activity, and EMSAs with E-box 2-containing probes did not yield distinct shifted bands (29). Using a candidate gene approach, based on known E-box binding activity (24, 38) and expression in the RPE (39, 40) of the microphthalmia-associated transcription factor (MITF), we found that MITF could transactivate the VMD2 promoter reporter in D407 cells in an E-box-dependent manner.

MITF is a member of the basic helix-loop-helix leucine zipper (bHLH-ZIP) family of transcription factors and is expressed in several cell lineages, including melanocytes, RPE, osteoclasts, and mast cells (24, 38, 41–45). In humans, heterozygous MITF mutations result in Waardenburg syndrome type IIA and Tietz syndrome, conditions characterized by hearing loss and pigmentation defects (46–48). Homozygous mutations, which might have a significant ocular phenotype, have not been identified in humans. In contrast, in Mitf mutant mice (Mitf⁴⁰¹/⁴⁰¹), the RPE becomes thickened, loses pigmentation, and forms a multilayered structure resembling the early stage of neural retina, indicating a critical role for MITF in RPE differentiation (39, 40, 49–52). In vitiligo mice (Mitfplacing/mi⁴⁰¹vii/Mitfplacing/vii), although the RPE and neural retina show grossly normal morphological development, the photoreceptors degenerate, with almost complete rod and cone loss by 2 years (53, 54). In mouse eye development, Mitf is expressed throughout the optic vesicle at embryonic day 9 (E9), but its expression is rapidly restricted to the presumptive RPE by E9.5 (40, 55). This restriction in expression appears to be developmentally important in that maintenance of the identity of the neural retina requires repression of Mitf by Chx10 (55, 56).

In addition to MITF, other members of the MITF-TFE (MiT) bHLH-ZIP subfamily are TFE3 (57), TFEB (58), and TFEC (59). All MiT members share an identical (basic) DNA binding domain and have highly homologous HLH and ZIP dimerization domains (38, 43). All can bind to E-box sequence in vitro as either homodimers or heterodimers with each other but not with other members of the bHLH-ZIP family (43). Besides MITF, TFE3 also stimulates the human Tyr and murine Tyrp1 promoter activities, and TFEB transactivates the murine Tyrp1 promoter in B16 mouse melanoma cells (60). TFE3 can collaborate with MITF to superactivate the TRAP promoter in osteoclasts in transfection assays (61), and MITF and TFE3 have functionally redundant roles in osteoclast development in mice (44). Tfe3 knock-out mice do not exhibit visible abnormal phenotypes; however, double knockouts for Tfe3 and Mitf develop severe osteopetrosis, a phenotype not seen in mice null for either gene alone (44). Knock-out mice for Tfeb die between E9.5 and E10.5 because of severe defects in placental vascularization (62). Tfec is only transiently expressed in the early developing RPE in the eye (56), and Tfec knock-out mice are phenotype normally (44).

To gain further insights into the molecular mechanisms regulating RPE gene expression, we have been trying to understand the VMD2 promoter in greater detail. In this study, we further define the minimal promoter region necessary for RPE-specific expression, present data indicating that the previously identified E-boxes 1 and 2 are both important in vivo, and provide evidence strengthening our earlier suggestion of the involvement of MITF in mediating the activity of the E-boxes. Based on the DNA binding and functional data presented, we suggest a model of MITF transactivation of VMD2 in which MITF acts on E-box 1 by direct DNA binding, whereas its effect on E-box 2 is mediated by an indirect mechanism, possibly involving a second E-box binding protein. Furthermore, we present evidence suggesting that TFE3 and TFEB are also involved in modulating the VMD2 promoter.

EXPERIMENTAL PROCEDURES

Plasmid Construction—VMD2 promoter/luciferase reporter constructs containing the fragments −154 to +38 and −104 to +38 bp in the pGL2-Basic vector, which contains firefly luciferase gene (Promega, Madison, WI), were generated previously (29). Mutated VMD2 promoter/luciferase constructs containing mutation (CANNTG to ACNNTA) in E-box 1 (−47 to −42 bp, designated m1), E-box 2 (−69 to −64 bp, m2), or both (m1m2) were made in the context of the −154 to +38 bp fragment as follows. Forward oligonucleotide that corresponds to −154 to −75 bp (5′-AGGCTTGCTAGCCGTGTCTTCTGAGCAGATTAAGAAGGGACCAAGACTCC-TTGGAGGAGTCTCTGCTAG-3′) was mixed at a 10:1 ratio with the previously generated −104 to +38 bp fragments containing mutations, m1, m2, and m1m2 (29), and the mixture was used as template to amplify the −154 to +38 bp fragments containing m1, m2, and m1m2 by PCR using a forward primer 5′-AGGCTGTGCTAGCCGTGTCTTCTGAGCAGATTAAGAAGGGACCAAGACTCC-TTGGAGGAGTCTCTGCTAG-3′ and a reverse primer 5′-GGTCTGCGACTAGGCTGTGTCTGCTAGCTGCTAG-3′. All fragments were blunt-ligated into SmaI site of pGL2-Basic vector and verified by sequencing.

Two VMD2 promoter/lacZ reporter constructs containing the −154 to +38 bp fragments, wild-type and m1m2, were made for transgenic mouse studies. Both fragments were generated by PCR as described above and blunt-ligated into the SmaI site upstream of lacZ gene in placF vector as described previously (29).

