Role of the Initiator Element in the Regulation of the Melanoma Cell Adhesion Molecule Gene

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The melanoma cell adhesion molecule is a membrane glycoprotein whose expression is associated with tumor progression and the development of metastatic potential. The mechanisms for upregulation of the melanoma cell adhesion molecule during melanoma progression are still poorly understood. In this study, we show further evidence that melanoma cell adhesion molecule expression is tightly regulated at the transcriptional level. Using a combination of chloramphenicol acetyl transferase reporter assays and DNA mobility shift experiments, we investigated the role played by three putative melanoma cell adhesion molecule regulatory elements, namely the initiator sequence, the SCA element, and the ASp element. The SCA and the ASp boxes can potentially interact with the transcription factors Sp1 and AP-2. Sp1 binding to both sites was confirmed, but only the SCA sequence could form a complex with AP-2. AP-2-driven downregulation of the melanoma cell adhesion molecule promoter, however, did not depend only on a functional SCA element. The pyrimidine-rich CTCACTTG initiator, which overlaps the RNA start site, was essential for promoter function and was shown to interact with proteins related to basic helix-loop-helix transcription factors. Binding in nonmetastatic melanoma cells was induced by cAMP. In metastatic cells, however, binding was constitutive, but could be markedly decreased upon treatment with phorbol esters. As melanoma cell adhesion molecule expression is modulated by cAMP and phorbol ester signaling, these results suggest that the initiator is the central element that mediates cAMP and phorbol ester sensitivity and initiates melanoma cell adhesion molecule overexpression in melanomas. ***Key words: AP-2/Inr/MCAM/transcription. J Invest Dermatol 115:668–673, 2000***

The cell surface glycoprotein MCAM (melanoma cell adhesion molecule), also known as MUC18, Mel-Cam, S-Endo-1, or CD146, has been identified as a marker of tumor progression in melanoma cells (Lehmann *et al*, 1987). *In vivo*, MCAM cannot be detected on normal melanocytes, normal adult skin, or in skin adjacent to melanocytic lesions (Kraus *et al*, 1997), but is expressed on a fraction of benign nevi and on the majority of malignant melanomas (Shih *et al*, 1994). The expression of MCAM by human melanoma cell lines correlates with their ability to grow and produce metastases in nude mice (Luca *et al*, 1993; Xie *et al*, 1997). It was recently demonstrated that MCAM influences later stages of the metastatic process, namely extravasation and establishment of new foci of growth (Schlagbauer-Wadl *et al*, 1999).

MCAM is a member of the immunoglobulin superfamily and shares both structural and sequence similarity with gicerin, a molecule mediating intercellular adhesion in the developing nervous system (Taira *et al*, 1994). In normal adult tissues, MCAM is essentially expressed by smooth muscles and vascular endothelium (Shih *et al*, 1994; Bardin *et al*, 1996) and functions as a cell adhesion molecule (Johnson *et al*, 1997; Shih *et al*, 1997). The MCAM extracellular domain is formed of five immunoglobulin-like domains and the cytoplasmic tail contains several potential recognition sites for protein kinases, suggesting the involvement of MCAM in cell signaling (Sers *et al*, 1993). Indeed, engagement of MCAM at the cell surface was shown to activate the $p59^{fyn}$- and $p125^{FAK}$-dependent pathways (Anfosso *et al*, 1998).

The promoter of the MCAM gene has been cloned and sequenced (Sers *et al*, 1993). It is a GC-rich promoter lacking the conventional TATA box. We recently demonstrated that most (if not all) cis-acting elements required for constitutive and inducible MCAM expression were located within 196 nucleotides (nt) from the RNA start site (Karlen and Braathen, 1999). This minimal promoter fragment is characterized by the presence of five GC boxes specific for the transcription factor Sp1, two putative AP-2 binding elements, and one cAMP response element binding (CREB) sequence. Most of these elements are grouped into two clusters, termed the SCA box and the ASp box, respectively. Both contain an Sp1 element and an AP-2 binding sequence, whereas the cAMP response element (CRE) site is associated with the SCA element only. In addition, the pyrimidine-rich CTCACTTG initiator sequence (Inr) is found overlapping the RNA start site (Smale and Baltimore, 1989). In promoters lacking a canonical TATA box, Inr elements were found to play a crucial role in the regulation of transcription (Zenzie-Gregory *et al*, 1992; Usheva and Shenk, 1994). The Inr core motif CACTTG is reminiscent of the E-box motif (CANNTG), which interacts with basic helix-loop-helix (bHLH) transcription factors (Roy *et al*, 1991). Such elements

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Abbreviations: Ad-MLP, adenovirus major late promoter; bHLH, basic helix-loop-helix; CRE, cAMP response element; CREB, cAMP response element binding protein; Inr, initiator; MCAM, melanoma cell adhesion molecule; MITF, melanocyte transcription factor microphthalmia.

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are present in the promoter of three melanocyte-specific genes, namely tyrosinase and tyrosinase-related proteins 1 and 2, and were found to interact with the bHLH melanocyte transcription factor microphthalmia (MITF) (Bentley *et al*, 1994; Bertolotto *et al*, 1998a).

