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Molecular Cloning of cDNA Encoding a Novel Microphthalmia-Associated Transcription Factor Isoform with a Distinct Amino-Terminus[1]

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Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix-leucine zipper protein, and plays an important role in the development of various cell types, such as neural-crest-derived melanocytes and optic-cup-derived retinal pigment epithelium. Three isoforms of MITF with distinct amino-termini have been described. These include melanocyte lineage-specific MITF-M, heart-type MITF-H, and the recently identified MITF-A. Here we identify a fourth isoform, MITF-C, with a unique amino-terminus of 34 amino acid residues, which shares about 43% sequence identity with putative transactivation segments of two previously identified leukemogenic factors, ENL and AF-9. Reverse transcription-polymerase chain reaction analysis revealed that MITF-C mRNA is expressed in many cell types, including retinal pigment epithelium, but is undetectable in melanocyte-lineage cells. In contrast, MITF-A and MITF-H mRNAs are coexpressed in all cell types examined. Transient cotransfection assays suggested that MITF-C, like other MITF isoforms, functions as a transcriptional activator of certain target genes, but its transactivation specificity for the target promoters is different from those of other MITF isoforms. Therefore, isoform multiplicity provides MITF with differential expression patterns as well as functional diversity.

Key words: melanocyte, microphthalmia, retinal pigment epithelium, tyrosinase, and Waardenburg syndrome.

Microphthalmia-associated transcription factor (Mitf) is a basic helix-loop-helix-leucine zipper (bHLH-LZ) protein encoded by the mouse *microphthalmia* locus (*1, 2*). Mitf and its human homolog, MITF (*3*), share amino acid similarity with several transcription factors containing a bHLH-LZ structure, such as TFE3, TFEB, and TFEC (*4-7*). Mutations at the *Mitf* alleles affect the development of many cell types, including retinal pigment epithelium (RPE), melanocytes, mast cells, and osteoclasts (*1, 8, 9*). Thus, Mitf provides a unique example of a bHLH-LZ transcription factor in which the phenotypic consequences of structural alterations have been well characterized (*1, 9*). Mice with certain mutations at the *Mitf* locus have been considered an animal model for Waardenburg syndrome type 2 (WS2), a typical auditory-pigmentary syndrome (*10*). WS2 is an autosomal dominant disorder characterized by varying combinations of sensorineural hearing loss, heterochromia iridis, and patchy abnormal pigmentation of the hair and skin (*11*). The MITF gene has been mapped to human chromosome 3p12.3-14.1 (*3*), and mutations in the MITF gene have been identified in 20% of cases with definite WS2 (*12*).

Recently, we identified a novel Mitf/MITF isoform, named Mitf-A/MITF-A (*13*), which differs in the amino-terminus from the authentic melanocyte-type isoform (Mitf-M/MITF-M) and the heart-type isoform (Mitf-H/MITF-H) (*9*). MITF-A is widely expressed in many cell types, whereas MITF-M is exclusively expressed in pigmented melanoma cells and melanocytes (*13*). MITF-A and MITF-H possess unique amino-termini, termed domains A and H, consisting of 35 and 19 amino acid residues, respectively (*13*). Domain A or domain H is followed by a common domain of 83 amino acids (*13*), named domain B1b (*14*). Domain B1b shows about 45% amino acid similarity to the equivalent portions of TFEB (*13*) and TFE3 (*14*), and domain A shows 37% similarity to the amino-terminal

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Abbreviations: MITF, microphthalmia-associated transcription factor; bHLH-LZ, basic helix-loop-helix-leucine zipper; WS2, Waardenburg syndrome type 2; RPE, retinal pigment epithelium; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pairs; kb, kilobase pairs.

region of TFE3 (*14*). These three MITF isoforms share the entire carboxyl region, including a transactivation domain (*15*) and a bHLH-LZ structure. MITF-A, like MITF-M, is able to transactivate the two melanogenesis genes, tyrosinase and tyrosinase-related protein 1, in melanoma cells (*13*).

In this study, we have identified a novel MITF isoform, named MITF-C, whose amino-terminus is different from that of MITF-A, MITF-H, or MITF-M. The expression patterns of four MITF isoforms and their possible functions as transcriptional regulators have been analyzed.

MATERIALS AND METHODS

Northern Blot Analysis—Total RNA (about 20 μg) from human cell lines was glyoxylated and electrophoresed in agarose gels (1.5%) with phosphate buffer (pH 7.0). The RNA was transferred to a Zeta-Probe blotting membrane (Bio-Rad), and fixed with UV Stratalinker 1800 (Stratagene). The subsequent procedures were detailed previously (*16*). To detect MITF transcripts, the blot was exposed to X-ray film for 3 days at −70°C. The hybridization probes used were the *Xho*I-*Eco*RI fragment (positions 996 to 1634) derived from a subclone phMI-9 coding for MITF (*17*), now renamed MITF-M (*13*), the region of which is common to all MITF isoforms, and the *Nae*I-*Apa*LI fragment (positions 99 to 1751) derived from a human β-actin cDNA. These cDNA probes were labeled with [α-^{32}P]-dCTP by the random priming method. RNA from HMC-1 mast cell leukemia cells (*18*) was a gift from Hiroshi Ohtsu, Tohoku University School of Medicine.

