Regulation of Tyrosinase Gene Expression by cAMP in B16 Melanoma Cells Involves Two CATGTG Motifs Surrounding the TATA Box: Implication of the Microphthalmia Gene Product

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Abstract. In melanocytes and in melanoma cells, up-regulation of melanogenesis, by cAMP elevating agents, results from a stimulation of tyrosinase activity that has been ascribed to an increase in tyrosinase protein and messenger amount. However, the mechanism by which cAMP elevating agents increase tyrosinase mRNA remains to be elucidated. In this study, using a luciferase reporter plasmid containing the 2.2-kb fragment 5′ of the transcriptional start site of the mouse tyrosinase gene, we showed that cAMP elevating agents lead to a strong stimulation (20-fold) of transcriptional activity of the tyrosinase promoter. Deletions and mutations in the mouse tyrosinase promoter showed that the M-box 70-bp upstream from the TATA-box and the E-box located downstream the TATA-box, near to the initiator site, are involved in the regulation of the tyrosinase promoter activity by cAMP. Additionally, we showed that microphthalmia, a b-HLH transcription factor associated with pigmentation disorders in mouse, binds to these regulatory elements and modulates the transcriptional activity of the tyrosinase promoter. Since cAMP stimulates the binding of microphthalmia to the M-box and to the E-box; it is tempting to propose that microphthalmia, through its interaction with *cis*-acting elements surrounding the TATA-box, plays a key role in the regulation of the mouse tyrosinase gene expression by cAMP.

SKIN melanocytes originate from the neural crest, from which they migrate into the basal layer of epidermis and proliferate as precursor melanoblasts (Le Douarin, 1982). Subsequently, these cells differentiate to melanin-producing melanocytes and acquire a specific enzymatic machinery responsible for melanin synthesis. Tyrosinase, the rate-limiting enzyme in melanogenesis, catalyzes the two initial steps of this process, hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA)[1] and oxidation of DOPA to DOPA quinone (Hearing and Jimenez, 1987; Prota, 1988; Hearing and Jimenez, 1989; Hearing and Tsukamoto, 1991). Two other enzymes, tyrosinase-related protein 1 (TRP-1), possessing a 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase activity (Kobayashi et al., 1994) and tyrosinase-related protein 2 (TRP-2) endowed with a DOPA chrome tautomerase activity (Jackson et al., 1992; Kameyama et al., 1993; Yokoyama et al., 1994*a*) are involved in melanin production (Abdel-Malek et al., 1993). The expression of these enzymes is restricted to melanocytes, suggesting the presence in their promoter of regulatory elements responsible for tissue-specific expression. Sequencing of TRP-1, TRP-2, and tyrosinase promoters has revealed the presence of a 10-bp sequence (GTCATGTGCT) termed the M-box, which was thought to be involved in cell-specific expression (Lowings et al., 1992; Ganss et al., 1994; Yokoyama et al., 1994*b*). The CATGTG motif matches with the core hexamer sequence CANNTG (E-box) that is recognized by the basic-helix-loop-helix (b-HLH) transcription factor family. This transcription factor family includes ubiquitously expressed proteins such as myc, max (Blackwood and Eisenman, 1991), upstream stimulatory factor, USF (Gregor et al., 1990), and tissue-specific expressed proteins such as myogenic factors (Edmondson and Olson, 1993). Recently, pigmentation disorders observed in microphthalmic mouse have been associated with mutations of a b-HLH transcription factor encoded by the microphthalmia gene (Hodgkinson et al., 1993; Hughes et al., 1993). Similarly, MITF, the human homologue of the mouse microphthalmia gene, has been linked to abnormal pigmentation observed in Waardenburg Syndrome type 2 (Hughes et al., 1994).

In vivo or in cultured cells, melanogenesis can be stimu-

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1. *Abbreviations used in this paper*: CTx, cholera toxin; DOPA, 3,4-dihydroxyphenylalanine; b-HLH, basic-helix-loop-helix; IBMX, isobutylmethylxanthine; αMSH, αmelanocyte-stimulating hormone; pRB, retinoblastoma protein; SV40, simian virus 40; TRP, tyrosinase-related protein.

lated by ultraviolet B light (Friedmann and Gilchrest, 1987; Agin et al., 1991; Aberdam et al., 1993) and by cAMP elevating agents such as forskolin, a direct activator of adenylate cyclase, Isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, and α melanocyte stimulating hormone (αMSH) that activates adenylate cyclase through the binding to a receptor coupled to α_s (Wong and Pawelek, 1975; Hunt et al., 1993). Upregulation of melanogenesis by cAMP elevating agents results from a stimulation of tyrosinase activity that has been ascribed to an augmentation of tyrosinase protein and messenger amount (Aroca et al., 1993; Kuzumaki et al., 1993). The mechanism by which cAMP elevating agents increase tyrosinase mRNA remains controversial. Indeed, it has been recently proposed that the regulation of tyrosinase mRNA by cAMP occurs by a posttranscriptional mechanism such as mRNA stabilization (Ganss et al., 1994). On the other hand, the augmentation of tyrosinase expression by αMSH was inhibited by α-amanitin suggesting a transcriptional regulation of the tyrosinase gene expression (Fuller et al., 1987). Sequencing of the 5′-flanking region of the mouse tyrosinase gene has failed to identify canonical cAMP responsive elements (CRE) (Borrelli et al., 1992). Nevertheless, other enhancer elements such as ultraviolet responsive element (URE) (Ronai et al., 1994), TPA responsive element (TRE) (Angel et al., 1987), and AP2-binding sequence (Imagawa et al., 1987) have been found in the tyrosinase promoter and might be the target of transcription factors activated following the rise of the cAMP content in melanocytes.

