Regulation of the Melanoma Cell Adhesion Molecule Gene in Melanoma: Modulation of mRNA Synthesis by Cyclic Adenosine Monophosphate, Phorbol Ester, and Stem Cell Factor/c-Kit Signaling

Stéphane Karlen and Lasse R. Braathen

Dermatological Clinic, University of Bern, Inselspital, Bern, Switzerland

The melanoma cell adhesion molecule was identified as a human melanoma-associated antigen that increases in expression as tumors increase in thickness and begin to acquire metastatic potential. Clinical and experimental evidences suggest that the development of metastatic capacity might be the consequence of increased melanoma cell adhesion molecule expression. The mechanisms for upregulation of the melanoma cell adhesion molecule during melanoma progression are, however, still poorly understood. In this study, we show that melanoma cell adhesion molecule expression is tightly regulated at the transcriptional level. Using a combination of CAT reporter assays and semiquantitative reverse transcriptase–polymerase chain reaction, we observed that cyclic adenosine monophosphate significantly increases transcription of the melanoma cell adhesion molecule in nonmetastatic melanoma cells. In metastatic cells, transcription of the gene was constitutive and could not be further increased by cyclic adenosine monophosphate. On the other hand, melanoma cell adhesion molecule promoter activity was impeded upon treatment with phorbol esters or in the presence of stem cell factor, a phenomenon which was protein kinase C-dependent. Promoter-deletion studies demonstrated that the first 196 nt of the melanoma cell adhesion molecule promoter region are sufficient to get full expression in metastatic melanoma cells. This fragment contains five binding sites for the transcription factor Sp1 and DNA mobility shift experiments showed direct binding of Sp1 to the promoter. In conclusion, our results indicate that Sp1 is sufficient to drive constitutive melanoma cell adhesion molecule expression in metastatic melanoma cells. In nonmetastatic cells, however, melanoma cell adhesion molecule expression is repressed and we speculate that stem cell factor/c-Kit signaling might be responsible for the control of melanoma cell adhesion molecule synthesis, and thus, perhaps, of melanoma progression and metastasis. ***Key words: melanoma/melanoma cell adhesion molecule/Sp1/stem cell factor/c-Kit. J Invest Dermatol 113:711–719, 1999***

Malignant melanomas are invasive and highly metastatic tumors which arise from dendritic pigmented melanocytes (Mancianti and Herlyn, 1989). The cell surface glycoprotein melanoma cell adhesion molecule (MCAM) (previously known as MUC18, Mel-cam, or CD146) was identified as a human melanoma-associated antigen that increases in expression as tumors increase in vertical thickness and begin to acquire metastatic potential (Lehmann *et al*, 1987). No expression of MCAM antigen has been detected in normal melanocytes, normal adult skin, or skin adjacent to melanocytic lesions (Shih *et al*, 1994a, b; Kraus *et al*, 1997). MCAM antigen, however, can be detected on nevi, but at lower levels than on primary or metastatic melanoma (Shih *et al*, 1994a). 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). Moreover, enforced expression of MCAM in low-tumorigenic, nonmetastatic melanoma cells significantly increases their tumorigenicity and metastatic potential in nude mice (Xie *et al*, 1997a) and the production of tumorigenic variants from a nontumorigenic melanoma cell line is accompanied by MCAM upregulation (Bani *et al*, 1996). Finally, transfection of AP-2 regulatory factors into highly metastasic melanoma cells inhibits their tumor growth and metastatic potential in nude mice through downregulation of MCAM expression (Jean *et al*, 1998a). These various lines of evidence support the hypothesis that the development of metastatic capacity in melanoma may result from increased MCAM expression.

The mechanisms for upregulation of MCAM expression during melanoma progression are unknown. There is no evidence that the expression of MCAM by malignant tumors is associated either with chromosomal translocation, mutation, or gene amplification (Luca *et al*, 1993). MCAM expression is essentially restricted to tumors of the neuroectodermal lineage and in cutaneous melanoma it appears to increase with tumor thickness and progression. These

Manuscript received February 9, 1999; revised June 14, 1999; accepted for publication July 22, 1999.

Reprint requests to: Dr. Stéphane Karlen, Dermatological Clinic, Inselspital, CH-3010 Bern, Switzerland. Email: stephane.karlen@insel.ch

Abbreviations: CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CREB, cAMP response element binding protein; GAPDH, glyceraldehyde-3-phosphate-dihydrogenase; MCAM, melanoma cell adhesion molecule; SCF, stem cell factor.

0022-202X/99/$14.00 · Copyright © 1999 by The Society for Investigative Dermatology, Inc.

two observations indicate that the regulation of MCAM synthesis is transcriptional (Grimm and Johnson, 1995). An increase in intracellular cyclic adenosine monophosphate (cAMP) levels leads to an upregulation of cell surface MCAM due to increased mRNA synthesis (Rummel *et al*, 1996). In contrast, exposure of the cells to phorbol esters reduces expression to background levels by 24 h. This downregulation is associated with a significant decrease in mRNA levels. These later observations point to a protein kinase C (PKC)-activated pathway that could repress MCAM expression. In malignant tumors this pathway would be no longer functional.

The proto-oncogene c-Kit encodes a transmembrane tyrosine-protein kinase receptor. Whereas normal function of c-Kit is required for human melanocyte development, its expression progressively decreases during local tumor growth and invasion of human melanoma (Lassam and Bickford, 1992; Natali *et al*, 1992). The ligand for c-Kit, stem cell factor (SCF), has a growth inhibitory effect on c-Kit expressing melanoma cells (Funasaka *et al*, 1992; Zakut *et al*, 1993) by inducing apoptosis (Huang *et al*, 1996). As SCF has been shown to be a regulator of PKC activity (Blume-Jensen *et al*, 1993, 1995), it seems possible that MCAM expression may be controlled by signaling through the c-Kit receptor.

Sequence analysis on the MCAM promoter region reveals several interesting properties (Sers *et al*, 1993). The 150 bp fragment found immediately upstream of the transcription site is 88% (G + C)-rich. No TATA or CAAT boxes can be identified, but the pyrimidine-rich initiator sequence (Inr) CTCACTT (Smale and Baltimore, 1989) is found overlapping the RNA start site. Five GC boxes which bind the transcription factor Sp1 (Dynan and Tjian, 1983) are clustered within 130 bp from the Inr. The MCAM promoter also contains four putative AP-2 binding elements. AP-2 plays an important part in the control of gene expression in melanomas. Upregulation of MCAM (Jean *et al*, 1998a) and downregulation of c-Kit expression (Huang *et al*, 1998) in highly metastatic cells correlate with loss of expression of the transcription factor AP-2. A consensus motif for the cAMP-response element (CRE) is found at position –32 in the control region. The CRE-binding factor, CREB, has been recently found to be involved in the malignant transformation of melanoma cells (Jean *et al*, 1998b). As mentioned above, MCAM expression can be modulated by cAMP, thus this particular CRE motif could account for MCAM upregulation in response to cAMP-inducing agents.

In this study, we developed a transient transfection assay for measuring the activity of MCAM-CAT reporter gene constructs in human melanoma cells. We demonstrated that MCAM expression is tightly regulated at the transcriptional level. We also provide evidence that MCAM mRNA synthesis can be downregulated through activation of the SCF/c-Kit regulatory pathway.

MATERIALS AND METHODS

Cell lines and culture conditions The human melanoma cell line SK-Mel2 was purchased from ATCC (catalog no. HTB68) and is highly metastatic in nude mice (Fogh *et al*, 1977). 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 (Verschraegen *et al*, 1991; Luca *et al*, 1993; Singh *et al*, 1995).