Cloning and Characterization of MITF Isoform cDNAs—The 5′-ends of MITF isoform cDNAs were cloned from Marathon-Ready human kidney cDNA from Clontech. The cDNA segments were amplified by polymerase chain reaction (PCR) with adaptor primer 1 (Clontech) and an anti-sense primer *MITF-2* (5′-TCTTGCTTCAGACTCTG-TGGG-3′), which spans nucleotides 691-711 of the MITF-M cDNA (*3*). The underlined 7 bases at the 3′ end of primer *MITF-2* are complimentary to the 3′ end of the 18-base insert coding for ACIFPT, generated by alternative usage of the upstream splicing acceptor site (*11, 14*). Incidentally, *MITF-2* is able to hybridize to MITF cDNAs without the 18-bp insert (20/21 matches) (*13, 14*), since the 3′ sequence underlined is also complimentary to the sequence 5′-CTCACAG-3′, located at the front of the 18-bp insert, except for the T residue. Thus, both types of MITF cDNAs with and without the 18-bp insert were amplified by PCR with *MITF-2*. The MITF cDNA segments were amplified by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The resulting products were then amplified with adaptor primer 2 (Clontech) and an anti-sense primer *3′B1b* (5′-CTTAAGGACTTCCATCGGCACCTG-3′) (see Fig. 2B). The amplified cDNA segments were subcloned into the *Sma*I site of pBluescript II SK+ vector (Stratagene), and sequenced in both directions. The nucleotide sequences of MITF-C cDNA segments were determined using three independent clones.

The entire MITF-C cDNA segment was then amplified by PCR from Marathon-Ready human kidney cDNA under the same conditions as described above. The sets of upper and lower primers were primer *5′C1-1* (positions 14 to 34 of MITF-C cDNA) and adaptor primer 1 (Clontech), also located at the 3′ end of cDNAs, for the first PCR, and primer *5′C1-2* (positions 50 to 70) and anti-sense primer *E9R* (5′-ACAAGTGTGCTCCGTCTCTTC-3′) for the second PCR. Primer *E9R* is complimentary to the cDNA segment coding for the carboxy-terminus of MITF isoforms.

Reverse Transcription (RT)-PCR Analysis of the Expression of MITF Isoform mRNAs—Total RNA from human cell lines and kidney was reverse-transcribed using oligo-dT_{12-18}. PCR was carried out as described above, except for the primer sets (see legend to Fig. 3) and the annealing temperature (50°C for MITF-C and MITF-H, 66°C for MITF-A, and 52°C for MITF-M). The sets of upper and lower primers used for each amplification reaction were primers *5′C1-1* (positions 14 to 34) and *MITF-2* or *Primer No. 1* (5′-GTCATCGATTACATCATCCA-3′) for MITF-C cDNA, primers *5′H1* (5′-GCAGAACACCTTAAAGGAA-AA-3′) and *3′ B1b* for MITF-H cDNA, and primers *5′M1* (5′-ACCGTCTCTCACTGGATTGGT-3′; positions 82-102) and *MITF-2* for MITF-M cDNA. The primer set for MITF-A cDNA comprised Mipr2 (positions 37 to 57) and Mipr3 (positions 207 to 227) (*13*). In the case of MITF-C, a second PCR amplification was performed using the primers *5′C1-1* and *3′B1b*.

Plasmid Construction—Expression plasmids pRc/CMV-MITF-A and pRc/CMV-MITF-H contain full-length MITF-A and MITF-H cDNA, respectively, in the pRc/CMV eukaryotic expression vector (Invitrogen) (*13*). pRc/CMV-MITF-M, coding for MITF-M with a 6-amino-acid insert, was constructed as described previously (*17*). An expression plasmid, pRc/CMV-MITF-C, was constructed from pRc/CMV-MITF-A by replacing the 5′ portion of MITF-A cDNA with the 5′ portion of MITF-C cDNA. Thus, pRc/CMV-MITF-C contains the full-length MITF-C cDNA under the control of the cytomegalovirus promoter of pRc/CMV. The 5′ end of the cDNA insert is the first residue shown in Fig. 2B. A truncated MITF construct, pRc/CMV-MITF-ΔN, which lacks the amino-terminal region including domain A, C, or H, was constructed as follows. The *Xba*I-*Afl*II fragment, coding for the amino-terminal portion of domain B1b, was isolated from a subclone, pH-B1b-BS, that carries the PCR fragment coding for domain B1b. The 5′ *Xba*I site represents a vector sequence of pBluescript II SK+, and the *Afl*II site is located near the 3′ end of the exon sequence for domain B1b. The *Xba*I-*Afl*II fragment was ligated to the larger fragment of pRc/CMV-MITF-A linearized with *Xba*I and *Afl*II, yielding pRc/CMV-MITF-ΔN. This *Xba*I site represents the vector sequence, located upstream from the 5′ end of MITF-A cDNA.

Transient Transfection Assays and Western Blot Analysis—HeLa cervical cancer cells were grown in Minimum Essential Medium, supplemented with 10% fetal calf serum. HeLa cells, seeded to about 70% confluency in 15-cm dishes, were transfected with each MITF isoform cDNA (50 μg) by the calcium phosphate-precipitation method as described previously (*17, 19*). Nuclear extracts were prepared from transfected HeLa cells by the method of Schreiber *et al.* (*20*). Nuclear extracts (100 μg protein) were mixed with 2×SDS loading buffer, consisting of 100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol, and separated in an SDS-polyacrylamide gel (8%). Western blot analysis

was performed as described previously (*21*). The specific immunocomplexes were detected with a western blot kit (ECL Plus, Amersham). The pET-28a(+) vector (Novagen) was used to express His-Tag-MITF-M in *E. coli* BL21(DE3). Anti-MITF polyclonal antibody, which will be fully described elsewhere, was produced in a rabbit using His-Tag-MITF-M as an antigen and affinity-purified using protein A agarose (Bio-Rad). As a positive control, total cell extracts were prepared from HMV-II melanoma cells and subjected to Western blot analysis (*21*).

