IDENTIFICATION OF THE ELEMENTS REGULATING THE EXPRESSION OF THE
CELL ADHESION MOLECULE MCAM/MUC18. LOSS OF AP-2 IS NOT REQUIRED
FOR MCAM EXPRESSION IN MELANOMA CELL LINES

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Running Title: Transcriptional Regulation of MCAM

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SUMMARY

The cell adhesion molecule MCAM/MUC18/CD146 is specifically upregulated on tumors of neuroectodermal origin and in animal models confers metastatic capacity to human melanoma cells. To identify critical regions regulating MCAM expression in melanomas, 1 kb of the MCAM 5’ region was analyzed for promoter activity and transcription factor binding in 1 glioma, 1 carcinoma and 4 melanoma cell lines. The minimal MCAM promoter (−106/+22 bp) consists of 4 Sp-1 sites, two AP-2 elements, one cAMP responsive element and the initiator surrounding the transcriptional start site. Analysis of mutated constructs indicated that the CRE element is a major transcriptional activator in the majority of cell lines. Site directed mutagenesis revealed that, in AP-2 expressing cells, the AP-2 site within the core promoter (−23bp) has an inhibitory influence on MCAM expression while the AP-2 sites at -131bp and -302bp are activating. Functional AP-2 was observed in both MCAM positive and MCAM negative melanoma cell lines indicating that expression of MCAM does not require loss of this transcription factor. Furthermore, all MCAM constructs were strongly expressed in MCAM negative as well as MCAM positive cells, indicating that the expression of this gene is not controlled solely by the presence of transactivating factors binding to the investigated region.
INTRODUCTION

Adhesion molecules mediate cell-cell and cell-matrix interactions and also function as signal transducers thereby playing fundamental roles in many physiological and pathological processes (1-3). Alterations in the adhesive properties of tumor cells have been implicated in tumorigenesis and in the biological behavior of many tumors (2,3). In human malignant melanoma the development of advanced and metastatic disease is associated with the induction or upregulation of the 113 kD highly glycosylated integral membrane protein, MCAM/MUC18 (CD146). MCAM is predominantly expressed by smooth muscle and vascular endothelium in normal adult tissues (4,5) and has been shown to function as a cell adhesion molecule, mediating cation independent adhesion through interaction with an unidentified ligand (6,7).

MCAM was originally identified as a melanoma associated antigen and is only rarely observed on carcinomas (8,9). In melanoma MCAM is a progression antigen, being strongly expressed only in malignant melanocytic lesions and increasing in frequency and strength with increasing vertical thickness of primary tumors. The strongest expression is observed in metastatic lesions (4,10). Studies in animal models suggest that MCAM may in fact play a role in tumor growth or metastasis formation in vivo. De novo expression of MCAM cDNA conferred metastatic potential, as assessed in experimental metastasis assays, to two independent MCAM negative human melanoma cell lines (11,12). In unrelated experiments, the generation of highly tumorigenic variants by insertional mutagenesis in the slow growing melanoma
cell line WM35 was also associated with an induction or upregulation of MCAM (13). Inasmuch as MCAM expression by melanomas appears to be associated with tumor growth and/or metastatic capacity, it is important to understand how its expression in these cells is regulated. The MCAM 5′ region contains multiple potential binding sites for Sp1 and AP-2 and a CRE (14) and two recent studies have examined the role of these elements in regulating expression in melanomas. Jean et al. (15) reported that constitutive MCAM expression in melanoma cells is due to the loss of the transcription factor AP-2 which represses MCAM promoter activity. Karlen and Braathen (16) were unable to identify a role for AP-2 and concluded that Sp1 is necessary and sufficient to account for constitutive MCAM expression in melanoma cell lines. In the study presented here, approximately 1 kb of the 5′ region of the human MCAM gene has been analysed for promoter activity and for transcription factor binding in 3 MCAM positive and 3 MCAM negative cells lines. Deletion and site specific mutational analyses indicate that Sp1, CRE and AP-2 are all important regulators of MCAM transcription in melanoma cell lines. Functional AP-2 was observed in both MCAM expressing and non expressing cells indicating that MCAM expression does not require the loss of this transcription factor. Furthermore in this larger panel of cells, 5′ region activity as assessed in transient transfection assays did not correlate with MCAM expression. These results indicate that additional elements are involved in regulating MCAM expression in melanoma cells.
EXPERIMENTAL PROCEDURES

Cell culture

Cell lines were purchased from the American Type Culture collection (Manassas, VA), obtained through exchange, or established in our laboratory. All cell lines were cultured in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 1 mM sodium pyruvate, and antibiotics.

