# Characterization of a Transcription Factor Binding Site, Specifically Activating MIA Transcription in Melanoma

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**We have previously isolated the protein MIA, which is secreted from melanoma cells, and identified highly restricted expression patterns in melanocytic tumors. Preliminary studies of the human MIA gene provided evidence that the promoter is specifically activated in melanoma cells but is silent in nonmelanocytic cells and benign melanocytes. In this study we aimed to identify cis-regulatory promoter elements that mediate promoter activation during malignant transformation of melanocytes. We therefore subcloned 1.4 kb of the murine MIA promoter and a series of 5′ deletion constructs into a luciferase reporter plasmid and identified the most active cis-regulatory element between nucleic acids –230 and –130. Cloning oligomeric fragments of this promoter region in front of a minimal TK promoter revealed a 30 bp enhancer element mediating expression of the reporter gene in melanoma cells but not in melanocytes or nonmelanocytic cells. Gel mobility shift assays and southwestern blots led to the identification of specific DNA-protein complexes in melanoma cells. Fine mutational analysis of the cis-regulatory promoter element showed that two critical nucleic acid residues are essential for both transcriptional activity and formation of the band shift complexes. By an initial small-scale affinity purification we were able to isolate a protein approximately 32 kDa in size from melanoma cells, which we refer to as MATF (''melanoma-associated transcription factor''). Our study identified for the first time MATF, a transcription factor that is upregulated or activated during malignant transformation of melanocytes.** ***Key words: cell-specific expression/melanoma/MIA/transcription factor.***

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The study of molecular mechanisms of malignant transformation has identified an accumulation of gene mutations leading to defects in tumor suppressor genes as one important principle of carcinogenesis. In parallel, the progression of transformed cell clones to a systemic tumor disease by invasion and formation of distant organ metastases frequently involves reactivation or *de novo* expression of intact endogenous genes. It is therefore of great interest to identify transcriptional mechanisms involved in tumor-specific gene expression.

In melanomas several promoter regions of genes that are strongly and/or specifically expressed in melanocytic tumors have been investigated. In the case of the tyrosinase promoter several cis-regulatory promoter elements were identified. The ''tyrosinase distal element'', TDE (Shibata *et al*, 1992; Yasumoto *et al*, 1994), involves DNA-sequence-specific binding of the transcription factor MITF (microphthalmia-associated transcription factor) (Hodgkinson *et al*, 1993; Aberdam *et al*, 1998; Fang and Setaluri, 1999). Subsequent investigations described at least three further promoter regions mediating specific expression of tyrosinase in melanocytic cells (Lowings *et al*, 1992; Ganss *et al*, 1994a, b). Specific promoter regions have also been characterized for other proteins including TRP-1 (Yavuzer and Goding, 1994; Carreira *et al*, 1998), TRP-2 (Yokoyama *et al*, 1994), and melanotransferrin (Angel *et al*, 1987). None of these studies, however, has identified a transcriptional activator mediating overexpression or *de novo* expression in malignant but not benign cells of melanocytic origin.

We have previously isolated a small soluble protein, MIA, which is secreted from melanoma cells, and have identified highly restricted expression patterns in melanocytic tumors (Blesch *et al*, 1994; Bosserhoff *et al*, 1996, 1997a). Expression levels of MIA paralleled closely the progression of melanocytic tumors. In non-neoplastic tissues MIA expression was detected foremost in cartilage (Bosserhoff *et al*, 1997b). Preliminary studies of the human MIA gene provided evidence that the promoter is specifically activated in melanoma cells but silent in nonmelanocytic cells and benign melanocytes (Bosserhoff *et al*, 1996). In this study we therefore aimed to identify cis-regulatory promoter elements that mediate promoter activation during malignant transformation of melanocytes.

## MATERIALS AND METHODS

**Cell lines and culture conditions** Human primary melanocytes derived from normal skin were cultivated in melanocyte medium MGM-3 (Gibco, Eggenstein, Germany) under a humified atmosphere of 8% $CO_2$ at 37°C. Cells were used in passages 6–10 and not later than 3 d after trypsinization. Cells were detached for subcultivation or assay with 0.1% trypsin, 0.04% ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline.

