Characterization of the MN/CA 9 promoter proximal region: a role for specificity protein (SP) and activator protein 1 (AP1) factors

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MN/CA IX (MN) is a tumour-associated isoenzyme of the carbonic anhydrase family. Previous deletion analysis of the MN promoter established that protected regions (PRs) 1 and 2 are crucial for its transcriptional activity. Computer-assisted searching indicated putative binding sites for activator protein (AP) 2 and specificity protein (SP) 1 transcription factors, plus a CACCC box in PR1 and an AP1 site in PR2. PR1 produced four complexes in electrophoretic mobility-shift assay (EMSA) with HeLa nuclear extracts. Of these, three were completely competed with the SP1 and transforming growth factor-β retinoblastoma control-element CACCC box (RCE) probes, whereas the AP2 probe competed against the same three complexes partially. Supershift EMSA identified SP1 in the complex 1 and SP3 in the complexes 2 and 4. Point mutations in the SP1 site abrogated the PR1 function, while mutations affecting the overlapping CACCC box/AP2 site in PR1 had minor effect on MN promoter activity. Block-replaced MN promoter mutants that had a consensus binding site (SP1 or AP2) or the RCE in place of PR1 demonstrated the stringent selectivity of the PR1 position as only the SP1 mutant reconstituted the MN promoter activity. The consensus SP1 probe generated the same SP1 and SP3 complexes as PR1 in EMSA; therefore we conclude that SP activity is both necessary and sufficient in the PR1 position. The critical role of AP1 in the PR2 position was confirmed by supershift of the PR2 complex with c-Fos antibody and markedly decreased activity of the construct with a mutated AP1 site. Detailed deletion analysis proved that PR1+PR2 account for 90% of the MN promoter activity, while neither PR1 nor PR2 on their own are sufficient for transactivation. Thus, synergistic co-operation between SP and AP1 factors bound to the adjacent PR1 and PR2, respectively, is necessary for MN transcriptional activity. The PR1+PR2 module also stimulated transcription from a heterologous promoter. The modulation of AP1 activity with PMA stimulated MN expression and activated the MN promoter, whereas inhibition of protein kinase C activity had no effect on MN expression in HeLa cells.

Key words: mutagenesis, SP1, SP3, transcriptional analysis.

INTRODUCTION

MN/CA IX (MN), classified as an isoenzyme of the carbonic anhydrase (CA) family [1], is strongly associated with tumours. To date, MN has been found in cervical carcinomas and cervical intra-epithelial squamous and glandular neoplasia [2], renal cell carcinoma [3], oesophageal tumours [4], colorectal tumours [5] and non-small cell lung cancer [6], but not in the corresponding normal tissues. MN was also detected in diverse carcinoma-derived cell lines [7]. Until recently, the only normal tissues known to express MN were of gastrointestinal origin, e.g. the gastric, intestinal and biliary mucosa [8], and the pancreas [9]. However, a recent detailed study revealed a more wide-spread MN expression in normal tissues. In addition to gastrointestinal tissues, samples prepared from testis, ovary, basal cells of hair follicle, choroid plexus and cells lining body cavities also stained positively for MN expression [10]. In HeLa cells, MN expression is density-dependent *in vitro*: the protein is absent in sparse, rapidly proliferating cells and its synthesis is induced in dense, overcrowded cultures [1].

MN is a transmembrane glycoprotein that displays CA activity, binds zinc and can be detected on Western blots as twin bands of 54 and 58 kDa [1]. Although the exact role of CA activity in carcinogenesis is not known, recently it was hypothesized that tumour-associated transmembrane isoenzymes (MN and CA XII) may play a role in acidification of the extracellular milieu surrounding cancer cells, thus facilitating tumour growth and spreading [11]. The significance of CA activity for the invading capacity of renal cancer cell lines was confirmed by experiments *in vitro* with acetazolamide, a potent CA inhibitor [12].

The *MN* gene consists of 11 exons and covers about 10 kb. The molecular organization of its coding region corresponds to the proposed domain composition of MN protein [13]. Due to unique N- and C-terminal domains, *MN* is considered to be a chimaeric gene, assembled by exon shuffling [13].

Functional analysis of the *MN* upstream region led to the definition of the MN promoter in the -173 to $+31$ region [14]. Within the promoter five protected regions (PRs) were detected; of these, PR4 was confirmed to function as a promoter-, position- and orientation-independent silencer. In comparison with non-tumorigenic CGL1 cells, significantly decreased levels of the PR4-binding repressor could be responsible for MN up-regulation in tumorigenic CGL3 cells [14]. The remaining four PRs were identified as positive-acting *cis* elements. Removal of either PR1 or PR2 led to a dramatic decrease of promoter activity; therefore it was proposed that synergistic co-operation

Abbreviations used: AP, activator protein; SP, specificity protein; BIM, bis-indolylmaleimide; CA, carbonic anhydrase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; HRE, hypoxia-response element; HSV tk, herpes simplex virus thymidine kinase; MN, MN/CA IX; NE, nuclear extract; PKC, protein kinase C; PR, protected region; RCE, transforming growth factor-β retinoblastoma control-element CACCC box; VEGF, vascular endothelial growth factor.

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between transcription factors bound to these *cis* elements is essential for *MN* transcriptional activity [14].

Recent studies revealed a hypoxia-response element (HRE) in the MN promoter [15]. Tight regulation of this element by the hypoxia-inducible factor/von Hippel–Lindau factor system was reflected in the pattern of MN expression within tumours [15].

The relation between the p53 tumour suppressor and MN expression was investigated in two cell lines. There was no effect of up-regulated p53 on MN expression in HeLa cells [16]. In MaTu cells, however, up-regulated endogenous or ectopically expressed wild-type p53 had a potent repressing activity on the MN promoter, whereas the V143A p53 mutant had a modest stimulatory effect [17].