Construction of MCAM promoter plasmids

A 934-bp SalI-XmaIII MCAM genomic DNA fragment, isolated from a human leukocyte genomic library (14), in the expression vector pXP-2 (17) was used as a source for all promoter-luciferase constructs (accession number X68264). A SacI restriction site which was necessary for subsequent 5’-3’ unidirectional digestion by Exonuclease III [Erase a base system from Promega, (Madison, WI)] was introduced by polymerase chain reaction (PCR) directly 5’ of the MCAM DNA fragment. The deletion construct mcluc-173 was generated by exonuclease III digestion. All other constructs were prepared by restriction enzyme digestion (mcluc-106, mcluc-62, mcluc-7) or by PCR amplification (mcluc-37 and mcluc-del) with sequence specific oligonucleotides containing the desired restriction sites. Site specific mutations were introduced using mutated oligonucleotides. For some of the mutations (mcluc-mAP2-131, mcluc-mAP2-302) the Quick change system (Strategene, La Jolla, CA) was used and the mutated region was subsequently subcloned into the full length 5’ region construct mcluc-912. Because of the high GC content of the MCAM
promoter, all PCR reactions contained 20% Q solution (Qiagen, Hilden, Germany).

Mutant constructs were produced using the following specific primers: mcluc-CREmut (-32 bp, TGACGTCA to TCAGGACA ) 5'

CAGCTCGAGGCTGCCGGGGGCTGTCCCTGAGGG 3'; mcluc-mAP2-23 (-23bp, CCCCCCGCC to TTCCCTTC in the context of the deletion construct mcluc-106)

5’CAGCTCGAGGCTGAAGGGAAGTAACGTACGTACGTCG 3'; mcluc-mAP2+14 (+14bp, CCTCCCGGC to TTTCTTTC ; 5’GCTCAGATCTCCGGGAAGGAAAGCGAGAGCGAGGC-3’)

mcluc-mAP2-131 (-131bp, CCGGAGCCCG to CTGTGCCCCG; 5’-

GCTGGCGGCGCCTGTGCCGCCCCCTAGGGCT-3’ and 5’-

AGCCCTAGGGGCGCAGCAGCCCGCCCGAGCC-3’), mcluc-mAP2-302 (-302bp, CCGGAGCCCG to CTGGCT; 5’-

GGCTGCTTCGACTCGGCTCAGCTCGGCGGCATCC-3’ and 5’-

GGATGCCCGAGGAGCTCGAGGCGAGGCTCGAGGAGCGCAAGCCAGGC-3’). All constructs used in this study were the intended deletions and mutations were introduced.

**Transient transfection**

For transfection of the reporter constructs, the FuGENE6 transfection reagent (Roche Molecular Biochemicals, Mannheim, FRG) was used according to the manufacturer’s recommendations. 2.5 x 10^5 cells were transfected with 3 ul FuGENE6 reagent and 1 µg DNA and assayed 48 h later using luciferase lysis and assay reagents from Promega. 20 µl cell lysate was measured with 100µl luciferase assay reagent. Expression of luciferase in cells transfected with the promoterless pXP-2 vector and the pXP-2 plasmid containing the RSV promoter, were used as
negative and positive controls, respectively.

To standardize the measured relative light units (RLU), the protein content of the lysates was determined using the Biorad protein assay reagent (Biorad, Hercules, CA) and luciferase activity was presented as RLU/µg protein. Transfections were carried out in triplicate and at least three independent experiments were performed, using different batches of DNA. DNA was prepared using the JETSTAR 2.0 Plasmid kit (Genomed Inc., Bad Oeyenhausen, FRG).

Preparation of nuclear extracts

Nuclear extracts were prepared according to a modified method of Schreiber et al. (18). Approximately 5 x 10^7 cells were washed 3x in PBS and lysed in 2 ml low salt buffer A (10 mM Hepes-KOH pH 7.6, 15 mM KCl, 2 mM MgCl2, 0.1 mM EDTA pH 8.0, 1 mM dithiothreitol, complete mini EDTA free protease inhibitor cocktail tablet (Roche)) +1% NP40. Nuclei were pelleted by centrifugation for 5 min at 2200 rpm, 4°C, and washed once with 2 ml buffer A+NP40. 300 µl of buffer C (25 mM Hepes-KOH pH 7.6, 50 mM KCl, 0.1 mM EDTA pH 8.0, 10% glycerol, 1 mM dithiothreitol, Complete Mini EDTA free protease inhibitor cocktail) was added to 500 µl pellet volume. The salt concentration of the nuclear suspension was adjusted to 0.4 mM NaCl and nuclear protein was extracted on ice for 45 - 60 min. Debris was pelleted by centrifugation and the supernatants aliquoted and stored at -80°C. Protein concentrations were determined using the Bio-Rad protein assay reagent.

