Distinct Co-regulation of Endogenous versus Transfected MITF-Dependent Tyrosinase Promoter

(microphthalmia / MITF / tyrosinase / melanoma / E1A)

B. ŠESTÁKOVÁ, J. VACHTENHEIM

Laboratory of Molecular Biology, University Hospital, 3rd Faculty of Medicine, Charles University, Prague, Czech Republic

Abstract. The tissue-specific control of gene activation in melanocytes is directed by the microphthalmia-associated transcription factor (MITF), a master regulator of melanocyte development and differentiation. Tyrosinase is a rate-limiting enzyme in melanin biosynthesis and a prototypic MITF target. While the expression of tyrosinase is restricted to pigmented cells, the transfected tyrosinase promoter is active in a broad range of cell types if ectopic MITF is co-expressed. Here we used the E1A oncoprotein and its mutants as repressors of both the transiently transfected and endogenous tyrosinase promoter. We report that the requirement of the E1A N-terminus for repression of the MITF-activated tyrosinase promoter and the sensitivity to derepression by the histone deacetylase inhibitor trichostatin A are distinct when the activity of the transiently transfected or the endogenous promoter is analysed in U2-OS cells. Thus, for transiently transfected versus chromatin-embedded promoter, the activity of obligatory MITF seems to be executed through different mechanisms of transcriptional co-activation.

Activation of many lineage-specific genes requires the ordered events at the chromatin-embedded promoters, involving the recruitment of transcription factors and general transcription machinery, covalent modification of histones, and nucleosome remodelling. Melanocytes evolve from the neural crest and are characterized by the presence of the microphthalmia-associated transcription factor (MITF), a master regulator of the melanocyte-specific differentiation programme (Goding, 2000; Steingrimsson et al., 2004). MITF is a tissue-restricted transcriptional activator essential for differentiation of melanocytes and at least three other cell lineages: pigmented cells of the retina, mast cells, and osteoclasts (Nechushtan and Razin, 2002; Steingrimsson et al., 2004). These tissues express various isoforms of MITF which differ in their N-termini and are expressed from different promoters (Shibahara et al., 2001). The melanocyte-specific isoform (MITF-M) is transcribed from a promoter which is active exlusively in melanocytes and melanoma cells (Fuse et al., 1996). Although the genes of the melanogenic pathway, which are MITF-M-dependent, are expressed in a strictly specific fashion only in melanin-producing tissues, the distal tyrosinase promoter, if transfected in a reporter plasmid and co-transfected with the MITF vector, is activated in a broad spectrum of cell lines of various origin (Bentley et al., 1994; Yavuzer et al., 1995; Wu et al., 2000; Vachtenheim and Drdová, 2004; data not shown). It is unclear whether this difference could be explained by the presence of an upstream regulatory element in the tyrosinase gene (Yasumoto et al., 1994) and the expression of melanocyte-specific MITF isoform, and an additional mechanism such as utilization of different coactivators might be involved. Conversely, it is exceptional for non-melanocyte cells to express the endogenous melanocyte-specific MITF targets when challenged with ectopic MITF. For instance, the mouse NIH3T3 fibroblasts begin to activate transcription of several pigment cell markers after expressing MITF ectopically (Tachibana et al.,1996; de la Serna et al., 2006). Of human cells, osteosarcoma cell line U2-OS triggers the transcription of tyrosinase if the exogenous MITF is expressed, but other melanogenic markers remain silent (Vachtenheim et al., 2001; Vachtenheim and Drdová, 2004). Because such induction is not possible in melanocytes where MITF and all its downstream targets are constitutively expressed, these rare non-melanocyte cell models are useful for exploring the mechanisms that underlie the transactivation of pigment cell-specific genes in chromatin. Athough transcription coactivators p300 and CBP associate with MITF and are thought to act as its cofactors (Sato et al., 1997; Price et al., 1998), it is unclear whether this association is physiologically relevant for transcription of target genes packed in chromatin. Recently, MITF has been shown to utilize the SWI/SNF chromatin remodelling complex to activate some, but not all, of its endogenous targets (de la Serna et al., 2006). Here, by