An expression vector containing human MITF-M cDNA in pcDNA3.1/Myc-His(−) B vector (Invitrogen) was made previously (29). Using this construct as a backbone, four mutated MITF-M expression vectors containing mutations from Mitf mutant strains were newly generated. Mitf mutant alleles from which mutations were used are as follows: Mitf⁴⁰¹⁴⁰¹ (allele name, white; T to A transition at nucleotide 764), Mitf⁴⁰¹⁴⁰¹ (spotted; insertion of a C residue at a splice acceptor site, leading to a loss of
VMD2 Is Regulated by the MIT Family

exon 6a, Mitf

(enu198) (vitiligo; G to A transition at nucleotide 793), and Mitf

(enu198) (A to G transition at nucleotide 749) (Mouse Genome Informatics, The Jackson Laboratory) (63, 64). A point mutation (Mitf

, Mitf

, and Mitf

) was introduced into the corresponding position of the wild-type human MITF-M expression vector using QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following primers. For mutation of Mitf

, forward primer 5'-GGC-AGGAGAAGGACAATCACACCTGAATGAGCAAAGAA-3' and reverse primer 5'-CTTCTCTCTCCGTTCAATT-CAGGTTGTGATTTGCTCTTTTTCGCC-3'; for Mitf

, forward primer 5'-CGAAGAGAAGAAGATTACATAAATA-ACCCGATATAAGAAGACTAGG-3' and reverse primer 5'-CTTCTTTTTATGCGGTTATTTATGTTAAATCTTCTTTCG-3', and for Mitf

, forward primer 5'-GGCAAAGAGGGCAAGAAAAGGCAATCAACCTGTAGTTG-3' and reverse primer 5'-CAATCAGGATGTGATGCTCCCTTTTCTGCTCCTTTTGGC-3'. MITF-M cDNA with an 18-bp deletion leading to a loss of exon 6a (Mitf

) was obtained among the natural splice variants from human RPE RNA by reverse transcription (RT)-PCR and subcloned into pcDNA3.1/Myc-His(−) B vector as described previously (29).

To construct expression vectors for human TFE3 and TFEB, cDNAs were generated by RT-PCR using human RPE total RNA as template and the following primers and inserted into EcoRV/HindIII and EcoRI/HindIII sites, respectively, in pcDNA3.1/Myc- His(−) B vector as described previously (29). The primers used were as follows: for TFE3, forward containing EcoRV site 5'-ACTGATATCGGCGTCATGTCTC-ATCGGGCC-3' and reverse containing HindIII site 5'-AGAAAGCTTGAGGACCTCTCTCTTCCGTTGAGA-3'; for TFEB, forward containing EcoRI site 5'-ACTGAATTCGCCACCAT-GGCGTCAGGCATA-3' and reverse containing HindIII site 5'-AGAAAGCTTGACGTACAAGCCGCCCTCTCCCATC-3'.

Cell Cultures—D407 human RPE cells were cultured as reported (65). Five human melanoma cell lines were screened for the expression of VMD2 by RT-PCR as described previously (29). The expression of MITF-M in these melanoma cells was also checked using a forward primer 5'-CATCCGGCTCCTCTGATGATTG-3' and a reverse primer 5'-CATTCCATCTCTGCATAAGG-3'. Based on these results, SK-MEL-5 cells (66) was obtained among the double-stranded cDNAs followed by size fractionation using cDNA synthesis kit (ChilP) and maintained in the medium recommended by the American Type Culture Collection (ATCC, Manassas, VA).

Transient Transfection—Transient transfection assays were performed as described previously (29), except that dual luciferase assays were used instead of single luciferase and β-galactosidase assays. Plasmid DNA for each 60-mm dish included 3 μg of a firefly luciferase construct and 0.1 ng of pRL-CMV containing Renilla luciferase gene (Promega) as an internal control for transfection efficiency. Because we found that MITF-M activates the cytomegalovirus promoter in control pRL-CMV, we instead utilized pRL-TK containing Renilla luciferase gene driven by the thymidine kinase promoter (Promega), which is not affected by MITF-M, as control for co-transfection studies involving MITF-M. For these studies, DNA mixture for each dish included 3 μg of a firefly luciferase construct, 2.5 μg of a human MITF-M expression vector or an empty pcDNA3.1 vector as control, and 1 ng of pRL-TK. Transfections were performed four independent times in duplicate each time. Cell lysates were prepared using 300 μl of Passive Lysis Buffer (Promega) for each dish and analyzed using Dual-Luciferase Reporter System (Promega) according to the company’s protocols. Firefly luciferase activities were normalized by Renilla luciferase activities, and relative luciferase activities were calculated as the ratio of the normalized luciferase activity with constructs containing VMD2 promoter fragments to that with empty pGL2-Basic vector. To assess the effect of MITF-M on the VMD2 promoter, relative luciferase activities (fold increase) were calculated as the ratio of the normalized luciferase activity with the MITF-M expression vector to that with empty pcDNA3.1 vector. To evaluate the effects of MITF mutations, percent luciferase activities were calculated as the percentage of the normalized luciferase activity with mutated MITF to that with wild-type MITF (presented as 100%).

In Vivo Electroporation—The development of an in vivo electroporation method to introduce plasmid DNA directly into adult mouse RPE and its application to analysis of the VMD2 promoter were described elsewhere (67, 68). Using 6–8-week-old BALB/cj mice, in vivo electroporation following subretinal injection was performed with 1 μl of phosphate-buffered saline containing 0.5 μg of a VMD2 promoter/luciferase construct and 0.25 ng of pRL-CMV as control to normalize electroporation variability among eyes. Mice were euthanized 3 days after electroporation, eyes were dissected to remove cornea and lens, and cell lysates were prepared from the posterior portion of the eye using 100 μl of Reporter Lysis Buffer (Promega) (68). Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter System, and relative luciferase activities were calculated as described above.

Generation and Analysis of Transgenic Mice—Transgenic mice carrying a construct containing the VMD2 –154 to +38 bp fragment, either wild-type or with m1m2, fused to a lacZ reporter (VMD2 promoter/lacZ) were generated and analyzed as described previously (29).

Yeast One-Hybrid (YOH) Screen—A hybrid library of bovine RPE cDNAs fused to the activation domain (AD) of yeast transcription factor GAL4 was constructed using 5 μg of poly(A)+ RNA purified by RNasey and Oligotex (Qiagen, Valencia, CA). An oligo(dT) primer containing an Xhol site was used for first-strand cDNA synthesis, an EcoRI adapter was attached to the 5'-end of the double-stranded cDNAs followed by size fractionation using cDNA synthesis kit (Stratagene), and the double-stranded cDNAs were inserted into EcoRI/Xhol sites downstream of GAL4-AD in pGADT7 carrying the LEU2 gene for nutritional selection (Clontech). Electroporation was used to transform Escherichia coli (E. coli) by the ligated library plasmids. The number of independent clones was 3.1 × 10⁶, 3.0 × 10⁶, 2.9 × 10⁶, and 3.1 × 10⁶ in Fractions I, II, III, and IV, respectively. The average size of inserts evaluated by colony PCR was 2.0, 2.0, 1.5, and 1.5 kb for Fractions I, II, III, and IV, respectively.