AP-2 plays an important role in the control of several genes involved in melanoma progression. Upregulation of MCAM (Jean *et al*, 1998) and MMP2 (Takahashi *et al*, 1999) and downregulation of c-Kit (Huang *et al*, 1998) or E-cadherin (Hsu *et al*, 1996) in highly metastatic cells correlate with loss of expression of the AP-2 transcription factor. Transfection of highly metastatic melanoma cells (MCAM positive, AP-2 negative) with the AP-2 gene resulted in a decrease in endogenous MCAM mRNA and protein expression (Jean *et al*, 1998).

In this study, we assessed the contribution of the Inr sequence and of the SCA and ASp elements in the regulation of the MCAM gene. Using chloramphenicol acetyl transferase (CAT) reporter constructs containing the 196 nt promoter fragment with intact or mutated SCA and ASp elements, we demonstrated that both elements regulate MCAM transcription. Co-transfection studies with an AP-2 expression vector, however, indicated that AP-2 downregulation might be indirect and not mediated through the SCA and ASp regulatory boxes. Finally, the Inr sequence was shown to interact with bHLH regulatory factors and to respond to phorbol 12-myristate 13-acetate (PMA) and forskolin stimulation.

MATERIALS AND METHODS

Cell lines and culture conditions The human melanoma cell line SK-Mel2 was purchased from ATCC (cat. no. HTB68) and is highly metastatic in nude mice (Fogh *et al*, 1977). This cell line was maintained in culture as adherent monolayers in Earle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, 2-fold vitamin solution, and penicillin-streptomycin (Gibco-Life Technologies, Basel, Switzerland). The SB2 cell line was isolated from a primary cutaneous lesion and was a gift from Dr. B. Giovanella (The Stehlin Foundation for Cancer Research, Houston, TX). In nude mice, the SB2 cell line is poorly tumorigenic and nonmetastatic. The SB2 cells were maintained in MCDB153 medium (Sigma, St. Louis, MO) supplemented with LB15 medium (4 parts MCDB and 1 part LB15), 2% fetal bovine serum, L-glutamine, and penicillin-streptomycin. For stimulation studies, PMA and forskolin (both purchased from Sigma) were used at concentrations of 50 ng per ml and 20 μM, respectively.

Plasmids The construction of pMCAM-IV-CAT, pMCAM-II-CAT, and pMCAM-del4-CAT has been described previously (Karlen and Braathen, 1999). To produce the constructs SCA/S-mut, SCA/SC-mut, and SCA/SA-mut, the 72 bp fragment located between the sites Xho*I* (nt −11) and Bss*HII* (nt −105) in pMCAM-II-CAT was replaced by a synthetic linker containing mutations destroying the Sp1, the Sp1/CRE, and the Sp1/AP-2 binding sites, respectively (**Fig 2**). The mutations generated were the same as those listed for SCA-binding studies in Karlen and Braathen (1999). Similarly, ASp/S-mut, ASp/AS-mut, and ASp/SCA-2xmut were produced by replacing the Bss*HII*-Nar*I* fragment in pMCAM-II-CAT or SCA/SA-mut by a synthetic sequence in which the Sp1 and the AP-2/Sp1 binding elements of the ASp box were mutated. The fragment Xho*I*(nt −11)-Bgl*II* (site in the polylinker of the pCAT3 basic vector; Promega, Madison, WI) was removed from pMCAM-II-CAT and replaced by the sequence TCGAGCTGC**TTGCTAG**CGTCTCG (complementary strand: GATCCGAGACG**CTAGCAA**GCAGC) to produce the plasmid Inr-mut, in which the putative Inr element (in bold) was altered by three mutations (underlined) (see **Fig 5*A***).

The pVI-AP2-CV expression vector (Meier *et al*, 1995) was kindly provided by Dr. P. Mitchell (Pennsylvania State University, University Park, PA). pVI was obtained by removing by Hind*III* digestion the AP-2 coding sequence from pVI-AP2-CV. The MipEBB expressing vector (Hemesath *et al*, 1994) was a generous gift of Dr. D.E. Fischer (Harvard Medical School, Boston, MA).

Transient transfection and CAT assays Transfection reactions were carried out as described before (Karlen and Braathen, 1999). Briefly, melanoma cells (8×10^5) were transfected into 60 mm culture dishes with Fugene6 reagent (Roche, Basel, Switzerland). One microgram of the various MCAM-CAT or thymidine kinase-CAT constructs was cotransfected with either 1 μg of pEE6-HCMV DNA (Stephens and Cockett, 1989) or, in the case of AP-2 transactivation studies, 0.1 μg of pVI or pVI-AP2-CV DNA. For stimulation studies, PMA and forskolin were added either alone or in combination and the cells were incubated for 48–72 h at 37°C. The CAT assay was then performed, analyzed, and normalized as described before (Karlen and Braathen, 1999).