Electrophoretic mobility shift assays (EMSA)
CRE binding reactions were performed at RT for 30 min in a total volume of 15 µl containing 5 µg nuclear extract and 10,000 cpm of ds MCAM specific oligonucleotide (5′CCCCTGACGTCAGCC3′) in binding buffer [120 mM NaCl, 10 mM Tris-Cl pH 7.5, 5% glycerol, 5 mM DTT, 1 mM EDTA, 1 mM Pefabloc (Sigma, St. Louis, MO), 2 µg poly (dl-dC)]. For supershift assays, proteins were incubated with DNA for 20 min and anti-CREB-1 antibody (1 µl) or control serum (1 µl) added, for a further 20 min (RT). Sp1 binding reactions were carried out on ice for 30 min in a 40 µl reaction volume containing 5 µg nuclear extract and 40,000 cpm labeled MCAM specific oligonucleotide (5′CCTCCCCGCCCCCCC3′) in binding buffer (20 mM Hepes-KOH pH 7.9, 12% glycerol, 0.5 mM dithiothreitol, 5 µg BSA, 1 µg poly (dl-dC), 50 mM KCl, 1 mM MgCl2, 0.5 mM Pefabloc). In assays with antibodies, the standard binding reaction was performed for 15 min on ice and anti-Sp1 antibody (2 µl) or control serum (2 µl) was added and incubation was continued for another 25 min.

AP-2 mobility shift reactions contained 17 µl of the premixed incubation buffer (Stratagene, La Jolla, CA), 50,000 cpm oligonucleotide (5′GAACTGACAGCCCCCGGCAGCCCG3′, MCAM specific sequence is underlined) and 10 µg nuclear extract in a reaction volume of 25 µl. The reaction was incubated for 20 min on ice. For supershift reactions, antibodies (4 µl anti-AP-2 or 4 µl control serum) were added for the last 20 min of a 40 min incubation of protein with DNA. Double stranded MCAM specific oligonucleotides containing the CRE-, Sp1- and AP-2 sites in the core promoter were end labeled with γ-32P using T4- Polynucleotide-kinase (New England Biolabs, Beverly, MA). Nonlabeled competitor oligonucleotides, when used, were added at the same time as the labeled oligomers. Antibodies to
transcription factors and the oligonucleotides containing the consensus recognition motifs used in EMSA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant AP-2 was purchased from Promega. The contents of the binding reactions were electrophoresed at room temperature on a 4% nondenaturing polyacrylamide gel in 0.25 x Tris-Borate-EDTA running buffer for 2-3 h. Gels were dried and exposed to x-ray film.
**Immunoprecipitation and western blotting**

For MCAM western blotting, cells were lysed in PBS containing 1% NP40 (Calbiochem, San Diego, CA) and protease inhibitors. The protein content of the samples was measured using the Lowry protein assay (Biorad). 0.5 mg of protein lysate was precleared by overnight incubation with horse serum-saturated Sepharose. After preclearing, the samples were exposed to anti-MCAM MoAb MUC18BA.3, bound to Protein G Agarose (Sigma), overnight at 4°C. The bound material was eluted from the washed immunosorbents (95°C, 5 min), separated on a 7.5% SDS-PAGE gel under reducing conditions and electrophoretically transferred to nitrocellulose membranes. The filters were blocked for 1h in 5 % skim milk in PBS and then incubated for 1 h with MoAb MUC18BA.3 followed by horseradish peroxidase-linked rabbit anti mouse immunoglobulin (anti-IgM+IgG) 1:5000 (Dako, Hamburg, FRG). The membranes were washed in PBS + 0.1% Tween and the bound antibody was visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

For the detection of AP-2 protein, 30 µg of nuclear extract was separated on a 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 1% casein in PBS and incubated with anti-AP-2 serum (C-18, 1:500, Santa Cruz Biotechnology) followed by horseradish peroxidase-linked goat anti-rabbit immunoglobulin 1: 8000 (Dako).
**RT-PCR for mRNA detection**

Total RNA was isolated using the RNeasy extraction kit from Qiagen. The RNA preparation was subjected to DNaseI digestion and phenol extraction followed by RNA precipitation with 100% ethanol overnight at -20°C. 5 µg of total RNA was used for reverse transcription (RT)-polymerase chain reactions (PCR). First strand cDNA synthesis was performed in a reaction volume of 20µl using Superscript II Reverse Transcriptase (Life Technologies, Paisley, UK) according to the manufacturer's instructions. 5% of the first strand reaction was used for PCR. Samples were subjected to 35 rounds of temperature cycling (94°C for 35 s, 58°C for 55 s, 70°C for 50 s).

