Transcriptional Regulation of the MN/CA 9 Gene Coding for the Tumor-associated Carbonic Anhydrase IX

IDENTIFICATION AND CHARACTERIZATION OF A PROXIMAL SILENCER ELEMENT*

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The MN/CA 9 (MN) gene encodes a tumor-associated isoenzyme of the carbonic anhydrase family. Functional characterization of the 3.5-kilobase pair MN 5’ upstream region by deletion analysis led to the identification of the -173 to +31 fragment as the MN promoter. In vitro DNase I footprinting revealed the presence of five protected regions (PRs) within the MN promoter. Detailed deletion analysis of the promoter identified PR1 and PR2 (numbered from the transcription start) as the most critical for transcriptional activity. PR4 negatively affected transcription, since its deletion led to increased promoter activity and was confirmed to function as a promoter-, position-, and orientation-independent silencer element. Mutational analysis indicated that the direct repeat AGGGGAGGGC is required for efficient repressor binding. Two components of the repressor complex (35 and 42 kDa) were found to be in direct contact with PR4 by UV cross-linking. Increased cell density, known to induce MN expression, did not affect levels of PR4 binding in HeLa cells. Significantly reduced repressor level seems to be responsible for MN up-regulation in the case of tumorigenic CGL3 as compared with nontumorigenic CGL1 HeLa x normal fibroblast hybrid cells.

MN/CA IX (MN),† one of the most recently described isoenzymes of the carbonic anhydrase (CA) family, apparently exhibits features that make it unique within this family (1). Most importantly, MN was found in several types of tumors such as cervical carcinomas and cervical intraepithelial squamous and glandular neoplasia (2), renal cell carcinoma (RCC) (3, 4), esophageal tumors (5), and carcinoma-derived cell lines (6) but not in the corresponding normal tissues. The only normal tissues expressing MN identified to date are the gastric, intestinal, and biliary mucosa (7). In HeLa x human fibroblast hybrid cells, prepared by Stanbridge et al. (8), expression of MN correlated with tumorigenicity; nontumorigenic hybrid CGL1 was found negative for MN expression, while tumorigenic segregant CGL3 was MN-positive (6). When expressed in murine NIH 3T3 fibroblasts, MN displayed transformation potential (1). Expression of MN in HeLa cells is density-dependent; the protein is absent in sparse, rapidly proliferating cells, and its synthesis is induced in dense, overcrowded cultures (1).

CA XII, another member of the CA family, was also found to be associated with at least lung cancer and RCC. In 10% of RCC patients, the corresponding transcript was expressed at much higher levels in neoplastic tissue than in surrounding normal tissues (9).

MN is a transmembrane glycoprotein, present on Western blot as twin bands of 54 and 58 kDa. As expected from the presence of CA core, MN displays CA activity and binds zinc (1). The corresponding gene consists of 11 exons, covers 10.9 kb (including 3.5 kb of 5’ upstream sequence), and the molecular organization of its coding region corresponds to the proposed domain composition of MN protein (10). Due to unique N- and C-terminal domains, unrelated to other CA isoenzymes, MN is considered to be a chimeric gene, assembled by exon shuffling (10). The MN transcription start was localized by rapid amplification of cDNA ends (1) and unanimously confirmed by RNase protection assay (10). These results, as well as presence of a single transcript on Northern blot (1), pointed out to the existence of a single MN promoter. No TATA box was found within the region immediately upstream of the transcription start. Neither of the two initiator elements (PyPyCAPyPyPyPyPyPy) in the immediate upstream region overlaps with the transcription start. Despite the fact that the -507 to +1 region contains almost 60% GC residues, it does not exhibit additional features of typical TATA-less promoters of housekeeping genes (10, 11).

A PuPuPuC/A/T(T/A/G)PyPyPy putative p53 binding site (12), indicated by SignalScan search (13) at positions -46 to -37, was protected in an in vitro DNase I footprinting assay. However, neither of the complexes generated in an electromobility shift assay (EMSA) contained the p53 protein. Tetracycline-inducible antisense expression of human papillomavirus 18 E6 in HeLa (human cervical carcinoma cell line) resulted in an increased level of p53 but did not affect MN levels (14).

Another member of the tumor suppressor family, the von Hippel-Lindau factor, was recently described to down-regulate MN in RCC cell lines, although the molecular mechanism in operation is not well understood (15).