Three yeast reporter vectors (pHIS1, pHIS1-1, and pLaCZ1) were constructed with a tetramer of the VMD2 promoter region from –60 to –36 bp containing E-box 1 (29) using the Matchmaker One-Hybrid System (Clontech) (69). A 124-base
sense-strand oligonucleotide containing the tetramer, which also included an EcoRI site at the 5’-end and Sall and XbaI sites at the 3’-end, was entirely synthesized along with a 15-base antisense oligonucleotide complementary to the 3’-end of the sense-strand. After annealing, the antisense strand was extended by Klenow fragment, and the resultant double-stranded DNA was directionally cloned into EcoRI/XbaI sites of pPHSi and pPHSi-1 and into EcoRI/Sall sites of pLaCZi. Then the yeast reporter constructs were linearized and used for transforming yeast strain YM4271 by a polyethylene glycol/lithium acetate method (70), and yeast transformants were obtained by nutritional selection. After testing the background expression of HIS3 and lacZ, a dual reporter strain containing both HIS3 and lacZ constructs was generated.

The yeast dual reporter strain was transformed with plasmid DNA of the hybrid library and plated on selection media as described previously (69). Using 30 µg of DNA of both Fractions I and III of the library, 14.3 × 10⁶ yeast colonies were screened for each fraction, and growing colonies were stained by X-gal as a secondary screen. Crude plasmid DNAs were extracted from yeast mini liquid cultures and used to transform E. coli by electroporation, and the resultant purified plasmid DNAs were used for sequencing.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed according to standard methods as described previously (29, 71), except that proteins to be tested (MITF-M, TFEB, and control luciferase proteins) were generated by in vitro transcription/translation using the TNT T7 Quick-Coupled Transcription-Translation System (Promega). The efficiency of protein production was checked in a separate set of reactions by labeling proteins with [35S]methionine. Two oligonucleotide probes, one corresponding to −55 to −34 bp that contains E-box 1 (probe 1) and the other corresponding to −77 to −56 bp that contains E-box 2 (probe 2), were generated together with probes containing mutation of E-box 1 and 14.3 bp containing 1 µl of in vitro translated proteins in binding solution (10 mM Tris-Cl, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5% glycerol) containing 1 µg of poly(dI-dC) on ice for 30 min and analyzed. For supershift analyses, proteins were incubated with 2 µl of each antibody on ice for 2 h before addition of a labeled probe. Antibodies used were anti-MITF mouse monoclonal antibodies (Ab-1, Lab Vision, Fremont, CA) and D5 (Ab-2, Lab Vision) (72, 73). For cold oligomer competition experiments, [32P]-labeled probe 1 was first mixed with 10-, 100-, and 1,000-fold molar excess of unlabeled annealed oligonucleotides, and then added to binding solutions containing 1 µl of MITF-M protein.

Chromatin Immunoprecipitation—ChIP with SK-MEL-5 cells was performed using 2 × 10⁷ cells as described previously (74) with minor modifications, and precipitated chromatin-protein-antibody complexes were collected by protein G-agarose (Upstate, Charlottesville, VA). The antibodies used were as follows: a mixture of C5 and D5, sc-10999 (Santa Cruz Biotechnology, Santa Cruz, CA), and sc-11002 (Santa Cruz Biotechnology) for MITF; ab2753 (Abcam, Cambridge, UK) and 554263 (Pharmingen) for TF3; and ab2636 (Abcam), sc-11004 (Santa Cruz Biotechnology), sc-11005 (Santa Cruz Biotechnology), and AB4149 (Chemicon International, Temecula, CA) for TFEB. The final DNA precipitates were analyzed by PCR using primers specific for the VMD2 promoter. As positive control, the tyrosinase proximal promoter (TYR-P) and distal enhancer (TYR-D) and the tyrosinase-related protein 1 (TYRP1) promoter were analyzed. The rhodopsin (RHO) and albumin (ALB) promoters were used as negative control. The primers used were as follows: for VMD2, forward 5’-AAGGACTCCTTTTGAGAGTT-3’ and reverse 5’-GGCTCT-GCCGACTAGGGTTCT-3’; and TYRP1, forward 5’-CTACCTCTC-ATTGGCAGTCT-3’ and reverse 5’-CCAGGAACATTCTC-CTCTAG-3’; TYR-D, forward 5’-AGTGCGCTGAAAGGCA-3’ and reverse 5’-TTGACGAGTACATGAGACAGT-3’; and RHO, forward 5’-AGCTGTTCAAGGAGATCA-3’ and reverse 5’-GGTAATGAAATAGTACATCG-3’ and reverse 5’-GGTTACCACCTCAAGTGC-3’. siRNA Transfection—siRNAs were transfected into 1 × 10⁵ SK-MEL-5 cells in 24-well plates using 1.5 µl/well TransIT-siQUEST reagent (Mirus Bio, Madison, WI) following the company’s instructions. To check the dose dependence of gene knockdown, siRNAs targeting at MITF, TF3, and TFEB (ON-TARGETplus SMARTpool siRNAs, Dharmaco, Lafayette, CO) were initially transfected at 0, 10, 20, 40, 60, 80, and 100 nM. Based on these results, each siRNA was used at 60 nM for siRNA combination experiments. Throughout these experiments, control siRNA (CONTROL Non-Targeting siRNA 1; Dharmacon) was also included. Different siRNAs targeting at MITF, TF3, and TFEB were also obtained from Sigma-Prologi (Woodlands, TX) and used to confirm the initial results, to help rule out possible off-target effects. To measure expression levels of siRNA targets and VMD2, total RNAs were extracted 24 h post-transfection using TRIzol reagent (Invitrogen). To analyze the effects of siRNAs on VMD2 promoter activity, double transfections were performed sequentially, first with siRNAs and then 24 h later with plasmid DNA, including 0.3 µg of a VMD2 promoter/luciferase construct, and 0.05 ng of PRL-CMV as control for each well, using Lipofectamine PLUS (Invitrogen). Cell lysates were prepared 48 h after plasmid transfection using 50 µl/well Passive Lysis Buffer, and dual luciferase assays were performed as described earlier. In one set of experiments, the same siRNAs or the same combinations of siRNAs were transfected in three wells, one for RNA extraction and two for double transfection, and the same set was repeated three independent times. To evaluate the effects of TF3 and TFEB on VMD2 promoter activity under the condition of MITF knockdown, double transfection was performed using 60 nM MITF siRNA in the same manner as described above, except that plasmid DNA included additional 0.3 µg of a pcDNA3.1 expression vector containing TF3 or TFEB cDNA or no insert.