In this report, we first investigated whether cAMP regulates the mouse tyrosinase gene expression through a transcriptional mechanism. In this aim, we constructed a reporter plasmid containing the 2.2-kb fragment 5′ of the transcriptional start site of the mouse tyrosinase gene. We showed that cAMP increases the transcriptional activity of the mouse tyrosinase promoter. A M-box 70-bp upstream from the TATA-box and the E-box located downstream the TATA-box, near to the initiator site, are involved in the regulation of the tyrosinase promoter activity by cAMP. Moreover, our data suggest that microphthalmia, through the binding to these regulatory elements, mediates the effect of cAMP on the tyrosinase gene expression.

Materials and Methods

Materials

Dulbecco's modified Eagles medium (DMEM), 4-norleucine 7-D-phenylalanine-α-melanocyte stimulating hormone ([Nle^4, D-Phe^7]-αMSH), IBMX, forskolin, bovine serum albumin (BSA), 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF), aprotinin, and leupeptin were purchased from Sigma Chem. Co. (St. Louis, MO). γ-[^{32}P]ATP (3,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL) and lipofectamine reagent from GIBCO-BRL (Gaithersburg, MD). Klenow fragment, T4 polynucleotide kinase, and T4 DNA ligase were from Biolabs. Synthetic oligonucleotides were purchased from Genset or Oligo express. Anti-microphthalmia antibody was obtained by injection of a rabbit with a peptide (NH_2-T-S-S-R-R-S-S-M-S-A-E-E-T-E-H-A-C-COOH) corresponding to the COOH terminus part of microphthalmia (Hodgkinson et al., 1993) coupled to keyhole limpet hemocyanine.

Cell Cultures

B16/F10 murine melanoma cells (from Dr. V.J. Hearing, NIH, Bethesda, MD), S91 murine melanoma cells and NIH3T3 fibroblast cells were grown at 37°C under 5% CO_2 in DMEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. G361 human melanoma cells were grown under the same conditions in Mc Coy's medium.

Transfections and Luciferase Assays

B16 melanoma cells, in 24-well dishes, were transfected with 0.25 μg of the test plasmid and 0.05 μg of pCMVβGal to control the variability in transfection efficiency, using 2 μl of lipofectamine in a 200-μl final volume. In the experiences with microphthalmia the transfection was performed in the same conditions using, 0.25 μg of the test plasmid, 0.01 μg of pCMVβGal, and 0.04 μg of pCDNA3 expression vector, empty or containing the microphthalmia coding sequence. After 6 h, the transfection medium was changed and the cells were incubated with the different cAMP elevating agents for the indicated time. Then, cells were washed with a saline phosphate buffer and lysed with 25 mM Tris-phosphate (pH 7.8) buffer containing 1% Triton X-100, 2 mM EDTA, and 2 mM DTT. Soluble extracts were harvested and assayed for luciferase and β-galactosidase activity. All transfections were repeated at least five times using different plasmid preparations and gave similar results. To obtain stably transfected B16 cells, we used a 10:1 molar ratio of pMT2.2 or pMT0.27 and pCDNA3. Stable transfected B16 cells were selected in medium containing 800 μg/ml G418. Individual clones were isolated and characterized after 3 wk. NIH3T3 cells stably expressing microphthalmia were obtained by transfection with pCDNA3 encoding microphthalmia and selection with 500 μg/ml G418. Individual clones were isolated and characterized after 3 wk.

Construction of the Reporter Plasmid

A 2.2-kb fragment 5′ of the transcriptional start site of the mouse tyrosinase gene was isolated from mouse genomic DNA by polymerase chain reaction (PCR) using the following primers: 5′-TTCAACCCTTTTC-TATGTCC-3′ (−2236/−2217) and 5′-TCATACAAgATCTGCACCAA-3′ (+63/+42). The transcription initiation site was numbered +1 according to the report of Kikuchi et al. (1989). BglII recognition site was introduced in the lower primer to facilitate the cloning. The PCR product was initially introduced into the TA cloning vector and sequenced (Invitrogen, Deschelp, Netherlands). Subsequently, the XhoI–BglII fragment was cloned into the unique XhoI–BglII sites of pGL2-basic vector (PGL_2B), upstream the luciferase coding sequence (pMT2.2; −2236/+59). Two deletion plasmids, pMT1.8 (−1789/+59) and pMT1.3 (−1317/+59), were obtained by exonuclease III digestion using the Erase-a-base system (Promega, Madison, WI). For the other deletions, pMT2.2 was first linearized with KpnI, and then digested with either NcoI, AvrII, or NsiI. Plasmids were filled in with Klenow fragment and self-ligated with T4 DNA ligase, giving respectively the following plasmids: pMT1.1 (−1100/+59), pMT0.9 (−986/+59), and pMT0.5 (−517/+59). pMT0.27 (−270/+59) was obtained by subcloning the 270-base pairs (bp) XbaI–BglII fragment of the mouse tyrosinase gene, into the NheI–BglII sites of pGL_2B. pMT0.08 (−80/+59) was constructed by subcloning the HindIII (converted to blunt end)–BglII fragment isolated from pMT0.27 into the SmaI–BglII sites of pGL_2B. The 2.2-kb fragment (XhoI–BglII) 5′ of the transcriptional start site of the mouse tyrosinase gene was also cloned into the unique XhoI–BglII sites of pGL_2-promoter vector (PGL_2P) which contains the simian virus 40 (SV40) enhancerless early promoter linked to the luciferase gene (pSVMT2.2). pMT0.1 (−126/+59) was constructed by subcloning the RsaI–BglII restriction fragment isolated from pSVMT2.2 into the unique SmaI–BglII sites of pGL_2B. pMTΔ0.1 and pMT0.04 and all the mutants were constructed with the Transformer™ site-directed mutagenesis kit (Clontech, Palo Alto, CA). Deletions and mutations of all constructs were verified by plasmid sequencing. A cDNA encoding the microphthalmia gene product was isolated by reverse transcription of B16 melanoma cell RNA and PCR using the following primers: 5′AAGTGGTCTGCGGTGTCTCC3′ and 5′AAGGCAGGCTCGCTAACACG3′. The PCR product (1.3 kb) was initially cloned into pSKbluescript and sequenced. A clone that was found to be 100% identical to the published microphthalmia sequence (Hodgkinson et al., 1993) was then inserted as a HindIII–NotI restriction fragment into the unique HindIII–NotI sites of the $pCDNA_3$ expression vector. All constructs were purified using the silica column from Quiagen (Hylden, Germany).