While PR1 generated multiple bands when probed with HeLa nuclear extracts (NEs) in electrophoretic mobility-shift assay (EMSA), PR2 produced a single band [14]. Confirming the observed complex-binding propensity of PR1, computer-aided searching indicated putative binding sites for multiple transcription factors in this sequence. Because of the crucial role of PR1 and PR2 in *MN* transcriptional activity, we studied further the transcription factors that bind to these positions. In this study we investigated transcription factors binding PR1 by competition and supershift EMSA and by point and block-replacement mutagenesis. The PR2-binding factor was characterized further by supershift EMSA and point mutations. Activity of the PR1 + PR2 module was tested on a heterologous promoter. Effects of pharmacological modulators of activator protein (AP) 1 and protein kinase C (PKC) activity were tested on the levels of endogenous MN and MN promoter.

MATERIALS AND METHODS

Enzymes, kits and reagents were used according to the manufacturers' recommendations.

Plasmids

Numbers in parentheses indicate position relative to the *MN* transcription start site [13]. pBMN5 contains the −173 to +31 *MN* fragment in pBLCAT6 [14]. Preparation of pBMN5 derivatives with internally deleted PR1 (−PR1) or PR2 (−PR2) was described previously [18].

PR1AP2mut, PR1SP1mut and PR2AP1mut constructs were pBMN5 derivatives containing mutations in PR1 and PR2 (Table 1), and were prepared by splicing with the overlap-extension procedure [19] on the −173 to +31 *MN* fragment in pBKS (Stratagene). The primary and secondary PCRs (25 μl), containing 20 μM of each primer, 0.2 mM of each of the four dNTPs and 1 unit of recombinant *Pfu* polymerase (Stratagene) in 1 × recombinant *Pfu* polymerase reaction buffer, were amplified in a GeneATAQ Controller (Amersham Pharmacia Biotech). In primary reactions, the M13 universal and reverse primers (Table 1) were combined with the antisense and sense mutagenic primers, respectively, and 1 ng of template was amplified for 25 cycles: 95 °C for 30 s, 42 °C for 30 s and 72 °C for 30 s. After purification, the primary reaction products (1 ng) were combined and amplified with the M13 universal and reverse primers in the secondary reaction: 95 °C for 30 s, 42 °C for 2 min and 72 °C for 30 s (2 cycles); 95 °C for 30 s, 42 °C for 30 s and 72 °C for 30 s (25 cycles). The products were purified with the QIAquick PCR Purification Kit (Qiagen) and subcloned into pBLCAT6. The PR1, PR2 and PR1 + PR2 constructs contained the −45 to +31, −72 to −53/−24 to +31, and −72 to +31 *MN* fragments, respectively, in pBLCAT6.

Block-replacement mutagenesis of PR1 was carried out in two steps. In the first, PR1 was deleted by inverse PCR with −24 to −4 (ESPs) and −54 to −72 (PR2a) primers (where s and a stand for sense and antisense polarity of the primers, respectively; Table 1) [18]. In the second, AP2, specificity protein (SP) 1, a CACCC box containing transforming growth factor-β retinoblastoma control element (RCE) and PR1 double-stranded oligonucleotides (Table 1) were ligated to the purified PCR product to provide the block-replacement PR1 → AP2, PR1 → SP1, PR1 → RCE and PR1 → −PR1 mutants, with AP2, SP1, RCE and inverted PR1 in the PR1 position, respectively. (PR1 + PR2) HSV tk contains the −72 to −24 *MN* fragment upstream of the herpes simplex virus thymidine kinase (HSV tk) promoter in pBLCAT5 [20]. All recombinant clones were sequenced in their *MN* regions with the T7 sequencing kit™ (Amersham Pharmacia Biotech) to check for correct joining and random mutations. Plasmid DNAs for transfections were purified with the Qiagen Plasmid Midi Kit (Qiagen).

Table 1 Sequences of the oligonucleotides used in this study

Sequences of the sense strands are written in the 5′ → 3′ direction, and mutations against the wild-type sequence are underlined. Sequences of ESP, PR1 and PR2 are from [14]; nucleotides not protected in the DNase I footprinting assay in PR2 are in italics. Sequences of AP1-, AP2- and SP1-binding oligonucleotides are according to [26], [25] and [24], respectively. RCE is according to [44].

Oligonucleotide	Sequence
ESP (−24 to −4)	GCTCTCGTTTCCAATGCACG
PR1 (−45 to −24)	GGCTTGCTCCTCCCCCACCCAG
PR1AP2mut	GGCTTGCTCCTCCCTTACCCAG
PR1SP1mut	GGCTTGCTCCTAACCCACCCAG
PR2 (−72 to −54)	*CGC*TCTGTGAGTCAGCCTG*CTCCC*
PR2AP1mut	*CGC*TCTGTGAGTTGGCCTG*CTCCC*
AP1	CGCTTGATGAGTCAGCCGGAA
AP2	GATCGAACTGACCGCCCGCGGCCCGT
SP1	ATTCGATCGGGGCGGGGCGAGC
RCE	CGCCCCCGGCCCCACCCCAGGAGG
M13 universal	GTAAAACGACGGCCAGT
M13 reverse	GGAAACAGCTATGACCATG

Cell culture and transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (both from Life Technologies) and 0.16 mg/ml gentamicin (Sigma). 50–80% confluent cells on 3.5 cm plates were exposed to a mixture of plasmid DNA (2 μg) and 12 μl of GenePORTER™ 2 Transfection Reagent (Gene Therapy Systems). Transfected cells were harvested 72 h post-transfection, and the chloramphenicol acetyltransferase (CAT) activity was assayed with a CAT ELISA kit (Roche). The protein concentrations were determined with BCA Protein Assay Reagent (Pierce). For PMA experiments HeLa cells were maintained in medium containing 0.75% foetal calf serum for 24 h and for an additional 24 h in the absence or presence of PMA (Calbiochem). For bis-indolylmaleimide (BIM) experiments HeLa cells were kept for 48 h in sparse culture (less than 40% confluency), seeded in dense culture (more than 90% confluency) and then incubated in the absence or presence of BIM (Calbiochem) for 24 h.