DNA mobility shift experiments Melanoma nuclear extracts were obtained from a small number of cells (10^7) as described previously (Karlen and Braathen, 1999). The HeLa nuclear extracts, as well as the Sp1 and AP-2 purified factors, were purchased from Promega. Standard binding reactions contained 2.5 μg of nuclear extracts (supplemented with 300 ng of purified AP-2 protein in the case of HeLa extracts) or approximately 300 ng (1 footprinting unit) of purified Sp1 or AP-2 factor, 60 mM KCl, 5 mM $MgCl_2$, 12 mM HEPES buffer pH 7.9, 12% glycerol, 1 mM dithiothreitol, 1 μg poly(dI-dC), and 25 fmol end-labeled oligonucleotide probe. The binding reactions were left on ice for 20 min. Protein-DNA complexes were resolved at 4°C on a 5% (7% for the Inr probe) non-denaturing polyacrylamide gel. The gels were dried and exposed to a phosphoimager screen and analyzed with the Storm 860 instrument (Molecular Dynamics, Sunnyvale, CA). For competition assays, unlabeled oligonucleotides were added 20 min before adding the probe. The SCA and ASp probes, as well as the Inr binding sequence and its corresponding mutants, are described in **Figs 3(*A*)**, **4(*A*)**, and **5(*A*)**. The sequence of the AP-1 oligonucleotide was 5′-CGCTTG-ATGACTCAGCCGGAA-3′. The sequence from the adenovirus major late promoter (Ad-MLP) competing oligonucleotide, which contains a consensus E-box motif for bHLH transcription factors (**Fig 4*A***), was adapted from Yavuzer and Goding (1994). The oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). The Sp1 and AP-2 antibodies (Santa-Cruz Biotechnology, Santa Cruz, CA) were used in supershift analysis as previously described by Karlen *et al* (1996).

RESULTS

Proximal MCAM promoter activity MCAM is not expressed in normal melanocytes (Kraus *et al*, 1997) or in the nonmetastatic SB2 melanoma cells (Bar-Eli, 1997). Changes in cAMP intracellular levels, however, markedly increase expression of the gene (Rummel *et al*, 1996). In contrast, in metastatic melanoma cells (such as SK-Mel2), MCAM expression is constitutive. As little as 196 bp of the human MCAM promoter were sufficient to drive constitutive and inducible regulation in SK-Mel2 and SB2 cells, respectively (**Fig 1*A***; Karlen and Braathen, 1999). As shown in **Fig 1(*B*)**, the activity of the pMCAM-II-CAT construct was strong in unstimulated SK-Mel2 cells and could only be slightly increased upon treatment with forskolin (a potent activator of adenylate cyclase and therefore of cAMP). In the presence of PMA, MCAM promoter activity was considerably reduced. In SB2 cells, the MCAM promoter was silent and required forskolin to be activated (**Fig 1*C***). Forskolin-induced promoter activity, however, could be strongly attenuated in the presence of PMA.

The 196 bp MCAM promoter fragment, which drives CAT expression in the pMCAM-II-CAT construct, contains two putative AP-2 binding elements associated with the SCA and the ASp elements, respectively. Jean *et al* (1998) showed that transfection of AP-2 into highly metastatic A375 melanoma cells strongly inhibits MCAM expression at the transcriptional level. To investigate whether AP-2 mediates MCAM downregulation through the 196 nt fragment, the plasmid pMCAM-II-CAT was transfected into unstimulated SK-Mel2 cells or into SB2 cells treated with forskolin, together with a vector expressing the AP-2 transcription factor. As shown in **Fig 1(*B*, *C*)**, the activity of the 196 nt MCAM promoter was abolished upon expression of AP-2.

Interestingly, the MCAM promoter lacks a TATA box, but the pyrimidine-rich Inr sequence CTCACTTG is found overlapping the start site (**Fig 5*A***). Deletion of this sequence resulted in a complete loss of promoter activity (**Fig 1*A***).

Regulatory elements in the MCAM proximal promoter region Previous promoter deletion studies showed that the ASp and SCA elements were important regulators of MCAM promoter activity (Karlen and Braathen, 1999). Regulation by forskolin and PMA, however, appeared not to be mediated by these two sites. To assess the individual contribution of the SCA and ASp boxes in the control of MCAM promoter activity, point mutations destroying the binding potential of both elements were introduced in