Immunofluorescence Studies

Cells were washed with PBS and fixed at −20°C for 10-min with metha-

nol/acetone (3:7, vol/vol). After a 10-min rehydratation at 25°C in PBS containing 3% BSA (PBS/BSA), fixed cells were incubated with the primary antibody directed to the COOH terminus part of microphthalmia for 60 min at 25°C. Cells were then washed five times with PBS/BSA and incubated in PBS/BSA for 60 min at 25°C with fluorescein isothiocyanate–conjugated secondary antibody (anti–rabbit, 1:100). Finally, cells were washed five times with PBS/BSA and examined with a Zeiss Axiophot microscope.

In Vitro Transcription and Translation

In vitro translations of microphthalmia were carried out using the linked T7 transcription-translation system from Amersham. Microphthalmia translation was monitored using [^{35}S]methionine and SDS-PAGE analysis.

Nuclear Extracts and Gel Mobility Shift Assay

B16 cells were stimulated with forskolin and the nuclear extracts were prepared essentially as previously described (Dignam et al., 1983). Double-stranded synthetic M-box, 5′-GAAAAAGTTAGTCATGTGCTTTGCAGAAGA-3′ or iE-box 5′-GGTCTTAGCCAAAACATGTGATAGTCACTCCAG-3′ was γ^{32}P end-labeled with the T4 polynucleotide kinase. 10 μg of nuclear proteins or 4 μl of the in vitro translated microphthalmia were preincubated in binding buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, 80 μg/ml of salmon sperm DNA, 0.5 μg poly (dIdC), 0.2% BSA, 2 mM $MgCl_2$, and 2 mM spermidine for 30 min at room temperature. Then, 150,000–200,000 cpm of ^{32}P probe was added to the reaction mixture for 20 min at 37°C. DNA-protein complexes were resolved by electrophoresis on 5% polyacrylamide gel (37.5:1 Acrylamide-Bisacrylamide) in TAE buffer (10 mM Tris, 9 mM sodium acetate/acetic acid, 275 mM EDTA, pH 8) for 6 h at 150 V. When indicated, an excess of cold competitor oligonucleotides was added during preincubation. In mutated M-box and iE-box the CATGTG motif was converted to CGGATC. For supershift assays, the antibodies were preincubated with nuclear extracts in binding reaction buffer for 60 min before adding the labeled probe.

Results

Regulation of Mouse Tyrosinase Promoter Activity by cAMP Elevating Agents

Initially, a plasmid containing the 2.2-kb 5′ of the transcriptional start site of the mouse tyrosinase gene upstream from the firefly luciferase coding sequence (pMT2.2; −2236/+59) was transiently transfected in B16 mouse melanoma and in NIH3T3 fibroblasts. Consistent with previous reports (Klüppel et al., 1991; Bentley et al., 1994), expression of luciferase driven by the mouse tyrosinase promoter was 50 times higher in B16 melanoma cells than in NIH3T3 cells (data not shown), demonstrating the presence in this part of the promoter of regulatory elements accountable for cell-specific expression of mouse tyrosinase gene. To study the effects of intracellular cAMP elevation on the mouse tyrosinase promoter activity, B16 cells were transiently transfected with pMT2.2 and exposed to forskolin, a direct activator of adenylate cyclase. We observed a fourfold stimulation of luciferase activity after 6 h with forskolin. A much stronger stimulation of luciferase activity (15–18-fold) was obtained after 24 h or 30 h forskolin treatment (Fig. 1 *A*). Next, we studied the effects of various melanogenic agents that also increased the intracellular cAMP level on mouse tyrosinase promoter activity. B16 melanoma cells were transfected with pMT2.2, and then treated for 24 h with forskolin, Cholera toxin (CTx), αMSH, IBMX or αMSH plus IBMX. CTx led to a 10-fold stimulation of the luciferase activity. αMSH or IBMX treatment caused a fivefold stimulation while αMSH

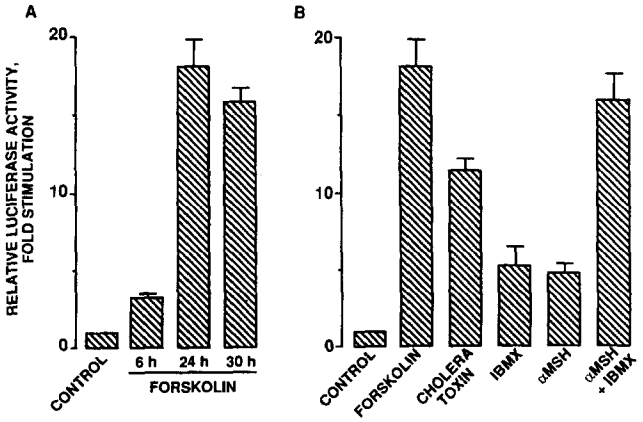

Figure 1. cAMP-elevating agents stimulate the mouse tyrosinase promoter activity in B16 cells. B16 cells were transiently transfected with pMT2.2, a plasmid which contains a 2.2-kb fragment 5′ of the transcriptional start site of the mouse tyrosinase gene cloned upstream the luciferase coding sequence. After transfection, cells were incubated with 10 μM forskolin for 6, 24, or 30 h (*A*), or with 1 μM [Nle^4, D-Phe^7] αMSH (αMSH), 100 μM IBMX, 10 μM cholera toxin (CTx), or αMSH+IBMX for 24 h (*B*). Then, cells were solubilized and luciferase activity was assayed. Luciferase activity was normalized by the β-galactosidase activity and the results were expressed as fold stimulation of the basal luciferase activity from unstimulated cells (CONT). Data are means ± SE of five experiments performed in triplicate.

in combination with IBMX stimulated about 17-fold the luciferase activity (Fig. 1 *B*). Stimulation of the mouse tyrosinase promoter activity by cAMP elevating agents was also observed in S91 mouse melanoma cells and in G361 human melanoma cells (Table I). Interestingly, similar experiments performed in NIH3T3 cells showed that a forskolin treatment was unable to stimulate the transcriptional activity of the mouse tyrosinase promoter (Table I), indicating that a cell type–specific mechanism triggers cAMP response in melanoma cells. These results indicate that the 2.2-kb promoter fragment contains regulatory elements involved in the transcriptional regulation of the mouse tyrosinase promoter by cAMP.