Western blotting

Total cellular protein was prepared from the control and appropriately treated cells as described previously [17].

EMSA

NE from 1×10^8 HeLa cells was prepared as described in [21]. Binding reactions (25 μl) contained 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol, 1 μg of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech), 10 μg of NE and 0.4 μM double-stranded competitor. The sequences of PR1, SP1, PR1SP1mut, AP2, PR1AP2mut, RCE and AP1 competitors are given in Table 1. After 10 min at room temperature, 30000 c.p.m. of end-labelled double-stranded PR1 probe was added and the mixture was incubated for an additional 25 min at room temperature. In the supershift experiments the antibody (1 μl) was added to the reaction mixture and incubated at 4 °C for 1 h. SP1 (PEP2), SP3 and AP2α rabbit polyclonal antibodies were from Santa Cruz Biotechnology; the c-Fos antibody αFos 2.2 was a kind gift from Dr Tom Curran (Roche Institute of Molecular Biology, Nutley, NJ, U.S.A.). For the electrophoretic separation of the DNA–protein complexes, non-denaturing 5% (w/v) polyacrylamide gel in 0.5 × TBE (44 mM Tris/Cl, pH 8/44 mM boric acid/1 mM EDTA) was used. The gel was pre-run at 4 °C for 1 h at 20 V/cm and complexes separated for 3 h under the same conditions. The positions of complexes were detected by autoradiography of the dried gel.

RESULTS

Analysis of PR1 interactions by EMSA

SignalScan [22] suggested multiple putative *cis* elements in PR1: a CCCMNSSS AP2 binding site (CCCACCC, sense orientation), a CACCC box (CCCCACCC, sense orientation) and a GGGMGGM SP1 site (GGGAGGA, antisense orientation). Initially, we tested involvement of these prospective *cis* elements in PR1 binding by competition EMSA with a set of competitors, comprising AP2, SP1, PR1SP1mut (carrying GTTAGGA mutations) [23], PR1AP2mut (carrying CTTACCC mutations; mutations against the wild-type sequence are underlined) [24], a CACCC box (RCE) and AP1 as a non-specific competitor. The PR1 probe generated four complexes with HeLa NEs (Figure 1A). These complexes appear to be specific, as they could be totally competed with excess of unlabelled PR1. The SP1 probe competed for complexes 1, 2 and 4 (Figure 1A), while the PR1SP1mut probe did not compete against PR1 binding. The AP2 probe competed for complexes 1, 2 and 4, albeit less efficiently than the SP1 probe. The PR1AP2mut and RCE probes were as efficient as the SP1 probe in competition against PR1 binding (Figure 1A). The AP1 probe (negative control) did not have any effect on PR1 binding.

The SP1, PR1AP2mut and RCE probes were equally efficient in the competition for PR1 binding. They all competed for complexes 1, 2 and 4 and left complex 3. It appears that with respect to competition capacity these three probes must harbour structurally similar core sequences, most likely encompassing the GGGCGGG (SP1), GGGAGGA (PR1AP2mut) and GGGTGGG (RCE) sequences.

The PR1SP1mut probe with the mutated putative SP1 site could not compete for complexes 1, 2 and 4, suggesting that binding in these complexes is mediated by the GGGAGGA sequence. The PR1SP1mut probe retains the putative AP2 site and it should therefore compete for a common PR1 complex with the AP2 probe. As this was not the case (Figure 1A), the participation of AP2 in PR1 binding was less likely, although at this stage we could not rule out the possibility of SP1-site-dependent AP2 binding in PR1.

Despite the fact that competition EMSA did not provide any conclusive results about the identity of the PR1 complexes, it helped us distinguish two categories. In the first are complexes 1, 2 and 4, whose cognate factors appear to recognize the putative