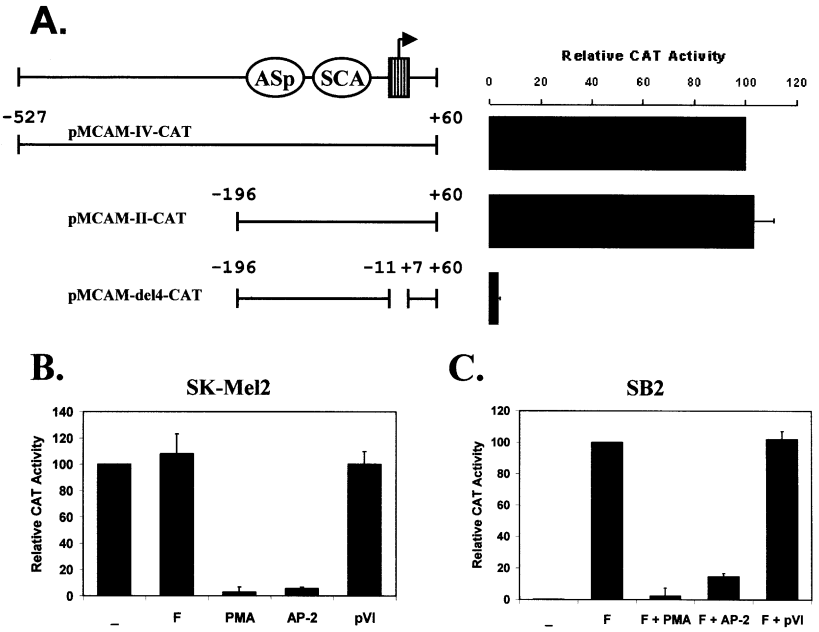

Figure 1. MCAM proximal promoter activity in melanoma cells. (*A*) CAT activity of the MCAM promoter region. SK-Mel2 cells were transfected with (i) pMCAM-IV-CAT (construct –527 to +60), (ii) pMCAM-II-CAT (construct –96 to +60), and (iii) pMCAM-del4-CAT (construct –196 to +60 in which the initiator sequence (Inr) has been deleted) as described in *Materials and Methods*. CAT assays were carried out 48 h later and analyzed by liquid scintillation counting (LSC). Results are expressed relative to the activity found in cells transfected with the pMCAM-IV-CAT construct and are normalized for transfection efficiency. Data are means (SEM of three experiments in duplicate). (*B, C*) Activity of the 196 nt promoter fragment (pMCAM-II-CAT construct) in SK-Mel2 cells and SB2 cells. Where indicated, the transfected cells were treated with 50 ng per ml PMA and 20 μM forskolin (F), or co-transfected either with the pVI-AP2-CV expression vector (AP-2) or with the empty vector pVI. Results are expressed relative to the activity found in unstimulated (–) SK-Mel2 cells (*B*) or in forskolin-treated SB2 cells (*C*).

pMCAM-II-CAT (**Fig 2*A***) and the resulting constructs were tested for CAT activity in SK-Mel2 (**Fig 2*B***) or SB2 cells (data not shown). The alteration of the Sp1 site associated with the SCA element (construct SCA/S-mut) resulted in a small but significant increase in MCAM promoter activity (**Fig 2*B***, *lane 2*), which was further drastically enhanced (*lane 4*) with the additional mutation of the AP-2 binding element (construct SCA/SC-mut). These results, together with our previously published data based on internal deletion constructs (Karlen and Braathen, 1999), indicate that a potential repressor site is associated with the SCA element. In contrast, the point mutation analysis confirmed that the CRE element was not involved in the regulation of the MCAM promoter (**Fig 2*B***, *lane 3*; data not shown). Following mutation of the Sp1 site found in the ASp element (construct ASp/S-mut), however, MCAM promoter activity was sensibly reduced (*lane 5*), but was not further affected upon mutagenesis of the AP-2 binding sequence (*lane 6*). The high levels of CAT activity produced by the construct SCA/SA-mut (*lane 4*) were also markedly reduced after alteration of the ASp box (*lane 7*, construct ASp/SCA-2xmut), confirming the activator function of this element. Finally, MCAM promoter activity was reduced to background levels when the Inr was destroyed by mutations (*lane 8*). In the presence of PMA, the transcriptional activity of all the ASp and SCA single or double promoter mutants was sensibly reduced.

All the mutant constructs described above were also tested in SB2 cells. As expected, low or no promoter activity could be detected in unstimulated cells. In forskolin-treated cells, however, the promoter activities were proportionally similar to the activities observed in unstimulated SK-Mel2 cells, and addition of PMA resulted in a marked decrease in CAT production (data not shown). Taken together, these data indicate that the sensitivity to both PMA and forskolin might be mediated via regulatory elements independent from the ASp and SCA boxes.

Finally, the activity of the SCA/SA-mut and ASp/SCA-2xmut constructs, in which the AP-2 binding sequences are destroyed, was sensibly lower following transient expression of AP-2 in SK-Mel2 cells (**Fig 2*C***). This suggests that the effects of AP-2 might be indirect.

AP-2 interacts with the SCA element Several observations by Jean *et al* (1998) indicated that AP-2 might downregulate promoter activity through specific interactions with the MCAM regulatory region. DNA mobility shift experiments performed with nuclear extracts obtained from SB2 and SK-Mel2 melanoma cells failed to show AP-2 binding to the SCA box (Karlen and Braathen, 1999). In order to clearly establish the role of AP-2, DNA mobility shift experiments were performed in the presence of HeLa extracts supplemented with AP-2 factors (**Fig 3*B***). These extracts were shown to contain high levels of binding activities for Sp1 AP-2 and CREB (**Fig 3*B***, *left panel*). The formation of two DNA-protein complexes (C_1 and C_2) on the SCA probe could be observed (**Fig 3*B***, *right panel*). Competition experiments in which excess amounts of competing Sp1, CRE, and AP-2 oligonucleotides were used in various combinations showed that Sp1 was present in the C_1 complex and that AP-2 was part of the C_2 complex. In contrast, CREB could not associate with the SCA element, even though CRE binding activities were found to be present in the protein extracts used in the assay. The formation of two DNA binding complexes was also observed in the presence of SB2 nuclear extracts (**Fig 3*C***). Interestingly, the binding pattern was not altered upon treatment with forskolin and was identical to the pattern observed with SK-Mel2 nuclear extracts in the presence or absence of PMA. The two complexes formed in the presence of SB2 extracts had a migration profile similar to the C_1 and C_2 complexes obtained with HeLa extracts supplemented with AP-2 factors (**Fig 3*D***).