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Corresponding author: Jiří Vachtenheim, Laboratory of Molecular Biology, University Hospital, Budínova 2, 180 00 Prague 8, Czech Republic, Phone: (+420) 266 082 272; Fax: (+420) 284 840 840; e-mail: jivach@upn.anet.cz

Abbreviations: MITF – microphthalmia-associated transcription factor; TSA – trichostatin A, wt – wild-type.

using the U2-OS cell line and the adenoviral protein E1A and its mutants, we demonstrate that the E1A N-terminus is dispensable for repression of the endogenous gene, but required for repression of the transfected tyrosinase promoter, and trans-repression of the transfected versus endogenous template by a p300 non-binding E1A mutant is differently sensitive to trichostatin A. Thus, while MITF is required for the promoter activity in both instances, the data strongly suggest that different mechanisms are involved in the transcriptional co-activation.

Material and Methods

Plasmid constructs

The wild-type (wt) E1A 12S protein, its mutants, the human tyrosinase promoter-luciferase construct, and the expression vector for the FLAG-tagged human melanocyte-specific isoform of MITF (MITF-M) have been described (Drdová and Vachtenheim, 2004; Vachtenheim and Drdová, 2004; Vachtenheim et al., submitted). All constructs were verified by dideoxynucleotide sequencing of both strands. The vector pBJ-Brg1 was kindly provided by Dr. G. Crabtree.

Cells, transfections, and RT-PCR

U2-OS, C33A (human cervical carcinoma), H1299 (human lung cell carcinoma) and other human tumour cell lines were cultivated in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, antibiotics, and L-glutamine. Cells transfected by the calcium phosphate method were harvested for analysis after 48 hours and luciferase activity was determined according to manufacturer´s instructions (Promega, Madison, WI). Transfection efficiency was normalized by determining the β-galactosidase activity expressed from pCMV-β. All transfection experiments were repeated at least three times in duplicate samples. To detect the endogenous MITF-induced tyrosinase transcript, semi-quantitative RT-PCR was carried out as described previously (Vachtenheim and Drdová, 2004). The amount of RNA normalized to transfection efficiency was reverse-transcribed with the reverse transcriptase Superscript II (InVitrogen, Carlsbad, CA) followed by 18 to 22 cycles of PCR, Southern blotting, and hybridization as described (Vachtenheim and Drdová, 2004). Alternatively, in some experiments, the PCR products were directly stained in the agarose gel after 28 cycles of PCR. Similar results were obtained by using both approaches. These RT-PCR assays were performed independently with two distinct sense primers and a common antisense primer. Either the sense primer spanning the splicing site between exons 1 and 2 of human tyrosinase cDNA (5´-TTGGCAGATTGTCTGTAGCC) or an exon 1-derived sense primer (5´-CCAGAAGCTGACAGGAGATG) was used with the antisense primer (5´-AGGCATTGTGCATGCTGCTT) derived from exon 3, resulting in products 284 bp or 431 bp long, respectively. Similar results were obtained by using both primer sets and only data with the longer PCR product are presented. PCR for GAPDH with primers from Clontech was used as a positive control and a test for RNA integrity in all experiments.

Immunoblotting and antibodies

For detection of expressed E1A and MITF proteins, the extract was normalized for transfection efficiency, electrophoresed in 10% SDS-PAGE gel, transferred, and probed with anti-E1A M73 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-FLAG M2 antibody (Sigma, St. Louis, MO) using standard procedures, utilizing the ECL detection system (Amersham Biosciences, Uppsala, Sweden).

Results

Tyrosinase promoter-reporter is activated by ectopic MITF in many cell types while triggering the endogenous targets is rare in non-melanocytes