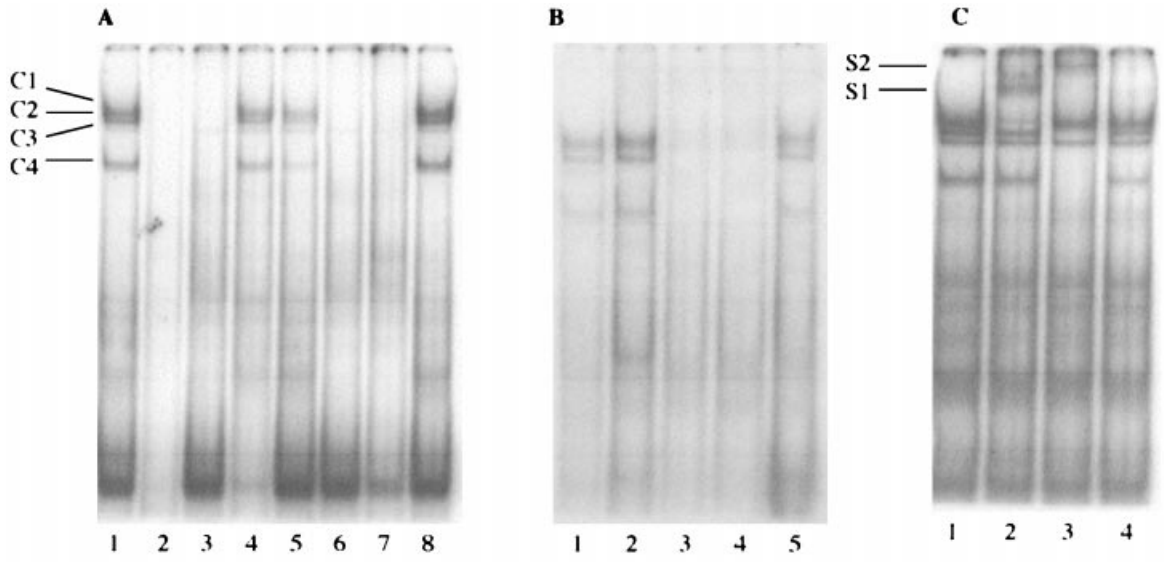

Figure 1 EMSA of PR1

Binding reactions contained end-labelled PR1 (SP1) probe and 10 μg of HeLa NEs. Competitors were used in 100-fold excess. Complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gels in 0.5 × TBE at 4 °C. (**A**) Competition EMSA of PR1. PR1 complexes were numbered from top to bottom and designated C1–C4. PR1 binding was tested in the presence of the following competitors: lane 1, no competitor; lane 2, PR1; lane 3, SP1; lane 4, PR1SP1mut; lane 5, AP2; lane 6, PR1AP2mut; lane 7, RCE; lane 8, AP1. (**B**) Comparative EMSA of PR1 and SP1. Lane 1, PR1 probe only; lane 2, SP1 probe only; lane 3, SP1 probe and SP1 competitor; lane 4, SP1 probe and PR1 competitor; lane 5, SP1 probe and PR1SP1mut competitor. (**C**) Supershift EMSA of PR1. PR1 binding was tested in the presence of 1 μl of the following antibodies: lane 1, no antibody; lane 2, SP1 antibody; lane 3, SP3 antibody; lane 4, AP2 antibody. S1 and S2 indicate supershift complexes with SP1 and SP3 antibodies, respectively.

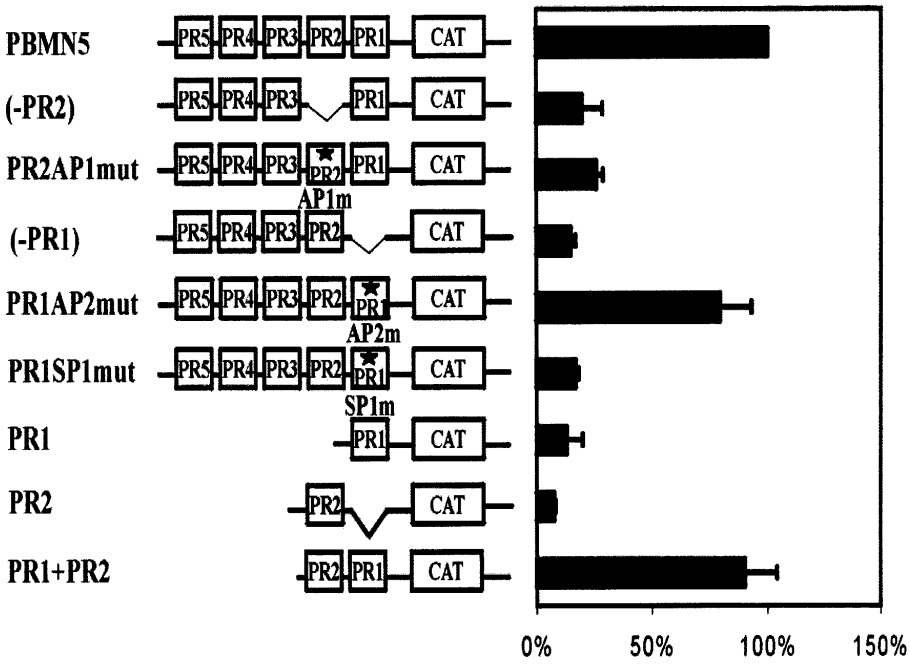

Figure 2 Point-mutation and deletion analysis of the MN promoter proximal region

Activity of MN promoter mutants was tested in transiently transfected HeLa cells. The CAT activity obtained with pBMN5 construct was normalized against protein concentration and set at 100%. The activity of other constructs is expressed as a percentage of the pBMN5 value. Each of the bars represents the mean ± S.D. of the CAT activity from at least three individual experiments.

SP1 site. Formation of complex 3, which could not be competed with any probe (except PR1 itself), is sensitive to the introduced mutations at both positions (SP1 and the putative AP2 site), and its cognate-factor binding site appears to comprise the SP1 site (at least part of it) and the downstream flanking sequence.

Next we wished to reveal the identity of the factors in complexes 1, 2 and 4. In a recent paper SP1 probes derived from the vascular endothelial growth factor (VEGF) promoter generated three complexes in EMSA (one SP1 and two SP3) [25]. Direct comparison of the consensus SP1 and PR1 probes by EMSA revealed that, with the exception of the PR1 complex 3, both probes generated the same binding pattern with HeLa NEs (Figure 1B). Finally, we performed supershift analysis of the PR1 complexes with antibodies against SP1, SP3 and AP2. The SP1 antibody supershifted complex 1, while the SP3 antibody supershifted complexes 2 and 4 (Figure 1C). The AP2 antibody failed to recognize any of the PR1 complexes (Figure 1C). In order to show the functionality of the AP2 antibody and the presence of AP2 in the NEs used, we successfully supershifted the complex produced by the AP2 probe (results not shown). These results proved conclusively that most of the PR1 binding is SP1-related and that SP1 and SP3 factors bind PR1 *in vitro*.

Supershift EMSA also definitively ruled out participation of AP2 in PR1 binding. The observed partial competition of the AP2 probe against the SP-related PR1 binding could be explained in terms of sequence similarity. The AP2 probe harbours the GGGCGG sequence (− orientation, Table 1) that shares significant sequence similarity with the SP1 site and could thus be responsible for competition. Similarly, the RCE probe could compete against the SP-related binding by virtue of its GGGTGGG sequence (− orientation, Table 1). In summary, EMSA has established the GGGAGGA sequence within PR1 as a functional SP1 site, allowed the identification of SP1 and SP3 as factors binding PR1 and indicated the presence of another factor in complex 3.