The SK-Mel2 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 cells were maintained in MCDB153 medium (Sigma, St Louis, MO) supplemented with LB15 medium (four parts MCDB and one part LB15), 2% fetal bovine serum, L-glutamine, and penicillin–streptomycin. For stimulation studies, phorbol 12-myristate 13-acetate (PMA) and forskolin (both purchased from Sigma) were used at concentrations of 10 ng per ml and 20 μM, respectively. The human recombinant SCF was obtained from Roche Diagnostics (Basel, Switzerland) and was used at a final concentration of 50 ng per ml. The cAMP-dependent protein kinase inhibitor H89 and the PKC inhibitor GF109203X were purchased from Calbiochem (La Jolla, CA) and used at a concentration of 5 μM.

Plasmid construct To generate the MCAM promoter CAT construct (pMCAM-IV-CAT; **Fig 1**), the following strategy was used. The 5′-flanking region of the MCAM gene (nt +5 to –527) was amplified by PCR, using the Clontech Advantage-GC genomic PCR system (Palo Alto, CA). The RNA initiation site was designated +1. The PCR product was subcloned upstream of the CAT gene into the pCAT3 basic vector (Promega, Madison, WI) by using the *Sac*I and *Bgl*II sites. The sequence of the promoter was verified by sequencing. The construct pMCAM-III-CAT was obtained by cutting pMCAM-IV-CAT at the *Sac*I site (site in the polylinker) and *Tth*III1 (nt –392), filling the ends with T4 DNA, and religating. Similarly, pMCAM-II-CAT, pMCAM-I-CAT and pMCAM-0-CAT were made by cutting the pMCAM-IV-CAT construct at the *Sac*I site and the *Bst*XI (nt –195), *Srf*I (nt –65), or *Xho*I (nt –11), respectively, making the ends blunt and religating. pMCAM-del1-CAT, pMCAM-del2-CAT, and pMCAM-del3-CAT were obtained by removing from pMCAM-IV-CAT the *Aat*II–*Xho*I fragment (nt –11 to –30) the *Bss*HII–*Aat*II fragment (nt –26 to –105) and the *Nar*I–*Aat*II fragment (nt –26 to –134), respectively. Finally, pMCAM-del4-CAT was made by cutting pMCAM-IV-CAT with *Xho*I (nt –11) and *Bgl*II (site in the polylinker), filling the ends and religating. The pSV-βGal vector was purchased from Promega.

Transient transfections and CAT assays Melanoma cells (8×10^5) were transfected with 2.5 μg of the various MCAM CAT constructs and 0.5 μg of pSV-βGal DNA with Fugene6 reagent (Roche Diagnostics). Two hours post-transfection, PMA, forskolin, and SCF were added, either alone or in various combinations, and the cells were incubated further for 22 h at 37°C. The cells were then washed with phosphate-buffered saline (PBS) and harvested in 1 × Reporter Lysis Buffer (Promega). MCAM promoter activity was determined by measuring CAT activity. One hundred and twenty-five microliters of cell extract (preheated for 10 min at 60°C) were incubated for 3 h at 37°C in the presence of 5 μg per ml of n-Butyril-CoA and 150 μCi of D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol (Amersham). CAT activities were visualized by thin layer chromatography assay or quantitated by liquid scintillation counting (LSC assay). Normalization of transfection efficiency was done using a cotransfected pSV-βGal vector and measuring β-galactosidase activity. The efficiency of transfection in SB2 cells was 20 times lower than in SK-Mel2 cells. Fold activation or inhibition of CAT activity were calculated relative to control cells which were given the reference value of 100. Results are the mean of three independent experiments. Error bars represent SEM.

RNA analysis Total RNA was extracted from 10^6 cells using the SV total RNA isolation system from Promega. Detection of MCAM, c-Kit, and GAPDH mRNA was performed using the one tube/two enzymes Access reverse transcriptase–PCR system developed by Promega and 100 ng of total RNA for standard reactions. The following oligonucleotides were used as primers. MCAM sense: (nt 185–206) 5′-CCAAGGCAACCTCAG-CCATGTC-3′; MCAM anti-sense (nt 598–622) 5′-CTCGACTCCACA-GTCTGGGACGACT-3′. c-Kit sense (nt 1326–1347) 5′-GCC-CACAATAGATTGGTATTT-3′; c-Kit anti-sense (nt 1875–1896) 5′-AGCATCTTTACAGCGACAGTC-3′. GAPDH sense (nt 85–106) 5′-AACGGATTTGGTCGTATTGGGC-3′ and anti-sense (nt 663–684) 5′-AGGGATGATGTTCTGGAGAGCC-3′. PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. For semiquantitative reverse transcriptase–PCR analysis of MCAM mRNA, we used the following strategy (Yawalkar *et al*, 1998). A 1:4 serial dilution from each RNA extraction was prepared, with the concentration of the first sample being set to 100 ng per μl. An aliquot from each dilution was then subjected to 32 cycles of PCR amplification (20 s at 95°C, 20 s at 52°C and 30 s at 72°C in a Perkin Elmer 9600 thermocycler) with the appropriate primers. The resulting products were analyzed as described above. The exposed films obtained from the gels were evaluated with an optometric scanner and the integrated optical density in arbitrary units of each amplified fragment was determined. The dilution at which a 50% reduction of the signal was obtained is determined for both the MCAM and the GAPDH messengers. The amounts of MCAM transcripts, relative to the GAPDH control, was calculated and the results were expressed as the relative amount of mRNA. Results are the mean of three independent experiments. Error bars represent SEM.

DNA mobility shift experiments Melanoma nuclear cell extracts were obtained from a small number of cells (10^7) using the method described by Schreiber *et al* (1993) with the following modifications: Complete Protease Inhibitor Cocktail solution (Roche Diagnostics), specific for serine, cysteine, and metalloproteases, was added to the reaction buffers just before lysis. Nuclear protein extracts were assayed for protein content by the BioRad (BioRad Laboratories, Hercules, CA) protein assay reagent

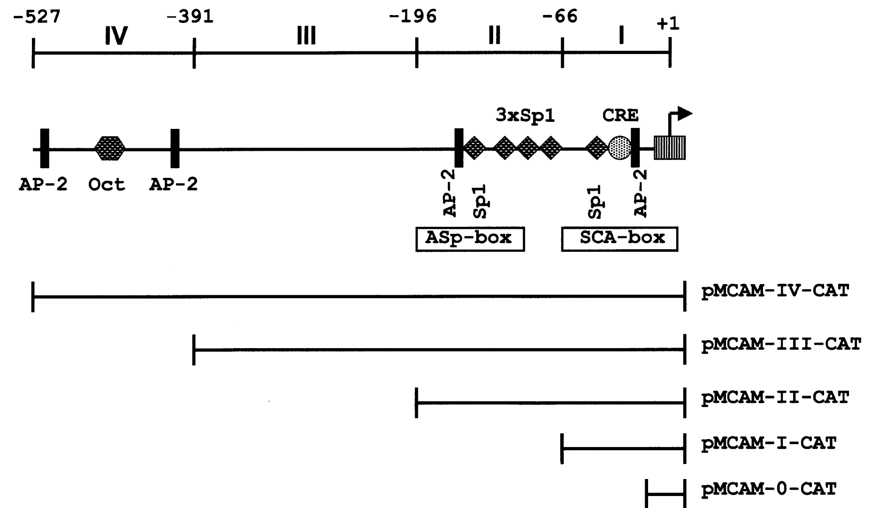

Figure 1. A diagrammatic representation of the MCAM promoter region. The RNA initiation site (*arrow*) was designated +1. Regulatory elements which have been previously described and which are potentially involved in the expression of the gene (Sers *et al*, 1993) are indicated. Promoter/deletion constructs used in the study are shown.

kit. Standard binding reaction contained 2.5 μg of nuclear protein, 60 mM KCl, 8 mM $MgCl_2$, 12 mM HEPES buffer pH 7.9, 12% glycerol, 1 mM dithiothreitol, 3 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% nondenaturing 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 probe was a 35 bp oligonucleotide which covers the MCAM promoter region from position –12 to –46 and includes the Sp1 site at nt –41, the CRE site at nt –32, and the AP-2 site at position –24. The 23 bp ASP probe covers the promoter sequence from nt –113 to –135 and contains the AP-2 site at position –130 and the Sp1 site at position –124. Competitor oligonucleotides with high-affinity binding sequences for either Sp1, CREB/ATF, or AP-2 were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA). The mutated SCA oligonucleotides used in competition studies are described in **Fig 9** and the mutated ASP oligonucleotides are shown in **Fig 8**. Unless otherwise stated, the oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Anti-Sp1, CREB/AT-1, ATF-2 and AP-2 antibodies (Santa-Cruz) were used in supershift analyses as previously described by Karlen *et al* (1996).