Functional Analysis of MITF Isoforms—HeLa cells, seeded to about 70% confluency in 6-well plates, were cotransfected as described above. The test fusion genes were pHTL12 (*22*) and pHHOSVL15, which contain the human tyrosinase and heme oxygenase-1 gene promoters (*23*) respectively, upstream from the firefly luciferase gene (*24*). pHTL12M5 contains the ACTGTG motif instead of the CATGTG motif at the tyrosinase initiator E box (*25*). The tyrosinase fusion plasmids, containing three copies of TDE (pHTL8/TDE3) or TDEM containing the altered CATGTG motif in TDE (pHTL8/TDEM3), have been described previously (*17*). TDE represents a distal enhancer element required for the efficient expression of a reporter gene in pigmented melanoma cells. The amounts of DNA used for cotransfection were 2 μg of fusion gene (see Fig. 5), 2 μg of test MITF plasmid, and 0.01 μg of pRL-SV40 as an internal control. pRL-SV40 contains the SV40 promoter region upstream of Renilla luciferase. Transfected cells were then incubated for 24 h at 37°C, and harvested. Firefly luciferase activity was normalized by dividing by the Renilla luciferase activity, and are shown as the ratio to the value with vector plasmid. Expression of a reporter gene and an internal control was determined by the Dual-Luciferase™ Reporter Assay System (Promega).

Accession Numbers—The nucleotide sequence data shown have been deposited in the GSDB/DDBJ/EMBL/NCBI DNA databases with the following accession number: AB 006988 for the exons coding for domains C and B1b.

RESULTS

Multiple MITF Transcripts in Various Human Cell Lines—Northern blot analysis with the cDNA probe common to the MITF isoforms showed the expression of multiple MITF transcripts in various human cell lines (Fig. 1). This Northern blot was overexposed to detect the lower expression levels of MITF mRNAs in non melanin-producing cells. The transcripts detected in non-melanoma cells may represent MITF-A, MITF-H, and/or hitherto unidentified isoform mRNAs, since MITF-M mRNA expression is restricted to melanocyte-lineage cells (*13*) (see also Fig. 3). MITF transcripts were detected in HeLa cervical cancer cells (Fig. 1, lane 2), U-937 myelomonocytic leukemia cells (lane 4), K-562 erythroleukemia cells (lane 5), KU-812-F basophilic leukemia cells (lane 6), A-172 and T98G glioblastoma cells (lanes 8 and 9), and DLD-1 colorectal cancer cells (lane 10). MITF transcripts were undetectable in THP-1 myelomonocytic leukemia (lane 3) and HMC-1 mast cell leukemia RNAs (lane 7), but were detectable by RT-PCR (see Fig. 3B).

Molecular Cloning of the 5′ Portion of MITF-C cDNA—We suggested the presence of hitherto unknown MITF transcripts by S1 nuclease mapping analysis (*13*), which prompted us to search for novel MITF isoforms. Thus, the cDNA segments coding for MITF-C were isolated from human kidney cDNA by the method for rapid amplification of 5′ cDNA ends (Fig. 2A). Using two sets of PCR primers, we then cloned the entire MITF-C cDNA and confirmed its identity by determining the nucleotide sequence of the cDNA segments. The initiation Met codon of MITF-C was assigned based on the consensus for eukaryotic translation initiation site (*26*), which is also supported by the presence of an in-frame termination codon, TGA, 6 bp upstream from the assigned Met codon (Fig. 2B). MITF-C is predicted to consist of 519 amino acid residues, excluding 6 amino acids (ACIFPT), which are present in the front of the basic region of some species of MITF-M (*3, 14*). The calculated molecular mass of MITF-C is 58.0 kDa.

MITF-C possesses a unique amino-terminus of 34 amino acid residues, termed domain C (Fig. 2, A and B). Domain C is followed by domain B1b comprising 83 amino acid residues, which is also shared by MITF-A and MITF-H. Domain C shows no similarity to domain A or H. Interestingly, domain C shares about 43 and 33% amino acid similarity with the putative transactivation segments located at the carboxyl termini of the two leukemogenic factors, ENL (eleven-nineteen leukemia) (*27, 28*) and AF-9 (*29*), respectively (Fig. 2C). ENL and AF-9 share a significant overall homology and represent fusion partners of the HRX protein, the human homolog of *Drosophila* trithorax protein, involved in the pathogenesis of some leukemias with 11q23 chromosomal translocations.

Recently, Mochii *et al.* (*30*) have cloned two species of chicken Mitf cDNAs from embryonic RPE and heart. One of them is a chicken homolog of Mitf-A and other is an isoform encoded by cmi9 whose mammalian homolog has not been identified yet. Domain A is identical to chicken domain A,