Real Time PCR—The knockdown of siRNA target genes as well as the expression changes of endogenous VMD2 were evaluated by real time RT-PCR using RNAs extracted as described above. First-strand cDNA was synthesized from 1 µg of total RNA.
with oligo(dT) primer using SuperScript III reverse transcriptase (Invitrogen), and quantitative PCR was performed with the following primers and IQ SYBR Green Supermix (Bio-Rad) using iQ5 Real-Time PCR Detection System (Bio-Rad). The primers used were as follows: for GAPDH (control), forward 5’CTGACTTCAACAGCGACACC-3’ and reverse 5’-TAGCCAAATTCGTTGTCATACC-3’; for RPLP0 (control), forward 5’-CGACCTGGAAGTCCAACACTAC-3’ and reverse 5’-ATCTGCTGACATCTGCTTTG-3’; for MITF (all isoforms), forward 5’-TGAATCGGATCATCAAGC-3’ and reverse 5’-CGCTAACAAAGTGTGCTCCGT-TC-3’; for TFE3, forward 5’-TCCTGAAGGCCTCTGTGGA-T-3’ and reverse 5’-AGTCCACAAGGGCATCTGA-3’; for TFB, forward 5’-AAATGCAGATGCCCACACGT-3’ and reverse 5’-ATTCCCCAATCTGATGGCAT-3’; and for VMD2, forward 5’-CATAGACACAAAGACAAAAGC-3’ and reverse 5’-GTGCTTCTACCTGTTTTCC-3’.

Based on the cross-over point (threshold cycle, Ct) values, the expression level of each gene was normalized by that of GAPDH, and percent expression was calculated as the percentage of the normalized expression level with siRNA to that without siRNA (presented as 100%).

RESULTS

−154 to +38 bp Region of VMD2 Is Sufficient to Drive RPE Expression—To define with more precision the regulatory region of the VMD2 promoter sufficient for RPE expression, we generated transgenic mice with a VMD2 promoter/ lacZ construct containing the region −154 to +38 bp. The resultant mice were analyzed by X-gal staining of RPE/choroid flat-mounds and eye sections. Six transgenic founder animals were obtained, and from these we were able to successfully establish four independent lines. Of the four, three demonstrated lacZ expression in their RPE. Of the three positive lines, line 1 showed strong X-gal staining (Fig. 1, A and E), with a staining pattern that was RPE-specific in the eye except for areas of weak blue staining in the ciliary body. The other two lines demonstrated milder RPE X-gal staining, with line 2 showing scattered blue dots (Fig. 1, B and F) and line 3 showing weak homogeneous staining (Fig. 1, C and G). None of the control mice showed positive X-gal staining (Fig. 1, D and H). These results indicate that the DNA region from −154 to +38 bp contains sufficient information to drive expression in the RPE and that within the eye this expression is largely RPE-specific.

Yeast One-Hybrid Screen with E-box 1 Bait Identified MITF, TFE3, and TFEB—Based on our previous studies suggesting that an important element is located in the −56 to −42 bp region that contains a canonical E-box (E-box 1, −47 to −42 bp), and that bovine RPE nuclear extract contains E-box 1 binding activity (29), we used a YOH approach to screen for factors that bind to this E-box 1-containing region. A bovine RPE cDNA/GAL4-AD fusion library was screened using a tetramer of the −60 to −36 bp region as bait. Of a total of 130 positive clones successfully identified by sequencing, 13, 11, and 8 clones encoded TFE3, TFEB, and MITF, respectively. Based on our prior candidate gene-based data implicating MITF as one of the factors that binds to E-box 1 (29), the well known role of MITF in RPE differentiation (39, 40, 52), and its preferential expression in the RPE compared with the broader expression patterns of TFE3 and TFEB (75), we decided to initially focus our studies on MITF.

MITF-M Protein Binds to E-box 1 but Only Marginally to E-box 2—Biochemical analysis was carried out to test the ability of MITF protein to bind to E-box 1. Because of conflicting data about the role of E-box 2 (in straight transfection-based promoter studies E-box 1 appears to be significantly more important than E-box 2, whereas in MITF-mediated transactivation assays mutation of either E-box has a similar effect (29)), we also included E-box 2 in the binding studies. EMSAs were performed with human MITF-M and control luciferase proteins generated by in vitro transcription/translation, and two annealed oligonucleotide probes, probes 1 (−55 to −34 bp containing E-box 1) and 2 (−77 to −56 bp containing E-box 2), as well as probes containing mutation of E-box 1 (probe m1) and E-box 2 (probe m2). Probe 1 yielded a prominent shifted band with MITF-M protein, which was supershifted by two anti-MITF antibodies, C5 and D5 (film exposure 9 h) (Fig. 2A, left panel). A similar prominent band was not observed with luciferase protein, and mutation in E-box 1 completely abolished the band shifts. A minor band that was seen in all lanes with probe 1 was most likely because of endogenous protein(s) present in the in vitro translation extract. In contrast to the results with E-box 1, EMSA with E-box 2 (probe 2) did not yield prominent shifted bands even after exposure for 10 days. A faint supershifted band was observed with probe 2 and MITF-M protein (Fig. 2A, right panel).

To compare the relative binding strength of MITF-M to E-boxes 1 and 2, a cold oligomer competition experiment was performed using 32P-labeled probe 1 and unlabeled “competitor” oligonucleotides corresponding to probes 1, m1, and m2. The strongest competition was observed with E-box 1 competitor, and the concentration of E-box 2 competitor necessary to achieve a similar level of competition was 10–100-fold higher than that of E-box 1 competitor (Fig. 2B). Mutated competitor, either m1 or m2, did not significantly compete with probe 1. These results indicate that MITF-M protein binds preferentially to E-box 1 and only marginally to E-box 2.