Point-mutation analysis of PR1

EMSA confirmed that at least three different factors can bind PR1 *in vitro* (SP1, SP3 and the complex 3-forming factor). In order to assess the importance of the SP1-related binding in *MN* transcriptional regulation, point-mutation analysis was used. The MN promoter mutant with disrupted SP1-related binding was prepared by means of the PR1SP1mut oligonucleotide (Table 1). In another construct SP1 binding was preserved, and the formation of complex 3 was eliminated by means of the PR1AP2mut oligonucleotide (Table 1). The effect of these mutations on promoter activity was investigated in transiently transfected HeLa cells. As can be seen in Figure 2, the PR1SP1-mut had a considerable effect on promoter activity. Reporter activities generated from the PR1SP1mut and −PR1 constructs were comparable, thus relating the effect of the mutated SP1 site within PR1 to that of the removal of the whole PR1. This is in good agreement with EMSA conclusions and strongly supports the critical role of the SP1 site in PR1 for *MN* transcriptional activation. Conversely, the PR1AP2mut had a minor effect on reporter activity (Figure 2). We therefore conclude that complex 3 has only a marginal role in *MN* transcriptional activation.

Block-replacement mutagenesis of PR1

Point-mutation analysis demonstrated the importance of SP1-related binding in the PR1 position. We wished to characterize further PR1 in the MN promoter by testing its selectivity. The AP2 and RCE probes (both GC-rich) could compete against

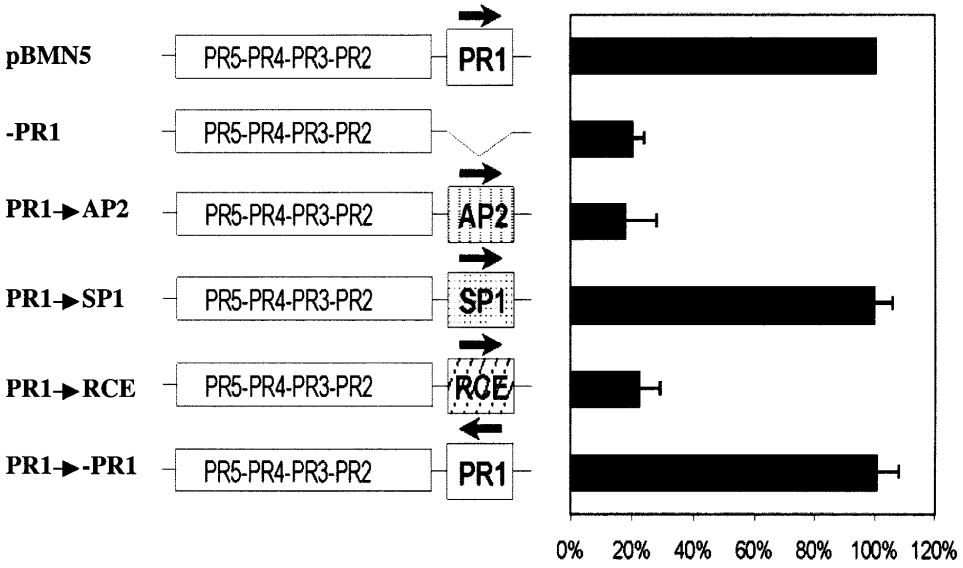

Figure 3 Block-replacement mutagenesis of PR1

The whole PR1 sequence within pBMN5 was replaced with the consensus binding site for AP2 or SP1 transcription factors, RCE or inverted. Mutant constructs were tested in transiently transfected HeLa cells. The activity of each construct is expressed as a percentage of the control activity (pBMN5), normalized against protein concentration. Each of the bars represents the mean ± S.D. of the CAT activity from at least three individual experiments.

PR1 binding to various extents, so therefore we investigated the compatibility of their cognate transcription factors in the PR1 position with the other activators assembled on the MN promoter. For this purpose, we used block-replacement mutagenesis and replaced the whole PR1 with a distinct *cis* element (PR1 → AP2 and PR1 → RCE mutants) in a two-step process. In addition to SP1-related complexes, PR1 binds another factor, complex 3. Therefore we replaced PR1 with the consensus SP1-binding sequence (PR1 → SP1 mutant) to compare directly the activity of PR1 and SP1 sites *in vivo*. Mutant promoter constructs were again tested in HeLa cells, and the results of transient transfections are shown in Figure 3. Only the PR1 → SP1 mutant restored the full promoter activity; the PR1 → AP2 and PR1 → RCE mutants produced activity comparable with that of the −PR1 construct. The block-replacement approach allowed us to prove the orientation-independent function of PR1, as inversion of PR1 led to a full restoration of promoter activity (Figure 3). In this way we demonstrated that even in the absence of the complex 3-forming factor, the SP factors in the PR1 position can productively co-operate with other factors on their own. This conclusively proves the critical role of SP factors in transactivation of the MN promoter. Complex 3 is dispensable for transactivation, and PR1 and SP1 sites are functionally equivalent *in vivo*. At the same time, the inability of the AP2 and RCE mutants to restore MN promoter activity clearly demonstrates the selectivity of the PR1 position. Factors binding to these types of element cannot enter into productive co-operation with other MN-promoter-binding factors. Despite the strong competition of the RCE probe against PR1 binding, the result of block-replacement mutagenesis argues strongly against the functionality of the CACCC-box-like sequence in PR1.

AP1 activity is necessary for PR2 function

Analysis of the PR2 sequence with the SignalScan program indicated the presence of a canonical TGAGTCAG AP1 site. An unrelated probe with a consensus AP1 site completely eliminated PR2 binding in competition EMSA [14]. To prove that AP1 indeed binds to PR2, supershift EMSA with a c-Fos antibody was performed. This antibody recognized the PR2 complex and produced a supershift (Figure 4). The role of AP1 in *MN* transcriptional activation was also investigated by point-

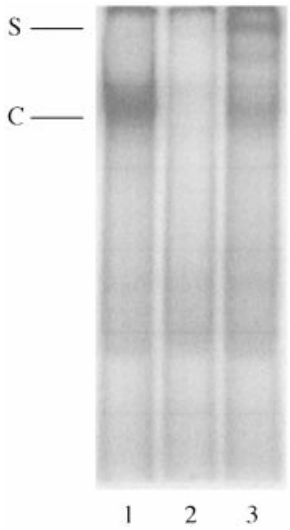

Figure 4 Supershift EMSA of PR2

Binding reactions contained end-labelled PR2 probe and 10 μg of HeLa NEs. Complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gel in 0.5 × TBE at 4 °C. Lane 1, probe only; lane 2, probe and 100-fold excess of the AP1 competitor; lane 3, probe and 1 μl of c-Fos antibody. C and S mark positions of the PR2 complex and the c-Fos-supershifted PR2 complex, respectively.