The short proximal tyrosinase promoter sequence (a region about 120-bp upstream of the transcription start) is sufficient to provide cell-specific transcription in melanocytes. This sequence contains the E-box at the initiator and the extended E-box (M-box) upstream of the TATA box. Both these E-boxes are bound by MITF, and the initiator E-box is required for the promoter activity while the M-box substantially augments transcription (Bentley et al., 1994). Moreover, the tyrosinase distal element (TDE), about 2-kb upstream, further enhances tyrosinase expression (Yasumoto et al., 1994). We used the proximal tyrosinase promoter-reporter in assays with the co-transfected MITF expression vector in a range of human cell lines. Fig. 1A (upper panel) shows that an increasing amount of the MITF plasmid activates the promoter activity in U2-OS, C33A, and H1299 cells by about 2- to 2.5-fold. Similarly, we have observed activation in eight other cell lines (from 2- to 5-fold, data not shown). Previously, we demonstrated that the ectopic MITF is capable of eliciting strong expression of endogenous tyrosinase in the U2-OS cell line, which is otherwise silent in these cells (Vachtenheim and Drdová, 2004). Indeed, as shown in Fig. 1A (lower panel), transfection of the MITF-encoding plasmid results in the appearance of the tyrosinase mRNA band in a dose-dependent fashion in U2-OS cells, while no expression is seen in C33A cells and a weak response is noted in H1299 cells. These three cell lines have a comparable transfection efficiency and U2-OS cells are the least transfectable, yet displaying the highest signal. The ATPases Brg1 and Brm are important components of the SWI/SNF nucleosome remodelling complex, which has recently been shown to be required for the activation of MITF targets in chromatin (de la Serna et al., 2006). While U2-OS cells express all components of

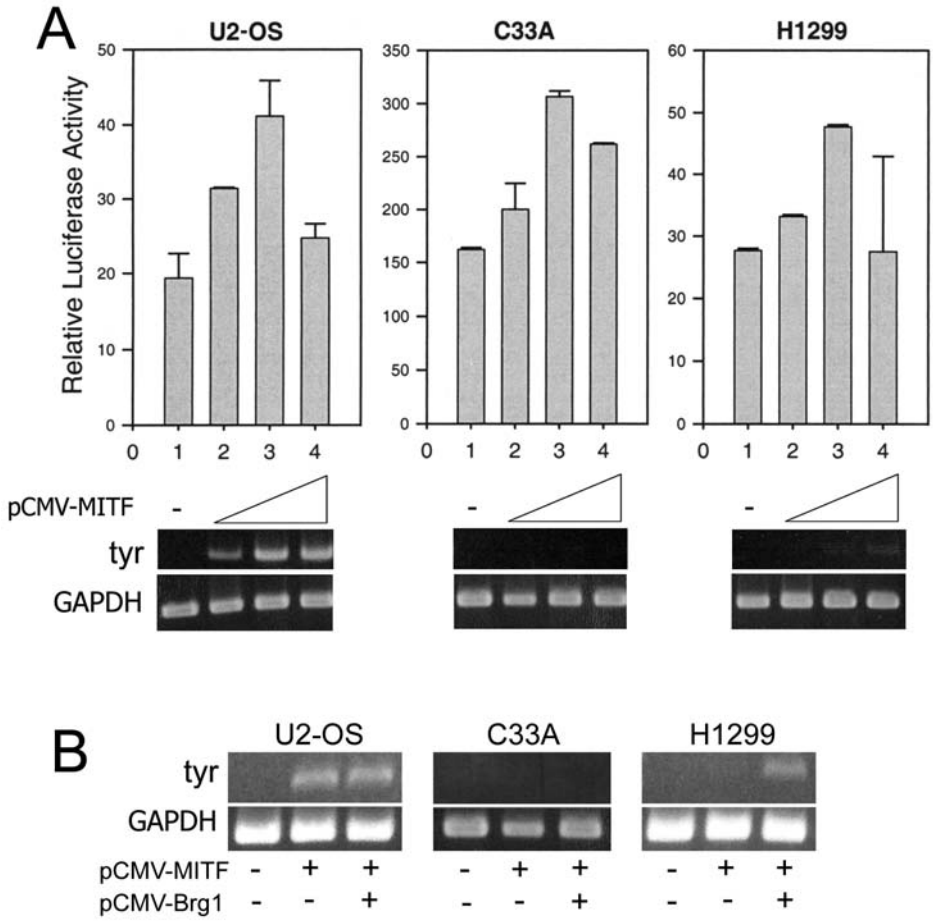

Fig. 1. A. Exogenous MITF activates tyrosinase promoter-reporter, but the endogenous tyrosinase is triggered selectively only in U2-OS cells. Cells were transfected with increasing amounts (0 μg, 0.5 μg, 1.0 μg, and 2.0 μg) of MITF expression vector together with the tyrosinase-luciferase reporter plasmid (0.2 μg) and pCMV-β to normalize luciferase activity. Luciferase values (upper panel) are means of duplicates +SE. Expression of tyrosinase was determined by RT-PCR (lower panel) as described in Material and Methods. RT-PCR for glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control (20 cycles). All experiments were repeated at least twice. B. Co-expression of Brg1 and MITF can trigger expression of tyrosinase in H1299 cells but not in C33A cells. The experimental setting was the same as in A. MITF and Brg1 proteins were properly expressed (data not shown).