RESULTS

MCAM promoter activity in melanoma cells In an attempt to elucidate further the molecular mechanisms controlling MCAM transcription, the activity of the CAT reporter gene driven by the MCAM promoter (nt +5 to –527; **Fig 1**) was analyzed in the highly metastatic SK-Mel2 melanoma cell line. The results are summarized in **Fig 2(*a*, *b*)**. MCAM promoter activity was strong in unstimulated SK-Mel2 cells (**Fig 2*b***, *lane 1*). Upon treatment with forskolin (a potent activator of adenylate cyclase and, therefore, of cAMP), however, MCAM promoter activity was only marginally increased (*lane 2*). The high level of MCAM promoter activity in unstimulated cells was not surprising. Like the majority of the melanoma cell lines, SK-Mel2 is tumorigenic and metastatic in nude mice (Fogh *et al*, 1977) and consequently might be expected to express a high amount of MCAM mRNA. Indeed, and as demonstrated in a semiquantitative reverse transcriptase–PCR assay (**Fig 2*c*, *d***), MCAM transcripts were highly expressed in unstimulated SK-Mel2 cells. Similarly, MCAM mRNA synthesis could only be slightly increased in the presence of forskolin. These data indicate that MCAM production in SK-Mel2 cells is constitutive and that the high level of MCAM expression is the consequence of increased transcriptional activity.

Downregulation of MCAM promoter activity by phorbol esters Previous experiments by Rummel *et al* (1996) indicated that MCAM mRNA levels were reduced in melanoma cells treated with PMA. Similarly, we observed a significant decrease in endogenous MCAM transcripts in SK-Mel2 cells in the presence of PMA (**Fig 3*a***). The block imposed by PMA on MCAM mRNA synthesis was maintained even when cells were costimulated with forskolin. **Figure 3(*b*)** demonstrates that PMA acted at the transcriptional level. Indeed, the activity shown by the MCAM promoter in unstimulated or forskolin-treated cells was greatly reduced when the cells were costimulated with PMA.

MCAM expression in SB2-cells To confirm that MCAM expression is regulated at the transcriptional level, the activity of pMCAM-IV-CAT was analyzed in the nonmetastatic MCAM negative SB2 melanoma cells (**Fig 4**). Very low to undetectable CAT activity could be observed in unstimulated SB2 cells (**Fig 4*a***). In contrast to that observed with SK-Mel2 cells, the MCAM promoter reacted strongly to forskolin treatment, demonstrating that the MCAM promoter can respond to cAMP elevation. MCAM transcriptional activities in stimulated SB2 cells, however, were still significantly lower than in the metastatic SK-Mel2 cells. MCAM mRNA synthesis was hardly detectable in the nonmetastatic SB2 cells when compared with the abundant production of MCAM transcripts observed in the SK-Mel2 cell line (**Fig 4*b***). Upon forskolin treatment, however, the amounts of MCAM mRNA produced by the two cell lines were comparable (**Fig 4*c***).

The effects of forskolin could be strongly attenuated in the presence of H89, a specific inhibitor of cAMP-dependent protein kinases (Song *et al*, 1998) (**Fig 5*a***). Similarly, the production of MCAM transcripts was severely impaired by PMA at induction with forskolin. The blocking effect of PMA, however, was released upon addition of GF109203X, a specific inhibitor of PKC (Song *et al*, 1998). Interestingly, SCF, the ligand for the c-Kit receptor, had a similar effect to PMA on MCAM RNA synthesis in c-Kit positive SB2 cells treated with forskolin (**Fig 5*a*, *b***). Like in the PMA costimulation studies, the block was released upon addition of GF109203X. SCF had no effect on MCAM transcription in the c-Kit negative SK-Mel2 cell line and expression of c-Kit was not induced in those cells after treatment with forskolin (data not shown). Taken together, these data suggest that MCAM expression may be controlled by alternative antagonist stimulatory pathways.

Deletion analysis of the MCAM promoter region The MCAM promoter fragment used in the transfection experiments appears to harbor sufficient genetic information to drive constitutive expression in highly metastatic melanoma cells. We were therefore interested to identify the regulatory elements required for constitutive MCAM expression. This fragment contains, in particular, four sequence motifs at position –24, –130, –410, and –505 identical to the core region of an AP-2 binding element (**Fig 1**). Five GC boxes, at position –41, –72, –77, –99, and –124, respectively, with a binding site for the transcription factor Sp1 have been mapped within 130 bp upstream of the RNA initiation site. A consensus sequence motif for the cAMP-response element CRE was found at position –32. To assess the role played by these putative *cis*-

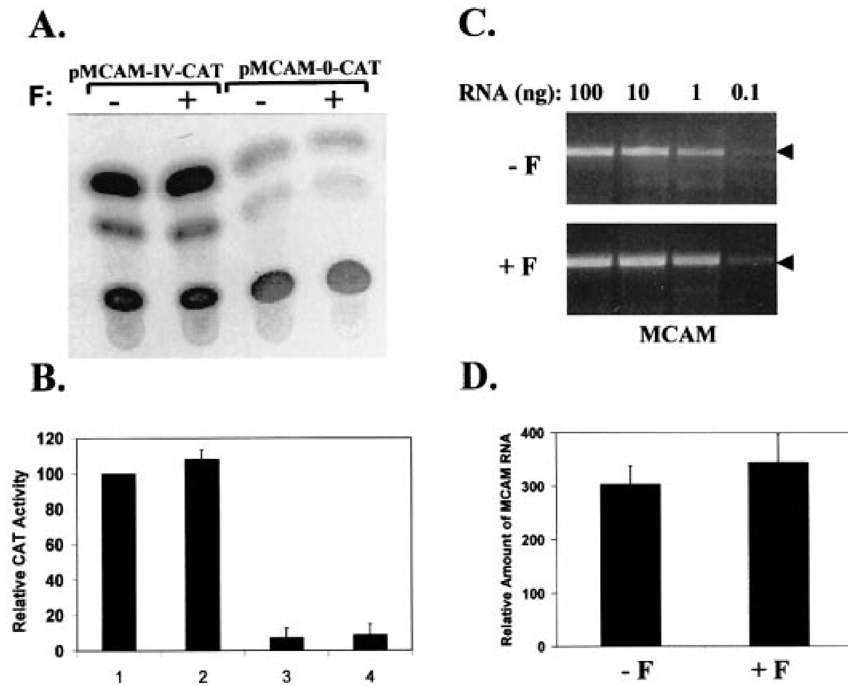

Figure 2. Activity of the MCAM promoter is constitutive in metastatic melanoma cells. (*A*, *B*) CAT activities expressed from the MCAM promoter region. SK-Mel2 cells were transfected with 2.5 μg of CAT constructs and 0.5 μg of pSVβGal in the absence (–) or in the presence (+) of 20 μM forskolin (F). CAT assays were carried out 24 h later and analyzed by thin layer chromatography (*A*) or LSC (*B*). In the LSC assay, results are expressed relative to the activity found in cells transfected with the pMCAM-IV-CAT DNA and are normalized for transfection efficiency. Data are mean ± SEM of three experiments in duplicates. (*C*) Access reverse transcriptase–PCR analysis of MCAM expression. MCAM transcripts were specifically amplified from unstimulated (–F) or forskolin-treated (+F) SK-Mel2 cells. Standard reactions contained 100 ng of total RNA (or where indicated 10, 1, and 0.1 ng). After 32 cycles of amplification, the products were analyzed by electrophoresis on 2% agarose and stained with ethidium bromide. The *arrow* shows the 438 bp MCAM product. (*D*) Semiquantitative reverse transcriptase–PCR analysis. Amplified products of MCAM were electrophoresed on 2% agarose, visualized with ethidium bromide, and analyzed by densitometric scanner. Semiquantitative analysis was performed using the levels of GAPDH transcripts as internal reference. Y-axes show the relative amount of MCAM mRNA in percentage of GAPDH. *Scale*: mean ± SEM.