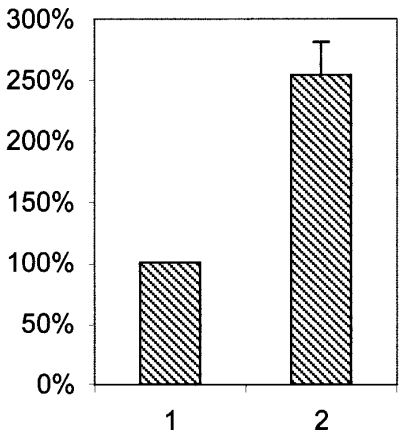

Figure 5 The effect of the *MN* PR1 + PR2 on the HSV tk promoter

Lane 1: the CAT activity obtained with the HSV tk-promoter-driven pBLCAT5 in transiently transfected HeLa cells was normalized against protein concentration (100%). Lane 2: the activity of the (PR1 + PR2) HSV tk is expressed as a percentage of the control value. The bar represents the mean ± S.D. of the CAT activity from three individual experiments.

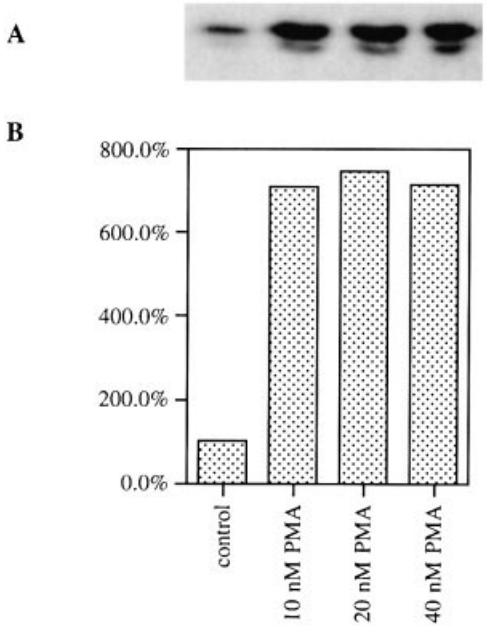

Figure 6 The effect of PMA on (A) endogenous MN protein and (B) MN promoter activity in HeLa cells

Cells were cultivated in medium containing 0.75% foetal calf serum for 24 h and for additional 24 h in the presence or absence of PMA. In (**B**) the CAT activity obtained with the pBMN5 construct was normalized against protein concentration and set as 100%. The promoter activity in the presence of PMA is expressed as a percentage of the control activity.

mutation analysis. Two point mutations, TGAGTTGG, known to specifically disrupt AP1 binding [26], were introduced into the PR2 sequence (Table 1). The PR2AP1mut MN promoter construct was tested against controls in transiently transfected HeLa cells and the results are shown in Figure 2. Indicated point mutations had a profound effect on the promoter, and the PR2AP1mut construct produced about 20% of the control (pBMN5) activity. This activity was only marginally above that of the −PR2 construct, suggesting that the elimination of AP1 binding in the PR2 position has an effect comparable with the removal of the whole PR2 *cis* element. Thus, AP1 activity in the PR2 position is essential for MN promoter activity.

PR1 + PR2 represent an enhancer module

Deletion analysis confirmed the critical role of PR1 and PR2 for *MN* transcriptional activity (Figure 2). Even if PR3–PR5 are deleted, the remaining PR1 + PR2 still produce as much as 90% of the pBMN5 activity (Figure 2). Further deletion analysis demonstrated that either of the two *cis* elements on its own can drive only fractional reporter activity (Figure 2). It is thus the synergistic interaction between transcription factors bound to PR2, PR1 and the downstream HRE that is primarily responsible for MN promoter activity. We also wished to test whether the combination of PR1 and PR2 could stimulate transcription from an unrelated heterologous promoter. As the MN promoter is a typical TATA-less promoter [13], the PR1 + PR2 fragment was cloned in front of the more distant TATA box containing the HSV tk promoter in pBLCAT5. Transient transfections in HeLa cells showed that the PR1 + PR2 module indeed stimulated transcription from the HSV tk promoter and functions as an enhancer sequence (Figure 5).

Effect of modulation of AP1 activity via PKC

Treatment with phorbol ester, a diacylglycerol analogue and activator of PKC, induces AP1 activity that results in enhanced expression of the genes with AP1-binding sites [26]. Therefore we studied the effect of PMA treatment on the protein and promoter levels of MN expression in HeLa cells. Cells were exposed to various concentrations of PMA for 24 h, and MN protein levels were investigated by Western blotting. PMA stimulated MN expression as shown in Figure 6(A). A similar effect was observed in cells transiently transfected with the MN promoter construct. In this case PMA treatment stimulated the reporter activity 6–7 times above control levels (Figure 6B). Together these experiments confirmed that MN expression is sensitive to PMA and that this effect is mediated specifically at the level of the MN promoter. The involvement of PKC in regulation of MN expression was tested with BIM, an inhibitor of PKC [27]. This inhibitor failed to elicit detectable changes to MN levels in HeLa cultures (results not shown). Absence of a BIM effect was also corroborated at the level of the promoter construct. HeLa cells transiently transfected with pBMN5 and treated with 1 μM BIM produced 95% of the control activity. Thus MN expression, at least in the cell line studied, is not regulated by PKC-mediated modulation of AP1 activity.

