Repression of the Melanocyte-Specific Promoter of the Microphthalmia-Associated Transcription Factor by the Adenoviral E1A 12S Oncoprotein

(E1A / microphthalmia / MITF / melanoma / repression)

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Abstract. Melanocytes express MITF, which is crucial for the development of the melanocyte lineage and is overexpressed in malignant melanomas. Adenoviral E1A protein-expressing melanocytes are unpigmented, with the expression of MITF being silenced. We tested here a direct repression of the melanocyte-specific MITF promoter by E1A and its mutants. We found that the extreme N-terminus and conserved region 1 are required for repression. In contrast, the motif in conserved region 2 (a.a. 122–126), as well as amino acids 26–35 at the N-terminus, are not necessary. As these two later motifs mediate E1A binding to the retinoblastoma protein or to the transcriptional co-activator TRRAP, respectively, and are important for transformation by E1A in cooperation with other oncogenes, the results suggest that the transformation-defective E1A can still efficiently repress the MITF promoter. The CREB binding motif-mutated promoter had lower activity, but was also repressed by the same E1A mutants in human melanoma cells. The E1A protein is known to also exert an antitumour activity, which is associated with its transcription repression function and the ability to induce apoptosis, and is a potential antimelanoma agent. Since recent data suggest that MITF may be a survival factor for melanoma cells, the E1A mutants described here might constitute a good targeting agent for antimelanoma therapy.

The adenovirus E1A gene encodes nuclear proteins that are necessary for productive viral infection. Two splicing variants, the E1A 12S and 13S proteins, share the nonconserved N-terminus and conserved regions (CR) 1 and 2. However, unlike the E1A 13S protein, E1A 12S does not contain the zinc finger activation domain CR3. A prominent feature of E1A proteins is the ability to repress transcription. The transcription repressory function underlies many biological effects of E1A such as immortalization of primary rodent cells, block of differentiation, and cooperation with E1B or oncogenic ras in malignant transformation (Gallimore and Turnell, 2001; Frisch and Mymryk, 2002).

Mouse melanocytes are one of the cell lineages that can be immortalized upon expression of E1A and these immortalized melanocytes show characteristics of totally dedifferentiated cells with lineage-specific markers being transcriptionally silenced (Dotto et al., 1989; Yavuzer et al., 1995; Halaban et al., 1996). A basic-helix-loop-helix-leucine-zipper transcription factor microphthalmia (MITF, for microphthalmia-associated transcription factor) is essential for the development and differentiation of the melanocyte lineage from the neural crest (Opdecamp et al., 1997; reviewed by Goding, 2000). MITF binds to the E-box present in promoters of several pigment cell-specific genes and melanoma antigens (tyrosinase, tyrosinase-related proteins [TRP] 1 and 2, MLANA, SILV, and melastatin), and activates their transcription (Bentley et al., 1994; Bertolotto et al., 1998; Du et al., 2003; Miller et al., 2004). Whereas melanocytes express the melanocyte isoform MITF-M, several other tissues like osteoclasts, mast cells, and retinal pigmented cells express other tissue-specific MITF isoforms differing from MITF-M in the first exon, while the downstream exons are common to all isoforms (Udono et al., 2000; Shibahara et al., 2001). Each of these isoforms (-A, -B-, -E, -M, and -H) utilizes its own tissue-specific promoter for transcription, suggesting a lineage-specific transcriptional regulation. Melanocyte-specific isoform MITF-M is expressed exclusively in melanocytes and its promoter has been shown to be regulated by four transcription factors: CREB, LEF-1, Pax3 and Sox10 (Watanabe et al., 1998; Potterf et al., 2000; Bondurand et al., 2000; Lee et al., 2000; Takeda et al., 2000; Verastegui et al.,

Received May 5, 2004. Accepted June 16, 2004.

The authors acknowledge the support of grant (NC/6804-3) and institutional research project (MZ00000064211) from the Ministry of Health, Czech Republic.

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Abbreviations: CBP – CREB binding protein, CR – conserved region, CREB – cAMP response element binding protein, MITF – microphthalmia-associated transcription factor, PKA – protein kinase A, Rb – retinoblastoma, wt – wild-type.

2000; Yasumoto et al., 2002). Moreover, MITF-M can potentiate its own expression through the interaction with LEF-1 (Saito et al., 2002). Recently, it has been suggested that the Sox10 binding site, besides the canonical CRE motif, is also required for the cAMP pathway-dependent, forskolin or melanocyte-stimulating hormone-activated transcription of MITF-M in melanocytes (Huber et al., 2003). The β-catenin signalling, which is often upregulated in malignant melanocytes, promotes the survival of melanoma cells and requires MITF as a downstream effector (Widlund et al., 2002). Together, the reported data indicate the importance of the cAMP/PKA and Wnt/β-catenin/LEF-1 pathways in MITF expression and melanocyte differentiation. Additionally, recent data suggest that MITF might be a critical survival factor not only for embryonic melanocytes, but also for melanoma cells (McGill et al., 2002; Widlund et al., 2003). This is supported by a previous observation that MITF expression is retained in virtually all malignant melanoma specimens (King et al., 1999).