acting element in MCAM transcription, the promoter region was divided in four "so-called" control domains. Domain I contains the RNA initiation site and the Sp1/CRE/AP-2 binding site cluster around position –32 (SCA box). Domain II extends from position –66 to –196 and is characterized by the presence of three Sp1 sites and by the Asp box, an hybrid element made of the Sp1 element found at position –124 partially overlapped by the AP-2 binding element located at position –130. Finally, domain III (nt –196 to –391) harbors a putative c-Myb regulatory element and domain IV (nt –391 to –527) contains two additional AP-2 binding sites. Deletion constructs were produced in which those domains were removed sequentially, transfected in SK-Mel2 and CAT activity was measured (**Fig 6*a***). The deletion of domains IV and III (constructs pMCAM-III-CAT and pMCAM-II-CAT, respectively) did not significantly affect MCAM promoter activity. Removal of domain II, which eliminated the ASp cluster and the majority of the Sp1 sites, reduced promoter activity to around 30% of that obtained with the full length construct. Finally, upon removal of domain I (construct pMCAM-0-CAT), which contains the SCA element, promoter activity was reduced to background levels. The activity of all the deletion constructs was significantly reduced in cells treated with PMA. The MCAM 5′-deletion constructs described above were then tested in SB2 cells (**Fig 6*b***). In unstimulated cells, low or no activity could be detected. In forskolin-treated cells, however, the promoter activities were proportionally similar to the activities observed in unstimulated SK-Mel2 cells. Addition of PMA resulted in a marked decrease in CAT production. The activation of a cAMP-dependent/PMA-sensitive pathway appears therefore to be necessary to get induction of the MCAM gene.

These data suggested that most (if not all) *cis*-acting elements required for constitutive MCAM expression are located within 196 nt from the RNA start site. In order to identify those elements, additional internal deletions were introduced in pMCAM-IV-CAT (**Fig 7**). A similar level of CAT activity was maintained in cells transfected with pMCAMdel1. In this construct, the deletion (–11 to –30) has destroyed the CRE (nt –32) and AP-2 components of the SCA box. A 2-fold increase in MCAM promoter activity was observed in cells transfected with pMCAM-del2 (deletion –26 to –105), in which four Sp1 sites and the CRE element were removed. The further deletion of the AP-2/Sp1 cluster (ASP box) around position –124 (construct pMCAM-del3) was, however, characterized by a significant decrease in MCAM promoter function. Finally the deletion in pMCAM-del4 of the Inr element, which overlaps the RNA start site, led to almost a complete loss in MCAM promoter activity. The transcriptional activity of the three internal deletion constructs (pMCAM-del1, -del2, and -del3; **Fig 7**) was markedly reduced in the presence of PMA. As for the 5′-deletion constructs, the promoter activities of the internal mutants in forskolin-treated cells were proportionally similar to those obtained in SK-Mel2 cells (data not shown).

Binding to the ASp element In the MCAM promoter, the region extending from –118 to –130 is characterized by the presence of the ASp box. This sequence was found to be critical for MCAM promoter activity (**Fig 7**). Probably due to the high GC content of the MCAM proximal promoter region, we were unable to use site-directed mutagenesis to assess the specific contribution of each of the components of the ASp element. Thus, DNA mobility shift experiments with extracts from SK-Mel2 cells were performed in order to identify factors binding to that region. A 23 bp oligonucleotide, which covers the MCAM promoter region from position –113 to –135 and includes the ASp element, was used in the assay. The formation of a major DNA/protein complex could be observed (**Fig 8*a***). Binding appeared to be specific for the ASp box, because it could be efficiently inhibited with an excess of ASp oligonucleotide, but not with a similar excess of AP-1 binding sequence. The binding pattern was identical with extracts from SK-Mel2 and SB2 cells, treated or not with PMA or forskolin (data not shown).

The ASp element has the potential to bind proteins belonging to the AP-2 and Sp1 families of transcription factors. Oligonucleotides with high-affinity sites for AP-2 or Sp1, respectively, were used as competitor sequences in the DNA mobility shift assay. Binding to the ASp element was totally abolished by a 25–125-fold molar excess of Sp1 binding sequences, whereas a similar excess of AP-2 oligonucleotide had no effect on binding (**Fig 8*b***). Mutations destroying either the AP-2 or the Sp1 binding motifs were introduced in the MCAM ASp element. These oligonucleotides were used in competition experiments. Binding to the ASp element was severely impaired in the presence of ASp-mut2 oligonucleotides (mutation destroying the putative AP-2 binding moiety), but was not affected by an excess of ASp-mut1 sequence (mutation altering the Sp1 moiety) (**Fig 8*b***). Finally, the formation of the ASp

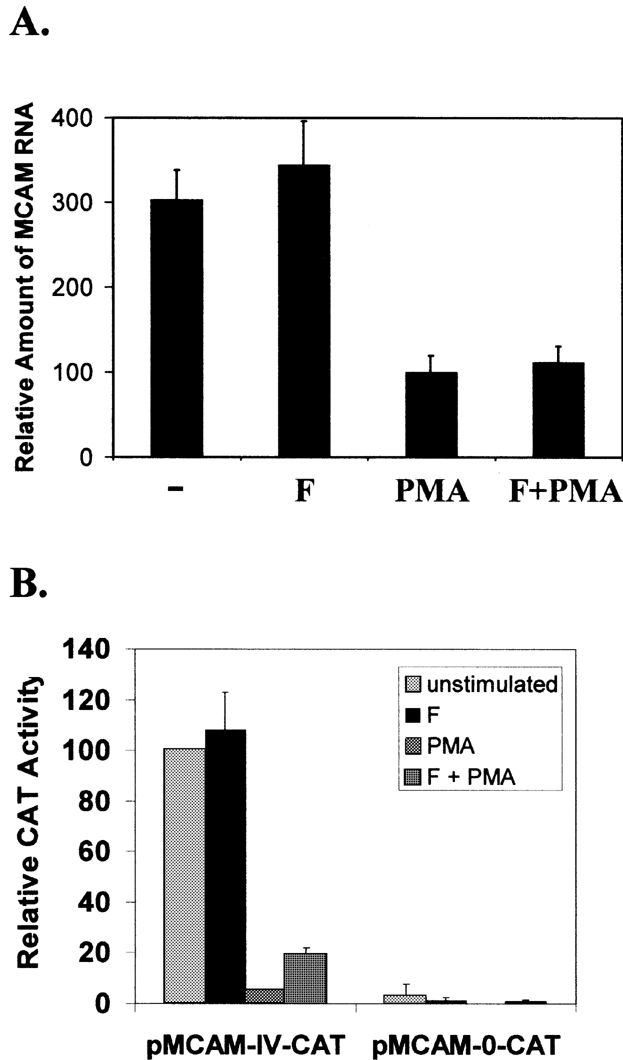

Figure 3. PMA downregulates MCAM expression in melanoma cells. (*A*) Semi-quantitative reverse transcriptase–PCR analysis of MCAM RNA in unstimulated cells (–) or in cells treated either with forskolin (F), PMA (10 ng per ml), or a combination of the two (*F* + PMA). Assays were carried out as described in **Fig 2**(***d***) and materials and methods. Y-axes show the relative amount of MCAM RNA in percentage of the GAPDH control. (*B*) PMA downregulates the activity of the MCAM promoter. The pMCAM-IV-CAT construct was transfected into SK-Mel2 cells in the presence or not of forskolin (F) and PMA, either alone or in combination. Cell transfections and CAT assays were performed as described in **Fig 2**(***a***). pMCAM-0-CAT DNA was used as the negative control.