SWI/SNF (not shown), several MITF-nonresponsive tumour cell lines we used, including C33A and H1299, are Brg1 null (Yamamichi et al., 2005). We therefore wanted to know whether the inability of these cell lines to trigger tyrosinase upon the transfection of MITF is caused by the incomplete expression of SWI/SNF components. We co-transfected a Brg1 expression vector together with MITF into the same three cells lines and found that reconstitution of Brg1 expression strongly potentiated the MITF-dependent expression of tyrosinase in H1299 cells, while no response was observed in C33A and U2-OS cells (Fig. 1B). Brg1 without co-transfected MITF did not have any effect (not shown). Thus, although Brg1 is a critical SWI/SNF component, which is missing in the H1299 cell line and is required for MITF-dependent transcription of tyrosinase in these cells, reconstitution of the defective SWI/SNF complex can not generally cooperate with MITF to elicit expression of this target in non-melanocytic cells. Furthermore, unlike U2-OS, several additional cell lines with the integral SWI/SNF complex did not reveal any appearance of tyrosinase (data not shown). Together, loss of some SWI/SNF components cannot explain that the non-melanocyte cell lines do not respond to exogenous MITF and the melanocyte-like response of U2-OS cells may possibly require a specific chromatin configuration at the target promoters.

Endogenous and transfected tyrosinase promoter is differently repressed by E1A mutants and derepressed by trichostatin A

We next tested whether the MIFT-dependent transcription could be affected by co-expressing the adenoviral E1A 12S protein. The E1A is capable of inhibiting not only the MITF promoter (Drdová and Vachtenheim,