MITF Binds to the VMD2 Proximal Promoter in Vivo—We next analyzed MITF binding to the VMD2 promoter in vivo using ChIP. For this analysis, we chose SK-MEL-5 melanoma
tyrosinase-related protein 1 (TYRP1) promoter, which are known to be direct targets of MITF, were also positive in the ChIP results, whereas the negative controls rhodopsin (RHO) and albumin (ALB) promoters were not enriched by ChIP. Antibody sc-10999 did not significantly precipitate any of the promoter regions tested, and thus it served as a control for nonspecific binding.

Effects of MITF Mutations on the VMD2 Promoter Are Correlated with the Severity of Phenotypes of Mitf Mutant Mice—Given the accumulating evidence that MITF plays a role in regulating VMD2 expression, we wanted to explore how known mutations of MITF affect its ability to modulate VMD2 promoter activity. A total of 26 phenotypic alleles have been identified at the mouse microphthalmia (mi) locus, where Mitf resides, including spontaneous, chemically or radiation-induced, and transgenic alleles (Mouse Genome Informatics (38)), and mouse strains containing these alleles demonstrate a wide range of phenotypes, including eye phenotypes such as microphthalmia, aberrant RPE, and photoreceptor degeneration. Mutations that affect different domains of the MITF protein can be arranged in an allelic series in which phenotypes range from near normal to severely affected (24, 38, 43, 63). We chose four Mitf mutant alleles, Mitf<sup>Mi-wh</sup> (white) (63), Mitf<sup>Mi-sp</sup> (spotted) (63), Mitf<sup>Mi-tw</sup> (vitiligo) (63), and Mitf<sup>Mi-enu198</sup> (enu198) (64), and we performed co-transfection assays using MITF-M expression vectors containing the mutations of these alleles with VMD2 promoter/luciferase constructs. All mutations significantly decreased the transactivating ability of MITF, with the order of the retained activity (high to low) being wild-type, Mitf<sup>Mi-sp</sup>, Mitf<sup>Mi-tw</sup>, Mitf<sup>Mi-enu198</sup>, and Mitf<sup>Mi-wh</sup> (Fig. 3B). The degree of biochemical abnormality thus corresponds to the degree of phenotypic abnormality, suggesting that the integrity of MITF is critical for the transactivation of the VMD2 promoter in a manner similar to that for known mouse phenotypes.

Transactivation of the VMD2 Promoter by MITF-M Requires Two Proximal E-box Sites—Because of the conflicting data cited above about the roles of E-boxes 1 and 2 in regulating VMD2 expression, additional experiments were performed to reassess and dissect further the relative activities of E-boxes. In the earlier D407 RPE cell line studies, the effects of E-box 1 (m1) and 2 (m2) mutations were tested in the context of −104 to +38 and −71 to +38 bp reporter constructs (29). However, because transgenic mouse studies indicated that the −154 to +38 bp region is sufficient to drive expression in the RPE, we decided to use this promoter region exclusively for the experiments described in this study. MITF-M transactivated the VMD2 −154 to +38 bp promoter in a dose-dependent manner similar to that seen in the previous studies (29) (data not shown), and mutation of either E-box, or mutation of both, eliminated essentially all MITF-mediated transactivation (Fig. 3C).

Proximal E-box Sites Are Important for VMD2 Promoter Activity; Transient Transfection—We next reassessed the importance of the proximal E-boxes in the base-line activity (without added MITF) of the −154 to +38 bp reporter construct. Similar to our previous results with the shorter constructs, the m1 mutation reduced activity by 85%, whereas the m2 reduced activity by only 46% in D407 cells (Fig. 3D). Because cells of the following: 1) they express MITF-M at high levels, in addition to detectable levels of VMD2 expression, and 2) results of transient transfection assays with these cells appear to reflect more accurately the in vivo situation than do assays with D407 cells (see transfection and in vivo electroporation results below). Of the three anti-MITF antibodies used to precipitate chromatin-MITF complexes, a mixture of C5 and D5 (C5 + D5) and sc-11002 gave clear precipitation of the VMD2 proximal promoter (Fig. 3A). The positive controls tyrosinase proximal promoter (TYRP-P) and distal enhancer (TYRP-D), and

![Figure 2](image-url)
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FIGURE 3. The VMD2 proximal promoter is bound and transactivated by MITF and requires two E-box sites for its activity in vivo. A, chromatin immunoprecipitation. ChIP analysis was performed using SK-MEL-5 cells with anti-MITF antibodies, a mixture of CS and DS (CS + DS, lane 3), sc-10999 (lane 4), and sc-11002 (lane 5). The chromatin precipitates were analyzed by PCR using primers specific for the upstream regions of the indicated genes. PCR was also performed for input DNA (lane 1), ChIP with no antibody (lane 2), and human genomic DNA (lane 6). B, effects of MITF mutations on VMD2 promoter activity. Luciferase constructs containing the VMD2−154 to +38 and −104 to +38 bp fragments were co-transfected into D407 cells with a human MITF-M expression vector in pcDNA3.1, either wild-type (labeled as MITF) or with mutations in the MITF-CDNA, together with pRL-TK containing Renilla luciferase gene as control for transfection efficiency. The mutations analyzed are derived from mouse Miff mutant alleles, Miffmut-sp (labeled as mi-sp), Miffmut-vit (mi-vit), Miffmut-enu198 (mi-enu198), and Miffmut-wh (Mi-Wh). An empty pcDNA3.1 vector was used as control. Firefly luciferase activities were normalized by Renilla luciferase activities, and percent luciferase activities were calculated as the percentage of the normalized luciferase activity with mutated MITF to that with wild-type MITF (presented as 100%). The values represent the means and S.E. (bar). C, transactivation of the VMD2 promoter by MITF-M. Luciferase constructs containing the VMD2−154 to +38 bp fragments, either wild-type or with mutation of E-box sites (m1, m2, or m1m2), were co-transfected into D407 cells with a human MITF-M expression vector or empty pcDNA3.1 vector together with pRL-TK. Relative luciferase activities (fold increase) were calculated as the ratio of the normalized luciferase activity with MITF to that without MITF (labeled as pcDNA). The values represent the means and S.D. (bar). D, transient transfection in D407. The same luciferase constructs used in C and pGL2-Basic vector were transfected into D407 cells together with pRL-CMV. Relative luciferase activities were calculated as the ratio of the normalized luciferase activity with constructs containing VMD2 promoter fragments to that with pGL2-Basic vector. The values represent the means and S.E. (bar). E, transient transfection in SK-MEL-5. Transfection experiments were performed in the same manner as in D except that SK-MEL-5 cells were used. Relative luciferase activities were calculated and presented as described in D. F, in vivo electroporation in mouse RPE. The same luciferase constructs as used in D were introduced into mouse RPE cells using in vivo electroporation together with pRL-CMV to normalize the variability of subretinal injection and in vivo electroporation among eyes. Relative luciferase activities were calculated and presented as described in D.