Human and murine melanoma cell lines, Mel Im (Jacob *et al*, 1995; 1998) and B16 (ATCC CRL6322), were maintained in Dulbecco's

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Abbreviations: HCR, highly conserved region; MATF, melanoma-associated transcription factor.

modified Eagle's medium supplemented with penicillin (400 U per ml), streptomycin (50 μg per ml), L-glutamine (300 μg per ml), and 10% fetal bovine serum (Sigma, Deisenhofen, Germany) and split 1:5 every 3 d. The murine fibroblast cell line NIH 3T3 (ACC 59) and the cell lines COS7 (ACC 60) and HeLa (ACC 57) were kept under the same conditions as the melanoma cell lines. The melanocytic cell line MelanA was kindly provided by Dr. Ian Hart (Hammersmith Hospital, London, U.K.) and maintained as described previously (Jacob *et al*, 1998).

**Promoter constructs, transient transfections, luciferase assays, and site-directed mutagenesis** The 5′ flanking region of the murine MIA gene spanning nucleic acid residues –1400 to –1 with respect to the ATG protein start codon was polymerase chain reaction (PCR) amplified and inserted into the promoterless luciferase plasmid pGL3-basic (Promega, Madison, WI). Also 5′ deletion constructs spanning promoter fragments from –400 to –1, –300 to –1, –230 to –1, and –130 to –1, respectively, were generated by PCR amplification. Point mutations were excluded by resequencing all promoter fragments. Shorter promoter constructs were generated by ligating double-stranded oligomeres containing the respective sequence (**Fig 2*a***) into the BglII restriction site of the plasmid pGL3 promoter (Promega) carrying a 200 bp fragment of the SV40 promoter.

A total of $2 \times 10^5$ cells were seeded into each well of a six well plate and transiently transfected with 1 μg plasmid DNA using the lipofectamine plus method (Gibco) according to the manufacturer's instructions. The cells were lyzed 24 h after transfection and the luciferase activity in the lysate was quantified by a luminometric assay (Promega). Transfection efficiency was normalized according to renilla luciferase activity by cotransfecting 0.5 μg of the plasmid pRL-TK (Promega). All transfections were repeated at least four times.

Mutations in the 1.4 kb murine MIA promoter region were introduced using a site-directed mutagenesis kit (Clontech, Heidelberg, Germany) following the manufacturer's instructions precisely.

**Gel mobility shift assays and southwestern blots** The double-stranded oligomeric binding sites oligo c (5′-GGC TCG AGT AGG CAT TTT CTT TGG CCC ATA-3′) and HCR (5′-GAG TAG GCA TTT TCT-3′) were phospholabeled and used for gel mobility shift assays. Oligo c was also used to probe southwestern blots. Nuclear extracts were prepared from primary melanocytes, Mel Im, B16, NIH 3T3, HeLa, and MelanA cells, and gel shifts were performed as described previously (Terranova *et al*, 1986). Competition experiments were performed using a 50-fold excess of the unlabeled binding site or the mutated binding site mut 3 (5′-CTC GAG TAG CCT TTT TCT TTG-3′).

For southwestern blots nuclear extracts of the cell lines Mel Im, B16, MelanA, NIH 3T3, HeLa, and primary melanocytes were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted onto PVDF membranes. Unspecific binding was blocked by incubation in SW buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 6 mM $MgCl_2$) supplemented with 1% blocking reagent (Roche, Mannheim, Germany) for 30 min. Then the blot was incubated with $1 \times 10^7$ cpm per ml of the phospholabeled oligomeric binding site oligo c in SW buffer at 4°C for 16 h. After washing three times in SW buffer at 4°C for 15 min each, the signals were detected by autoradiography.

**Protein purification by affinity chromatography** To isolate transcription factors binding to the specific promoter region a DNA-binding protein purification kit (Roche) was used as suggested by the manufacturer. The multimerized oligo HCR served as the binding site. Concatamers were generated by self-primed PCR (94°C 45 s, 65°C 45 s, 72°C 2 min for 10 cycles) using the oligonucleotide 5′-GAG TAG GCA TTT GAG TAG GCA TTT GAG TAG GCA TTT-3′. Binding of the nuclear proteins to the concatamer was performed with 100 μg of the nuclear extract in binding buffer (5 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 5 mM dithiothreitol, 10% glycerol) at 4°C for 60 min. Unspecific binding was repressed by competition with the mutated binding site (mut 3). Bound proteins were eluted by adding binding buffer plus 2 M KCl. Isolated proteins were separated on a 12% SDS-PAGE gel and silver-stained.