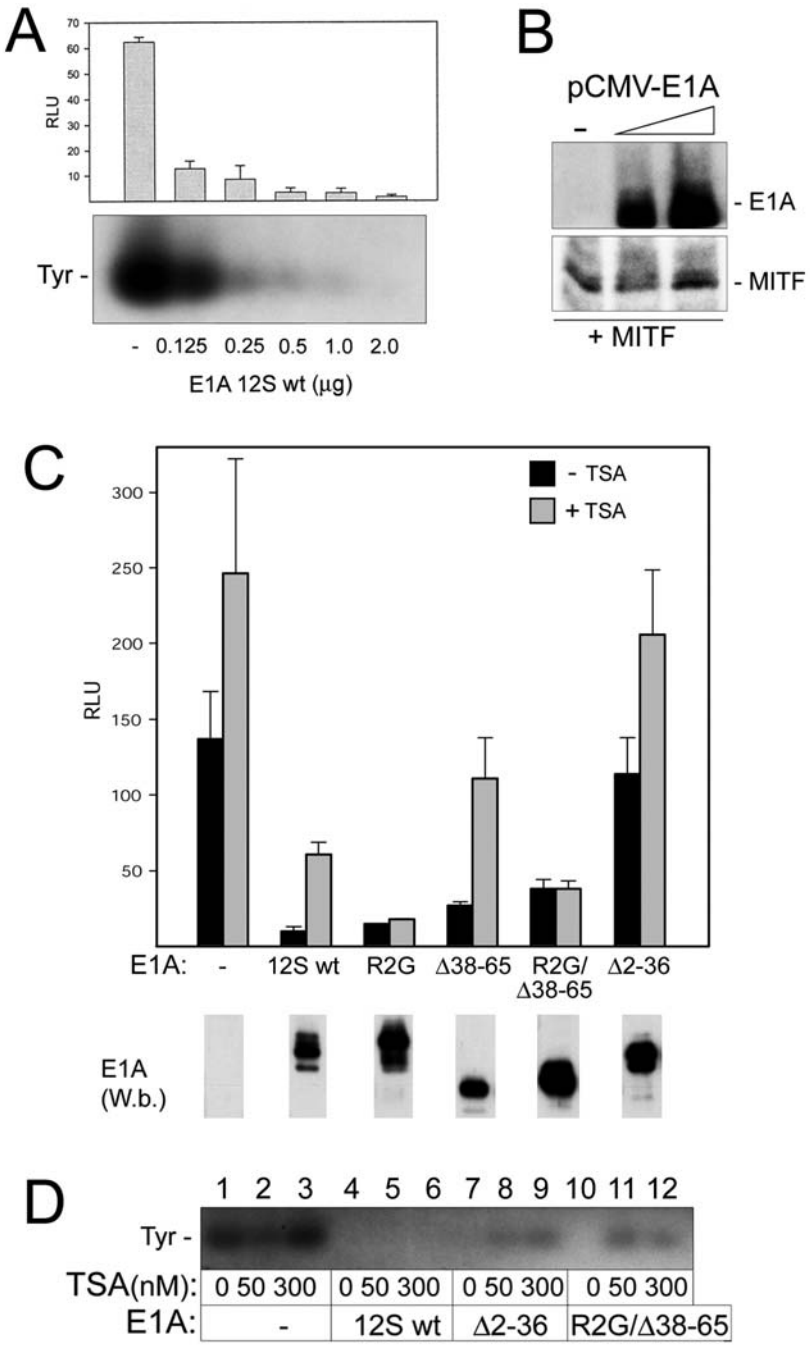

Fig. 2. Differential sensitivity of the endogenous versus transfected tyrosinase promoter to derepression by TSA in U2-OS cells. A. Dose-dependent inhibition of MITF-activated transcription by the E1A 12S protein. One μg of MITF expression plasmid was transiently transfected with the indicated amounts of the E1A 12S expression plasmid. Luciferase was detected in cell extracts and RNA was isolated, normalized for transfection efficiency, and subjected to RT-PCR followed by hybridization to the tyrosinase cDNA probe. B. Increasing levels of the expressed E1A protein do not alter the expression of exogenous MITF in U2-OS cells. Two μg of MITF expression plasmid were co-transfected with 6 μg of control vector or 3 μg or 6 μg of pCMV-E1A and both proteins were detected by Western blot. C. Differential sensitivity of E1A mutants to derepression by TSA in the promoter-reporter assay. Cells were transfected as above and treated, or not treated, with TSA (final concentration 200 nM) for 20 h before harvesting, and luciferase activity was determined. D. Sensitivity of the endogenous promoter repression to TSA. U2-OS cells were transfected with the MITF (1 μg) and E1A (or mutant E1A) (2 μg) expression vectors as above and medium containing varying final concentrations of TSA was applied to cells 20 h before harvesting. Tyrosinase expression was estimated by RT-PCR and hybridization. RT-PCR for GAPDH revealed similar band intensity as in Fig. 1 (not shown). Experiments were repeated twice with similar results.

2004), but can also affect the MITF-responsive promoters (Vachtenheim et al., submitted). When the pCMV-MITF is co-transfected with E1A into B16 mouse melanoma cells, a fraction of E1A is engaged in inhibiting the transcription of the endogenous MITF itself (Yavuzer et al., 1995) and, therefore, is not available for suppressing MITF targets (data not shown). These considerations are important because high doses of MITF can relieve the repressive effect of E1A on the MITF-activated tyrosinase promoter-reporter in melanoma cells (not shown). We therefore used U2-OS cells further and compared the effect of E1A on the reporter and endogenous promoter in the same MITF-transfected samples. Both promoters were inhibited by E1A in a dose-dependent manner (Fig. 2A). Then, we co-transfected a high dose of E1A suppressing the MITF activity fully (2 μg of E1A plasmid) with the MITF vector and investigated the effect of wt and four E1A mutants on both promoters. We also confirmed that the increasing amounts of E1A do not alter the MITF expression (Fig. 2B). As shown in Fig. 2C, the wt E1A 12S protein and the p300/CBP non-biding R2G mutant repressed the luciferase activity, while the Δ38-65 mutant (lacking most of the CR1 region) and the combined R2G/Δ38-65 mutant were slightly less effective in suppressing the promoter activity. The mutant Δ2-36 lacking the N-terminus was completely inactive. Surprisingly, the wt E1A and mutants R2G/Δ38-65 and Δ2-36 all repressed the endogenous