DISCUSSION

The analysis of *cis*-acting elements and their cognate *trans* factors governing transcription of the gene coding for the tumour-associated MN protein will ultimately unveil the mechanism(s) behind the unique MN expression pattern. Essential *cis* elements in the MN promoter were described previously [14,15]. In this study we focused on the identification of the factors binding to PR1 and PR2 and the assessment of their role in *MN* transcriptional activation.

Initially we probed PR1 binding by competition EMSA with two sets of competitors. The first set (AP2, SP1 and CACCC box) was selected on the basis of computer analysis of the PR1 sequence; the second set (PR1AP2mut and PR1SP1mut) contained specific point mutations in the putative AP2- and SP1-binding sites within PR1. The SP1 site and CACCC box

containing RCE probes displayed powerful competition against PR1 binding, whereas the AP2 probe competed partially. Further analysis confirmed that three out of the four PR1 complexes were also generated with the consensus SP1 probe. SP1 and SP3 antibodies supershifted complex 1 and complexes 2 and 4, respectively, whereas the AP2 antibody failed to recognize any of the PR1 complexes. EMSA did not provide any information on the identity of complex 3, except that it requires both SP1 and the downstream flanking sequence.

EMSA proved that PR1 is recognized by three different transcription factors (SP1, SP3 and the complex 3-forming factor). To analyse the role of these factors in *MN* transcriptional activation, we designed a simple method for functional dissection of multiple factor complexes. The method is based on the well-documented co-operation (additive or synergistic) between transcription factors that is primarily responsible for transcriptional activation [28]. We investigated whether the replacement of the multi-factor complex with a complex consisting of a previously characterized transcription factor (or family of factors) can provide data on the role of this factor in transcriptional activation. In the context of the promoter construct, the other factors are removed by block replacement of the whole original *cis* element with a new one, containing a consensus binding site for the tested factor. By the elimination of other factors from the examined position, we can functionally test (in transfected cells) to what extent the tested factor is capable of productive co-operation with factors bound to the neighbouring *cis* elements. The block-replacement strategy retains a single factor in the tested position and eliminates all the others at the same time; thus in essence it is the 'reverse' of the point-mutation approach, as point mutations are usually introduced with the aim of destroying a single binding site while preserving others. The role of the factor that has been removed from the complex in transcriptional activation is then inferred from the effect of the mutation on the promoter activity.

In the present study the results of PR1 block-replacement mutagenesis complemented the results of point mutagenesis and provided compelling evidence for the critical role of SP factors in the PR1 position. Activities of all mutant constructs were related to two controls, the full-length wild-type (-173 to $+31$) MN promoter (pBMN5) and the $-$PR1 construct with PR1 deleted. The PR1SP1mut (with its SP1 site eliminated by point mutations) had a profound effect on the promoter activity, the magnitude of which was comparable with the deletion of PR1, and hence removal of all factors from this position. It should be stressed here that the PR1SP1mut construct lost not only SP1-related binding in the PR1 position, but also its capacity to form complex 3 as well. The PR1 $\rightarrow$ SP1 mutant, having a consensus SP1 site (GC box) in place of PR1, lacks complex 3 but binds SP factors. In this mutant, the importance of SP factors in this position was manifested in the full restoration of promoter activity.

The functionality of the putative AP2-binding site in PR1 was ruled out by supershift analysis. However, at least part of this sequence was required for formation of complex 3. Functionally, the PR1AP2mut (AP2/CACCC-box site eliminated by point mutations) construct should be the same as the PR1 $\rightarrow$ SP1 mutant, both lacking complex 3 in the PR1 position. Indeed, the PR1AP2mut had only a limited effect on the promoter activity, indicating that these point mutations did not affect binding of a critical factor. Both the block-replacement and point-mutation approach are thus in favour of only a marginal role for complex 3 in *MN* transcriptional activation.

The PR1 $\rightarrow$ AP2 and PR1 $\rightarrow$ RCE block-replacement constructs demonstrated the selectivity of the PR1 position in the MN promoter. Factors bound to these *cis* elements are unable to synergize with the *MN* transcriptional complex and substitution of PR1 with any of these elements did not provide activity higher than the control $-$PR1 construct. The low activity of the PR1 $\rightarrow$ RCE mutant was rather surprising, as SP1 was identified as one of the three factors binding to this *cis* element [29]. The strong competition of the CACCC box against PR1 binding could thus be the result of its affinity for SP factors. Its non-functionality in the PR1 position can be explained in terms of previous findings that the CACCC box was unable to synergize with other transcription factors, notably AP1, in the promoter-element compatibility tests [30]. As AP1 appears to be the critical factor in PR2 (the neighbouring *cis* element in the MN promoter), it is likely that even in the context of the MN promoter, AP1- and CACCC-box-binding factors cannot produce the synergistic effect required for *MN* transcriptional activation. Results of point- and block-replacement mutagenesis confirm that the CACCC-box-like sequence in PR1 is non-functional. Finally, the block-replacement strategy allowed us to invert PR1 and find out that the PR1 function in the MN promoter is orientation-independent.

Although EMSA indicated the presence of another factor binding PR1 (complex 3), mutation analyses firmly established that it is dispensable for *MN* transcriptional activation. The results of PR1 mutation analysis are in full agreement with competition EMSA, and therefore we made the following conclusions: (i) SP1 and SP3 activity is essential in the PR1 position and (ii) synergistic co-operation of SP factors with *MN* transcriptional machinery does not require other factors. Whereas SP factors appear to be the critical effector factor, at present we are unable to assign a role for the complex 3-forming factor in PR1 and more work is required before this function is defined.