AP-2 α cDNA-specific primers (5’GTTACCCTGCTCACATCACTAGTAG-3’ and 5’GGCCTCGGTAGATAGTTCTGCAG-3’) are expected to yield a 359 bp fragment. AP-2B cDNA-specific primers (5’GTTACCCTGCTCACATCACTAGTAG-3’ and 5’GGAAGAAGGATGGAGGTATAGGAG3’) are expected to yield a 223 bp fragment.
RESULTS

The MCAM 5’ region contains no TATA or CAAT boxes, but instead an initiator sequence surrounding the transcriptional start 29 bp upstream of the first ATG (14). The 150 bp region immediately upstream of the initiator is highly GC-rich and contains five potential recognition sequences for the transcription factor Sp1 (-41/-36bp, -72/-67bp, -77/-72bp, -100/-95bp and -125/-119bp), two for AP-2 (-23 bp, -131 bp) and a consensus sequence motif for the cAMP response element at position -32. Additional AP-2 elements are found further upstream at -302, -505 and downstream at position +14. At positions -208 and -591 CArG-box motifs (5’CC (A or T)6GG) are present and at position -236 is a binding element for the transcription factor MyoD. In order to examine the role of elements in the 5’ region of the MCAM gene in regulating tissue restricted expression, 934bp (-912/+22bp) of this region were fused to the promterless firefly luciferase reporter gene. This construct as well as serially 5’ truncated fragments and constructs carrying deletions and site directed mutations (Fig 1a) were assessed for activity by transient transfection in a panel of cell lines. The cell lines examined consisted of four melanomas, a glioma and a colon carcinoma. MCAM expression in these cell lines as determined by Western blotting is shown in Fig. 1b. MCAM specific bands at 113 kD and at 100 kD can be seen in the melanoma cell lines MelJuSo (lane 1) and WM2664 (lane 2) and in the glioma cell line LN215 (lane 6), but not in the melanoma cell lines SB-2 (lane 3) and Mel888 (lane 4) or in the carcinoma cell line Colo320 (lane 5). This MCAM expression pattern was confirmed using immune fluorescence and RT-PCR (data not shown).
The luciferase activity driven by the 934 bp MCAM 5' region (mcluc-912), in each of these cell lines is shown in Fig.1c. The promoterless plasmid pXP-2 was used as negative control and luciferase activity is presented as RLU/µg protein. Expression ranged from 800 fold (SB-2) to $10^4$ fold (Mel888) over that observed for pXP-2. Luciferase activity driven by the RSV promoter was higher but showed a similar expression pattern (data not shown), suggesting that the differences observed between the cells reflect differences in transfection efficiencies. The MCAM full length construct is strongly expressed in both MCAM expressing and MCAM non expressing cells indicating that MCAM expression is not solely determined by the transactivating factors which bind to this region.

The activity of a series of deletion constructs was similar in all cells and results are presented for LN215, Mel888 and WM2664 (Fig. 2). In these studies the luciferase activity of the various constructs is presented in relation to that of the full length mcluc-912 which was set at 100%. The expression of construct mcluc-del bearing an internal deletion of 151 bp directly upstream of the transcriptional start, was less than 10% of that observed with the intact 5' region (mcluc-912), indicating a critical role for this region in MCAM transcription. The luciferase activity driven by constructs mcluc-173, consisting solely of the deleted region plus the initiator, and mcluc-106 were similar to (LN215) or higher than (Mel888, WM2664) that observed with the full length 912bp region. These observations indicate that all of the information required for optimal promoter activity is present in the first 128 base pairs of the MCAM 5' region. Further deletion of Sp1 sites (mcluc-62, mcluc-37) reduced promoter activity.
to less than 20% of the full length clone indicating an important role of Sp1 as a transcriptional activator of MCAM expression. The additional deletion of the putative CRE (-32 bp) and the AP2 site at -23 bp (mcluc-7) abolished the remaining activity in all examined cell lines.

The minimal region of the MCAM promoter required to drive transcription, is therefore included in the 128 bp region from -106bp to +22bp. This region contains 4 Sp1 sites, a CRE site, two AP2-binding sites and the initiator surrounding the transcription start.

DNA mobility shift assays, using an MCAM specific DNA fragment (-47bp/-33bp) containing the proximal Sp1 site produced a double band DNA-protein complex with nuclear extracts from all 6 cell lines. As shown for the cell line MelJuSo (Fig.3a), this complex was abolished by an excess of unlabeled probe (lane 2, 3) and by addition of unlabeled Sp1 consensus oligonucleotide (lane 4), but not by mutated Sp1 sequence (lane 5) or by an oligonucleotide containing a binding site for the CREB/ATF family (lane 6). An antibody directed against Sp1 specifically blocked formation of the DNA-protein complex (Fig 3b, lane 2) indicating that the protein binding to this site is immunologically related to Sp1.