individual cell lines only imperfectly reflect the genomic regulatory environment of their tissue of origin, we wanted to compare the relative effects of E-box 1 and 2 mutations in another cell line. We chose SK-MEL-5 melanoma cells for these studies because, as noted above, they express MITF-M at high levels and also express endogenous VMD2, and the ChIP experiments showed that their VMD2 promoter is bound by MITF. Transfections were performed with the −154 to +38 bp VMD2 promoter/luciferase construct in the presence and absence of E-box 1 and 2 mutations (Fig. 3E). VMD2 promoter activity was considerably higher in the SK-MEL-5 compared with the D407. More significantly, m1 reduced promoter activity by 97% (down to 1/30), m2 reduced activity by 95% (to 1/20), and the strongly suggest that E-box-binding factor(s) play critical roles in the regulation of VMD2 expression, and both E-boxes 1 and 2 are important in vivo.

Proximal E-box Sites Are Important for VMD2 Promoter Activity; Transgenic Mice—To complement the electroporation data, we also tested the activity of the proximal E-boxes in vivo in the context of chromatin structure. Although the ideal way to do this would have been to mutate the E-boxes in their normal chromosomal context through homologous recombination, for practical reasons we used a transgenic approach. For this experiment, given the profound influence that position effects can have on transgene expression patterns (76) and the difficulty in obtaining enough transgenic lines for statistically combined m1m2 mutation reduced activity by 99.8% (to 1/500). Thus, unlike in D407 cells, both E-boxes appear essential for promoter activity in the context of SK-MEL-5 cells.

Proximal E-box Sites Are Important for VMD2 Promoter Activity; in Vivo Electroporation—Because of this apparent discrepancy in terms of the relative activity of E-boxes 1 and 2 in transient transfections of D407 versus SK-MEL-5 cells, and the finding that in vivo MITF binds preferentially to E-box 1, we decided to try to assess the biological relevance of the E-boxes in more physiological experimental conditions. To achieve this goal, we employed an in vivo electroporation assay that allows transfer of plasmid DNA directly into adult mouse RPE and quantitative analysis of promoter activity (67, 68). We performed in vivo electroporation assays using the same −154 to +38 bp VMD2 promoter/luciferase constructs, with or without the m1, m2, or m1m2 mutations. The relative promoter activities in mouse RPE were intermediate in magnitude between the D407 (lowest) and SK-MEL-5 (highest) cells (Fig. 3F). In mouse RPE, m1, m2, and m1m2 decreased luciferase activity by 87% (down to 1/8), 82% (to 1/6), and 98% (to 1/50), respectively, suggesting that E-boxes 1 and 2 are both important in vivo, and that they function synergistically. These differences between the cell line and in vivo electroporation experiments most likely reflect differential expression of transcription factors and/or their interacting proteins between the various host cells. These results
significant results, we chose to look at the combined effect of alteration of E-boxes 1 and 2. Transgenic animals were generated with a −154 to +38 bp VMD2 promoter/\textit{lacZ} construct in which both E-boxes were mutated (m1m2). Eight founders were obtained, and from these six independent lines were established. Testing of multiple animals from each line by both RPE/choroid flat-mount and eye section staining, even with X-gal development times up to 72 h, failed to reveal any evidence of reporter activity in the RPE or neural retina of any of the lines (data not shown). Thus, the wild-type −154 to +38 bp VMD2 promoter/\textit{lacZ} construct revealed RPE expression in 3 out of 4 lines, whereas the −154 to +38 bp VMD2 promoter/\textit{lacZ} construct containing m1m2 revealed RPE expression in 0 out of 6 lines. The difference in RPE expression pattern between the wild-type and mutated constructs is statistically significant out of 6 lines. The difference in RPE expression pattern between the lac\textit{Z} expression level of the siRNA resulted in a dose-dependent reduction in the expression of target gene knockdown, we decided to look directly at the protein that were above physiological levels. To complement these studies, we wanted to determine whether reducing the level of MITF would lead to decreased VMD2 expression. We employed siRNA transfection to knock down MITF expression in SK-MEL-5 cells, and we analyzed the effects of decreased MITF expression on both VMD2 promoter activity in a luciferase reporter construct and endogenous VMD2 mRNA levels. First we checked the transfection efficiency using fluorescein-labeled double-stranded RNA and found that almost all cells contained fluorescent dots around nuclei (data not shown). Then we analyzed the knockdown efficiency of different concentrations of MITF siRNA using real time PCR. MITF mRNA levels were decreased by MITF siRNA in a dose-dependent manner up to 60 nM (Fig. 4A), but levels were not decreased further at 80 or 100 nM (data not shown). Treatment with MITF siRNA resulted in a dose-dependent reduction in the expression level of the −154 to +38 bp VMD2 promoter/luciferase construct, with 60 nM siRNA resulting in ~90% reduction (Fig. 4B). Perhaps even more importantly, the MITF siRNA significantly decreased expression of endogenous VMD2, with 60 nM siRNA resulting in ~50% reduction (Fig. 4C). An independent MITF siRNA sequence produced similar results, but control siRNA did not show any reduction in either assay (data not shown). Because our measurements of MITF levels in individual cell cultures indicated that even with the same concentration of siRNA there can be a fair amount of variability in degree of target gene knockdown, we decided to look directly at the correlation between MITF and endogenous VMD2 mRNA levels in different experiments (Fig. 4D). Analysis of 32 individual RNA samples from transfection with different concentrations of MITF siRNA revealed a surprisingly strong correlation (y = 0.95x + 10.2, R² = 0.7778). As a control to rule out possible effects of the siRNA that were not gene-specific, a similar comparison of MITF and RPLP0 levels did not reveal any evidence of correlation (y = 0.18x + 83.4, R² = 0.1233) (Fig. 4D). These results indicate that VMD2 expression is highly sensitive to (or tightly regulated by) the expression level of MITF at physiological RNA levels.