## RESULTS

**Characterization of a cis-regulatory element mediating MIA expression in melanoma cells** A previous preliminary promoter analysis testing 5′ deletion constructs of the human MIA gene promoter had indicated that the region between nucleic acid residues –230 and –170 is indispensible for promoter activity in melanoma cells (Bosserhoff *et al*, 1997b). To investigate whether potential cis-regulatory elements in this region are conserved between different species we cloned the murine MIA gene promoter into the promoterless luciferase reporter plasmid pGL3-basic and tested a similar series of 5′ deletion constructs in murine melanoma cells and melanocytes (B16 and MelanA). Our data summarized in **Fig 1** revealed that deletion of promoter sequences from –1400 to –230 diminished promoter activity in melanoma cells by approximately 35%. Further deletion of sequences between –230 and –130 led to complete loss of reporter gene expression in melanoma cells. None of the reporter plasmids including the full length –1400 bp construct supported luciferase expression in MelanA cells. From these data we concluded that a species-conserved promoter element between residues –230 and –130 supported MIA promoter activity specifically in malignant melanocytic cells.

To further fine map the MIA promoter we subcloned a series of synthetic oligomeres into the plasmid pGL3 promoter. As pGL3 promoter contains a basal SV40 promoter including the transcription initiation element these constructs allowed us to identify transposable enhancer elements of the MIA promoter. Results from transient transfection of a first series of promoter constructs indicated that a fragment spanning residues –268 to –181 but not fragments from –321 to –268 and –181 to –136 supported enhanced activity of the pGL3 promoter reporter plasmid (data not shown). We therefore further dissected the region between residues –268 and –181 and tested pGL3 promoter constructs carrying the oligonucleotides a, b, and c as displayed in **Fig 2(*a*)**. In contrast to

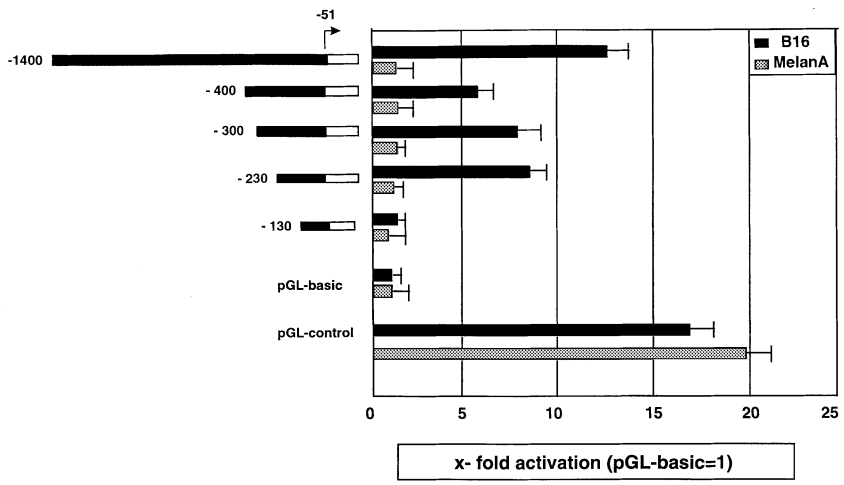


**Figure 1. Deletion analysis of the murine MIA promoter in melanoma cells and fibroblasts.** The 1.4 kb promoter fragment of the murine MIA promoter and 5′ deleted fragments generated by PCR were cloned into a pGL3-basic vector. The promoter fragments ranging from residues –1400 to –1 are indicated at the left. Luciferase activity resulting from transient transfections into B16 melanoma cells (*black bars*) and MelanA cells (*shadowed bars*) are indicated at the right. Basal activity resulting from the promoterless pGL3-basic vector was set arbitrarily as 1. Values indicate the average of at least three independent experiments.