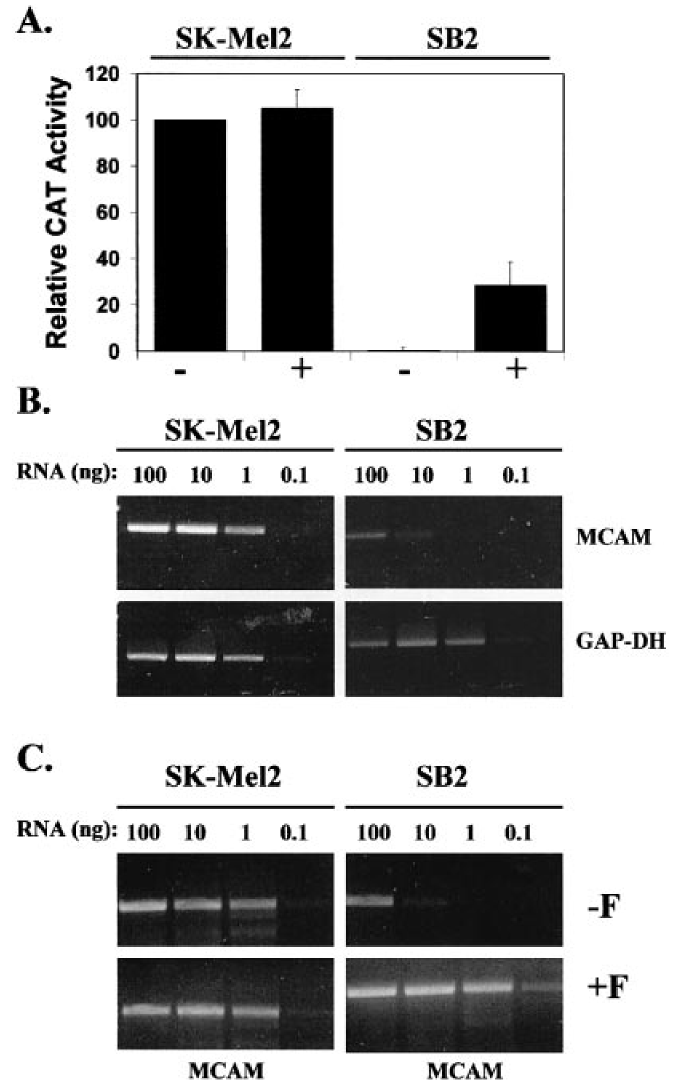

Figure 4. MCAM promoter activity in the nonmetastatic MCAM negative SB2 melanoma cell line. (*A*) Upregulation of MCAM promoter function by forskolin. The pMCAM-IV-CAT construct was transfected into SK-Mel2 or SB2 cells in the presence (+) or in the absence (–) of forskolin. CAT assays were performed 24 h after transfection and CAT activity measured by LSC. Results are expressed relative to the activity found in unstimulated SK-Mel2 cells and normalized for transfection efficiency. (*B*) Comparison of MCAM mRNA levels. Total RNA was extracted from SK-Mel2 and SB2 cells and subjected to reverse transcriptase–PCR analysis as described in **Fig 2**(***c***). Amplification of GAPDH (600 bp) was used as a control for mRNA integrity. (*C*) MCAM mRNA synthesis in forskolin-treated cells. MCAM transcripts were analyzed as described in above.

complex could only be altered upon addition of anti-Sp1-specific antibody (**Fig 8*c***). Taken together, these data indicate that Sp1 is the unique factor interacting with the ASp box.

Binding to the SCA element In the MCAM promoter, the region extending from –17 to –41 is characterized by the presence of the SCA box. This element has the potential to bind the Sp1, CREB/ATF, and AP-2 regulatory factors. Partial destruction of the SCA element resulted in a 2-fold increase in MCAM promoter activity (**Fig 7**). In order to identify factors interacting with the SCA sequence, a 35 bp oligonucleotide, which covers the MCAM promoter from position –12 to –46 was used in a DNA mobility shift assay with extracts from SK-Mel2 cells. The formation of two DNA/protein specific complexes (C1 and C2) could be observed (**Fig 9*a***). The binding pattern was similar in PMA or forskolin stimulated cells, or with extracts from SB2 cells (data not shown). Binding to the SCA element was not affected by an excess of CRE or AP-2 competing sequences, whereas a similar excess of Sp1 oligonucleotide strongly inhibited the formation of the top complex C1 (**Fig 9*a***). Mutations destroying the Sp1, CRE, and AP-2 binding motifs, either individually or in various combinations, were introduced in the MCAM SCA element. The resulting mutants were then used in competition experiment (**Fig 9*b***). Binding to the SCA box was not affected by an excess of mut1, mut4, mut5, and mut7 competing sequences. As Sp1 has been destroyed in those mutants, these data confirmed the important part played by this element in MCAM expression. On the opposite, and as shown in competition studies with the mut2, mut3, and mut6 oligonucleotides, the formation of the C1 and C2 complexes was completely abrogated by an SCA oligonucleotide containing an intact Sp1 binding sequence. Finally, only antibodies directed against the Sp1 factor could affect the formation of the C1 complex in binding experiments (data not shown). Taken together, these

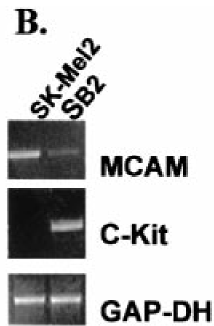

Figure 5. Modulation of MCAM expression by SCF. (*A*) PMA and SCF inhibits forskolin-driven MCAM expression. Semiquantitative reverse transcriptase–PCR analysis of MCAM transcripts in SB2 cells treated (where indicated) with 20 μM forskolin (F), 5 μM H89, 10 ng per ml PMA, 5 μM GF109203X and 50 ng per ml SCF. (*B*) SB2, but not SK-Mel2 expresses c-Kit, the receptor for SCF. Total RNA (100 ng) from both cell types was subjected to 32 cycles of reverse transcriptase–PCR amplification with primers specific for MCAM (438 bp), c-Kit (571 bp), and GAPDH (600 bp). Reactions were carried out as described in **Fig 2**(*c*).

Figure 6. Deletion analysis of the MCAM promoter region. SK-Mel2 (*A*) and SB2 cells (*B*) were transfected with 2.5 μg of the deletion constructs described in **Fig 1**, and 0.5 μg of psvβGal. Where indicated the cells were treated with PMA, forskolin (F) or a combination of the two (*F* + PMA). After 24 h, CAT assays were performed and analyzed by LSC. CAT activity was normalized to β-galactosidase activity and the results are expressed relative to the activity of pMCAM-IV-CAT. Data are mean ±SEM of three experiments in duplicates.

results indicate that Sp1 is the main factor involved in the formation of the C1 complex. They also show that the C2 complex is specific for the SCA oligonucleotide as none of the competitor could influence binding, except for the SCA oligonucleotides containing an intact Sp1 element. The factor associated to that complex may account for the 2-fold increase in CAT activity observed upon partial deletion of the SCA element

DISCUSSION

Although it has been shown that the melanoma cell adhesion molecule MCAM plays a part in the progression of malignant melanomas (Xie *et al*, 1997a; Meier *et al*, 1998), little is known about the mechanisms that regulate expression of MCAM in melanoma cells. cAMP has been involved in the regulation of several genes in melanocytes and melanomas (Eisen, 1996). Upregulation of cAMP pathway by forskolin induces melanocyte cell differentiation characterized by stimulation of melanin synthesis and dendrite formation (Bertolotto *et al*, 1998; Busca *et al*, 1998). MCAM is not expressed in normal melanocytes (Shih *et al*, 1994a,b Kraus *et al*, 1997) or in the nonmetastatic SB2 cells (**Fig 4*b***; Bar-Eli, 1997). Changes in cAMP intracellular levels, however, markedly increases expression of the gene (**Fig 4*c***; Rummel *et al*, 1996). In contrast, we found that in SK-Mel2 cells expression of the MCAM gene was constitutive, Sp1-dependent and could not be further induced by forskolin. Taken together, these observations suggest that MCAM expression in normal cells may be transcriptionally repressed. Upon stimulation of a cAMP-dependent pathway, the block would be released allowing RNA synthesis to take place. In malignant melanomas, the cells would be in a permanent de-repressed state, leading to constitutive production of MCAM RNA.