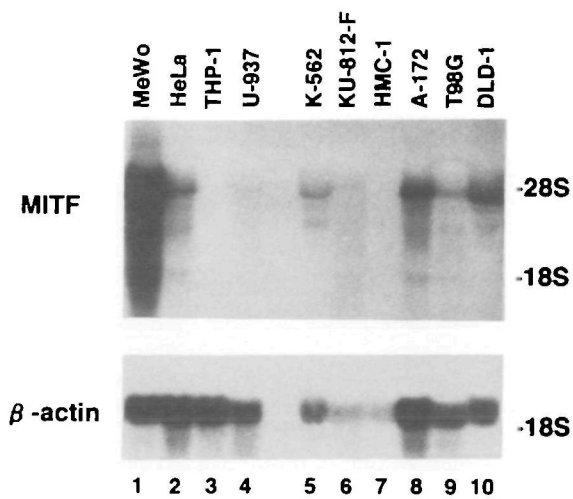

Fig. 1. **Northern blot analysis of MITF transcripts.** Shown is the autoradiogram of the RNA blot hybridized with a ^{32}P-labeled cDNA probe coding for a region common to all MITF isoforms. The blot was exposed to X-ray film for 3 days in order to show lower levels of MITF transcripts in non-melanoma cells. Human cell lines used were MeWo melanoma, HeLa cervical cancer, THP-1 and U-937 myelomonocytic leukemia, K-562 erythroleukemia, KU-812-F basophilic leukemia, HMC-1 mast cell leukemia, A-172 and T98G glioblastoma, and DLD-1 colorectal carcinoma. The bottom panel shows the expression of β-actin mRNA of about 2.2 kilobases.