oligonucleotides a and b, which did not significantly alter luciferase expression from the pGL3 promoter plasmid, oligo c harbored a strong transposable cis-regulatory element that enhanced transcription from the reporter approximately 10-fold in both human and murine melanoma cells (**Fig 2*b***). Trimerization of oligo c led to an even stronger activation in melanoma cells. Importantly, the element was insufficient to enhance promoter activity in the nonmalignant melanocytic cell line MelanA, in benign melanocytes cultured from human foreskin, and in NIH 3T3 fibroblasts. These results indicated that the murine MIA promoter harbors a cis-regulatory element between residues –210 and –181 that activates transcription specifically in malignant but not in benign melanocytes.

**Gel mobility shift assays and southwestern blots** As most transcription factors bind to DNA in a sequence-specific manner we next aimed to visualize specific DNA-protein complexes and used oligo c to perform gel mobility shift assays. When we challenged oligo c with nuclear extracts from the melanoma cell line B16, we detected a complex pattern of at least seven band shifts, three of which failed to form with nuclear extracts from MelanA cells (marked by arrows in **Fig 3*a***). These data indicated the presence of one or several proteins in B16 that are absent or fail to form a band shift complex in MelanA. Consistently, using radiolabeled oligo c to probe southwestern blots visualized interaction with a protein specifically expressed in malignant melanocytic cells. As shown in **Fig 3(*b*)** we detected a protein of approximately 32 kDa in the nuclear extract of both human and murine melanoma cells (Mel Im, B16) but not in the nuclear extracts of benign cells including primary human foreskin melanocytes, MelanA cells, and NIH 3T3 fibroblasts, or in the malignant nonmelanocytic cell line HeLa.

**Definition of a binding site for a melanoma-associated transcription factor (MATF)** Comparison of the murine oligo c with the corresponding human promoter region (Bosserhoff *et al*, 1996) indicated significant sequence conservation. In the central highly conserved region (HCR; boxed in **Fig 4*a***) eight of ten nucleic acid residues are identical (80%), suggesting sequence conservation of a putative transcription factor binding site. Inspection of oligo c with the MatInspector V2.2 program (Quandt *et al*, 1995), however, did not reveal any consensus binding site conserved between the murine and human promoter. Also an NF-kB binding site present in the human promoter (Bosserhoff *et al*, 1996) was not conserved in the murine sequence, and consistently we failed to detect binding of NF-kB to oligo c (data not shown). We therefore tested the importance of the HCR element by introducing specific mutations via site-directed mutagenesis in the context of the full 1.4 kb murine MIA promoter. Data summarized in **Fig 4(*b*)** show that a large mutation of six central residues in the HCR element (mut 1) as well as exchanges of three or two central residues (mut 3, 4) led to complete loss of promoter activity in melanoma cell lines. Further, single point mutations of the central residues GGCA reduced promoter activity between 20% and 50%, respectively. These data provide evidence that conservation of the central