The ASp element can also potentially interact with factors related to AP-2 or Sp1. Only Sp1 was found to form a complex with the ASp element in a DNA mobility shift assay (**Fig 4*B***). AP-2 failed to interact with the probe even though it could strongly associate with a consensus AP-2 motif. When nuclear extracts from SK-Mel2 or SB2 cells were used in the assay, the binding pattern was found to be similar and was not affected by the addition of PMA or forskolin (**Fig 4*C***), confirming the central role of Sp1 in ASp binding (Karlen and Braathen, 1999).

MCAM transcription is controlled by the Inr sequence Deletion or mutation of the MCAM Inr sequence resulted in a complete loss of promoter activity (**Figs 1*A*, 2*B***). Strong binding to the Inr element was observed in the presence of SK-Mel2 protein extracts (**Fig 5*B***). The interaction was specific as it could be completely abrogated with an excess of Inr competing sequences, but not with a similar excess of AP-1 binding sequence (**Fig 5*C***). Binding to the Inr oligonucleotide was restricted to the core sequence as the Inr-mut1 oligonucleotide, in which the CANNTG consensus E-box motif is destroyed by point mutations, failed to compete binding. In contrast, binding was inhibited in the presence of the Ad-MLP E-box motif (which shares the CACNTG motif with the Inr element) or in the presence of Inr-mut2 competing sequences, confirming that it is the Inr core motif that mediates the formation of the complex. Binding to the Inr element in SK-Mel2 cells was reduced in the presence of PMA to a level

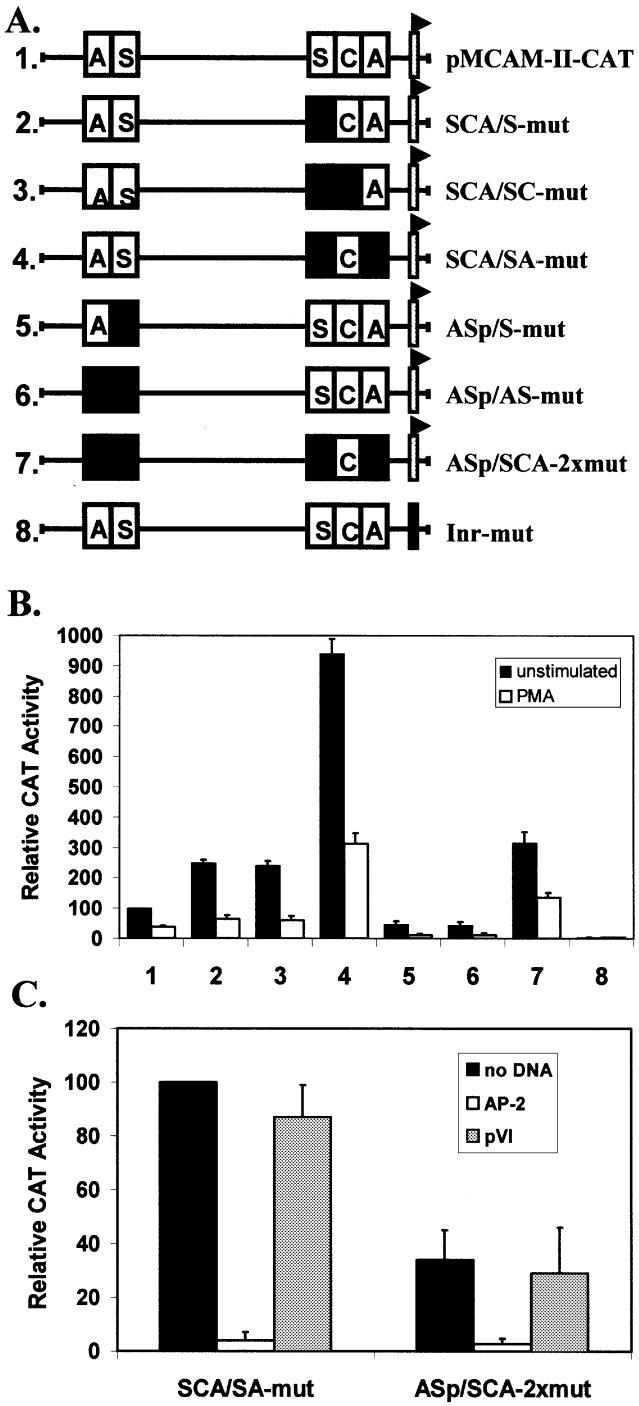

Figure 2. Mutagenesis analysis of the MCAM proximal promoter region. (*A*) Schematic representation of the SCA, ASp, and Inr mutants used in the study. (*B*) Promoter activities in SK-Mel2 melanoma cells. The cells were transfected with the constructs listed above and, where indicated, were treated with 50 ng per ml PMA. The assay was performed as described in **Fig 1**. Results are expressed relative to the activity found in cells transfected with the pMCAM-II-CAT construct. (*C*) Downregulation of the MCAM promoter by AP-2. The SCA/SA-mut and ASp/SCA-2xmut constructs were cotransfected into SK-Mel2 cells either with the pVI-AP2-CV plasmid (AP-2) or with the empty expression vector pVI as a control. The assay was performed as described in **Fig 1**. Results are expressed relative to the activity found in cells transfected with the SCA/SA-mut construct.