promoter (Fig. 2D, lanes 1,4,7,10). As the N-terminus (aa 2-36) binds and sequesters numerous transcription coactivators such as PCAF, hGCN5, TRRAP, p400, and p300/CBP (Frisch and Mymryk, 2002), their inactivation is probably not deleterious for the expression of tyrosinase in U2-OS cells, suggesting that another, as yet uncharacterized coactivator might be essential for MITF-driven transcription. To further delineate the differences in the mode of repression between the promoter-reporter and the endogenous gene, sensitivity to the histone deacetylase inhibitor trichostatin A (TSA) was evaluated. For the endogenous gene, wt E1A-dependent repression was entirely resistant to TSA and the suppression by E1A mutants Δ2-36 and R2G/Δ38-65 was only partially sensitive (Fig. 2D, lanes 2,3,5,6,8,9,11,12; the R2G mutant behaved as wt, not shown). In contrast, when the transfected promoter was tested, its activity was in part restored by TSA when wt E1A or the Δ38-65 mutant were employed for repression, whereas the R2G mutation conferred a complete TSA insensitivity, which was also retained in the combined mutant R2G/Δ38-65 (Fig. 2C). TSA slightly activated the reporter also in the absence of E1A (Fig. 2C). This might be due to the known upregulation of p21/WAF1 by TSA. We observed the co-activation of the tyrosinase promoter by ectopic p21/WAF1, while no restoration of the E1A-blocked promoter activity occured by p21/WAF1 (Šestáková et al., unpublished), suggesting that the relieving effect of TSA on represion by the wt E1A and the Δ38-65 mutant is indeed a result of inhibition of the deacetylase activities in the E1A-containing complexes and not an indirect effect of elevated p21/WAF1. Together, the requirement of the N-terminus for repression and the sensitivity to TSA are quite distinct when the transient versus the endogenous promoter is suppressed by E1A, strongly suggesting that different mechanisms are involved in their transcriptional co-activation. Conceivably, this distinction might reflect the ability of the tyrosinase reporter to be MITF-responsive in a range of cells of non-melanocytic origin, whereas the expression of natural MITF targets in pigmented cells is cell type-specific.

Discussion

In this study, we have demonstrated differential sensitivity of the E1A-repressed tyrosinase promoter-driven reporter and the endogenous gene to derepression by TSA, a histone deacetylase inhibitor. These observations suggest that, for the transfected promoter, sequestration of coactivators by wt E1A allows for deacetylation of histones, while in the case of the endogenous promoter, sequestration of cofactors without consequent histone deacetylation seems to be sufficient for repression. We have shown that both promoters are repressed by E1A mutant R2G that has been shown not to bind p300 *in vivo* in U2-OS cells and other cell lines (Fuchs et al. 2001), suggesting that sequestering another cofactor by E1A is crucial for repression. In support of this, we have observed that neither the p300 expression vector nor the p300 dominant negative construct lacking the E1A-interacting domain were able to efficiently counteract the repression induced by wt or R2G E1A (data not shown).

The avalability of tissue-specific transactivators on its own cannot fully explain the complexity of the gene expression pattern characterizing the differentiated cell type. We have noticed that although the complementation of missing components of the SWI/SNF chromatin remodelling complex could help trigger transcription of the MITF-dependent target in H1299 cells, the presence of an integral SWI/SNF complex together with ectopic MITF are generally not sufficient for melanocyte-like response in non-melanocytic cells. As described, the U2-OS cell line is exceptional as the tyrosinase transcription can be easily switched on solely by expressing exogenous MITF in these cells. Clearly, the differentiated state in melanocytes involves other yet unknown mechanisms that might possibly involve the configuration of chromatin at specific promoters. As MITF is believed to maintain the transcription of differentiation-specific markers in melanocytes, and is central in the network of pathways allowing sustained proliferation of melanoma cells, it will be crucial to understand which co-regulators are involved in the transcription of authentic MITF-activated promoters.

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