SP1 is a well-characterized, sequence-specific, DNA-binding protein that is important in the transcription of many cellular and viral genes that contain GC boxes in their promoters [31]. SP1 levels and function may change with differentiation, transformation and cell growth, suggesting that these changes have important biological consequences [32]. SP1 is expressed at vastly different levels in different tissues, and its transactivation potential may be enhanced by expression of oncogenes (v-Rel, Ras or Src) [33]. DNA-binding affinity of SP1 may be altered by growth factors (transforming growth factor β1, granulocyte-macrophage colony-stimulating factor and insulin-like growth factor) [32]. Constitutive VEGF transcription activation correlated with a high level of constitutive SP1 expression and activity in pancreatic cancer cell lines and cancer-tissue specimens [25]. Additional transcription factors (SP2, SP3 and SP4), similar in their structural and transcriptional properties to SP1, have been cloned and classified in the SP1 multigene family [31].

Here we show that SP1 and SP3 factors recognize PR1 in the MN promoter. Whereas SP1 stimulates transcriptional activity, the function of SP3 is less clear. SP3 has been shown to act as a dual-function regulator whose activity depends on the context of DNA-binding sites in a promoter [34]. Also, SP3 has the ability to repress SP1-mediated transcriptional activation of some genes, possibly by competing with SP1 for binding [35]. In our previous work we showed that NEs prepared from MN-non-expressing (sparse HeLa and non-tumorigenic hybrid CGL1 cells) and MN-expressing (dense HeLa and tumorigenic CGL3 cells) cultures bound PR1 with the same affinity, producing no observable differences in EMSA [14]. Therefore, at least in the two systems tested, activation of *MN* transcription is not associated with changes in SP1:SP3 ratio.

PR2 was defined as another important *cis* element in *MN* transcriptional regulation [14]. On the basis of 100% homology

and competition EMSA it appeared that the single *in vitro*-generated PR2 complex consists of AP1. Indeed, the PR2 complex was recognized by c-Fos antibody, and the elimination of AP1 binding in the PR2 position had the same effect as the deletion of the whole PR2. We therefore conclude that AP1 is the only factor binding to PR2. The widespread AP1 sites require co-operation with other transcription factor(s) for optimal activity in promoters of many genes, e.g. the neighbouring upstream regulatory sequence in the stromelysin promoter [36], polyoma virus enhancer domain element in the collagenase promoter [37], cAMP-response element in the cytotoxic serine protease B promoter [38], ETS1 (v-*ets* avian erythroblastosis virus E26 oncogene homologue 1) and nuclear factor κB in the granulocyte-macrophage colony-stimulating factor promoter [39] or SP1 in the involucrin [40] and VEGF [41] promoters. AP1, in an enhancer mode, was found to stimulate activity of nuclear factor 1, CP1, ATF/cAMP-response-element-binding protein and GC-box (SP1 site) element in promoter-element compatibility tests [30]. The rather promiscuous character of AP1 could cast some doubts on the results of the PR1 block-replacement mutagenesis, when diverse factors were combined with AP1 in artificial MN promoter constructs. In our opinion, the approach is validated by two independent facts: (i) in agreement with the conclusions of promoter-element compatibility tests [30], not all factors were capable of productive co-operation with AP1, and (ii) AP1 and SP1 factors were found to synergize in artificial promoter constructs [30], the involucrin [40] and VEGF [41] promoters; consequently it could be objected that the combination of SP1 and AP1 is likely to generate an enhancer element in the context of any promoter. Mutational analysis of the cytotoxic serine protease B gene promoter proved that this is not the case. In this promoter, an AP1 site and cAMP-response element were required for optimal activity, and the replacement of neither element with an SP1 site restored the activity [38]. Thus instead of entering into indiscriminate synergistic co-operation each time, the outcome of AP1 and SP1 combination depends on the promoter context.

The MN promoter proximal region closely resembles the strong activator element in the distal regulatory region of the involucrin gene. This element also consists of adjacent AP1 and SP1 sites and is necessary for optimal activity of the involucrin promoter [40]. The AP1 site appears to be absolutely required for transcriptional activation, whereas the neighbouring SP1 enhanced this activation and was required for optimal AP1 binding [40]. In contrast, both AP1 and SP1 sites are absolutely required for transcriptional activation of the MN promoter. Moreover, the EMSA showed that separated PR1 and PR2 can produce specific complexes, indicating that *in vitro* binding of either SP1 to PR1 or AP1 to PR2 is not dependent on the presence of the neighbouring *cis* element. The hypoxia-inducible VEGF promoter contains a combination of AP1, SP1 and HRE sites [41]. The layout of the MN promoter, which is also hypoxia-inducible [15], is essentially the same, and the combination of these *cis* elements may thus represent a paradigm for hypoxia-regulated genes.

In this study the PR2-binding factor was confirmed as AP1. Activation of the AP1 complex is one of the earliest nuclear responses to mitogenic stimuli, and decreased AP1 activity in old human fibroblasts was related to the inability of senescent cells to proliferate in response to mitogens [42]. AP1 activity is subject to regulation during the G_0–G_1 transition in NIH3T3 cells [43]. As with PR1, PR2 binding did not correlate with MN expression in sparse/dense HeLa cells or CGL1/CGL3 hybrids [14]. We therefore assume that in the course of MN induction *in vitro*, neither of the PR1- or PR2-binding factors is regulated and they are constitutively active in HeLa cells. Nevertheless, we investigated the effect of modulation of AP1 activity on MN expression in HeLa cells. Treatment of the serum-starved cells with PMA, a potent activator of PKC and AP1, increased the levels of endogenous MN in HeLa cells. Although activatable, it appears that AP1 represents an essential constitutive factor in MN expression. The fact that BIM, a highly selective cell-permeant PKC inhibitor, had no effect on MN expression in dense HeLa cells suggests that the PKC pathway is not implicated in the process of MN induction in dense cultures.

The results presented in this study substantially widen our knowledge of *MN* transcriptional regulation. Of several putative binding sites in the PR1 position, the SP1 site was shown to be critical. The activity of the PR1 + PR2 enhancer module depends on the synergistic co-operation between juxtaposed SP and AP1 transcription factors. How these factors interact functionally with hypoxia-inducible factor 1 in hypoxia-inducible expression is currently under study.

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