The E1A protein, paradoxically, has been shown to possess a tumour suppressor activity in human tumour cells, including melanoma (Deng et al., 2002; Frisch and Mymryk, 2002; Voigtlander et al., 2002). Apparently, this activity results from the ability of E1A to repress transcription of genes associated with the malignant phenotype. However, it has not been studied whether the downregulation of MITF is required for the E1A-mediated tumour repression in melanoma. Therefore, as a first step in addressing this issue, we studied here a direct repression of the melanocyte-specific MITF promoter by the E1A 12S protein. We show that larger deletions of the N-terminus or CR1 domain inactivate the repressive activity of E1A. However, smaller deletions affecting binding of the retinoblastoma (Rb) protein or TRRAP coactivator leave E1A fully functional. We further show that the CRE site-mutated promoter responds differently to E1A in human and mouse melanoma cells. Whereas this mutant promoter could be further downregulated by E1A in human melanoma cell lines, no effect of E1A was seen in malignant mouse melanocytes. Our results together suggest the usefulness of E1A mutants in targeting the melanocyte survival protein MITF in human melanoma cells.

Material and Methods

Plasmid constructs

Melanocyte-specific MITF promoter was amplified from human DNA by using the PromoterFinder kit (BD Biosciences, San Diego, CA) according to the instructions of the supplier. The gene-specific nested primer used in the second PCR step (5' ATACTTAAGCTTGACGGTAGACTATCCCTCCCT) contained the *Hind*III site. The Pfu polymerase-amplified promoter sequence (spanning nucleotides -2878 to +103 relative to the transcription start site) was then cloned into the *Mlu*I-*Hind*III sites of pGL3 basic (Promega, Madison, WI) coding for firefly luciferase, and verified by sequencing. The clone contained additional about 400 nucleotides at the 5'-end when compared to the original promoter clone isolated by Fuse et al. (Fuse et al., 1996). 5'-end deletions of the promoter were carried out by excising the fragments obtained after the digestion with *Mlu*I and appropriate second restriction enzyme (*Nde*I, *Msc*I, *Afl*II, *Pst*I, or *Eco*RV), followed by blunting and religation. In the CRE site-mutated promoter, the TGACGTCA motif positioned at -147 to -140 was deleted to TCA by PCR mutagenesis. All deletion constructs tested had a common 3' end.

E1A 12S wild-type (wt) cDNA (encoding the adenovirus type 5 E1A protein containing the 243 amino acids) and its various mutants were cloned as *Hind*III-*Xho*I inserts into the pcDNA3 (InVitrogen, Carlsbad, CA). The construction of the E1A mutants will be described in detail elsewhere (Vachtenheim et al., submitted). In brief, single amino acid substitutions, N-terminal deletion mutants and small deletions were prepared by PCR mutagenesis. To construct combined mutations, the two *Bst*XI sites and the *Cla*I site within the E1A sequence were utilized. We prepared an R2G mutant, amino acid deletions Δ2–36, Δ2–65, Δ4–15, Δ4–25, Δ16–25, Δ26–35, Δ38–65, Δ122–126, and combined deletion R2G/Δ38–65 in the full-length context of E1A. The details of clone constructions are available upon request. All constructs were verified by dideoxynucleotide sequencing of both strands.

Cell lines, transfections and luciferase assays

Human melanoma cell lines (MeWo, SK-MEL-2, SK-MEL-5, and Hbl) were cultured as described (Vachtenheim et al., 2001). B16-F1 mouse melanoma cells and H1299 cells (human lung cancer cell line) were maintained in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (InVitrogen, Carlsbad, CA), antibiotics, and L-glutamine. For transient transfections, cells were seeded in 24-well plates a day prior to transfection. All cell lines were transiently transfected, at 80% confluency, by mixing the plasmids (see Figure legends) with Lipofectamine 2000 (InVitrogen, Carlsbad, CA) in Opti-Mem medium, placing on cells for 4 to 6 h, and harvesting 48 h later. To normalize luciferase activities, 50 ng of pCMV-β (for cell lines MeWo, B16-F1, Hbl, and H1299) were co-transfected followed by estimation of β-galactosidase activity. For lines SK-MEL-2 and SK-MEL-5, 5 ng of the expression plasmid encoding Renilla luciferase were co-transfected and dual luciferase activity was determined as recommended by the supplier (Promega). The H1299 cell line was transfected by the calcium phosphate method in 12-well plates.

Immunoblotting

For detection of the expressed E1A proteins, the cell extracts used for reporter assays were immunoblotted directly. The amount of each of the extract was normalized for transfection efficiency, electrophoresed in a 10% SDS-PAGE gel, transferred onto the Immobilon-P membrane (Millipore, Bedford, MA), and probed with anti-E1A M73 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using standard procedures, utilizing the enhanced chemiluminescence detection system (Amersham Biosciences AB, Uppsala, Sweden).