**The cAMP-responsive site is an important element of the MCAM core promoter**

To assess the role of the cAMP responsive element at -32 bp in the regulation of basal MCAM expression, this site was mutated in the context of the full length construct and tested in transient transfection assays. As shown in Fig.4a. mutation of
the CRE site reduced reporter activity by approximately 70% compared to mcluc-912 in five of the six tested cell lines indicating that this site plays an important role in the MCAM core promoter. Interestingly no reduction of luciferase expression with the CRE mutant could be observed in the cell line WM2664. DNA shift analyses were performed with a MCAM specific oligonucleotide including this motif (-36bp/-22bp). A complex consisting of a double band was detected in nuclear extracts from all 6 cell lines (Fig 4b). Binding was abrogated by an excess of unlabeled oligonucleotide (lane 2) and by an excess of consensus CRE oligonucleotide (lane 3), but not by addition of a mutated CRE oligonucleotide (lane 4) or an AP-2 binding motif (lane 5). Identical results were obtained with nuclear extracts from WM2664 and MelJuSo. The observed DNA-protein complex could be partially supershifted with anti CREB-1 antibody (Fig.4c lane 2) indicating that one of the proteins binding to this sequence is CREB1. Although specific protein binding to the CRE site was also shown for the cell line WM2664 this regulatory element seems not to be as important in mediating transcriptional activation as in the other cell lines examined.

**The core promoter AP-2 site acts as a negative element while upstream AP-2 sites act as positive elements**

A perfect consensus AP-2 binding site is located at -23 bp within the MCAM core promoter. To test the role of this site in MCAM transcription it was mutated in the construct mcluc-106, which still shows optimal promoter activity. Luciferase activity driven by mcluc-106 and by the AP-2 mutant (mcluc-mAP2-23) were compared in the six cell lines. As seen in Fig. 5a, mutation of this AP-2 site increased luciferase
expression 2.5 - 5 fold in all four melanoma cell lines indicating that this AP-2 site acts as a negative element in basal expression. The AP-2 mutation had no effect on reporter activity in the carcinoma cell line Colo320 or in the glioma cell line LN215. Gel retardation assays were performed using an oligonucleotide containing the MCAM specific AP-2-23 binding site. This probe reacted with nuclear extracts from all the melanoma cell lines (Fig. 5b lanes 1, 6, 7, 10). This binding is specific as it was abrogated by an excess of unlabeled oligonucleotide (lane 2) and AP-2 consensus binding motif (lane 3), but not by a mutated AP-2 motif (lane 4) or by a Sp1 binding motif (lane 5). No complex formation was detected in the glioma cell line LN215 (lane 8) or in the carcinoma cell line Colo320 (lane 9), observations which are consistent with results of the reporter assays. Antibody supershift experiments identified AP-2α as the major regulatory factor interacting with the promoter proximal AP-2 binding site in the MCAM 5' region. As Fig. 5c. shows, anti-AP-2α antibody supershifted the DNA-protein complex (lane 2) while the control serum did not (lane 3). Incubation of recombinant human AP-2 (rh-AP-2) with the MCAM specific probe (lane 7) led to formation of a similar complex that was also supershifted with anti-AP-2α antibody (lane 8). Addition of an anti-AP-2β antibody (lane 4), or the same amount of control serum (lane 6) to the nuclear extracts did not alter the DNA-protein complex formation. However an anti-AP-2γ antibody resulted in a faint supershifted band suggesting that AP-2γ participates in the binding at this site.

In light of the observation that mutation of the AP-2 binding site at -23bp led to an increase in reporter gene activity, the putative AP-2 binding sites at +14, -131 and -302 were also individually mutated and examined for activity in Mel JuSo. As shown
in Figure 5d, mutation of the AP-2 binding site at +14 did not significantly affect the activity of the MCAM promoter, while mutation of the sites at -131 or -302 reduced promoter activity (by 70% and 44% respectively).

Mutations in the AP-2 binding sites led to changes in transcriptional activity only in the melanoma cells. The fact that AP-2 binding was also only observed in these cells, raises the question of whether or not AP-2 is expressed in the glioma and carcinoma cell lines. In order to determine this, all of the cell lines were examined for AP-2 expression using RT-PCR and Western blotting.

As shown in Fig.6a, AP-2α mRNA expression was observed in all cells except the carcinoma cell line Colo320 (lane 6). Compared to the melanoma cells, the glioma cells appear to express a lower level of AP-2 mRNA. RT-PCR analyses for AP-2B mRNA, a dominant-negative acting splice variant, indicated that it is expressed in all cells except the carcinoma cell line Colo320 (Fig.6d). Expression of AP-2 protein was analyzed by Western blot. 30µg nuclear protein were analyzed from each cell and the position of the AP-2 band was determined from the migration of recombinant AP-2 (Figure 6b, lane 1). Incubation of an identical blot with control serum indicated that the smaller band is not AP-2 related (data not shown). AP-2 protein was observed in all four melanoma cell lines (lane 3 - 6) but was undetectable in the glioma cell line LN215 (lane 7) and in the carcinoma cell line Colo320 (lane 2). The observed lack of AP-2 binding in LN215 and Colo320 can therefore be explained by the absence of detectable AP-2 protein. The highest AP-2 expression appears to be in the MCAM positive cell line WM2664 and in the MCAM negative cell line Mel888. These results show that the transcription factor AP-2 is expressed at similar levels in
MCAM positive and in MCAM negative melanoma cells.
DISCUSSION