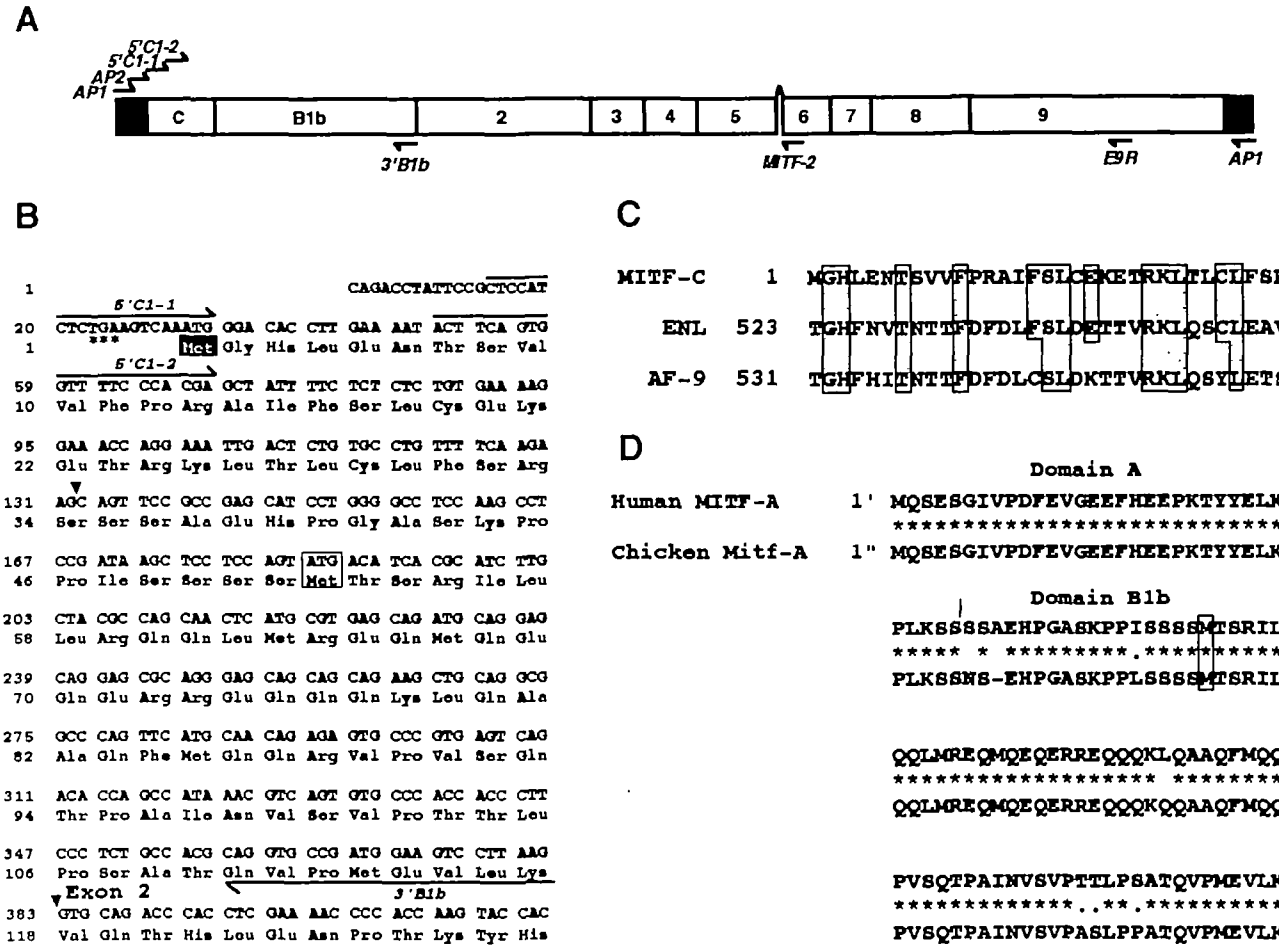

Fig. 2. **Molecular cloning of the cDNA coding for a novel MITF isoform.** A: Cloning strategy for MITF-C isoform cDNAs. Domains C and B1b represent the putative exons and the numbers (from 2 to 9) indicate the common exons identified in the *MITF* gene. The primers used for amplification of the 5′ ends of the MITF cDNAs were adaptor primers *AP1* and *AP2* and the anti-sense primers *MITF-2* and *3′B1b*. These adaptor primers are also present at the 3′ end of the cDNA, shown as closed boxes. The primers used for amplification of the entire MITF cDNA were *5′C1-1, 5′C1-2, AP1*, and *E9R*. Note that a gap, located at the 5′ end of exon 6, represents a deletion of 6 amino acids (ACIFPT). B: The nucleotide sequence of the 5′ portion of the MITF-C cDNA and the deduced amino acid sequence. The initiation Met codon is shown in reverse letters, and the upstream termination codon is indicated by asterisks. Arrowheads indicate the junctions between the putative exons. The 5′ end of exon 2 is also shown. The Met codon in domain B1b, corresponding to the initiation Met of MITF-Δ*N*, is boxed (see Fig. 4). The primers *5′C1-1, 5′C1-2*, and *3′B1b* are also indicated. C: Comparison of domain C with the transactivation segments of ENL and AF-9. Domain C is aligned with the carboxy-termini of ENL and AF-9. The numbers indicate the positions of the amino acid residues of MITF-C, ENL (*27*), and AF-9 (*29*). D: Alignment of the amino-terminal domains of MITF-A (*13*) and chicken Mitf-A (*29*). Shown are domains A and B1b. Note that human domain A is identical to chicken domain A. The initiation Met codons of MITF-Δ*N* and chicken cmi9-encoded Mitf (*30*) are boxed.

and domain B1b shares 92% identity with chicken domain B1b (Fig. 2D).

Differential Expression of MITF Isoform mRNAs—All MITF isoforms share the entire carboxyl portion (Fig. 3A), including a transcriptional activation domain (*15*) and a bHLH-LZ structure that is required for DNA binding and dimer formation. The amino-terminus of MITF-M is referred to as domain M, which comprises 11 amino acid residues and is encoded by exon 1M (*31*). In contrast to MITF-A mRNA, the expression of MITF-H mRNA was not detectable in many cell types by S1 nuclease mapping analysis (*13*). Likewise, we were unable to detect the expression of MITF-C mRNA by S1 nuclease mapping analysis (data not shown). Consequently, the expression patterns of MITF isoform mRNAs were analyzed by RT-PCR in various human cell lines and kidney (Fig. 3B). MITF-A and MITF-H mRNAs are coexpressed in all the cell lines examined and in kidney, whereas MITF-M mRNA expression was detected only in HMV-II and MeWo melanoma cells (lanes 1 and 2). The expression of MITF-C mRNA was detected in many cell types, including THP-1, KU-812-F, and HMC-1 leukemia cells (lanes 4-6), but was undetectable in melanoma cells (lanes 1 and 2).

The failure to detect MITF-C mRNA expression in melanoma cells even by RT-PCR prompted us to examine its expression in other melanocyte-lineage cells using a newly designed primer set (Fig. 3C). MITF-C mRNA was undetectable in HMV-II and 624-mel melanoma cells as well as in dermal melanocytes (lanes 2-4), whereas it was detected in three RPE cell lines, F-0202 (*32*), D407 (*33*), and ARPE-19 (*34*) (lanes 5-7).