Results

MITF promoter activity in mouse and human melanoma cell lines

Comparison of the mouse and human promoter sequences indicates that the regulatory elements for binding of transcription factors Sox10, Pax3, LEF-1 and CREB are entirely conserved between both species (Fig. 1A). As these transcription factors are thought to mediate the tissue-specific transcription in melanocytes, we first assessed the activity of the 5'-truncated promoter in mouse and human melanoma cells as compared to the human lung cancer cell line. As expected, a substantial decrease of activity occurred only after a more progressive truncation was introduced (down to nt -119 in MeWo human melanoma cells or to nt -382 in B16 mouse melanoma cells, Fig. 1B). No decrease in promoter activity was seen in the H1299 lung cancer cell line, even with the promoter containing only the TATA box (construct -119/+103), confirming that the regulatory elements, which are clustered within the proximal region and govern the MITF expression in melanocytes, do not further contribute to the activity mediated by a minimal promoter in non-melanocytic cells. Of note, in B16-F1 melanoma cells, some loss of activity was observable even with the promoter truncated more upstream (at nt -680 and -382), suggesting positive reg-

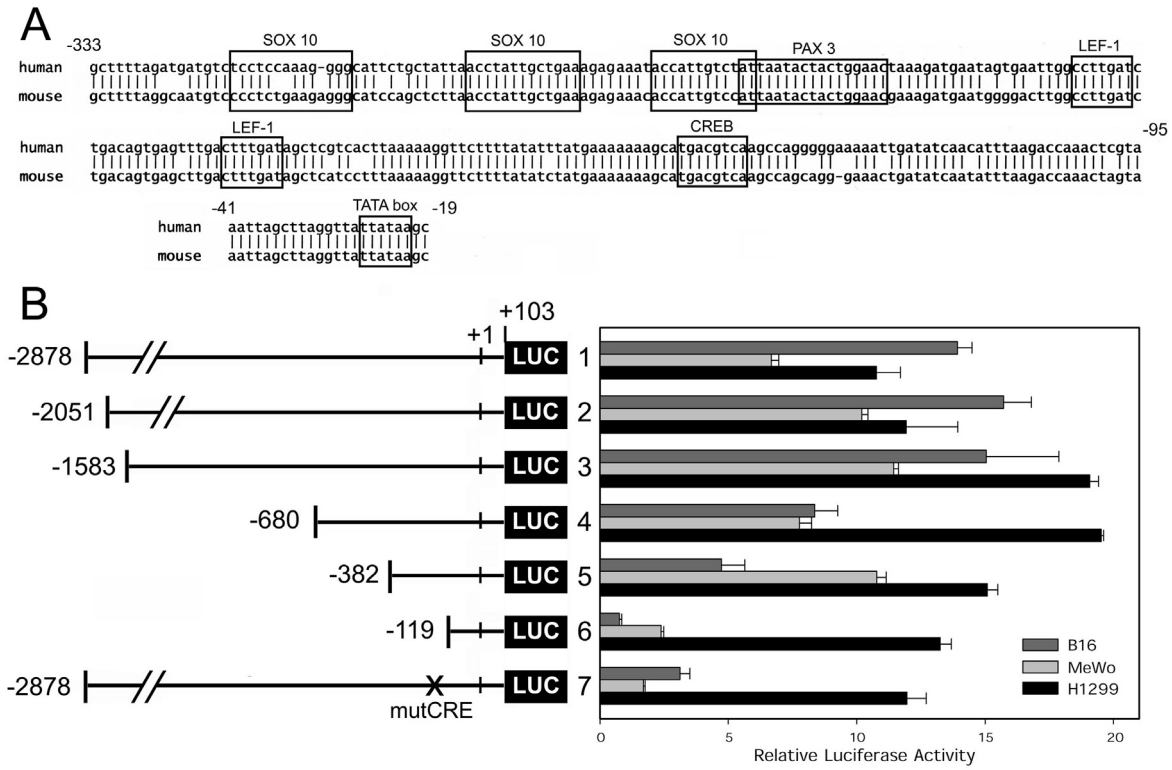

Fig. 1. Comparison of the human and mouse proximal promoter sequence and functional analysis of human MITF-M promoter constructs in various cell lines. A: Regulatory elements in the human and mouse melanocyte-specific MITF promoter. The consensus sites for MITF-regulating transcription factors (in boxes) are indicated for the TATA box, CRE site, LEF-1 sites, Pax3 binding motif, and Sox10 sites. Nucleotide numbers relative to the transcription start are given for the human promoter. The mouse promoter sequence was recovered from the mouse genome database (http://www.ncbi.nlm.nih.gov/genome/guide/mouse). The human MITF promoter sequence is from Genebank (AB009608). B: Reporter activities of the truncated MITF promoter. The amount of 0.3 μg of each indicated promoter construct was co-transfected together with pCMV-β (50 ng) into MeWo, B16-F1, or H1299 cells. Normalized luciferase values are shown. Three independent transfections gave similar results and one experiment is presented. Values are means of duplicates + SE.