The strong reactivity of the MCAM promoter to cAMP brings out a paradoxical situation, i.e., the stimulation of the MCAM gene in a cell type where it should not be expressed but which requires cAMP to differentiate fully. A possible explanation to that paradox would be that MCAM expression is controlled by alternative stimulatory pathway. Exposure of melanoma cells to phorbol esters (PMA) reduces MCAM surface expression to background levels (Rummel *et al*, 1996). This downregulation is associated with a significant decrease in mRNA levels that results from decreased MCAM promoter activity (**Figs 3*b*** and **5–7**). The effects of forskolin-induced activation and PMA-mediated downregulation on MCAM synthesis could be blocked by specific inhibitors of protein kinase A and PKC, H89 and GF109203X, respectively, suggesting that alternative independent regulatory pathways control expression of the MCAM gene. Opposite effects of PMA and forskolin treatment have been observed in other gene systems, especially in the case of cytokine production (Derig *et al*, 1990). In the case of interleukin-2, PMA and cAMP elevating agents were shown to act directly at the transcriptional level by targeting different sets of transcription factors to specific regulatory

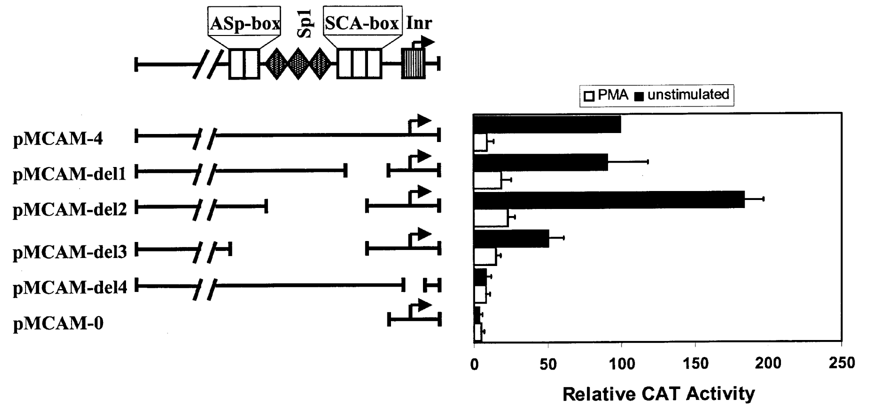

Figure 7. The SCA and ASp elements are involved in MCAM promoter function. SK-Mel2 cells were transfected with the following constructs: (i) pMCAM-del1 (deletion of the CRE and AP-2 consensus sites in the SCA box); (ii) pMCAM-del2 (deletion of the Sp1 and CRE motifs in the SCA element plus removal of three Sp1 putative binding sites); (iii) pMCAM-del3 (removal of the ASp element); and (iv) pMCAM-del4 (deletion of the Inr element overlapping the RNA initiation site). Experiments were performed as described in **Fig 6**. PMCAM-IV-CAT and pMCAM-0-CAT were used as positive and negative control, respectively.

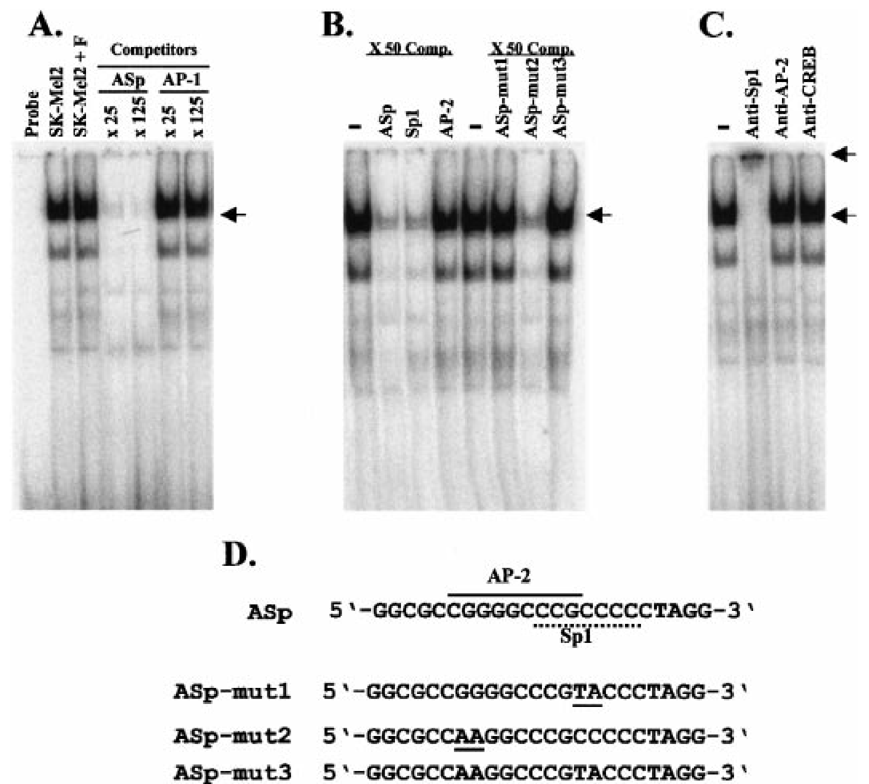

Figure 8. Binding of Sp1 to the ASp box. DNA mobility shift experiments were done with nuclear extracts from SK-Mel2 cells and a 23-mer end-labeled oligonucleotide (nt −113 to −135) containing the ASp box. (*A*) Specific binding to the ASp oligonucleotide. The probe was incubated either alone (probe) or with unstimulated (SK2) or forskolin-treated SK-Mel2 (SK2 + F) extracts. For competition experiments, the probe was incubated with unstimulated extracts and unlabeled homologous (ASp) or unrelated (AP-1) oligonucleotides were added in 25- and 125-fold excess. (*B*) Sp1 binds to the ASp box. The ASp probe was incubated with unstimulated SK-Mel2 nuclear extracts in the presence of competing oligonucleotides. For competition, homologous (ASp), AP-2 and Sp1 consensus and mutated (ASp-mut1, -mut2, and -mut3) oligonucleotides were added in 50-fold excess. (*C*) DNA supershift experiments. Where indicated, reactions were carried out in the presence of specific antibody directed against Sp1, AP-2, and CREB (anti-Sp1, anti-AP-2, and ant-CREB, respectively). (*D*) ASp oligonucleotide and corresponding mutants.

elements in the promoter of the gene (Chen and Rothenberg, 1994; Karlen *et al*, 1996).

MCAM mRNA synthesis in forskolin-treated SB2 cells was significantly reduced in the presence of SCF and PMA. This downregulatory effect was markedly attenuated by GF109203X, a specific inhibitor of PCK. As elegantly demonstrated by Blume-Jensen *et al* (1993, 1995), PKC can modulate the cellular response to SCF, indicating that SCF and phorbol esters may impede MCAM expression through a common PKC-dependent regulatory pathway. The majority of cell lines established from human melanomas do not express detectable levels of c-Kit mRNA or protein (Lassam and Bickford, 1992), making them insensitive to SCF stimulation. As SCF is produced by keratinocytes and other dermal cells in the skin (Longley *et al*, 1993), the loss of c-Kit receptor may allow MCAM expression to take place in malignant melanomas, hence contributing to tumor growth and eventually metastasis.