Localization of cis-acting Elements Responsible for cAMP Response

To identify the regulatory elements of the mouse tyrosinase promoter involved in the cAMP response, we constructed a series of reporter plasmids containing various deletions in the 5′-flanking region of the promoter. Previous reports demonstrated that the 270-bp 5′ of the transcriptional start site was sufficient to direct pigment cell-specific expression but was unresponsive to intracellular cAMP elevation (Ganss et al., 1994). Hence, we studied the effects of forskolin on deletion constructs spanning from 2.2 kb to 270 bp 5′ of the transcriptional start site. After transfection with pMT1.8 (−1789/+59), pMT1.3 (−1327/+59), pMT1.1 (−1100/+59), pMT0.9 (−986/+59), pMT0.5 (−517/+59), or pMT0.27 (−270/+59), a 24-h forskolin treatment stimulated 15–18-fold the luciferase activity (Fig. 2 *A*). The responsiveness of all deletion constructs to forskolin was similar to that observed with the

Table I. cAMP Stimulates the Transcriptional Activity of Tyrosinase Promoter in Melanoma Cell Lines but Not in NIH3T3

Cell lines	Fold stimulation induced by forskolin
B16	20 ± 2
S91	22 ± 3
G361	10 ± 1
NIH3T3	1.5 ± 1

Cells were transfected with pMT2.2, treated with forskolin for 24 h, and luciferase activity was measured. Results are means ± S.E. of three independent experiments done in triplicate.

initial reporter plasmid pMT2.2. Also, in stably transfected B16 cells with pMT2.2 or pMT0.27, forskolin lead respectively to a 15 ± 1 and 13 ± 2 (mean ± SE of three different experiments) fold increase in the tyrosinase promoter activity. These results indicate that cAMP responsive elements are present in the 270-bp fragment of the promoter. In an attempt to further characterize these elements, we constructed additional deletions in tyrosinase promoter, pMT0.1 (−126/+59), pMT0.08 (−80/+59), pMT0.04 (−40/+59), and pMTΔ0.1 corresponding to pMT0.1 in which the region between the HindIII (−80) restriction site and the TATA-box (−40) had been deleted. Luciferase activity of pMT0.1 was 17-fold stimulated by forskolin while, in the same conditions, after transfection with pMT0.08 or pMT0.04, forskolin caused only a fivefold stimulation of luciferase activity. pMTΔ0.1 showed a slightly decreased response (14-fold) to forskolin compared with pMT0.1 (Fig. 2 *B*). All of these constructs had a similar basal activity, except pMT0.08 and pMT0.04 which showed a fivefold decreased basal activity compared to the pMT2.2. These results suggest that important elements conferring cAMP responsiveness to mouse tyrosinase promoter in B16 mouse melanoma cells are located between RsaI (−126) and HindIII (−80) restriction sites.

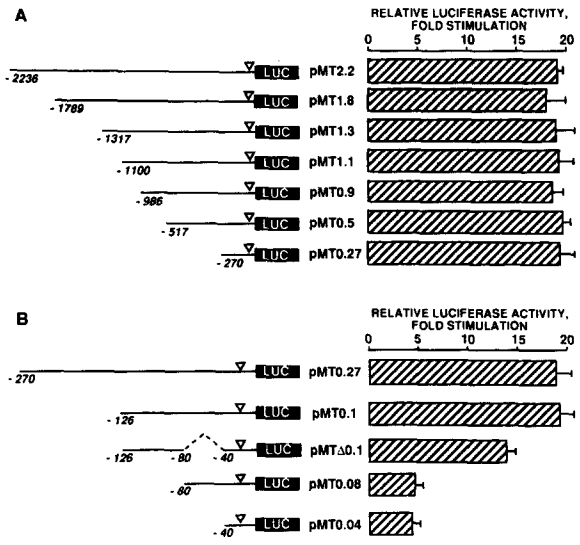

Figure 2. A cAMP regulatory element is localized between −126 and −80 in mouse tyrosinase promoter. B16 cells were transiently transfected with pMT2.2, pMT1.8, pMT1.3, pMT1.1, pMT0.9, pMT0.5, and pMT0.27 (*A*), or with pMT0.27, pMT0.1, pMT0.08, pMT0.04, and pMTΔ0.1 (*B*). After 24 h with forskolin, luciferase activity was assayed as previously described and normalized by the β-galactosidase activity. Numbers at the 5′ end of each construct indicate the deletion endpoints relative to the transcription initiation site (+1). Triangles indicate the TATA-box position. In pMTΔ0.1, the hatched lines indicate that the region between −80 and −40 has been deleted. Results are expressed as fold stimulation of the basal luciferase activity from unstimulated cells. Data are means ± SE of five experiments performed in triplicate.

Putative Enhancer Activity of cAMP Sensitive Regulatory Elements

The putative enhancer activity of regulatory elements involved in cAMP response of the mouse tyrosinase promoter was evaluated using an enhancerless expression vector containing the SV40 early promoter upstream from the luciferase coding sequence (pSV). Initially, we cloned the 2.2-kb fragment (pSVMT2.2) and the XbaI–HindIII (−270/−80) restriction fragment (pSVMT0.2) of the mouse tyrosinase promoter upstream the SV40 early promoter. Furthermore the sequence of the region between RsaI and HindIII (−126/−80) restriction sites conferring the cAMP responsiveness revealed the presence of a GTCATGTGCT motif (M-box). Thus, we introduced three copies of the M-box motif upstream the SV40 promoter into pSV (pSV3M). After transfection with pSV and pSV3M, luciferase activity was stimulated 2–3-fold by forskolin treatment (Fig. 3). With pSVMT2.2 and pSVMT0.2, the stimulation evoked by forskolin treatment (fivefold) was slightly increased, but this stimulation was markedly lower than that observed with pMT2.2. Taken together, these results indicate that the cAMP regulatory elements of the mouse tyrosinase promoter do not act as remote enhancer elements to confer cAMP responsiveness.