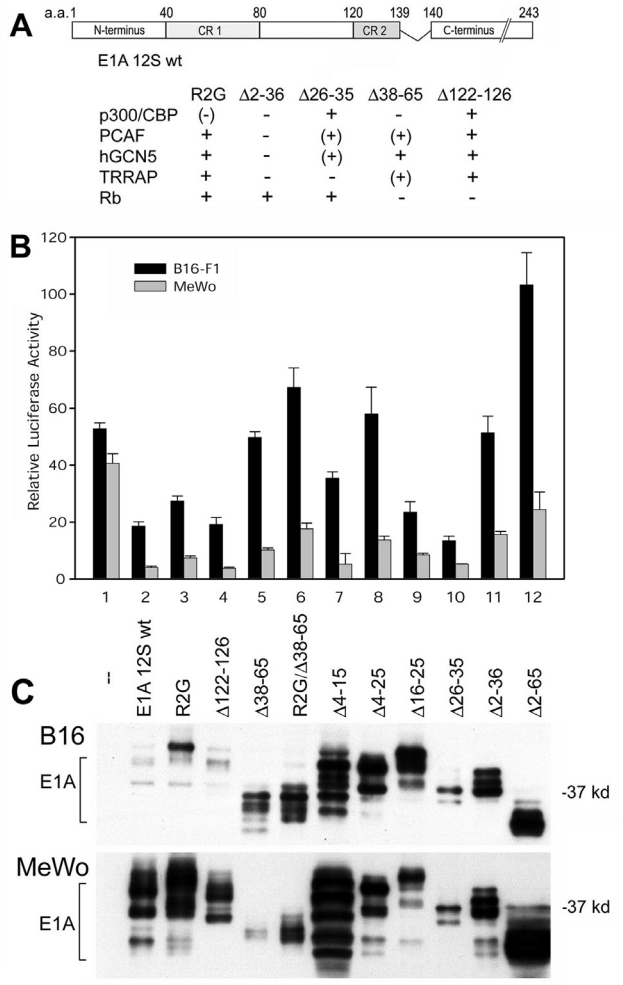

Fig. 2. E1A 12S and some of its mutants repress the MITF promoter. A: Schematic view of the E1A 12S protein molecule. Amino acids (a.a.) denote boundaries of the N-terminus and conserved regions 1 and 2, encoded by the first exon; the C-terminus is encoded by a second exon. Various mutations and deletions affect the binding of E1A to cellular proteins. +, binding; - loss of binding; (+) weak binding; (-) might interact very weakly. The data on E1A binding to cellular proteins are derived from earlier reports (Wang et al., 1993; Barbeau et al., 1994; Frisch and Mymryk 2002 and references herein). B: Transactivation of the MITF promoter is repressed by a panel of E1A mutant proteins in B16-F1 and MeWo cells. The amount of 0.3 μg of the MITF promoter-reporter was transiently transfected along with the pCMV-E1A expression vectors (0.5 μg) and pCMV-β (50 ng) to normalize transfection efficiency. Three independent transfections gave similar results and one experiment is presented. Values are means of duplicates + SE. C: Western blot of cell extracts. The membranes were probed with anti-E1A M73 monoclonal antibody. Upper panel, B16-F1 (mouse melanoma) cells; lower panel, MeWo (human melanoma) cells.

ulation through more distal sequences in mouse cells (Fig. 1B). No activity was noted with the promoterless plasmid in any cell line (data not shown). Our results with MeWo cells slightly differ from the original observation, where the truncation of upstream sequences (to nt -382) substantially decreased the promoter activity (Fuse et al., 1996), thus resembling the results with B16-F1 cells obtained here. Mutation of the CRE motif in the full-length promoter also abrogated the activity (Fig. 1B) and this effect differed among human melanoma cell lines (see below and Fig. 3). The effect of wt E1A 12S protein on the activity of the -2878/+103 promoter and shortened constructs (down to nt -382) was then tested in melanoma cells and it was found that E1A repressed all these constructs (data not shown). Therefore, the longest promoter construct was chosen for further analysis with E1A mutants.

E1A 12S repression of the MITF promoter requires the N-terminus and CR1 domain

Expression of the E1A 12S protein of the adenovirus type 5 results in dedifferentiation of mouse melanocytes accompanied by a phorbol ester-independent proliferation and loss of MITF expression (Dotto et al., 1989; Yavuzer et al., 1995; Halaban et al., 1996). To determine if E1A directly represses transcription from the melanocyte-specific MITF promoter, the -2878/+103 promoter-reporter construct was co-transfected together with wt E1A 12S or various E1A deletion mutants. The deletions introduced into E1A span the exon 1 sequences at the N-terminus, CR1 and CR2 domains. The N-terminal sequence (a.a. 2–36) is required for binding to a number of histone acetyltranferases (HATs) and transcription co-factors such as p300/CBP, PCAF, hGCN5, and TRRAP. The CR1 domain has been shown to contain motifs for the retinoblastoma (Rb) and p300/CBP bipartite binding sites as well as a site for binding to PCAF (Reid et al., 1998; Deleu et al., 2001; Gallimore and Turnell, 2001; Lang and Hearing, 2003), while CR2 is required for binding to the Rb protein family (Barbeau et al., 1994). Figure 2A shows a diagram of the E1A protein structure and mutation effects on E1A binding to cellular proteins. As depicted in Fig. 2B, wt E1A and mutants Δ122–126 and Δ26–35 were the most effective in repressing the MITF promoter in both MeWo and B16-F1 cells, while the mutants R2G and Δ16–25 were less effective. As the R2G mutation specifically disables the binding of E1A to the p300 co-activator *in vivo* (Wang et al., 1993) and deletion of a.a. 4–25 affects the N-terminal sequences also required for p300 association, the direct sequestration of p300 by E1A likely contributes to the repression. Other constructs appeared to be defective for repression. Further, the Δ122–126 construct had the lowest expression level in B16-F1 cells and repressed the reporter activity strongly, suggesting that the liberation of E1A from complexes with Rb family members allows for more