\textbf{TFE3 and TFEB Rescue the Effects of MITF Knockdown on the VMD2 Promoter}—Because TFE3 and TFEB were also identified by the YOH screen with the E-box 1-containing bait, and these proteins demonstrate the same DNA binding specificity as MITF, it seemed reasonable to speculate that TFE3 and TFEB might also be involved in the regulation of VMD2. To explore
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**FIGURE 5.** VMD2 expression correlates well with MITF mRNA levels, and TFE3 and TFEB can rescue the effects of MITF knockdown on the VMD2 promoter. A, effects of siRNAs on MITF mRNA levels. Sixty nm siRNAs targeting MITF, TFE3, and TFEB were transfected individually or in combination into SK-MEL-5 cells as indicated, and total RNAs were extracted 24 h post-transfection. The effects of siRNAs on MITF mRNA levels were evaluated and presented as described in Fig. 4A. B, effects of siRNAs on TFE3 mRNA levels. The same RNA samples used in A were analyzed for TFE3 mRNA levels, and the results were presented in the same manner as in A. C, effects of siRNAs on TFEB mRNA levels. The same RNA samples used in A were analyzed for TFEB mRNA levels, and the results were presented in the same manner as in A. D, effects of siRNAs on VMD2 promoter activity. Double transfections were performed sequentially, first with siRNAs and then 24 h later with plasmid DNA of the −154 to +38 bp VMD2 promoter/luciferase construct and control pRL-CMV. Cell lysates were prepared 48 h after plasmid transfection, and dual luciferase assays were performed. Percent luciferase activities were calculated and presented as described in Fig. 4B. E, effects of siRNAs on endogenous VMD2 expression. The same RNA samples used in A were analyzed using primers specific for VMD2, and the effects of siRNAs on VMD2 mRNA levels were presented in the same manner as in A. F, rescue of the effects of MITF knockdown by TFE3 and TFEB. Double transfections were performed sequentially, first with 60 nm MITF siRNA and then 24 h later with plasmid DNAs consisting of the −154 to +38 bp VMD2 promoter/luciferase construct, an expression vector containing TFE3 or TFEB cDNA or no insert (labeled as pcDNA), and control pRL-CMV. Dual luciferase assays were performed as described in D. Percent luciferase activities were calculated as the percentage of the normalized luciferase activity with siRNA and each expression vector to that without siRNA but with an empty pcDNA vector (presented as 100%). The values represent the means and S.D. (bar) of three independent experiments.

their biological roles, we first performed EMSA using in vitro translated TFE3 and TFEB proteins in the same manner as described earlier for MITF. The results showed that both TFE3 and TFEB proteins bind to E-box 1 but only faintly to E-box 2, the same binding profiles as observed with MITF (data not shown). Next, we carried out co-transfection assays in D407 and SK-MEL-5 cells. In distinction to the results with MITF, co-transfection with TFE3 and TFEB expression vectors failed to significantly increase (or decrease) −154 to +38 bp VMD2 promoter/luciferase activity (data not shown). Based on the possibility that this apparent lack of TFE3 and TFEB activity could be the result of masking by saturating amounts of MiT proteins, we tried straight siRNA experiments and also double transfections of SK-MEL-5 cells in the presence of MITF, TFE3, and TFEB siRNAs. Although MITF siRNA also reduced some-partially rescued the effects of MITF knockdown, revealing that they can, under appropriate conditions, transactivate the VMD2 promoter (Fig. 5F).

**TFE3 and TFEB Bind to the VMD2 Proximal Promoter in Vivo—**To extend the finding that TFE3 and TFEB can stimulate VMD2 promoter activity, we used ChIP with SK-MEL-5 cells to test whether TFE3 and TFEB bind to the endogenous VMD2 promoter. Of the two anti-TFE3 and four anti-TFEB antibodies used to precipitate chromatin-protein complexes, both anti-TFE3 (ab2753 and 554263) and two anti-TFEB (ab2636 and sc-11005) antibodies significantly enriched the VMD2 proximal promoter (Fig. 6). Of the known MITF targets used as control, TYR-P was precipitated by these anti-TFE3 and anti-TFEB antibodies in a somewhat similar pattern to that of VMD2, although the efficiency was much lower; TYR-D enrichment...
family factors identified by the YOH screen at their physiological expression levels.

The YOH screen isolated not only multiple MITF clones, supporting our previous hypothesis that MITF may be an important regulator of VMD2 expression, but also multiple clones for two other MiT family members, TFE3 and TFEB. Of these three factors, we decided to focus initially on MITF, because of its expression pattern restricted to the RPE in the eye and known essential roles in RPE differentiation (39, 40, 49–52). In vitro binding assays confirmed that MITF-M protein can bind well to E-box 1. In contrast, MITF-M binds marginally, if at all, to E-box 2, consistent with our previous EMSA results with bovine RPE nuclear extract (29). This binding preference is consistent with published data demonstrating that MITF binds well to both a CATGTG E-box motif flanked by a 5' T residue and a CACGTG E-box without a specific requirement for flanking residues, but not to atypical E-box motifs such as CACCTG and CAGGTG (77). The ChIP results, obtained with the SK-MEL-5 melanoma cell line, which expresses endogenous VMD2, provided further support for a role of MITF in regulating VMD2 expression; however, because of the close proximity of E-boxes 1 and 2 and the limited spatial resolution of the method, they did not provide any insight into the relative roles of the two E-boxes. Similarly, the transgenic results with the mutated −154 to +38 bp VMD2 promoter/lacZ construct indicated an important role for the proximal E-boxes in vivo but did not distinguish between them because in the construct both E-boxes were mutated.