similar to that observed in SB2 cells. The addition of forskolin was sufficient to restore binding in those cells. The latter observations strongly suggest that the modulation of MCAM promoter activity by PMA and forskolin might be mediated by the Inr element.

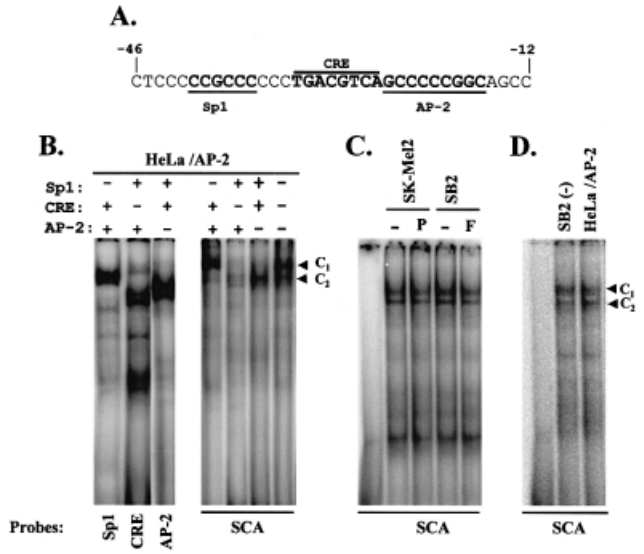

Figure 3. AP-2 binds to the SCA element. (*A*) Sequence of the SCA probe. The 35-mer oligonucleotide (nt −12 to 46) is shown with the binding sites for Sp1, CREB, and AP-2. (*B*, *C*, *D*) DNA mobility shift experiments. Binding reactions were performed as described in *Materials and Methods* with, where indicated, HeLa extracts supplemented with AP-2 factors (*B*, *D*), or with nuclear extracts from unstimulated (–) or PMA (p) treated SK-Mel2 cells or with extracts from unstimulated (–) and forskolin (F) treated SB2 cells (*C*, *D*). The arrows indicate the C_1 and the C_2 complexes, containing Sp1 and AP-2, respectively. For control experiments probes bearing consensus sites for Sp1, CREB, and AP-2 were used. HeLa extracts supplemented with AP-2 were shown to contain binding activities for the three control probes. Binding specificity was assessed by adding, where indicated, a 50-fold molar excess of Sp1, CRE, or AP-2 oligonucleotide.

DISCUSSION

Although it has been shown that MCAM production can be modulated by phorbol ester or cAMP signaling (Rummel *et al*, 1996; Karlen and Braathen, 1999), little is known about the mechanisms directing MCAM expression in melanoma cells. MCAM is not expressed in normal melanocytes (Kraus *et al*, 1997) or in the nonmetastatic SB2 cells (Bar-Eli, 1997). Changes in cAMP intracellular levels, however, markedly increase expression of the gene to a level comparable to that observed in metastatic melanoma cells such as SK-Mel2 (Karlen and Braathen, 1999). In contrast, melanoma cells treated with phorbol esters (PMA) lose MCAM surface expression (Rummel *et al*, 1996). The cAMP-induced activation and the PMA-driven downregulation of the MCAM gene are controlled at the transcriptional level (Karlen and Braathen, 1999). In this investigation, we show that both effects are mediated by the pyrimidine-rich CTCACTTG Inr sequence that overlaps the RNA start site.

The MCAM Inr element is essential for MCAM promoter activity (**Fig 1**). In promoters that lack a TATA box (such as the MCAM promoter), the Inr is functionally analogous to a TATA sequence, in that it is capable of directing basal transcription by RNA polymerase II and of determining the precise site of transcription initiation (Smale and Baltimore, 1989). The TATA-binding protein complex TFIID specifically interacts with the Inr and the interaction is dependent on Sp1 factors bound to upstream sites (Kaufmann and Smale, 1994). The 196 nt MCAM proximal promoter fragment is characterized by the presence of five putative Sp1 binding sites (Sers *et al*, 1993) and we could show evidence for the formation of SP1-containing complexes with at least two of them (**Figs 4**, **5**). Moreover, we recently demonstrated that MCAM transcription in melanoma cells was Sp1-dependent (Karlen and Braathen, 1999). Taken together, these observations confirm that the Inr is a key element in the regulation of the MCAM promoter.