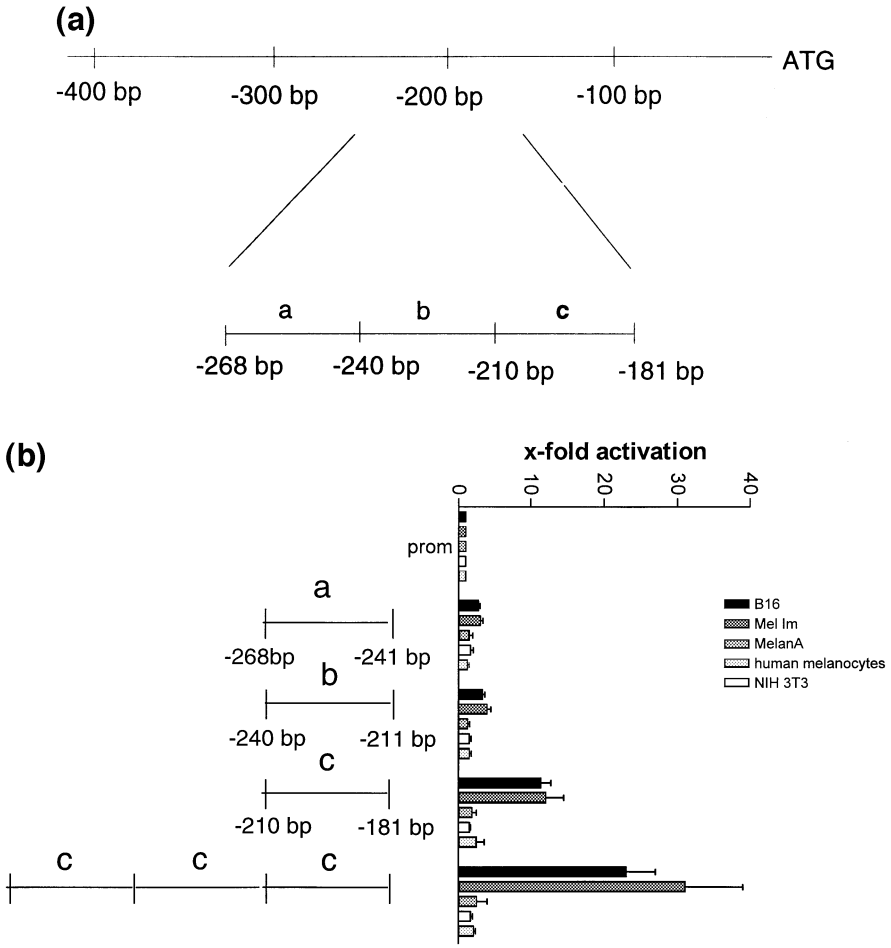


**Figure 2. Activity of MIA promoter fragments analyzed in pGL3 promoter.** Schematic illustration of the promoter fragments a, b, and c, which were synthesized as complementary oligonucleotides, hybridized, and subcloned into the vector pGL3 promoter. Luciferase activity supported by the short MIA promoter fragments in the plasmid pGL3 promoter after transient transfections into melanoma cell lines (Mel Im, B16), melanocytic cells (MelanA, primary melanocytes), and fibroblasts (NIH 3T3). Basal activity of the vector pGL3 promoter was set as 1.

nucleic acid residues in the HCR element is essential for MIA promoter activity both in human and murine melanoma cells.

Gel mobility shift assays revealed two distinct DNA-protein complexes when the highly conserved region (oligo HCR) was challenged with nuclear extracts of melanoma cells. The slower migrating band shift was detected also at very low intensity in nuclear extracts of MelanA cells but the faster shift was specifically observed in B16 melanoma cell extracts (**Fig 5**). Consistently, both band shifts were competed for by an excess of unlabeled oligo HCR, but using the competitor mut 3 failed to displace the faster migrating complex. From these data we concluded that the mutations in the sequence motif GGCA that led to inactivation of MIA promoter activity in melanoma cells resulted in disruption of the faster migrating band shift.