efficient binding to, and therefore sequestering of, the proteins associated with the amino-terminal region. A similar pattern of repression was observed with the same panel of E1A mutants in human melanoma cell line MeWo, albeit more pronounced inhibition was noted with all constructs relative to an empty vector (Fig. 2B). The expression of E1A proteins in MeWo cells was similar to that observed in B16-F1 cells with the wt E1A and Δ122–126 mutant being expressed at higher levels and Δ38–65 at a lower level in the MeWo cell line (Fig. 2C, lower panel). Together, the results indicate a strong repressive activity of E1A on the MITF-M promoter in melanoma cells, depending on the intact extreme N-terminal sequences and the CR1 domain, whereas the CR2 domain is dispensable for inhibition. As the deletion construct Δ122–126 does not bind the Rb protein family members Rb, p107, and p130 (Barbeau et al., 1994), and deletion of a.a. 26–35 removes the binding site for the transcription co-activator TRRAP, which is important for c-myc- and E1A-mediated transformation (McMahon et al., 1998; Deleu et al., 2001), the data strongly suggest that the Rb family proteins and TRRAP are not required for the promoter activity.

CRE-independent activity of the MITF promoter is suppressed by E1A in human but not mouse melanoma cells

Several signalling pathways converge on the MITF promoter (reviewed by Goding, 2000, and see above), each of which may contribute differently to the final promoter activity in various cell lines. The CRE motif mediates the cAMP/PKA signalling and has been shown to confer the promoter responsiveness upon stimulation with forskolin or α-MSH in melanoma cells, while it was insufficient for stimulation of the promoter activity in neuroblastoma cells (Huber et al., 2003). We tested whether abolishing this motif affects the activity of the promoter in unstimulated cells. The mutation of the CRE site resulted in activity that was reduced to levels similar to those obtained with the basal promoter (-119/+103) in MeWo cells. In B16-F1 cells, the activity of the mutCRE construct still remained about 4-fold higher than observed for the basal promoter (Fig. 1B). This would suggest that, in human melanoma cells, the whole promoter activity will depend entirely on the CRE site. To test this further, we compared the activity of the intact and CRE-mutated promoters in three other human melanoma cell lines, SK-MEL-2, SK-MEL-5, and Hbl. Abolishing the CRE site had a variable effect: in SK-MEL-2 cells, the promoter activity was reduced slightly, by 30 per cents; however, only about 10% of activity remained when SK-MEL-5 or Hbl melanoma cells were tested (Fig. 3A, compare the two vector lanes, of which the left is the activity with the intact promoter and the other with the CRE-mutated promoter). Thus, human melanoma cell lines may differ in the extent by which the cAMP/PKA/CREB pathway contributes to the total activity of the pharmacologically unstimulated MITF-M promoter. To test whether E1A inhibits the MITF promoter activity which is independent of cAMP signalling, we determined the responsiveness of the CRE motif-mutated promoter to the repressive action of wt E1A and mutants R2G Δ122–126, and Δ26–35. In all human melanoma cell lines tested (Hbl, SK-MEL-2, SK-MEL-5, MeWo), wt E1A and the three mutants retained the repressive activity to an extent similar to that observed with the intact promoter (Fig. 3A, grouped lanes of vector and E1A constructs, and Fig. 3B, right). Again, the R2G mutant, which binds p300 co-activator *in vivo* poorly, was less efficient in repression. In contrast, no repression could be achieved with wt E1A or mutants when the CRE-mutated promoter was tested in B16-F1 mouse melanoma cells, and constructs Δ122–126 and R2G even slightly promoted the activity (Fig. 3B, left panel). Therefore, mouse melanoma cells may differ from human cells in requirements of co-factors enhancing the CRE-independent promoter activity. The least affected, by abolishing the CRE site, was the MITF promoter activity in SK-MEL-2 melanoma cells (Fig. 3A), indicating that the β-catenin pathway together with transcription factors Pax3 and Sox10 contribute strongly to the promoter activity, which can be, nevertheless, still efficiently repressed by E1A. Expression of E1A in both B16-F1 and MeWo cells as well as in other cell lines was monitored by Western blotting of cell extracts and showed an expression level comparable for the tested E1A proteins (Fig. 3C, and data not shown).

Discussion

Aside from the function of an oncogene capable of immortalizing primary rodent cells, E1A has a strong tumour-suppressive activity in many types of human cancer cells. The powerful ability of E1A to repress transcription of a large number of genes underlies both these opposite effects. For instance, E1A can repress the *HER-2/neu* oncogene and thus acts as a tumour suppressor in breast cancer cells overexpressing neu, or downregulates expression of the extracellular matrix-degrading protease, resulting in a reduced propensity to metastasize (Frisch et al., 1990; Yan et al., 1991). Other antitumour activities of E1A include the conversion of some cultured cancer cells into epithelial, non-malignant phenotype, and the induction of apoptosis (reviewed by Frisch and Mymryk, 2002). The ability of E1A to repress transcription of a large number of genes is enabled by its affinity to transcriptional co-activators such as p300 and CBP. E1A binds strongly these two co-activators, both of which facilitate transcription of a number of transcription factors, and sequesters them

from transcription initiation complexes. Here we have observed that the E1A mutant R2G, which binds poorly to p300 in cells (Wang et al., 1993), nevertheless revealed only a partial reduction of repressive activity on the MITF promoter (Fig. 2). This indicates that sequestration of p300/CBP by wt E1A, mutant Δ122–126 and mutant Δ26–35 (all of which bind p300) can only partially explain their inhibitory activity. Both p300 and CBP are co-activators for CREB (reviewed by Goodman and Smolik, 2000), and have also been shown to promote the Wnt/β-catenin pathway (Hecht et al., 2000; Takemaru and Moon, 2000). The results suggesting that other yet-to-be defined co-factors, besides p300/CBP, participate in activation of the MITF promoter are supported by our observation that the co-expression of exogenous p300 could not restore the promoter activity repressed by wt E1A (data not shown). In addition, a short sequence within E1A CR1 of Ad12 has been implicated in binding to CBP (O'Connor et al., 1999). This region corresponds to the FPDSVML (a.a. 66 to 72) in E1A of adenovirus type 5. In accord with the present results, we did not observe any relieving effect on E1A-mediated repression when this sequence was mutated (data not shown).