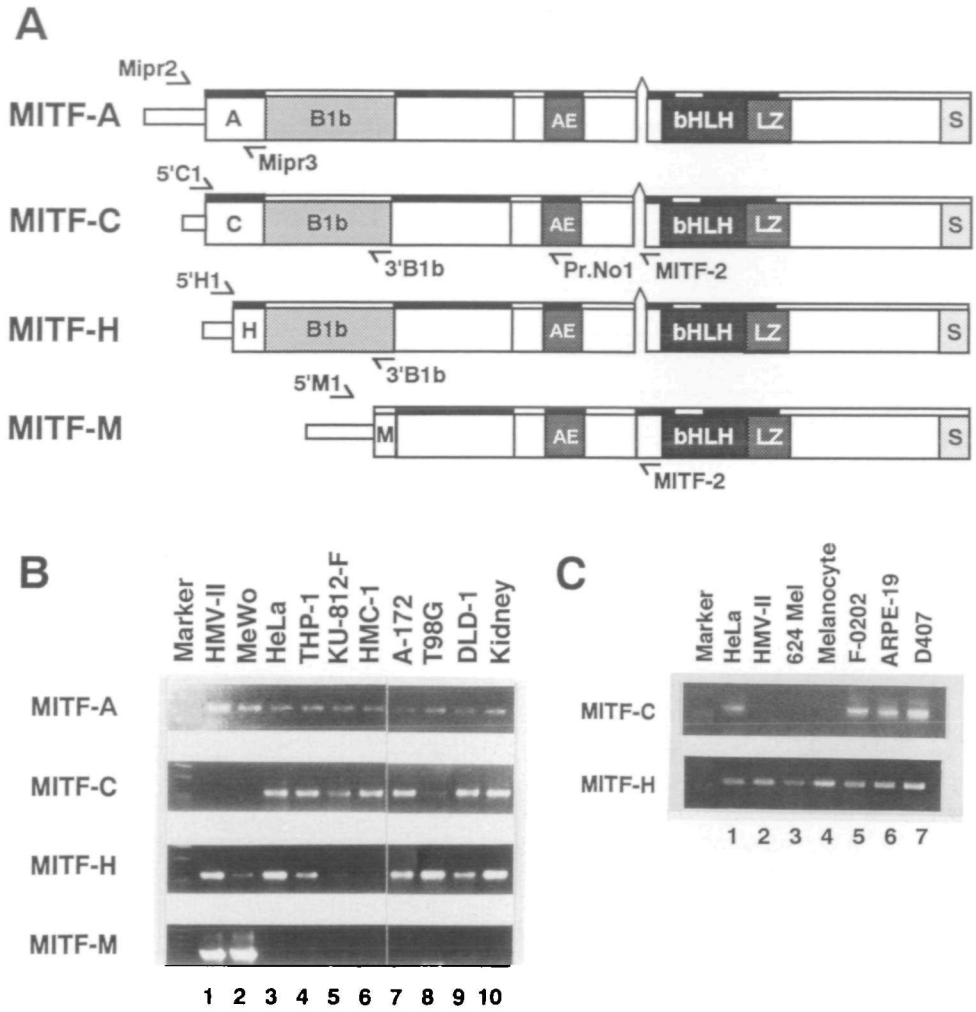

Fig. 3. **Differential expression of MITF isoform mRNAs in human cell lines.** A: Schematic representation of the structures of MITF isoform cDNAs. An activation domain (AE), the bHLH-LZ region, and the serine-rich region (S) are indicated. The positions for the PCR primers used are shown. B: Expression patterns of four MITF isoform mRNAs. Shown are the amplified DNA segments visualized by a ultraviolet lamp. For PCR of MITF-C and MITF-M cDNAs, the primer *MITF-2* was used as an anti-sense primer. The expected sizes of the target cDNA segments are 191 bp for MITF-A, 366 bp for MITF-C, 330 bp for MITF-H, and 630 bp for MITF-M. Total RNA was prepared from the indicated human cell lines (lanes 1-9) and kidney (lane 10). Human cell lines used were HMV-II and MeWo melanoma, HeLa cervical cancer, THP-1 myelomonocytic leukemia, KU-812-F basophilic leukemia, HMC-1 mast cell leukemia (*18*), A-172 and T98G glioblastoma, and DLD-1 colorectal carcinoma. The size markers used were ϕX174 replicative form DNA fragments generated by digestion with *Hae*III. Note that a visible band detected in T98G cDNA (lane 8) may represent unspecific PCR products amplified with the primers for MITF-M. C: Lack of MITF-C mRNA expression in melanocyte-lineage cells. Total RNA was prepared from 624-mel melanoma cells, dermal melanocytes, and three RPE cell lines, F-0202 (*32*), D407 (*33*), and ARPE-19 (*34*). In this series of experiments, an anti-sense primer, *Primer No. 1* (Pr.No1), was used for the first PCR amplification of MITF-C cDNA, instead of *MITF-2*. In contrast to MITF-C, MITF-H mRNA expression was detected in all cell lines (bottom panel).

Expression of MITF Isoform cDNAs in Transiently Transfected Cells—To confirm that each MITF isoform cDNA indeed encodes a predicted protein isoform, we transfected HeLa cells with a given MITF isoform cDNA and performed Western blot analysis using anti-MITF antibody (Fig. 4). For comparison, we also included an amino-terminally truncated mutant, MITF-ΔN, which contains an open reading-frame initiating with the Met codon in domain B1b (see Fig. 2, B and D). This Met codon corresponds to codon 53 of MITF-A, codon 52 of MITF-C, and codon 37 of MITF-H, and is also equivalent to the initiation Met of chicken cmi9-encoded Mitf (*30*). The calculated molecular masses of MITF-A, MITF-C, MITF-H, MITF-ΔN, and MITF-M are 58.2, 58.0, 56.4, 52.5, and 46.9 kDa, respectively.

A few faint bands of 60-65 kDa were detected in nuclear extracts of mock-transfected cells (lane 1) and may represent the endogenously expressed MITF isoforms in HeLa cells (see Figs. 1 and 3). A major band of about 57 kDa was detected in HMV-II melanoma cell extracts (lane 7) and in nuclear extracts prepared from HeLa cells transfected with MITF-M cDNA (lane 6). Such retarded mobility of MITF-M is consistent with previous reports of other investigators (*35*) and is possibly due to posttranslational modifications. Similarly, MITF-A was detected as a larger band of 65-66 kDa in nuclear extracts from cells transfected with MITF-A cDNA (lane 2). Thus, the antibody recognizes the common region of all MITF isoforms. Additional bands of 60 kDa seen in lane 2 may represent proteins translated from the downstream Met codon or partial degradation products of MITF-A. The expression of MITF-C and MITF-H was also detected as larger bands of 65 and 64 kDa compared to the expected sizes (lanes 3 and 4), although their expression levels were much lower than those of MITF-A. These results indicate that MITF-A, MITF-C, and MITF-H cDNAs are expressed in transiently transfected HeLa cells, but their expression levels vary depending on the isoform. Some faint bands were detected in cells transfected with MITF-ΔN cDNA (lane 5), but were not remarkably different from those seen in mock transfected cells (lane 1).

Functional Analysis of Four MITF Isoforms—We then assessed the functions of MITF-C in HeLa cells that lack MITF-M mRNA expression, since the transactivation activity of exogenous MITF isoforms, such as MITF-H, could be masked by the high contents of MITF-M in

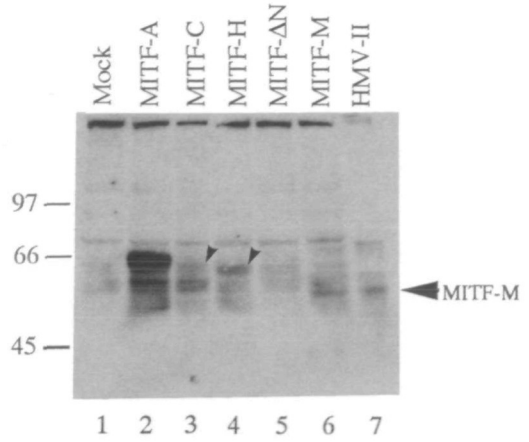

Fig. 4. **Expression of MITF isoform cDNAs in transfected cells.** HeLa cells were transiently transfected with each MITF isoform cDNA (lanes 2–6) or vector DNA pRc/CMV (lane 1) as indicated on top of the lanes. Expression of a given MITF isoform was determined by Western blot analysis. Note that lane 7 contained total cell extracts of HMV-II melanoma cells (10 μg protein), since MITF-M is abundant in melanoma cells (*13*). The arrowhead indicates MITF-M of 57 kDa, and small arrowheads indicate MITF-C of 65 kDa and MITF-H of 64 kDa. The biotinylated size markers are rabbit phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