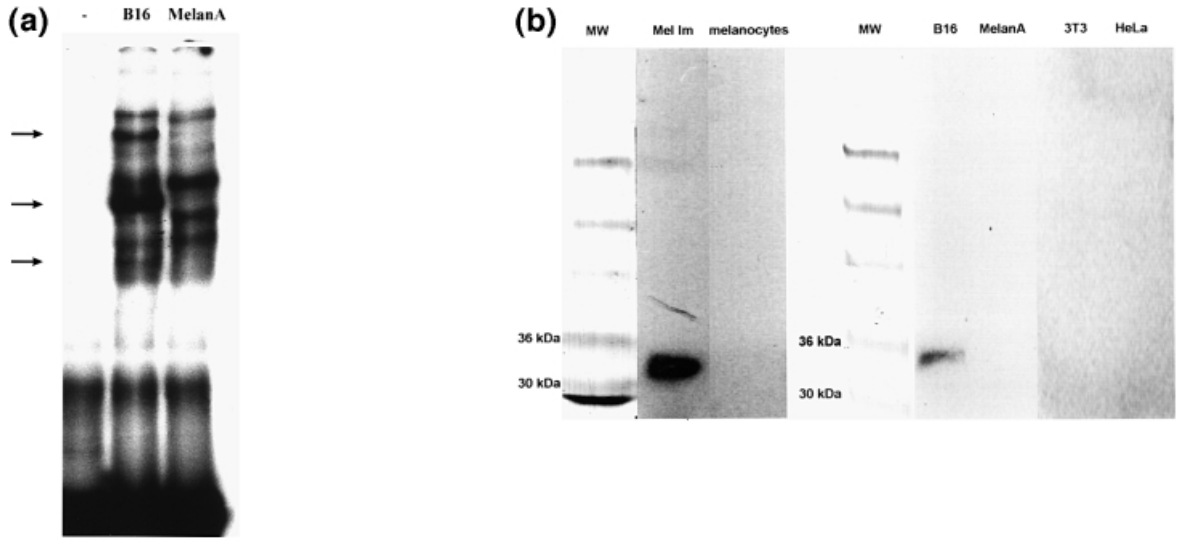


**Figure 3. Patterns of nuclear proteins interacting with oligo c.** Gel mobility shift analysis using oligo c of the murine MIA promoter. The phospholabeled binding site was mixed with albumin as a negative control (*lane* -) and B16 nuclear extract (*lane B16*) or MelanA nuclear extract (*lane MelanA*). Band shifts observed exclusively in B16 nuclear extract are marked by arrows. Southwestern blot of nuclear proteins extracted from melanoma cell lines (Mel Im, B16), melanocytic cells (MelanA, primary human melanocytes), and nonmelanocytic cells (NIH 3T3, HeLa) using the phospholabeled oligo c binding site as a probe. A single band of approximately 32 kDa was visualized.

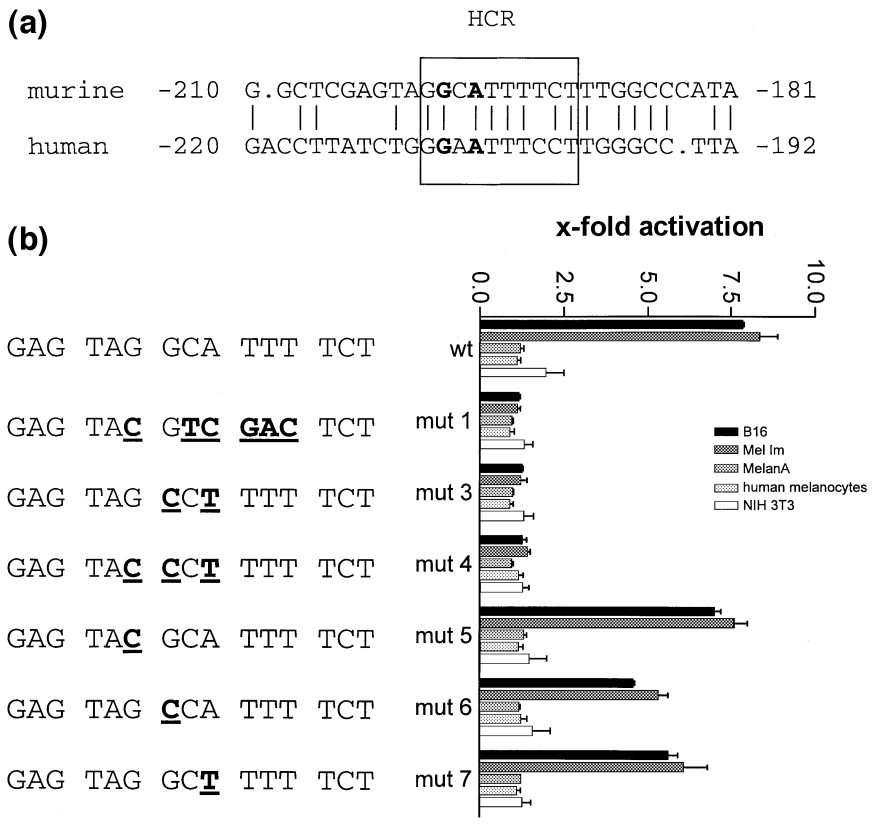


**Figure 4. Sequence comparison of the murine and the human oligo c MIA promoter region and functional analysis of the HCR.** *A*: Identical bases are indicated in the murine and the human oligo c region. The HCR is surrounded by a box. The two residues that are essential for promoter activity are shown in boldface. *B*: Schematic illustration of changes in the HCR inserted by site-directed mutagenesis into the murine 1400 bp MIA promoter (wt) and functional analysis of the mutated promoter constructs by transient transfections into melanoma cells (Mel Im, B16), melanocytic cells (MelanA, primary human melanocytes), and fibroblasts (NIH 3T3). The basal luciferase activity of the parental vector pGL3 promoter was set as 1.