Although the E1A-immortalized melanocytes are MITF-negative, they proliferate rapidly, apparently due to the immortalizing function of E1A, which thus overcomes the MITF deficiency. Such an MITF-independent growth has also been reported for two human melanoma cell lines in culture (Vachtenheim et al., 2001). In this case, MITF target genes became non-responsive to MITF expressed ectopically. Because MITF transcription is regulated by several pathways, blocking its expression by inhibiting one specific pathway is not feasible. We show here that the MITF promoter activity can be suppressed by expressing the E1A 12S protein in several human melanoma cell lines, independently of whether or not is the promoter mutated at the CRE site. In contrast, when tested in mouse melanoma cells, the CRE site-mutated promoter was

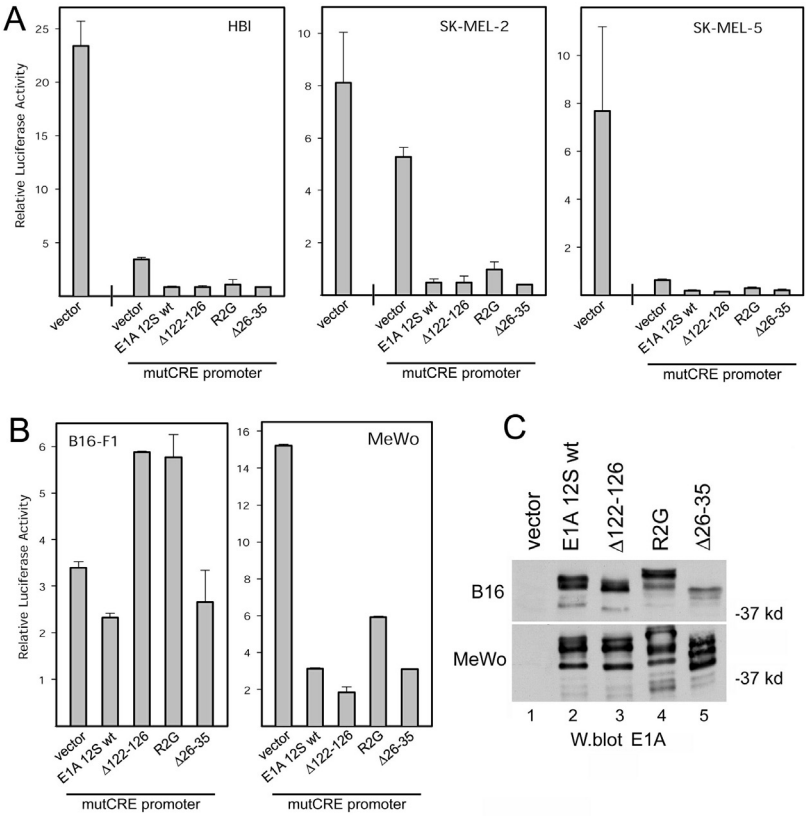

Fig. 3. E1A represses the CRE motif-mutated MITF promoter in human melanoma cell lines.
A: Relative reporter activities for the MITF promoter (left, separated vector lanes) and CRE site-mutated promoter (vector lanes, first in grouped lanes). Inhibition of mutated promoter-reporter activity by E1As is shown for Hbl, SK-MEL-2, and SK-MEL-5 human melanoma cells. B: Inhibition of CRE site-mutated promoter-reporter activity by E1As in B16-F1 and MeWo cells. In A and B, three independent transfections were performed with similar results and one experiment is presented. Luciferase activity was normalized by co-transfecting pCMV-β or Renilla luciferase expression plasmid and estimation of enzyme activities as described in Material and Methods. Values are means of duplicates + SE. C: Western blot of expressed E1A proteins in B16-F1 and MeWo cells. The expression was similar in other cell lines tested (data not shown).

refractory to inhibition by E1A. Thus, the cAMP-independent expression of MITF may be co-regulated differently in human and mouse melanoma cells. Taken collectively, our data show that it is possible to repress the activity of the melanocyte-specific promoter with E1A mutants which are known to be transformation-defective. Future studies are warranted to determine if the E1A harbouring such mutations (Δ122–126 and Δ26–35) could be used to block the expression of endogenous MITF and decrease the survival of human melanoma cells. The E1A has been demonstrated to be a potential therapeutic agent for melanoma (Dickopp et al., 2000; Deng et al., 2002). However, it has not been studied whether this E1A's activity requires downregulation of MITF in melanoma cells where the general antitumour activity and MITF-repressive activity of E1A might be additive. Independent studies have shown a possibility that MITF may be a crucial survival factor for melanoma cells (McGill et al., 2002; Widlund and Fisher, 2003). Therefore, the E1A mutants that retain the capacity to repress the MITF promoter might be more effective in eliminating melanoma cells. If MITF could be suppressed by overexpressing E1A in malignant melanocytes, and provided the E1A's immortalizing activity is abolished, E1A might become a powerfull tool for targeting MITF in antimelanoma therapy.