melanoma cells (*13, 25*). The tyrosinase and ubiquitously expressed heme oxygenase-1 gene promoters were used as test promoters (Fig. 5A), although the tyrosinase gene is not endogenously expressed in HeLa cells. A tyrosinase construct, pHTL12, contains the three *cis*-regulatory elements, each of which contains a CATGTG motif (*17*), and pHTL12M5 contains a mutation at the CATGTG motif of the initiator E box (*25*). The heme oxygenase-1 promoter contains at least two functional CANNTG motifs (*36*), known as E boxes, which are potential binding sites for a large family of transcription factors with bHLH-LZ structures.

All MITF isoforms transactivated the tyrosinase promoter (pHTL12). MITF-A, MITF-H, and MITF-M transactivated the tyrosinase promoter, possibly through the CATGTG motif at the initiator E box, because the degree of transactivation by these isoforms decreased remarkably when pHTL12M5 was used (Fig. 5A). In contrast, MITF-C was able to transactivate pHTL12M5 efficiently. Moreover, MITF-C showed no noticeable effects on the heme oxygenase-1 promoter, whereas other MITF isoforms exerted a marginal transactivation effect on it. Interestingly, MITF-ΔN, which lacks the amino-terminal region including domain A, C, or H, shows a transactivation function similar to that of MITF-A and MITF-H but not MITF-C, despite the low expression levels of the MITF-ΔN protein under similar conditions (see Fig. 4).

To further explore the functional difference between MITF isoforms, we then used test promoters containing three copies of TDE (pHTL8/TDE3) or TDEM with the altered CATGTG motif in TDE (pHTL8/TDEM3) (Fig. 5B). Both constructs contain the initiator E box of the human tyrosinase gene (*17*). Each of MITF isoforms significantly increased the expression of pHTL8/TDE3, whereas only MITF-C could increase the expression of pHTL8/TDEM3. Taken together, these results suggest that the transactivation specificity of MITF-C is different from that of other MITF isoform, and that domain C appears to be responsible for the selection of target gene promoters.

DISCUSSION

The present study has shown that MITF consists of at least four isoforms with distinct amino-termini. MITF-C mRNA is coexpressed with MITF-A and MITF-H mRNAs in many cell types, except for melanocyte-lineage cells, in which MITF-C mRNA expression was undetectable. In contrast, MITF-M mRNA is exclusively expressed in melanocyte-lineage cells. Such mutually exclusive expression patterns of MITF-C and MITF-M mRNAs may shed light on the regulatory mechanism of MITF gene transcription.

It is noteworthy that the tyrosinase promoter, a test promoter used here, was transactivated in HeLa cells by any of MITF isoforms, including MITF-C and MITF-H. These results suggest that MITF-C and MITF-H could function as transcriptional activators of certain target genes and may provide MITF with functional redundancy. On the other hand, MITF-C, unlike other isoforms, transactivated the tyrosinase promoters with a mutated initiator E box (pHTL12M5) and with three copies of the mutated TDEs (pHTL8/TDEM3). Moreover, using pHHOSVL15 containing the 4.5-kb 5′-flanking region of the human heme oxygenase-1 gene, we detected marginal transactivation activity for MITF-A, MITF-H, and MITF-M, but not for MITF-C. These results suggest that an amino-terminal domain, such as domain C, may specify the target gene of a given MITF isoform. However, the biological significance of MITF-C remains to be investigated, since the expression of MITF-C mRNA was detectable only after two rounds of RT-PCR. We predict that MITF-C may be expressed preferentially in certain cell types not examined in this study, or expressed during restricted periods of development.

MITF-ΔN represents an amino-terminal truncation mutant that is common to all MITF isoforms, since the initiation Met of MITF-ΔN is located in domain B1b. Incidentally, the initiation Met of MITF-ΔN is equivalent to that of the cmi9-encoded Mitf, which was cloned from chicken embryonic RPE cells and shown to increase the expression of the tyrosinase and mmp115 genes in cultured chicken RPE cells (*30*). The transactivation activity of MITF-ΔN with the test promoters was indistinguishable from that of MITF-A, MITF-H, or MITF-M (Fig. 5). Thus, MITF-ΔN may mimic the function of the hitherto unidentified human homolog of the cmi9-encoded Mitf.

Domain C shows a significant amino acid sequence similarity to putative activation segments of ENL and AF-9. Such activation segments are located at each carboxy terminus. Both ENL and AF-9 have been identified as fusion partners of the HRX protein seen in some leukemias with 11q23 chromosomal translocations, and provide the fusion proteins with transactivation capacity (*27–29*). It is therefore conceivable that domain C may be involved in the transcriptional activation capacity of MITF-C. This notion is consistent in part with the function of MITF-C detected by transient cotransfection assays (Fig. 5). In addition, domain A of MITF-A shows significant similarity to the