Expression of the cell adhesion molecule MCAM/MUC18 is characteristic of malignant melanomas where it increases in frequency and strength with increasing tumor growth and progression to metastatic disease. In light of the evidence that MCAM may actually play a role in tumor growth or metastasis formation in vivo (11-13), an understanding of how expression of this molecule is regulated is of great importance. In this study transient transfection, site-directed mutagenesis and transcription factor binding were used to define the major regulatory elements present in the 5’ region of the human MCAM gene. The MCAM core promoter was shown to comprise the region from -106bp to +22bp containing 4 Sp1 sites, 1 cAMP response element and two AP-2 sites near the initiator motif containing the transcriptional start (14). Using oligonucleotide competition experiments Karlen and Braathen (16) could demonstrate only Sp1 binding to the MCAM promoter and concluded that this transcription factor was sufficient to drive constitutive MCAM expression in melanomas. Confirmation that Sp1 is an important regulator of MCAM expression is seen here in the results of deletion analysis where removal of all putative Sp1 sites reduced the promoter activity by 80%. However, site directed mutagenesis also revealed important regulatory roles for the CRE element at -32 and for several of the putative AP-2 binding sites in the majority of cells studied, and CREB-1 and AP-2 were shown to specifically bind to the MCAM promoter in these cell lines. Mutation of the CRE element which was shown to bind CREB-1, reduced promoter activity by 70% in 5 of the 6 cells examined. A role for CRE in regulating constitutive MCAM expression was suggested by Xie et al (19) who observed that
transfection of the melanoma cell line MeWo with a dominant negative CREB mutant led to downregulation of MCAM expression. The CRE element does not play a crucial role in MCAM expression in all cells inasmuch as mutation of this element did not effect basal MCAM transcriptional activity in the melanoma cell line WM2664. MCAM expression can be upregulated by exposure to elevated cAMP levels (9), suggesting that this CRE element may also mediate inducible MCAM expression. A dual role of the CRE in promoting basal and inducible transcription has been described for other genes (20,21).

Site-directed mutation of the AP-2 sites at +14bp, -23 bp, -131bp and -302bp suggested that this transcription factor influences MCAM promoter activity in a complex manner. While mutation of the site at +14bp had no effect on promoter activity, mutation at -23bp increased reporter activity up to 5 fold and mutations at -131bp and -302bp decreased reporter activity by 70% and 55% respectively in melanoma cell lines. The activating role of the AP-2 site at -302bp is also apparent in the analysis of the deletion constructs as deletion of the region between -341 and -258bp leads to a reduction in transcriptional activity only in AP-2 positive cells (data not shown). The reduction in reporter activity between mcluc-173 and mcluc106 is observed in all cells suggesting that positively and negatively acting elements are present in this region. Functional AP-2 binding sites mediating transcriptional activation are frequently found upstream of the core promoter region (22,23) while AP-2 sites localized within the core promoter have been shown to mediate repression in a variety of genes (24,25). EMSAs and supershift analyses indicated specific binding of AP-2 complexes (predominantly AP-2α homodimers) to
the element at -23bp. The AP-2 mutations had no influence on reporter activity
assessed in the glioma or carcinoma cells lines and no AP-2 binding complexes
were observed in either cell. Both cells were shown by Western blot and RT-PCR
analysis to lack AP-2 expression.

The use of a larger panel of MCAM positive and MCAM negative cell lines in the
present study has led to conclusions which differ in several respects from previously
reported studies. Although Karlan and Blaathan (16) did not show that the conditions
used for their EMSAs were permissive for AP2 and CREB binding, their failure to
observe binding of these factors could also be due to the cell line (Sk Mel 2) studied.
Our examination indicates that the CRE and AP2 binding sites do not contribute to
basal MCAM expression in all cell lines. Previous reports also indicated that the
activity of the 900 bp MCAM 5’ region as assessed by transient transfection of CAT
reporter constructs correlates with MCAM expression (15,16). In both of these
studies, the melanoma cell line SB-2 was the sole MCAM negative cell examined. In
the present study of 3 MCAM positive and 3 MCAM negative cell lines, no correlation
could be observed between reporter activity driven by the 5’ region (-912bp or
deletion constructs) and the MCAM expression status of the cell lines. The reporter
activity was similar (10^3 - 10^4 over vector alone) in all lines tested. Although the
activity of this region was in fact lowest in the SB-2 cell line, it was highest in the
melanoma cell line Me888 which is also MCAM negative (Figure 1b). These results
suggest that the expression of MCAM is not solely dependent on the constellation of
trans-acting factors binding to the 900 bp MCAM 5’ region studied. The elements
relevant for repression of MCAM expression in MCAM negative cells may be located
elsewhere or need to be embedded in chromatin to accurately reflect MCAM expression.