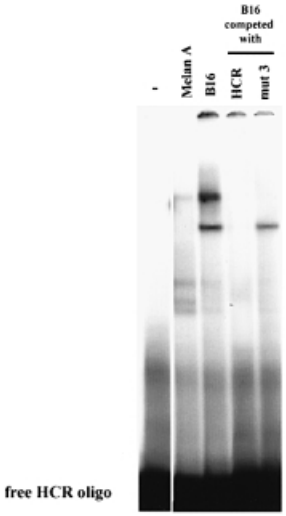


**Figure 5. Gel mobility shift analysis of the HCR.** The phospholabeled HCR oligomere was mixed with albumin (*lane 1*), MelanA nuclear extract (*lane 2*), and B16 nuclear extract (*lanes 3*, *4*, and *5*). Competition experiments were performed by adding 50-fold excess of the wildtype binding site (*lane 4*, HCR) or the mutated HCR binding site mut 3 as displayed in **Fig 4**(***B***) (*lane 5*, mut 3).

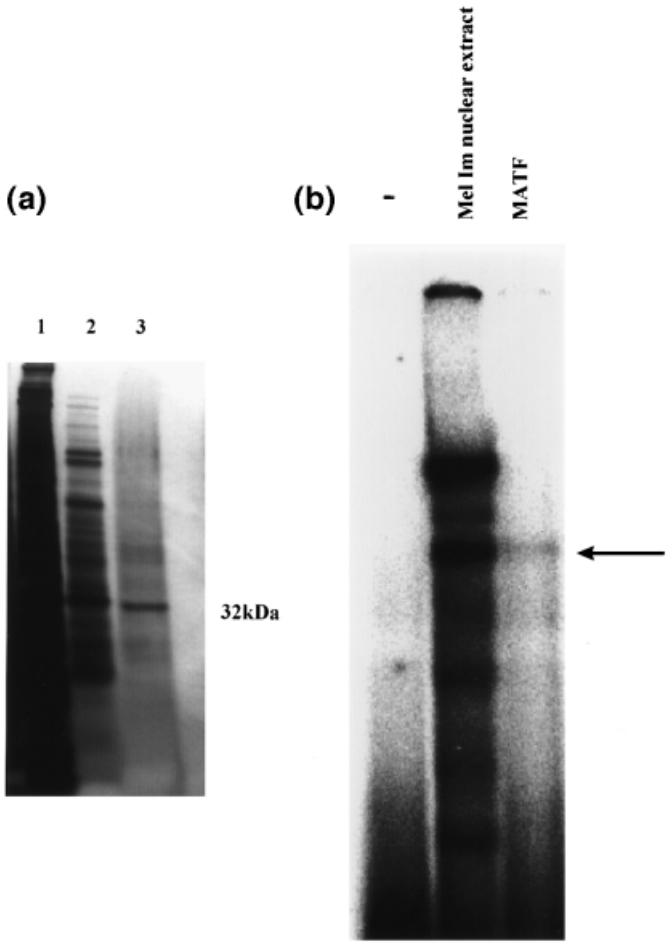


**Figure 6. Isolation of a nuclear protein interacting with the HCR.** *A*: SDS-PAGE of the nuclear proteins isolated by affinity chromatography with the HCR binding site: *lane 1*, Mel Im crude nuclear extract; *lane 2*, proteins eluted by washing with the mutated HCR binding site "mut 3"; *lane 3*, proteins eluted by washing with binding buffer supplemented with 2 M KCl. *B*: Gel mobility shift analysis of the HCR of the murine MIA promoter challenged with albumin (–), with Mel Im crude nuclear extract, or with the final protein eluate shown in *lane 3* of (*A*) (MATF)