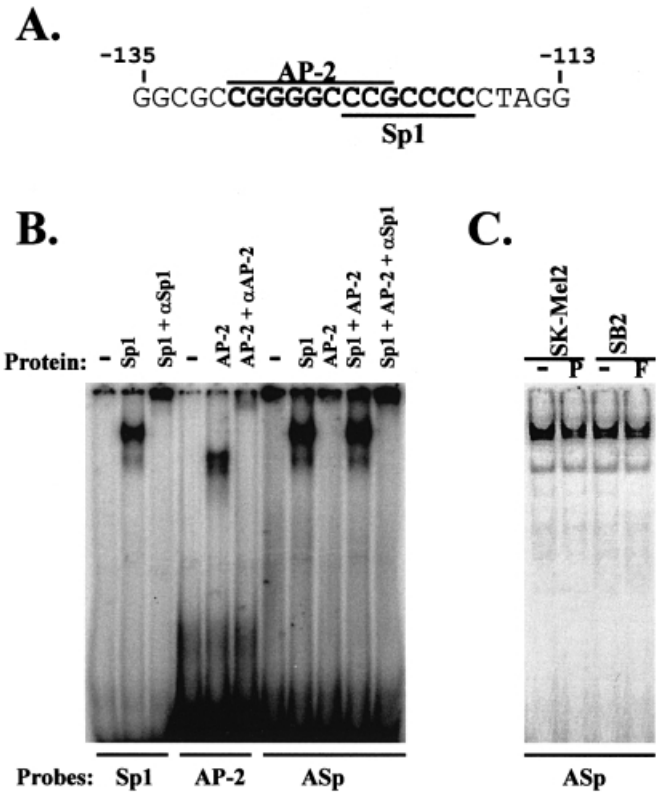

Figure 4. Sp1, but not AP-2, interacts with the ASp element. (*A*) Sequence of the ASp probe (nt −113 to −135) used in the assay. The overlapping AP-2 and Sp1 sites are indicated. (*B, C*) DNA mobility shift experiments were done with purified Sp1 or AP-2 factors (*B*), or with nuclear extracts from SK-Mel2 (with or without PMA) and SB2 cells (plus or minus forskolin) (*C*). For control experiments, Sp1 and AP-2 probes were used. Where indicated, αSp1 and αAP2 antibodies were used in supershift controls.

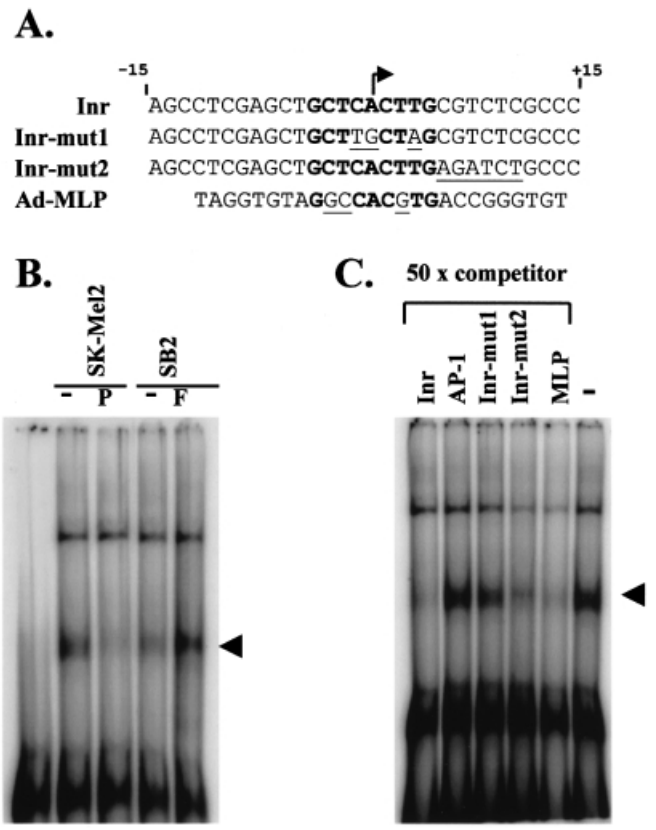

Figure 5. Binding to the Inr sequence. (*A*) Sequence of the Inr probe (nt −15 to +15) and its corresponding mutants and of the Ad-MLP initiator binding element. The MCAM Inr core binding motif is indicated in bold characters. The arrow shows the RNA start site (position +1). (*B*) Binding to the Inr sequence. DNA mobility shift experiments were performed using the Inr oligonucleotide as a probe together with nuclear extracts of SK-Mel2 or SB2 cells, treated where indicated with PMA or forskolin. (*C*) Competition studies. Binding reactions were performed with nuclear extracts obtained from unstimulated SK-Mel2 cells in the presence of a 50-fold molar excess of unlabeled oligonucleotide.