Identification of cis-acting Elements Involved in cAMP Response

To thoroughly study the role of the GTCATGTGCT motif (−120/−110) in the cAMP responsiveness of the mouse tyrosinase promoter, mutations were introduced into the core M-box and in its surrounding region in pMT0.1. Mutations of the three nucleotides upstream (M1) or downstream (M4) the M-box sequence and mutations of the

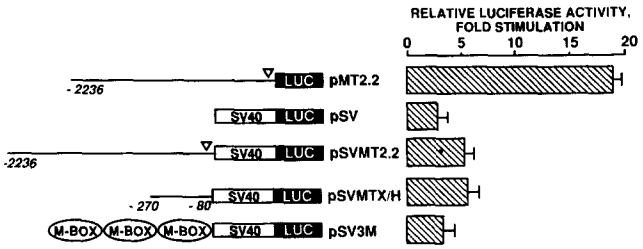

Figure 3. The *cis*-acting elements involved in cAMP responsiveness did not function as remote enhancer activity. B16 cells were transiently transfected with pMT2.2, pSV, pSVMT2.2, pSVMTX/H, or pSV3M. After 24 h with forskolin, luciferase activity was assayed as previously described and normalized by the β-galactosidase activity. Results are expressed as fold stimulation of the basal luciferase activity from unstimulated cells. Data are means ± SE of five experiments performed in triplicate.

three nucleotides at the end of the M-box (M3) did not impair the effect of forskolin on luciferase activity compared with the wild-type pMT0.1 (Fig. 4). Conversely, mutations in the first three nucleotides (M2) or in the core motif CATGTG (M5) of the M-box decreased markedly the cAMP response of the promoter (respectively, 9- and 5-fold stimulation). Also, when pMT2.2 containing the same mutated M-box (M6) was transfected, luciferase activity was only sixfold stimulated by forskolin treatment. The results show that this M-box plays a key role in cAMP responsiveness of the mouse tyrosinase promoter.

A second CATGTG motif (E-box) was found 10 bp below the TATA-box near the initiator elements. Hence, we studied the effects of mutations in this region of the promoter on the cAMP response. Mutations in pMT0.1 of the nucleotides upstream the E-box (E1, E2) did not impair the cAMP effect on the luciferase activity compared with the wild-type pMT0.1 (17–19-fold stimulation) (Fig. 5). Conversely, the cAMP response was markedly reduced (sevenfold) when mutations were introduced immediately downstream the CATGTG motif (E3) but unaffected in E4 mutant. Furthermore, mutations within the core E-box in both pMT0.1 (E5) and pMT2.2 (E6) led to a dramatic decrease in the cAMP effect on luciferase activity (threefold stimulation). Double mutation of both M-box and initiator E-box (iE-box) in pMT2.2 did not further decrease the cAMP response (threefold). These data indicate that this E-box is also involved in the cAMP responsiveness of the tyrosinase promoter. Additionally, it should be noted that mutation of the M-box, or of the iE-box, markedly decreased the basal activity of the promoter (respectively, 5- and 10-fold compared to pMT2.2) in B16 cells but in any case, these constructs still remained much more active than a promoter less construct. In NIH3T3, all these constructs were unresponsive to cAMP. However, mutations of the M-box or of the iE-box did not affect the basal activity of the promoter transfected in NIH3T3 cells.

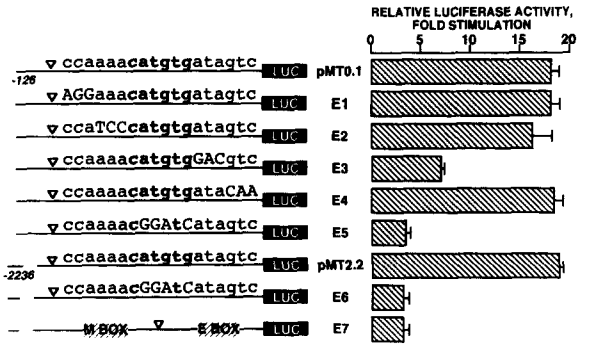

Figure 5. The initiator E-box is also involved in cAMP response of the mouse tyrosinase promoter. B16 cells were transiently transfected with promoter constructs mutated at the core iE-box motif (pMT2.2 and pMT0.1) or in its flanking regions (pMT0.1). B16 cells were also transfected with pMT2.2 containing mutated M-box and iE-box. The nucleotides mutated are indicated in capital letters. After 24 h with forskolin, luciferase activity was assayed as previously described and normalized by the β-galactosidase activity. Results are expressed as fold stimulation of the basal luciferase activity from unstimulated cells. Data are means ± SE of five experiments performed in triplicate.