Jean et al. reported an inverse correlation between AP-2 expression and MCAM expression in melanoma cell lines and have proposed that the presence of this transcription factor represses MCAM expression. While the results presented here support a negative effect of AP-2 binding at -23bp on MCAM promoter activity, all AP-2 expressing cells examined (Mel JuSo, WM-2664, SB2, Me888) did nevertheless show strong activity of the full length MCAM 5′ region. This may reflect a counter balance of the more upstream AP-2 sites, two of which have been shown to have an activating effect on MCAM expression. Furthermore Mel JuSo and WM-2664 express high levels of MCAM mRNA and protein and are tumorigenic in nude mice (15; B. Jansen, personal communication), despite the presence of significant levels of AP-2 protein. That the AP-2 present in the MCAM positive cells is functional is evident from gel retardation experiments and from the altered reporter gene expression observed when the AP-2 sites at -23bp, -131bp or -302bp are mutated. These results do not rule out a role for AP-2 in the regulation of MCAM expression but clearly indicate that AP-2 by itself cannot explain the constitutive MCAM expression in melanoma cell lines. Furthermore they indicate that expression of MCAM by melanoma cells does not require the loss or downregulation of the transcription factor AP-2.
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FOOTNOTES

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Abbreviations:
The abbreviations used are: MCAM, melanoma cell adhesion molecule; CRE, cAMP responsive element; CREB-1, cAMP responsive element binding protein-1; PBS, phosphate buffered saline; RLU, relative light units; rh, recombinant human; EMSA, electrophoretic mobility gel shift assay; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; ds, double stranded; MoAb, monoclonal antibody; RSV, rous sarcoma virus.
FIGURE LEGENDS

Fig. 1 Characterization of the cell lines and reporter constructs

(A). Schematic representation of the MCAM/MUC18 5’ region and of the constructs used in this study. Numbering is in relation to the transcription start site (+1). Putative transcription factor binding sites are indicated. Boxes to the right represent the luciferase gene. Small black boxes indicate point mutations. Dotted line indicates deleted region. Note that the sizes are not drawn to scale. Inr=initiator

(B). MCAM/MUC18 expression in melanoma and non melanoma cells. MCAM was immunoprecipitated from 0.5 mg protein, separated by SDS-PAGE and detected by Western blotting. Expression of MCAM protein was examined in the MCAM-positive melanoma cell lines MelJuSo (lane 1) and WM2664 (lane 2), in the MCAM-negative melanoma cell lines SB-2 (lane 3) and Mel888 (lane 4), in the MCAM positive glioma cell line LN215 (lane 6) and in the MCAM negative colon carcinoma cell line Colo320 (lane 5). MCAM specific bands at 113 kD and at 100 kD are indicated by an arrow, as is the heavy chain of the precipitating antibody.

(C). Activity of the MCAM/MUC18 5’ region in MCAM positive and negative cell lines. Reporter assays were performed as described in Experimental Procedures. Luciferase activity is shown in relative light units (RLU) per microgram of protein. Results represent mean and standard deviation of 3 independent experiments. Expression driven by the construct mcluc-912 is shown in black bars and that driven by the promoterless vector pXP-2 in gray.
**Fig. 2. Definition of the MCAM core promoter region** The indicated reporter constructs were transiently transfected as described in Experimental Procedures. Results are shown for LN215, Mel888 and WM664 cells. Results are presented as percent luciferase activity relative to the activity of the full length construct mcluc-912 which was set at 100%. The results are presented as the mean and standard deviation of 3 independent experiments.

**Fig. 3. Sp1 binds to the MCAM core promoter**

(A). EMSA indicating specific binding to an Sp1 element within the MCAM core promoter. 5µg of nuclear extract from the cell line MelJuSo was incubated with a radiolabeled MCAM specific Sp1-DNA fragment (-47 bp/-33 bp) in the absence (lane 1) or presence (lanes 2-6) of unlabeled competitor oligonucleotides. Lanes 2,3: MCAM specific Sp1; lane 4: Sp1 consensus; lane 5: mutated Sp1; lane 6: CRE motif oligonucleotide.

(B). Antibody depletion identifies Sp1 as the transacting factor binding to the MCAM Sp1-site. Specific DNA-protein complexes in the cell line MelJuSo (lane 1), were incubated in the presence of anti-Sp1 antibody (lane 2), or normal rabbit serum (lane 3). Probe alone (lane 4).

**Fig. 4 The CRE at –32 bp is required for optimal expression of the MCAM gene**

(A). Effects of mutation in the MCAM cAMP responsive element on reporter activity. Luciferase activity driven by the CRE-mutant (mcluc-CREmut, black bars) was
compared to that of the unmutated full length construct mcluc-912 (set to 100%; gray bars) in the cell lines Mel888, SB-2, LN215, Colo320, MelJuSo and WM2664. The results represent mean and standard deviation of 3 independent experiments.

(B). EMSA gel to determine specific protein binding to the CRE-element.