Deletion analysis indicated that the first 196 bp of the proximal control region were still sufficient to get full promoter activity in SK-Mel2 cells or in forskolin-treated SB2 cells. This fragment is characterized by the presence of two clusters of regulatory elements, the ASp box and the SCA box, respectively, separated by a series of Sp1 binding sites (**Fig 1**). Both clusters can potentially bind factors related to AP-2 and Sp1. As shown in binding experiments, Sp1 was found to be associated with both the ASp and SCA elements. Jean *et al* (1998a) recently demonstrated that upregulation of MCAM expression in highly metastatic cells correlates with loss of expression of AP-2. Accordingly, we did not find any evidence of AP-2 binding on neither the ASp box or the SCA element in SK-Mel2 cells, implying that Sp1 is necessary and sufficient to initiate MCAM transcription in metastatic cells. Surprisingly, we could not demonstrate AP-2 binding to the ASp and SCA elements in SB2 cells even though AP-2 binding activities were present in the extracts (data not shown).

The level of MCAM produced in SB2 cells could, however, still be dramatically increased in forskolin-treated cells, indicating that additional regulatory factors might be required for full activation of the gene. The cAMP-responsive transcription factor CREB has been shown to be a potent mediator of tumor growth and metastasis of human melanoma cells, acting probably as a survival factor (Jean *et al*, 1998b). In addition, MCAM synthesis was downregulated in melanoma cells transfected with a dominant negative CREB mutant (Xie *et al*, 1997b). Sequence analysis of the MCAM gene reveals the presence of a potential CRE binding element associated with

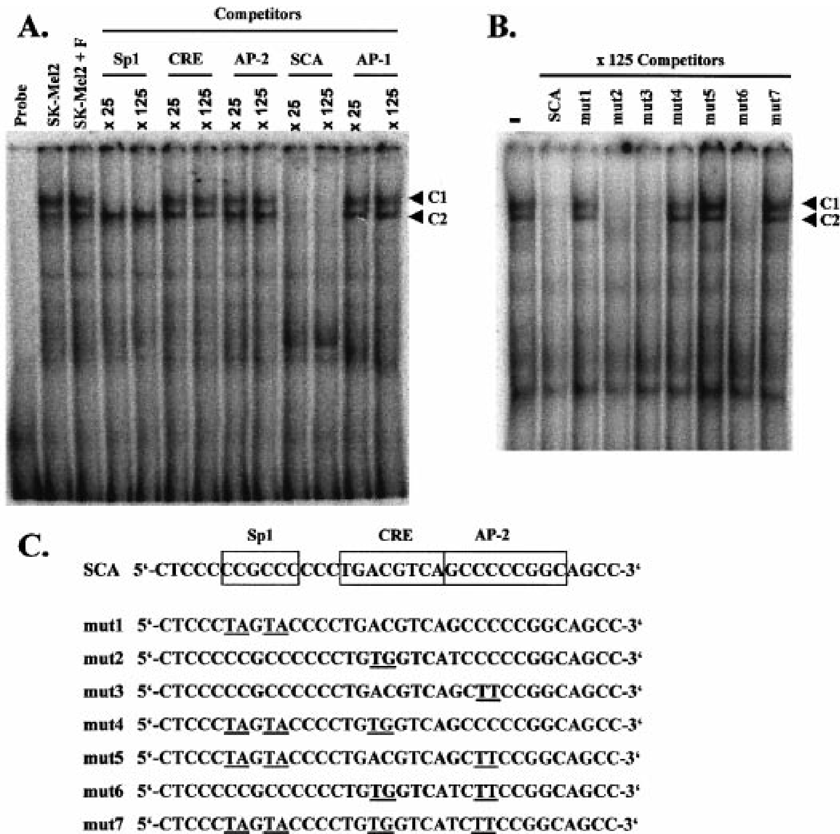

Figure 9. Binding to the SCA element. DNA mobility shift experiments were performed with a 35-mer oligonucleotide (nt –12 to –46) covering the MCAM promoter region and containing the SCA box and extracts from SK-Mel2 cells. (*A*) Formation of two complexes (C1 and C2). The probe was incubated either alone (probe) or in the presence of SK-mel2 extracts derived either from unstimulated cells (SK2) or from forskolin-treated cells (SK2 + F). Nuclear extracts from unstimulated cells were used in competition experiments in which a 25- and a 125-fold molar excess of unlabeled oligonucleotides was added prior binding. (*B*) C1 contains Sp1 and C2 is specific for the SCA probe. Competition experiments using the SCA probe were carried out with extracts from unstimulated SK-Mel2 cells and a 125-fold molar excess of homologous (SCA) or mutated (mut1 to mut7) SCA unlabeled oligonucleotide. (*C*) The SCA probe and the corresponding mutants.

the SCA box. We did not find any evidence of binding of CREB/ATF factors to the SCA element, however, even though CREB/ATF proteins were found to interact with a consensus CRE motif in both SK-Mel2 and forskolin-stimulated SB2 cells (data not shown). Moreover, upon deletion of the CRE motif, the MCAM promoter still responded to forskolin. The strong response of the MCAM gene to cAMP induction might be explained by a second CRE motif found at position +1126 in the first intron of the gene (Sers *et al*, 1993). This CRE element is missing in the CAT constructs used in this study. These constructs did respond to forskolin, but to a much lower extent than the endogenous MCAM gene, suggesting that the intronic CRE element might be required for full responsiveness to cAMP elevating agents. Alternatively, the effects of cAMP might be mediated by elements unrelated to CRE motifs. The MCAM promoter is characterized by an octamer binding motif located at position –456. The POU domain transcription factor Oct3/Brn2 has been involved in the regulation of melanocyte specific genes (Eisen *et al*, 1995). Phosphorylation of the Oct3/Brn2 POU domain by protein kinase A prevents DNA binding (Eisen, 1996). This strongly suggests that Oct3 may participate in MCAM regulation.

Finally, an intriguing question in the regulation of the MCAM gene remains, i.e., the lack of expression in melanocyte and nonmetastatic melanoma cells. AP-2 might be the key to the problem. AP-2 has the potential to downregulate MCAM expression (Jean *et al*, 1998a). In addition it is constitutively expressed by cell lines derived from primary cutaneous melanoma (such as SB2), which are low tumorigenic and nonmetastatic in nude mice. Preliminary data from our laboratory indicate that the activity of the 196 nt MCAM promoter fragment (construct pMCAM-II-CAT) is strongly reduced when AP-2 is expressed. The apparent lack of AP-2 binding on this fragment (which contains both the ASp and SCA elements) suggest, however, that the effects of AP-2 might be indirect, by activating critical regulators of MCAM expression. In that case, the c-Kit receptor would be an attractive candidate.

We thank Dr. Willy Hofstetter and Dr. Robert R. Friis for critical reading of the manuscript. Mrs. B. Rohrbach provided excellent technical assistance. We are most grateful to Dr. B. Giovanella and Dr. M. Herlyn for providing the SB melanoma cell lines and to Dr. K. Satyamoorthy for his invaluable advice on melanoma cell culture. These investigations were supported by a grant from the Bernese Cancer League to S.K.