In contrast to the binding studies, the co-transfection assays with an MITF-M expression vector and the mutated VMD2 promoter/luciferase constructs repeatedly pointed to a crucial role of E-box 2 for MITF-mediated transactivation. These conflicting results prompted us to reevaluate the functional role of the two proximal E-boxes using approaches closer to in vivo conditions. Using the same VMD2 promoter/luciferase constructs used in the D407 experiments, we analyzed luciferase expression in SK-MEL-5 cells and in murine RPE using in vivo electroporation (67, 68). Unlike with the D407 cells, in the absence of co-transfected MITF, both the SK-MEL-5 and murine RPE results suggested that for "base-line" activity both E-boxes are essential and of similar importance. One possible explanation for these differing results is that both native RPE and cultured SK-MEL-5 cells express considerably higher endogenous MITF than do D407 cells, with SK-MEL-5 cells expressing the highest level.6 Thus, the base-line activity we measured in the RPE and SK-MEL-5 cells may actually more closely reflect the MITF-stimulated phenomenon. Perhaps reflecting this MITF-stimulated state, luciferase activity was 300- and 20-fold higher in the SK-MEL-5 and RPE cells than that in the D407 cells, respectively. Taken together, our data suggest that MITF is an important regulator of the VMD2 promoter, that E-boxes 1 and 2 are both important for VMD2 expression, and that E-box 1 is important in mediating MITF activity, but our data leave open the question of what factor or factors bind to E-box 2 and mediate its activity.

6 N. Esumi, unpublished results.
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A number of MITF target promoters have been identified to date, and many of them contain multiple E-box sites in the region important for MITF regulation. For instance, three functional E-box sites are located in the 5′-upstream region of human TYR, two in the proximal promoter and one in the distal enhancer. However, in this case all three are bound by MITF in EMSA (22, 23). The TRPM1 (melastatin gene) promoter contains three E-box sites, all of which are bound by MITF and function cooperatively in melanocytes and melanoma (78). Of four E-boxes found in the CTSS (cathepsin K gene) promoter, three are bound by MITF and reduce transactivation by MITF when mutated, but the other site is neither bound by MITF nor does it change promoter activity when mutated (79). As far as we are aware, no MITF target promoter has been reported to contain an E-box that is not bound by MITF but is important for transactivation by MITF.

Cooperative binding and transcriptional regulation or cross-talk of multiple E-box sites have also been described for other bHLH transcription factors, such as members of the MYOD family, and MYOD1 binds to multiple functional binding sites in its target promoters (80–84). Unlike what is known about MITF, there are examples of different E-boxes being bound by different factors that function cooperatively on the same promoter or regulatory region. Human GLI1 contains two E-boxes critical for its regulation in the first intron, and TWIST1 activates GLI1 reporter expression through one E-box element but requires binding of USF proteins to a second E-box (85). As noted above, our results suggest the possibility that a similar scenario may exist for the MITF-mediated regulation of VMD2. However, we cannot exclude the possibility that other regulatory elements may overlap E-box 2 and its flanking sequence, and therefore our mutation of E-box 2 might be acting by disrupting the binding of a member of a different class of transcription factors. As one example of this possibility, there are overlapping cyclic AMP-responsive element and E-box motifs in the enhancer region of the bovine leukemia virus 5′-long terminal repeat that is critical for transcriptional activity of the viral promoter (86).

There are reports suggesting the existence of additional regulatory mechanisms that cooperate or synergize with MITF to control melanogenic gene expression. Using primary neural crest cell cultures, Hou et al. (87) demonstrated that the presence of MITF is not sufficient for Tyr expression in melanoblasts. Using adenovirus to express MITF in B16 mouse melanoma cells, it was shown that although wild-type MITF can activate Tyr promoter-reporter constructs, it cannot increase the expression of endogenous Tyr or TyrP1 (88). However, a dominant negative mutant of MITF inhibited the expression of endogenous TYR and TYRP1 proteins, indicating that MITF is required but is not sufficient to induce the expression of endogenous melanogenic enzymes (88). Of potential relevance to these reports, the expression of MITF-M is lost in a subset of human melanoma cell lines, and forced expression of MITF-M in these cells does not induce TYR, TYRP1, or dopachrome tautomerase expression to restore the melanogenic phenotype, suggesting that these cells lack other factors that are required for MITF function in melanocytes (89). Future studies will be needed to more directly assess whether a parallel situation exists in the regulation of VMD2 expression in the RPE, and what the putative bHLH or other transcription factors are that bind to E-box 2.

The data presented here also raise the possibility that TFE3 and TFEB, in addition to MITF, may be regulators of the VMD2 promoter. Of potential significance, transactivating activity was revealed only after using siRNA to significantly reduce the effects of MITF. This situation is in some ways analogous to the genetic studies, using MiT mutant and knock-out mice, that uncovered a role for Tfe3 in osteoclast development. The role of Tfe3 is functionally redundant with that of Mitf and was revealed only after obtaining Tfe3 null mice in an Mitf mutant background (44). Other examples of redundant functions shared by MiT family members include studies showing that the lethal effects of short hairpin RNA-mediated MITF knockdown on cell viability of clear cell sarcoma cells were rescued by co-transfecting plasmids encoding TFE3 or TFEB (90). Likewise, studies utilizing both trans dominant negative proteins and stem-loop RNA strategies demonstrated that TFE3 and TFEB are direct, physiological, and mutually redundant activators of CD40 ligand expression in activated CD4+ T cells (91). With increasing use of RNAi and other “knockdown” technologies, it seems likely that additional examples of redundant functions shared by MiT family members in different biological contexts will be identified.

Using siRNA transfection, we demonstrated a positive correlation of expression levels between endogenous MITF and VMD2 that was unexpectedly stronger than the correlation between endogenous MITF and its well known target TYR in SK-MEL-5 cells. Together with the ChIP results showing different binding profiles of MITF, TFE3, and TFEB for the promoters of VMD2 and the melanogenic genes TYR and TYRP1, and the requirement of E-box 2 for VMD2 transactivation by MITF, the results described in this study suggest that VMD2 is regulated by MiT family members in a different manner from that controlling melanogenic genes. VMD2 thus provides one more example of the complex interplay between DNA regulatory elements, transcription factors, and chromatin structure that underlies cell type-specific gene regulation. The role of the MiT family seems to provide a particularly interesting model for further study because the same family of transcription factors plays such essential but differing roles in two of the main types of pigmented cells in the body, melanocytes and RPE.

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