Acknowledgement

We thank Dr. Č. Vlček (Institute of Molecular Genetics, Prague) for plasmid sequencing.

References

Barbeau, D., Charbonneau, R., Whalen, S. G., Bayley, S. T., Branton, P. E. (1994) Functional interactions within adenovirus E1A protein complexes. *Oncogene* **9,** 359-373.

Bentley, N. J., Eisen, T., Goding, C. R. (1994) Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol. Cell Biol.* **14,** 7996-8006.

Bertolotto, C., Busca, R., Abbe, P., Bille, K., Aberdam, E., Ortonne, J. P., Ballotti, R. (1998) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol. Cell Biol.* **18,** 694-702.

Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Caignec, C. L., Wegner, M., Goossens, M. (2000) Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. *Hum. Mol. Genet.* **9,** 1907-1917.

Deleu, L., Shellard, S., Alevizopoulos, K., Amati, B., Land, H. (2001) Recruitment of TRRAP required for oncogenic transformation by E1A. *Oncogene* **20,** 8270-8275.

Deng, J., Kloosterbooer, F., Xia, W., Hung, M. C. (2002) The NH(2)-terminal and conserved region 2 domains of adenovirus E1A mediate two distinct mechanisms of tumor suppression. *Cancer Res.* **62,** 346-350.

Dickopp, A., Esche, H., Swart, G., Seeber, S., Kirch, H. C., Opalka, B. (2000) Transformation-defective adenovirus 5 E1A mutants exhibit antioncogenic properties in human BLM melanoma cells. *Cancer Gene Ther.* **7,** 1043-1050.

Dotto, G. P., Moellmann, G., Ghosh, S., Edwards, M., Halaban, R. (1989) Transformation of murine melanocytes by basic fibroblast growth factor cDNA and oncogenes and selective suppression of the transformed phenotype in a reconstituted cutaneous environment. *J. Cell Biol.* **109,** 3115-3128.

Du, J., Miller, A. J., Widlund, H. R., Horstmann, M. A., Ramaswamy, S., Fisher, D. E. (2003) MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am. J. Pathol.* **163,** 333-343.

Frisch, S. M., Mymryk, J. S. (2002) Adenovirus-5 E1A: paradox and paradigm. *Nat. Rev. Mol. Cell Biol.* **3,** 441-452.

Frisch, S. M., Reich, R., Collier, I. E., Genrich, L. T., Martin, G., Goldberg, G. I. (1990) Adenovirus E1A represses protease gene expression and inhibits metastasis of human tumor cells. *Oncogene* **5,** 75-83.

Fuse, N., Yasumoto, K., Suzuki, H., Takahashi, K., Shibahara, S. (1996) Identification of a melanocyte-type promoter of the microphthalmia-associated transcription factor gene. *Biochem. Biophys. Res. Commun.* **219,** 702-707.

Gallimore, P. H., Turnell, A. S. (2001) Adenovirus E1A: remodelling the host cell, a life or death experience. *Oncogene* **20,** 7824-7835.

Goding, C. R. (2000) Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev.* **14,** 1712-1728.

Goodman, R. H., Smolik, S. (2000) CBP/p300 in cell growth, transformation, and development. *Genes Dev.* **14,** 1553-1577.

Halaban, R., Bohm, M., Dotto, P., Moellmann, G., Cheng, E., Zhang, Y. (1996) Growth regulatory proteins that repress differentiation markers in melanocytes also downregulate the transcription factor microphthalmia. *J. Invest. Dermatol.* **106,** 1266-1272.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., Kemler, R. (2000) The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* **19,** 1839-1850.

Huber, W. E., Price, E. R., Widlund, H. R., Du, J., Davis, I. J., Wegner, M., Fisher, D. E. (2003) A tissue-restricted cAMP transcriptional response: SOX10 modulates alpha-melanocyte-stimulating hormone-triggered expression of microphthalmia-associated transcription factor in melanocytes. *J. Biol. Chem.* **278,** 45224-45230.

King, R., Weilbaecher, K. N., McGill, G., Cooley, E., Mihm, M., Fisher, D. E. (1999) Microphthalmia transcription factor. A sensitive and specific melanocyte marker for melanoma diagnosis. *Am. J. Pathol.* **155,** 731-738.

Lang, S. E., Hearing, P. (2003) The adenovirus E1A oncoprotein recruits the cellular TRRAP/GCN5 histone acetyltransferase complex. *Oncogene* **22,** 2836-2841.

Lee, M., Goodall, J., Verastegui, C., Ballotti, R., Goding, C. R. (2000) Direct regulation of the Microphthalmia promoter by Sox10 links Waardenburg-Shah syndrome (WS4)-associated hypopigmentation and deafness to WS2. *J. Biol. Chem.* **275,** 37978-37983.

McGill, G. G., Horstmann, M., Widlund, H. R., Du, J., Motyckova, G., Nishimura, E. K., Lin, Y. L., Ramaswamy, S., Avery, W., Ding, H. F., Jordan, S. A., Jackson, I. J., Korsmeyer, S. J., Golub, T. R., Fisher, D. E. (2002) Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell* **109,** 707-718.