REFERENCES

Bani MR, Rak J, Adachi D, Wiltshire R, Trent JM, Kerbel RS, Ben-David Y: Multiple features of advanced melanoma recapitulated in tumorigenic variants of early stage (radial growth phase) human melanoma cell lines: evidence for a dominant phenotype. *Cancer Res* 56:3075–3086, 1996

Bar-Eli M: Molecular mechanisms of melanoma metastasis. *J Cell Physiol* 173:275–278, 1997

Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP, Ballotti R: Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J Cell Biol* 142:827–835, 1998

Blume-Jensen P, Siegbahn A, Stabel S, Heldin CH, Ronnstrand L: Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. *EMBO J* 12:4199–4209, 1993

Blume-Jensen P, Wernstedt C, Heldin CH, Ronnstrand L: Identification of the major phosphorylation sites for protein kinase C in kit/stem cell factor receptor in vitro and in intact cells. *J Biol Chem* 270:14192–14200, 1995

Busca R, Bertolotto C, Abbe P, *et al*: Inhibition of Rho is required for cAMP-induced melanoma cell differentiation. *Mol Biol Cell* 9:1367–1378, 1998

Chen D, Rothenberg EV: Interleukin-2 transcription factors as molecular targets of cAMP inhibition: delayed inhibition kinetics and combinatorial transcription roles. *J Exp Med* 179:931–942, 1994

Derig HG, Burgess GS, Klinberg D, Dahreine TS, Mochizuku DY, Williams DE, Boswell HS: Role for cyclic AMP in the postreceptor control of cytokine stimulated stroma cell growth factor production. *Leukemia* 4:471–479, 1990

Dynan WS, Tjian R: The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* 35:79–87, 1983

Eisen TG: The control of gene expression in melanocytes and melanomas. *Melanoma Res* 6:277–284, 1996

Eisen T, Easty DJ, Bennett DC, Goding CR: The POU domain transcription factor Brn-2: elevated expression in malignant melanoma and regulation of melanocyte-specific gene expression. *Oncogene* 11:2157–2164, 1995

Fogh J, Fogh JM, Orfeo T: One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59:221–226, 1977

Funasaka Y, Boulton T, Cobb M, *et al*: c-Kit-kinase induces a cascade of protein tyrosine phosphorylation in normal human melanocytes in response to mast cell growth factor and stimulates mitogen-activated protein kinase but is down-regulated in melanomas. *Mol Biol Cell* 3:197–209, 1992

Grimm T, Johnson JP: Ectopic expression of carcinoembryonic antigen by a melanoma cell leads to changes in the transcription of two additional cell adhesion molecules. *Cancer Res* 55:3254–3257, 1995

Huang S, Luca M, Gutman M, McConkey DJ, Langley KE, Lyman SD, Bar-Eli M: Enforced c-KIT expression renders highly metastatic human melanoma cells susceptible to stem cell factor-induced apoptosis and inhibits their tumorigenic and metastatic potential. *Oncogene* 13:2339–2347, 1996

Huang S, Jean D, Luca M, Tainsky MA, Bar-Eli M: Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. *EMBO J* 17:4358–4369, 1998

Jean D, Gershenwald JE, Huang S, Luca M, Hudson MJ, Tainsky MA, Bar-Eli M: Loss of AP-2 results in up-regulation of MCAM/MUC18 and an increase in tumor growth and metastasis of human melanoma cells. *J Biol Chem* 273:16501–16508, 1998a

Jean D, Harbison M, McConkey DJ, Ronai Z, Bar-Eli M: CREB and its associated proteins act as survival factors for human melanoma cells. *J Biol Chem* 273:24884–24890, 1998b

Karlen S, D'Ercole M, Sanderson CJ: Two pathways can activate the interleukin-5 gene and induce binding to the conserved lymphokine element 0. *Blood* 88:211–221, 1996

Kraus A, Masat L, Johnson JP: Analysis of the expression of intercellular adhesion molecule-1 and MUC18 on benign and malignant melanocytic lesions using monoclonal antibodies directed against distinct epitopes and recognizing denatured, non-glycosylated antigen. *Melanoma Res* 7(Suppl. 2):S75–S81, 1997

Lassam N, Bickford S: Loss of c-kit expression in cultured melanoma cells. *Oncogene* 7:51–56, 1992

Lehmann JM, Holzmann B, Breitbart EW, Schmiegelow P, Riethmuller G, Johnson JP: Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. *Cancer Res* 47:841–845, 1987

Longley BJJ, Morganroth GS, Tyrrell L, Ding TG, Anderson DM, Williams DE, Halaban R: Altered metabolism of mast-cell growth factor (c-kit ligand) in cutaneous mastocytosis. *N Engl J Med* 328:1302–1307, 1993

Luca M, Hunt B, Bucana CD, Johnson JP, Fidler IJ, Bar-Eli M: Direct correlation between MUC18 expression and metastatic potential of human melanoma cells. *Melanoma Res* 3:35–41, 1993

Mancianti ML, Herlyn M: Tumor progression in melanoma: the biology of epidermal melanocytes in vitro. *Carcinog Compr Surv* 11:369–386, 1989

Meier F, Satyamoorthy K, Nesbit M, Hsu MY, Schittek B, Garbe C, Herlyn M: Molecular events in melanoma development and progression. *Front Biosci* 3:D1005–D1010, 1998

Natali PG, Nicotra MR, Sures I, Santoro E, Bigotti A, Ullrich A: Expression of c-kit receptor in normal and transformed human nonlymphoid tissues. *Cancer Res* 52:6139–6143, 1992

Rummel MM, Sers C, Johnson JP: Phorbol ester and cyclic AMP-mediated regulation of the melanoma-associated cell adhesion molecule MUC18/MCAM. *Cancer Res* 56:2218–2223, 1996

Schreiber E, Tobler A, Malipiero U, Schaffner W, Fontana A: cDNA cloning of human N-Oct3, a nervous-system specific POU domain transcription factor binding to the octamer DNA motif. *Nucleic Acids Res* 21:253–258, 1993

Sers C, Kirsch K, Rothbacher U, Riethmuller G, Johnson JP: Genomic organization of the melanoma-associated glycoprotein MUC18: implications for the evolution of the immunoglobulin domains. *Proc Natl Acad Sci USA* 90:8514–8518, 1993

Shih IM, Elder DE, Hsu MY, Herlyn M: Regulation of Mel-CAM/MUC18 expression on melanocytes of different stages of tumor progression by normal keratinocytes. *Am J Pathol* 145:837–845, 1994a

Shih IM, Elder DE, Speicher D, Johnson JP, Herlyn M: Isolation and functional characterization of the A32 melanoma-associated antigen. *Cancer Res* 54:2514–2520, 1994b

Singh RK, Gutman M, Reich R, Bar-Eli M: Ultraviolet B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8. *Cancer Res* 55:3669–3674, 1995

Smale ST, Baltimore D: The "initiator" as a transcription control element. *Cell* 57:103–113, 1989

Song S-K, Choi SY, Kim KT: Opposing effects of protein kinase A and C on capacitative calcium entry into HL-60 promyelocytes. *Biochem Pharmacol* 56:790–784, 1998

Verschraegen CF, Giovanella BC, Mendoza JT, Kozielski AJ, Stehlin JSJ: Specific organ metastases of human melanoma cells injected into the arterial circulation of nude mice. *Anticancer Res* 11:529–535, 1991

Xie S, Luca M, Huang S, Gutman M, Reich R, Johnson JP, Bar-Eli M: Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. *Cancer Res* 57:2295–2303, 1997a

Xie S, Price JE, Luca M, Jean D, Ronai Z, Bar-Eli M: Dominant-negative CREB inhibits tumor growth and metastasis of human melanoma cells. *Oncogene* 15:2069–2075, 1997b

Yawalkar N, Karlen S, Hunger R, Brand CU, Braathen LR: Expression of interleukin-12 is increased in psoriatic skin. *J Invest Dermatol* 111:1053–1057, 1998

Zakut R, Perlis R, Eliyahu S, Yarden Y, Givol D, Lyman SD, Halaban R: KIT ligand (mast cell growth factor) inhibits the growth of KIT-expressing melanoma cells. *Oncogene* 8:2221–2229, 1993