Characterization of anti-Microphthalmia Antibody

In recent reports, a b-HLH protein named microphthalmia was suspected to be involved in tissue-specific expression of tyrosinase through its interaction with the CATGTG sequences (Bentley et al., 1994; Hemesath et al., 1994; Yasumoto et al., 1994). To study the role of this transcription factor, we first raised an antibody against a peptide corresponding to the COOH terminus domain of microphthalmia. Immunofluorescence studies showed no signal with this antibody in NIH3T3 cells (Fig. 6 *A*). However, we observed a strong labeling of the nucleus in NIH3T3 cells stably transfected with pCDNA$_3$ encoding microphthalmia

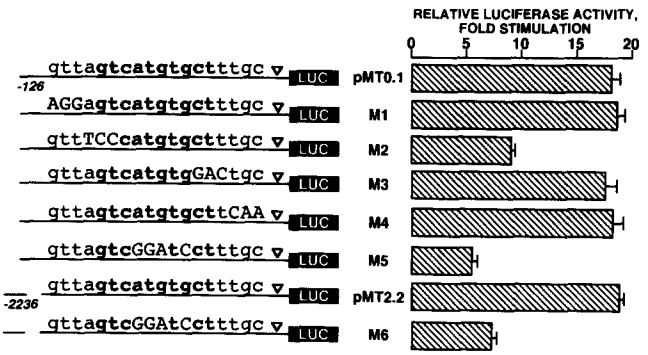

Figure 4. M-box upstream from the TATA-box is involved in cAMP response. B16 cells were transiently transfected with promoter constructs mutated at the core M-box motif (pMT2.2 and pMT0.1) or in its flanking regions (pMT0.1). The nucleotides mutated are indicated in capital letters. After 24 h with forskolin, luciferase activity was assayed as previously described and normalized by the β-galactosidase activity. Results are expressed as fold stimulation of the basal luciferase activity from unstimulated cells. Data are means ± SE of five experiments performed in triplicate.

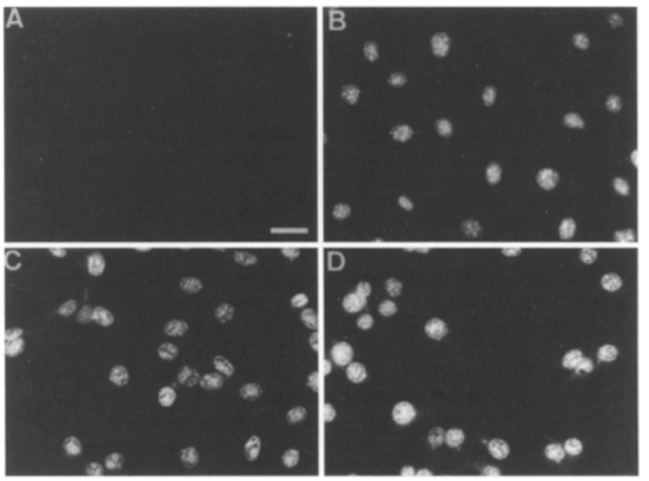

Figure 6. Immunolocalization of microphthalmia. Immunofluorescence labeling was performed with the anti-microphthalmia antibody. Nontransfected NIH3T3 cells (*A*), NIH3T3 cells transfected with the expression plasmid encoding microphthalmia (*B*), unstimulated B16 mouse melanoma cells (*C*), and forskolin-stimulated B16 mouse melanoma cells (*D*). Bar in *A* represents 10 μm.

(*B*). A strong labeling of nucleus by anti-microphthalmia antibodies was also observed in B16 melanoma cells (*C*), and this labeling was not modified by forskolin treatment (*D*). Then, we performed a gel shift assay with the in vitro transcribed/translated microphthalmia using labeled M-box as probe. In vitro transcribed/translated proteins from $pCDNA_3$ encoding microphthalmia formed a complex with the M-box. This complex was shifted by our antibody but unaffected by the preimmune serum. No complex was observed with in vitro transcribed/translated reactions performed in the absence of plasmid (Fig. 7). Taken together these results demonstrate the specificity of our anti-microphthalmia antibody.

Characterization of the Factor Interacting with cAMP Regulatory Elements of the Mouse Tyrosinase Promoter

To characterize the nuclear factors interacting with the cAMP regulatory elements of the tyrosinase promoter, we performed band shift assays using M-box or iE-box as probes (Fig. 8). M-box and iE box formed one major complex with nuclear extracts from basal- and forskolin-treated B16. M-box and iE-box complexes were displaced by unlabeled probe but was unaffected by mutant oligonucleotide. Furthermore, the nature of proteins bound to the M-box and to the iE-box was investigated by supershift experiments. M-box or iE-box complexes formed with B16 or NIH3T3 nuclear extracts were partially shifted by USF antibodies (not shown). Using antibodies to microphthalmia (Fig. 9 *A*), we showed that M-box or iE-box complexes from NIH3T3 nuclear extracts did not react with these antibodies. Per contra, M-box or iE-box complexes from B16 nuclear extracts were partially shifted by anti-microphthalmia antibody (Fig. 9 *B*). Interestingly, cAMP increased M-box or iE-box complexes shifted by anti-microphthalmia antibody. Also, band shift assays performed using immunopurified microphthalmia (Fig. 9 *C*) confirmed that cAMP increased the amount of microphthalmia complexed to the M-box or the iE-box. Taken together, these data demonstrate that microphthalmia is a B16 melanoma cell-specific transcription factor and that cAMP stimulates the binding of microphthalmia to its target sequences.

To further investigate the role of microphthalmia in the effect of cAMP on tyrosinase promoter, we cotransfected an expression vector encoding microphthalmia with pMT2.2 and pMT0.1. Microphthalmia induced a dramatic increase in the luciferase activity. This effect on basal transcription did not allow us to observe further stimulation when microphthalmia-transfected cells were treated with forskolin (not shown). Moreover, we carried out a study to compare the effect of microphthalmia on the different tyrosinase promoter constructs with their cAMP responsiveness. For this purpose, both parameters were represented on a single graph. cDNA encoding microphthalmia and different tyrosinase promoter constructs were cotransfected in B16 melanoma cells and the effect of microphthalmia expression, on the basal transcriptional activity of the constructs,

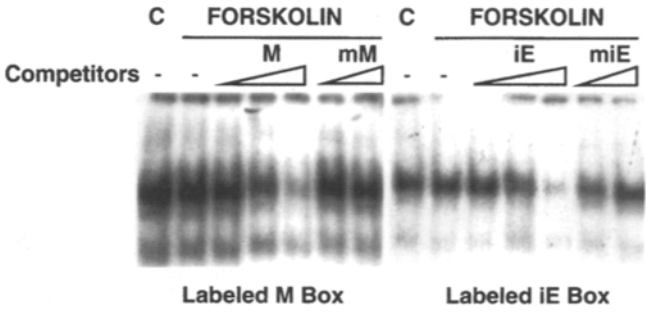

Figure 8. M-box and the iE-box–binding activities are present in B16 nuclear extracts. DNA-binding activity of 10 μg nuclear proteins from control, C, or forskolin-treated B16 cells was measured by gel mobility shift assay using labeled M-box or iE-box. Specificity of the complexes was tested by competition with increasing molar excess (3×, 10×, 50×) of unlabeled M-box or iE-box and with increasing molar excess (10×, 50×) of mutated M-box (mM-box) or iE-box (miE-box).