McMahon, S. B., Van Buskirk, H. A., Dugan, K. A., Copeland, T. D., Cole, M. D. (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* **94,** 363-374.

Miller, A. J., Du, J., Rowan, S., Hershey, C. L., Widlund, H. R., Fisher, D. E. (2004) Transcriptional regulation of the melanoma prognostic marker melastatin (TRPM1) by MITF in melanocytes and melanoma. *Cancer Res.* **64,** 509-516.

O'Connor, M. J., Zimmermann, H., Nielsen, S., Bernard, H. U., Kouzarides, T. (1999) Characterization of an E1A-CBP interaction defines a novel transcriptional adapter motif (TRAM) in CBP/p300. *J. Virol.* **73**, 3574-3581.

Opdecamp, K., Nakayama, A., Nguyen, M. T., Hodgkinson, C. A., Pavan, W. J., Arnheiter, H. (1997) Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. *Development* **124,** 2377-2386.

Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H., Pavan, W. J. (2000) Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. *Hum. Genet.* **107,** 1-6.

Reid, J. L., Bannister, A. J., Zegerman, P., Martinez-Balbas, M. A., Kouzarides, T. (1998) E1A directly binds and regulates the P/CAF acetyltransferase. *EMBO J.* **17,** 4469-4477.

Saito, H., Yasumoto, K., Takeda, K., Takahashi, K., Fukuzaki, A., Orikasa, S., Shibahara, S. (2002) Melanocyte-specific microphthalmia-associated transcription factor isoform activates its own gene promoter through physical interaction with lymphoid-enhancing factor 1. *J. Biol. Chem.* **277,** 28787-28794.

Shibahara, S., Takeda, K., Yasumoto, K., Udono, T., Watanabe, K., Saito, H., Takahashi, K. (2001) Microphthalmia-associated transcription factor (MITF): multiplicity in structure, function, and regulation. *J. Investig. Dermatol. Symp. Proc.* **6,** 99-104.

Takeda, K., Yasumoto, K., Takada, R., Takada, S., Watanabe, K., Udono, T., Saito, H., Takahashi, K., Shibahara, S. (2000) Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. *J. Biol. Chem.* **275,** 14013-14016.

Takemaru, K. I., Moon, R. T. (2000) The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J. Cell Biol.* **149,** 249-254.

Udono, T., Yasumoto, K., Takeda, K., Amae, S., Watanabe, K., Saito, H., Fuse, N., Tachibana, M., Takahashi, K., Tamai, M., Shibahara, S. (2000) Structural organization of the human microphthalmia-associated transcription factor gene containing four alternative promoters. *Biochim. Biophys. Acta* **1491,** 205-219.

Vachtenheim, J., Novotna, H., Ghanem, G. (2001) Transcriptional repression of the microphthalmia gene in melanoma cells correlates with the unresponsiveness of target genes to ectopic microphthalmia-associated transcription factor. *J. Invest. Dermatol.* **117,** 1505-1511.

Verastegui, C., Bille, K., Ortonne, J. P., Ballotti, R. (2000) Regulation of the microphthalmia-associated transcription factor gene by the Waardenburg syndrome type 4 gene, SOX10. *J. Biol. Chem.* **275,** 30757-30760.

Voigtlander, C., Rand, A., Liu, S. L., Wilson, T. J., Pittelkow, M. R., Getz, M. J., Kelm, R. J., Jr. (2002) Suppression of tissue factor expression, cofactor activity, and metastatic potential of murine melanoma cells by the N-terminal domain of adenovirus E1A 12S protein. *J. Cell Biochem.* **85,** 54-71.

Wang, H. G., Rikitake, Y., Carter, M. C., Yaciuk, P., Abraham, S. E., Zerler, B., Moran, E. (1993) Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and to the control of cell growth. *J. Virol.* **67,** 476-488.

Watanabe, A., Takeda, K., Ploplis, B., Tachibana, M. (1998) Epistatic relationship between Waardenburg syndrome genes MITF and PAX3. *Nat. Genet.* **18,** 283-286.

Widlund, H. R., Horstmann, M. A., Price, E. R., Cui, J., Lessnick, S. L., Wu, M., He, X., Fisher, D. E. (2002) Beta-catenin-induced melanoma growth requires the downstream target Microphthalmia-associated transcription factor. *J. Cell Biol.* **158,** 1079-1087.

Widlund, H. R., Fisher, D. E. (2003) Microphthalamia-associated transcription factor: a critical regulator of pigment cell development and survival. *Oncogene* **22,** 3035-3041.

Yan, D. H., Chang, L. S., Hung, M. C. (1991) Repressed expression of the HER-2/c-erbB-2 proto-oncogene by the adenovirus E1a gene products. *Oncogene* **6,** 343-345.

Yasumoto, K., Takeda, K., Saito, H., Watanabe, K., Takahashi, K., Shibahara, S. (2002) Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling. *EMBO J.* **21,** 2703-2714.

Yavuzer, U., Keenan, E., Lowings, P., Vachtenheim, J., Currie, G., Goding, C. R. (1995) The Microphthalmia gene product interacts with the retinoblastoma protein in vitro and is a target for deregulation of melanocyte-specific transcription. *Oncogene* **10,** 123-134.