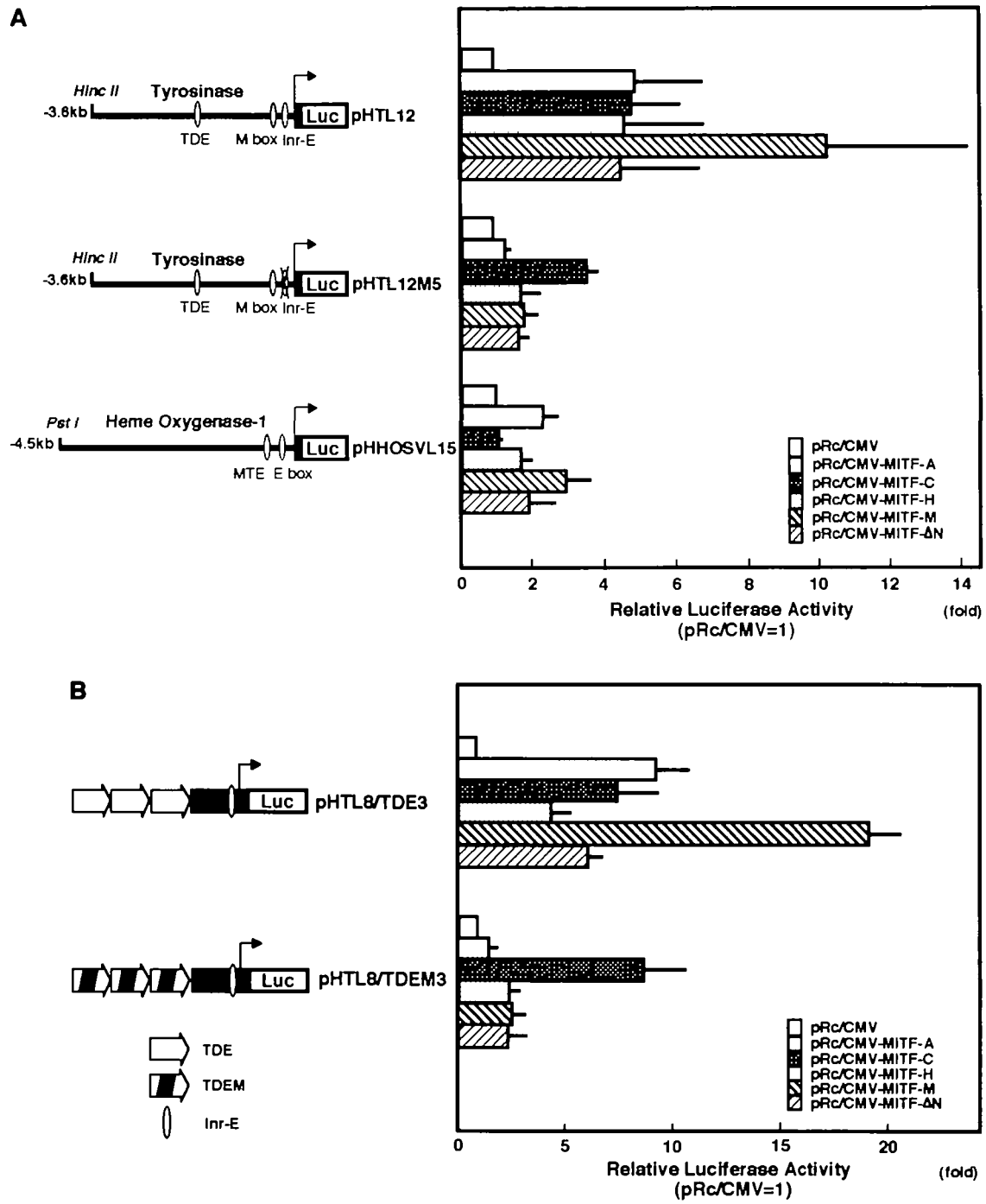

Fig. 5. **Functional analysis of MITF isoforms.** A: Comparison of the transactivation activity of MITF isoforms. HeLa cells were transfected with each MITF isoform cDNA and a test fusion gene as described under "MATERIALS AND METHODS." Among the MITF constructs used, only pRc/CMV-MITF-M contains the 18-bp insert, coding for ACIFPT. There are three *cis*-regulatory elements in the tyrosinase gene, TDE (positions −1861 to −1842), the M box (positions −104 to −94), and the initiator E box (positions −12 to −7) (*22*). Each *cis*-regulatory element contains a CATGTG motif (*17*). pHTL12M5 contains a mutation at the initiator E box (*25*). pHHOSVL15 contains the 4.5-kb 5′-flanking region of the heme oxygenase-1 gene, including at least two functional E boxes. MITF-ΔN contains an open reading-frame initiating with the Met codon in domain B1b (see Fig. 2D). The magnitude of activation is presented as the ratio of normalized luciferase activity obtained with each MITF isoform cDNA to that with vector DNA (induction ratio). The data shown are the means ± standard deviations of at least three independent experiments. B: Effects of MITF isoforms on the expression of fusion genes containing three copies of TDE (pHTL8/TDE3) or TDEM with the altered CATGTG motif in TDE (pHTL8/TDEM3). Both constructs contain the initiator E box. Other conditions were the same as in A.

amino-terminal region of TFE3 (*14*), which has been identified by analyzing some papillary renal cell carcinomas with the (X;1) (p11;q21) translocation (*37*). This translocation results in the fusion of the amino-terminal region of the PRCC (papillary renal cell carcinoma) protein to the entire TFE3 (*37, 38*). These facts suggest the possibility

that chromosomal translocations involving the MITF gene may be responsible for some types of leukemia or cancer.

Isoform multiplicity provides MITF not only with differential expression patterns, but also with functional diversity and redundancy. The present study may further the understanding of the molecular basis for the phenotypic variability of WS2, and will facilitate the search for factors that interact with the amino-terminal domains of MITF isoforms.

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