**Purification of a 32 kDa protein from melanoma cells binding to the HCR element** We next aimed to design an affinity chromatography approach in order to enrich nuclear proteins interacting specifically with the HCR element in melanoma cells. Therefore, the HCR element was multimerized by PCR and coupled to magnetic beads using a commercially available kit (DNA-binding protein purification kit; Roche). One hundred micrograms of nuclear extract from Mel Im cells were mixed with 500 ng beads loaded with the multimerized HCR binding site. Washing was performed in the presence of the mutated oligomeric binding site (mut 3) to suppress unspecific binding of nuclear proteins and then 2 M KCl was added to the washing buffer for elution. As displayed in **Fig 6**(***a***)**,** approximately 80% of the final protein eluate contained a single polypeptide. Intriguingly, the estimated molecular size of approximately 32 kDa matched precisely the size of the MATF protein that was visualized in melanoma cell lines by southwestern blotting. In order to establish that the protein enriched by affinity chromatography revealed the same binding properties as the previously characterized MATF we performed gel shift experiments. As shown in **Fig 6**(***b***) mixing 10% of the affinity purified material with the radiolabeled HCR binding site resulted in a band shift equivalent in electrophoretic mobility to the melanoma-cell-specific protein-DNA complex displayed in **Fig 5**. Due to the significantly longer exposure time, however, a number of additional very faint band shift complexes are visualized in **Fig 6**(***b***). Taken together, these data provide evidence that affinity chromatography resulted in enrichment of a 32 kDa protein, MATF, binding specifically to the HCR element in the MIA promoter.

## DISCUSSION

In this study we have analyzed the murine MIA promoter in malignant and benign melanocytic cells and defined the HCR binding site, a cis-regulatory promoter element that supports enhanced MIA gene expression in melanoma cells. We further describe an affinity chromatography approach that will allow us to enrich MATF, a 32 kDa melanoma-cell-specific protein, binding to the HCR enhancer. Previous analyses have shown that expression levels of MIA parallel the progression of melanocytic tumors closely. Expression of both mRNA and protein is absent or extremely low in benign skin melanocytes and benign melanocytic nevi but high in melanomas and metastases (Bosserhoff *et al*, 1996, 1997a, 1999). Investigating this melanoma-specific expression pattern we have performed a preliminary analysis of the human MIA promoter and shown that a promoter fragment of less than 230 bp is sufficient to mediate high levels of gene expression in melanomas (Bosserhoff *et al*, 1996). Thus, changes in transcriptional control of gene expression occur during malignant transformation of melanocytes and lead to specific activation of MIA mRNA expression.

Enhanced MIA expression is causally involved in acquisition of the malignant tumor cell phenotype as we observed in a different study that the capability of melanoma cells to form metastases is strictly dependent on the amount of MIA protein secretion. Therefore, we believe that different activities of the MIA gene promoter in benign and malignant melanocytic cells result from changes in transcriptional control of gene expression that are causally related with tumor progression. Importantly, the MIA HCR element will provide a useful tool to clone MATF and thereby elucidate the molecular mechanisms that mediate melanoma-cell-specific gene programs. Consistent with a specific role of MATF in malignant transformation of melanocytic cells, we were unable to detect MATF by southwestern probing of nuclear extracts prepared from benign human and murine melanocytes or from malignant cell lines that do not express MIA mRNA.

The HCR element defines a transposable enhancer that is necessary and sufficient for enhanced gene expression in melanoma

cells when juxtaposed to a basal promoter. In non-neoplastic cells strong MIA mRNA and protein expression occurs foremost in cartilage. Interestingly, the HCR element does not seem to play a role in mediating MIA expression in chondrocytes, as omission of the HCR by 5′ deletion analyses did not alter reporter gene expression in chondrocytes significantly (Xie *et al*, 1999). In contrast, an AP-2 site at position −475 and a sox9 binding site have been described as enhancer elements important for MIA expression in cartilage (Xie *et al*, 1998; 1999). These data indicate different transcriptional control of MIA expression in chondrocytes and melanoma cells and suggest that the HCR element of the MIA promoter may be exploited for cell-type-specific gene therapy of melanomas.

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