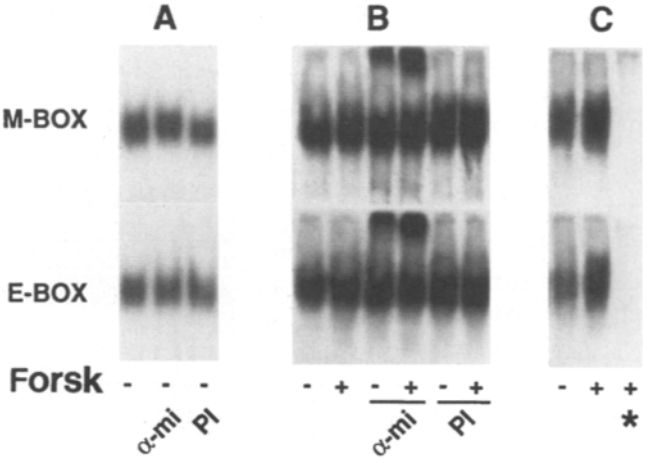

Figure 9. Microphthalmia is a B16-specific transcription factor that binds to M-box and iE-box. Gel shift assays were performed using labeled M-box or iE-box. (*A*) NIH3T3 nuclear extracts were incubated for 1 h with 1 μl of preimmune serum (*PI*) or specific antibodies against microphthalmia (α-MI). (*B*) B16 nuclear extracts from control (−) or forskolin-treated cells (+) were incubated for 1 h with 1 μl of preimmune serum (*PI*) or specific antibodies against microphthalmia (α-MI). (*C*) B16 nuclear extracts from control (−) or forskolin-treated cells (+) were immunoprecipitated with the anti-microphthalmia antibody, and then microphthalmia was eluted with 10 μM of COOH terminus peptide. Specificity of the complexes was tested by competition with molar excess of unlabeled M-box or iE-box (*).

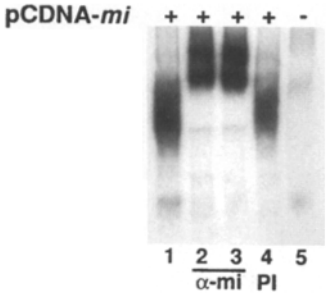

Figure 7. Anti-microphthalmia antibody recognizes in vitro translated microphthalmia. Transcription/translation was performed either in the presence of pCDNA3 encoding microphthalmia (lanes *1–4*) or in the absence of plasmid (lane *5*). The transcription/translation mix was used in a gel shift assay with labeled M-box. Lanes *1* and *5*, no serum; lanes *2* and *3*, respectively, 0.3 and 1 μl of anti-microphthalmia serum; lane *4*, 1 μl of preimmune serum.

was represented on the X axis. The effect of cAMP on these different constructs as determined in Figs. 4 and 5, was reported on the Y axis. This drawing allowed us to identify two groups of constructs (Fig. 10). In the first one the constructs showed a strong stimulation of the basal transcriptional activity (25–45-fold) in the presence of microphthalmia and a strong responsiveness to cAMP (17–24-fold). In the second group, the effect of microphthalmia (3–13-fold) and the cAMP responsiveness (3–8-fold) were markedly decreased. Hence, it appears that the effect of cAMP on the tyrosinase promoter activity correlates with the ability of microphthalmia to bind and transactivate the promoter.

Discussion

cAMP elevating agents stimulate melanogenesis through an augmentation of tyrosinase mRNA. However, it remains to elucidate whether cAMP stabilizes tyrosinase messenger or stimulates transcriptional activity of tyrosinase gene promoter. In the present report, we cloned a 2.2-kb fragment (−2236/+56) of the tyrosinase promoter upstream from the luciferase coding sequence (pMT2.2). Using this construct in transient transfection assays in B16 melanoma cells, we showed that melanogenic agents that increase cAMP level stimulate the transcriptional activity of the mouse tyrosinase promoter. This observation demonstrates that this fragment of the promoter contains regulatory elements involved in the cAMP response. Recent studies have tentatively characterized the regulatory elements involved in tissue-specific expression of the mouse tyrosinase promoter (Ganss et al., 1994; Lowings et al., 1992; Yokoyama et al., 1994). On the other hand, the responsive elements implicated in the acute regulation of tyrosinase expression by cAMP elevating agents remain to be identified. Experiments using deletion constructs of the pMT2.2 showed that the 270-bp promoter fragment 5′ of the transcription start site is highly responsive to cAMP in both transiently or stably transfected B16 cells. This observation is contradictory with a recent report indicating that the same promoter fragment was unresponsive to cAMP (Ganss et al., 1994). The reasons of this discrepancy appear difficult to understand since Ganss et al. (1994) used B16 melanoma cell line in which cAMP increase the expression of endogenous tyrosinase messengers.