Binding to the MCAM Inr sequence was observed in SK-Mel2 cells or in SB2 cells stimulated with forskolin. In contrast, binding was considerably reduced in untreated SB2 cells or in SK-Mel2 cells grown in the presence of PMA (**Fig 5**). Moreover, MCAM promoter activity is constitutive in SK-Mel2 cells and cAMP-dependent in SB2 cells, and, in both cases, can be inhibited by phorbol esters (Karlen and Braathen, 1999). Thus, both sets of data strongly suggest that the activation and the regulation of the MCAM gene are controlled by the Inr sequence. The Inr core motif CACTTG is reminiscent of the E-box consensus motif CANNTG (Sawadogo and Roeder, 1985), which is known to interact with bHLH transcription factors (Roy *et al*, 1991). Interestingly, in the melanocyte-specific tyrosinase promoter, the response to cAMP induction is partly mediated by an E-box motif located at the Inr site (Bentley *et al*, 1994). MITF, a bHLH transcription factor involved in melanocyte survival, development and differentiation (Hodgkinson *et al*, 1993), binds tightly to this site and is absolutely necessary for the cAMP response (Bertolotto *et al*, 1998a; 1998b). Competition experiments indicated that the MCAM Inr interacts with factors related to bHLH regulatory proteins (**Fig 5**). Thus, MITF was an obvious candidate for binding. Although higher levels of MITF mRNA were found in SK-Mel2 cells compared with SB2 cells, we did not find any evidence for MITF-mediated MCAM transactivation in a promoter assay in which the pMCAM-II-CAT construct was cotransfected with the MITF expressing vector MipEBB (data not shown). Thus, the identity of the factor associated with the MCAM Inr region remains unclear and the use of specific antibodies directed against various members of the bHLH transcription factor family will certainly be required to answer this question.

In addition to the Inr sequence, the MCAM promoter fragment harbors two other regulatory elements, namely the ASp and the SCA boxes, which have been shown to contribute to promoter function (Karlen and Braathen, 1999). Both elements are characterized by the presence of an Sp1 and an AP-2 binding site, whereas the SCA box contains in addition a CRE binding sequence. This CRE-like element appeared, however, not to be involved in cAMP sensitivity. Indeed, the activity of the SCA/SC-mut construct was not enhanced upon forskolin treatment (**Fig 2*B***) and we could not demonstrate CREB interaction with the SCA box (**Fig 3**). In addition, the response to PMA and forskolin treatment was independent of both the SCA and the ASp elements, confirming that both effects might be mediated by the Inr sequence.

AP-2 plays a major role in regulating tumor growth and metastasis of human melanoma. Loss of AP-2 results in upregulation of the MCAM gene and enhancement of melanoma tumorigenicity and metastasis, suggesting that AP-2 acts as a negative regulator of MCAM gene expression (Jean *et al*, 1998). Indeed, reexpression of AP-2 in the highly metastatic A375SM melanoma cell line results in downregulation of MCAM expression and in turn in inhibition of tumor growth and metastasis. AP-2 was shown to interact with the full-length promoter region and to inhibit in a dose-dependent manner MCAM promoter activity (Jean *et al*, 1998). Here, we observed that the activity of the 196 nt promoter fragment was still strongly reduced when AP-2 was expressed (**Fig 1**). Binding experiments confirmed that purified AP-2 protein could form a complex with the SCA element but was

unable to interact with the ASp sequence (**Figs 3**, **4**). Finally, by using CAT reporter constructs containing the individual SCA and ASp elements linked to a minimal thymidine kinase promoter, we observed that the regulatory functions of the SCA box, but not of the ASp sequence, could be downregulated by AP-2 in a dose-dependent manner (data not shown). Taken together all these data strongly suggest that AP-2 might impede MCAM expression by directly inhibiting transcription.

A couple of observations, however, let us postulate that MCAM downregulation might not be directly mediated by AP-2. The SCA C_2 complex formed in the presence of SB2 extracts had a mobility similar to a complex shown to contain the AP-2 transcription factor. The identity of the C_2 complex is not yet clearly established, however. Binding experiments performed in the presence of competing AP-2 oligonucleotides or AP-2 antibodies failed to detect the presence of AP-2 in the C_2 complex obtained with melanoma cell extracts (Karlen and Braathen, 1999). The same study also showed that the formation of the C_2 complex required the presence of an intact Sp1 binding site. Here we found that MCAM promoter activity was drastically increased after mutagenesis of the Sp1 and AP-2 binding sequences associated with the SCA element, suggesting that a repressor activity could be associated with the C_2 complex.

Finally, the binding pattern of the SCA element was always the same, whatever the type of melanoma cell extract (SB2 or SK-Mel2) or the type of stimulatory conditions used in the assay (**Fig 3*c***). Surprisingly, the MCAM-negative SB2 melanoma cell line maintained in our laboratory produces only very low levels of AP-2 proteins (the SK-Mel2 cells are, as expected (Bar-Eli, 1997), AP-2 negative). This means that either a small amount of AP-2 protein may be sufficient to switch off MCAM mRNA synthesis, or that AP-2 may activate an alternative regulatory pathway that would contribute to MCAM transcriptional downregulation. Indeed, Huang *et al* (1998) showed that AP-2 upregulates expression of the c-Kit receptor in melanoma cells, with as a result a marked decrease in tumor growth and metastasis. Interestingly, we recently reported that stem cell factor signaling through the c-Kit receptor could impede MCAM mRNA synthesis. Alternatively, melanocytes and nonmetastatic melanoma cells might express, besides AP-2, additional regulatory factors that would negatively control MCAM expression. The identification of such putative regulators would be of great interest, as they may provide new targets for the treatment of melanoma.

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