5µg of nuclear extract from the cell line WM2664 was incubated with MCAM specific CRE oligonucleotide (-36/-22 bp) in the absence (lane 1) or presence (lanes 2-5) of unlabeled competitor oligonucleotides. Lane 2, CRE-MCAM; lane 3, CRE consensus; lane 4, mutated CRE motif; lane 5, AP-2 binding motif. The formation of this specific double band (arrow) was also observed in the cell lines LN215 (lane 6), Mel888 (lane 7), SB-2 (lane 8), Colo320 (lane 9) and MelJuSo (lane 10).

(C). Immunochemical identification of the CRE binding factor.

EMSA were performed with the MCAM specific CRE oligonucleotide and 5µg of nuclear extract from the cell line MelJuSo alone (lane 1) or in the presence of anti-CREB-1 antibody (lane 2) or normal rabbit serum (lane 3). The supershifted specific complex is indicated by an arrow.
Fig. 5. AP-2 sites act as negative and positive regulators of MCAM expression in melanoma cells

(A). Mutation of AP-2 within the MCAM core promoter leads to enhanced reporter activity in melanoma cells. Luciferase expression (shown in per cent activity) driven by the AP-2 mutant (mcluc-mAP2-23; black bars) compared to that of the unmutated construct mcluc-106 (set at 100%; gray bars) in the cell lines MelJuSo, WM2664, SB-2, Mel888, Colo320 and LN215. Results are presented as mean and standard deviation of 3 independent experiments.

(B). Detection of nuclear factors binding to the AP-2 site at -23 bp
A radiolabeled probe containing the MCAM specific AP-2 site (~23 bp) was incubated with 10 µg nuclear extract from SB-2 cells in the absence (lane 1) or presence (lanes 2-5) of competitor oligonucleotides. Lane 2, MCAM-AP-2 oligonucleotide; lane 3, AP-2 consensus; lane 4, mutated AP-2; lane 5, Sp1 oligonucleotide; The specific complex is indicated by an arrow. Binding was also examined with nuclear extracts (10 µg each) from the cell lines MelJuSo (lane 6), WM2664 (lane 7), LN215 (lane 8), Colo320 (lane 9) and Mel888 (lane 10).

(C). AP-2 binds to the AP-2 element at –23 within the MCAM core promoter. 10 µg of nuclear extract from SB-2 cells were incubated with radiolabeled MCAM-AP-2 oligonucleotide alone (lane 1) or in the presence of anti-AP-2α antibody (lane 2), control normal rabbit serum (lane 3), anti-AP-2β antibody (lane 4), anti-AP-2γ antibody (lane 5) or with control normal goat serum (lane 6). 40 ng of human
recombinant AP-2 protein was incubated with the MCAM specific AP-2 probe in the absence (lane 7) or presence of anti-AP-2α antibody (lane 8).

(D). Mutation of upstream AP-2 sites reduces MCAM reporter activity in melanoma cell lines. Luciferase expression (shown in per cent activity) driven by the AP-2 mutant constructs compared to that of the unmutated full length construct mcluc-912 (set at 100%) in Mel JuSo. Results are presented as mean and standard deviation of 5-8 independent experiments.

Fig. 6. AP-2 expression in melanoma and non melanoma cells.

(A) RT-PCR analysis. RT-PCR for the detection of the AP-2α specific transcript in the cell lines MelJuSo (lane 1), WM2664 (lane 2), Mel888 (lane 3), SB-2 (lane 4), LN215 (lane 5) and Colo320 (lane 6). AP-2α specific primers generated a 359-bp product (arrowhead) as described in Material and Methods. Detection of AP-2B specific transcripts in the cell lines MelJuSo (lane 7), WM2664 (lane 8), Mel888 (lane 9), SB-2 (lane 10), LN215 (lane 11) and Colo320 (lane 12). AP-2B specific primers yield a 223 bp DNA-fragment (arrowhead) as described in Material and Methods.

(B). Western blot analysis of AP-2 protein expression. 30 ug of nuclear extract per lane from Colo320 (lane 2), MelJuSo (lane 3), WM2664 (lane 4), SB-2 (lane 5), Mel888 (lane 6) and LN215 cells (lane 7). 10 ng of recombinant human AP-2 was used as a positive control (lane 1). The AP-2 specific band at 52 kD is marked by an
arrow.
| SB-2 | none | 132x AP-2 MCAM | 77x AP-2 consensus | 64x AP-2 mutant | 142x Sp1 |
|------|------|----------------|--------------------|-----------------|---------|
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- **MelJuSo**: Lane 6
- **WM2664**: Lane 7
- **LN215**: Lane 8
- **Colo320**: Lane 9
- **Mel888**: Lane 10

**AP-2** band indicated by arrow.
Identification of the elements regulating the expression of the cell adhesion molecule MCAM/MUC18: Loss of AP-2 is not required for MCAM expression in melanoma cell lines

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