Additional deletions in the tyrosinase promoter showed that the cAMP response is dramatically reduced when the region spanning from −126 to −80 is removed. The absence in this region of canonical cAMP responsive elements (CRE) and of AP2-binding site, which was also shown to be responsive to cAMP (Imagawa et al., 1987), indicates that cAMP induces the stimulation of tyrosinase gene expression through undiscovered regulatory elements. Introduction of mutations in the fragment −126/−80 showed that the M-box and especially the core motif CATGTG are required for a full cAMP response of the tyrosinase promoter. Nevertheless, a 6–8-fold stimulation of luciferase activity was still observed in these mutants, suggesting that other regulatory elements involved in cAMP response might exist. Indeed, when mutations were introduced in the CATGTG motif of the initiator E-box (iE-box) only a threefold stimulation of the luciferase activity was obtained. Interestingly, mutations of both M-box and iE-box did not lead to a further decrease in the cAMP response compared to the iE-box single mutant. Furthermore, a weak effect of cAMP was observed on SV40 and on thymidine kinase promoters (threefold), suggesting that the residual effect of cAMP on the double mutant could be ascribed to an effect of cAMP on general transcription. However, this effect remains very low compared to the specific effect of cAMP on tyrosinase expression. Taken together these data indicate that M-box and iE-box are involved in the stimulation of tyrosinase promoter transcriptional activity by cAMP. These two elements act in synergy to give the tyrosinase promoter its strong cAMP sensitivity. Additionally, we cannot rule out the possibility that other elements of the tyrosinase promoter (between −126/+1) cooperate with M-box and iE-box.

The core motif CATGTG found in the M-box and in the iE-box was reported to interact with b-HLH transcription factors. Yet, until now it was not possible to identify a transcription factor specific of melanoma cells since USF, an ubiquitously expressed b-HLH transcription factor, was shown to interact in vitro with the M-box (Yavuzer and Goding, 1994). The binding of USF to the M-box could be ascribed to the extreme avidity of USF for all the CANNTG motifs and might not be relevant in intact cells. In this report, immunofluorescence studies show that microphthalmia is expressed in B16 melanoma cells but not in NIH3T3 cells, in which cAMP does not affect the tyrosinase promoter activity. Using an antibody to microphthalmia in band shift experiments we demonstrated that microphthalmia is a B16 melanoma cell-specific transcription factor. Further-

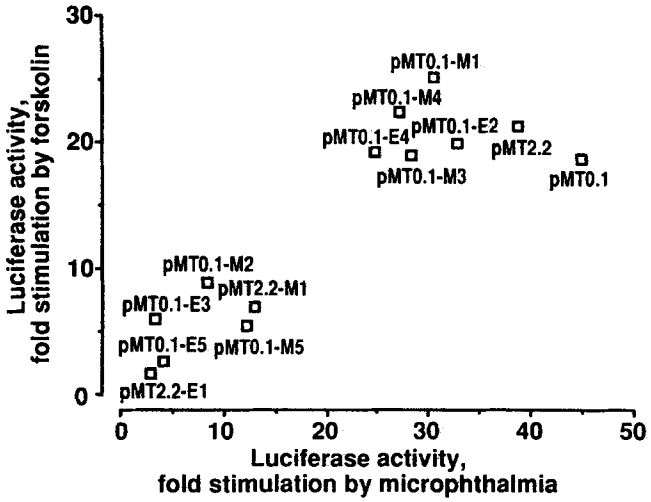

Figure 10. Transactivation by microphthalmia correlates with the cAMP responsiveness of the different promoter constructs. B16 cells were transiently cotransfected with 0.04 μg of pCDNA3 encoding microphthalmia and 0.25 μg of different tyrosinase promoter constructs. After 24 h, luciferase activity was assayed as previously described and normalized by the β-galactosidase activity. Results are represented on the X axis as fold stimulation of the basal luciferase activity in the absence of microphthalmia. Data are a mean of two separate experiments. On the Y axis are the effects of forskolin on the luciferase activity after transfection with the different constructs as indicated in Figs. 4 and 5.

more, we showed that cAMP increases its binding to the M-box and to the iE-box. The augmentation of the microphthalmia binding to its target sequences does not appear to be the consequence of an increased amount of microphthalmia. Indeed immunofluorescence labeling of B16 cells was not affected by cAMP. Thus, it is conceivable that cAMP stimulates the affinity of microphthalmia for the CATGTG motifs via posttranslational modifications. This could be achieved through the phosphorylation of microphthalmia by PKA or unidentified kinases. Additionally, the binding of microphthalmia to its target sequence could be regulated by its association with other proteins such as the retinoblastoma protein (pRB). Indeed, microphthalmia has been recently shown to interact with pRB (Yavuzer et al., 1995), and the phosphorylation of pRB has been reported to be inhibited by cAMP (Christofersenn et al., 1994). Thus, pRB could interact with microphthalmia in a phosphorylation-dependent manner and regulate microphthalmia binding. Finally, the correlation between the cAMP responsiveness of the different constructs and their transactivation by microphthalmia suggests that an interaction of microphthalmia with these *cis*-acting elements is required for the cAMP response. This result is in agreement with a recent report indicating that MITF, the human homologue of microphthalmia, transactivates the tyrosinase promoter through CATGTG motifs surrounding the TATA-box (Yasumoto et al., 1995).

Taken together our results suggest that microphthalmia is involved in the regulation of the tyrosinase gene expression by cAMP. Hence, it was tempting to propose that the lack of cAMP effect on the tyrosinase promoter in NIH3T3 cell was due to the absence of microphthalmia. However, cAMP was still unable to stimulate the tyrosinase promoter activity in NIH3T3 cells expressing microphthalmia (not shown). This result suggests that a B16 cell-specific signaling pathway is missing in NIH3T3 cells. Indeed it should be noted that cAMP was reported to inhibit the activation of MAP kinases in NIH3T3 cells (Burgering et al., 1993). On the contrary, in B16 melanoma cells we have shown that cAMP activated MAP kinase (ERK1) and induced its translocation to the nucleus (Englaro et al., 1995). The presence in microphthalmia of consensus sequence for MAP kinase phosphorylation (P-x-T/S-P) led us to propose that MAP kinases could phosphorylate and activate microphthalmia in the nucleus of B16 cells. This hypothesis is currently under investigation.

In summary, we demonstrated in this report that tyrosinase promoter was responsive to cAMP. Two CATGTG motifs surrounding the TATA-box are involved in this response and convey microphthalmia transactivating effect. Since we have shown that the binding of microphthalmia to these motifs is stimulated by cAMP, we hypothesized that microphthalmia is involved in the regulation of tyrosinase promoter by cAMP.

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