Despite several lines of evidence confirming its association with malignancies, no specific function for MN in tumorigenesis has been proven yet. In the absence of this role, characterization of critical factors governing expression could provide...
invaluable clues for understanding of processes leading to MN expression. For most eukaryotic genes, the tissue specificity and the level of expression are determined by elaborate interplay of cis-elements, usually located within the 5′-flanking sequences, and their cognate trans-acting factors (16). In this paper, we investigated transcriptional regulation of the MN gene. We report functional characterization of the 3.5-kb MN 5′ upstream region and detailed analysis of the −173 to +31 fragment. Cis-regulatory elements critically affecting the expression of MN have been identified, and a novel protected region 4 (PR4) silencer element, located at −134 to −110, has been characterized.

MATERIALS AND METHODS

Plasmid Construction—Position numbers indicate position relative to the MN transcription start (Ref. 10 and Fig. 3), and primers are written in the 5′-3′ direction. The MN−3506 to +31 fragment from a SuperCos cosmid clone (10) was subcloned into pBSK+ vector (Stratagene), and deletions were made either by the Erase-a-Base system (Promega) from the 5′ end or region distruption and subcloned into XbaI and BglII sites of pBLCAT6 (17). Constructs with internal deletions of PRs were prepared by amplification of the construct containing the −173 to +31 fragment in pBLCAT6 (pBMN5) by inverse polymerase chain reaction, using sense and antisense primers from the downstream and upstream PRs, respectively (deleted PR is indicated in parentheses): (−PR1) −24 to −4 sense and −54 to −72 antisense; (−PR2) −46 to −24 sense and −81 to −104 antisense; (−PR3) −74 to −56 sense and −106 to −133 antisense; (−PR4) −108 to −85 sense and −143 to −166 antisense; (−PR5) −137 to −110 sense and −143 to −166 antisense. Polymerease chain reactions (25 μl) contained 20 ng of template, a pair of phosphorylated primers at 0.2 μM each, a 200 μM concentration of each of the four dNTPs, 1 unit of Pwo DNA polymerase in 1X reaction buffer (Roche Molecular Biochemicals). Amplifications were carried out for 30 cycles: 94°C for 30 s, 56°C for 30 s (−PR1 and −PR3 constructs) or 68°C for 30 s (−PR2, −PR4, and −PR5 constructs) and 72°C for 3 min followed by a final extension at 72°C for 5 min.

Heterologous promoter constructs containing a single copy of PR4 were prepared by ligating double-stranded oligonucleotide GTACTGG-GAGAGGGCCAGACAGACACCG (sense) and GATCGGTTTGTGTCGGGCTTGTCCTTCCCA (antisense) into BglII or BamHI sites of pCAT reporter vector (Promega). Constructs with tandem repeats of PR4 were prepared as follows. −133 to −108 sense and antisense oligonucleotides (with TC and GA added to their 5′-ends to facilitate directional ligation) were annealed, kiaed, and subcloned into T4 DNA ligase, size-fractionation on an agarose gel, blunt-ended with Klenow fragment, and cloned into the EcoRV site of pBS108 (New England Biolabs). Inserts from recombinants containing four and eight tandem copies were subcloned into BglII or BamHI sites of the pCAT promoter.

Cell Culture and Transfections—HeLa, MaTu (18), CGL1 and CGL3 (8) cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies) and 0.16 mg/ml gentamicin (Sigma). Sparse and dense cultures were seeded at 1×10^5 and 2×10^5 cells/cm², respectively, and 50–80% confluency was seeded 24–48 h after transfection, and chloramphenicol acetyltransferase (CAT) quantities were measured by ligase-mediated quantification (Promega) and 1 μg of each construct. pSV2neo (CLONTECH Laboratories) was used as a positive control. 48 h after transfection, cells were collected in 600 μg/ml of G418 (Life Technologies) for 2 weeks. Resistant colonies were fixed with methanol and stained by Giemsa.

Preparation of Nuclear Extracts (NE)—NE from 1×10^6 cells were prepared as described (20), dispersed in aliquots, and stored at −80°C until needed.

In Vitro DNase I Footprinting Assay—The SureTrack footprinting kit (Amersham Pharmacia Biotech) was used to probe the −243 to +31 MN fragment with 40 μg of NE from CGL1 or CGL3 cells. Fragment was end-labeled with Klenow fragment, cut to produce labels at one end, and purified with an agarose gel. DNase I-treated samples and controls were analyzed on an 8% denaturing polyacrylamide gel.

EMSA—Double-stranded probes, corresponding to individual PRs, were prepared by annealing of the sense and antisense oligonucleotides corresponding to −46 to −24 (PR4), −74 to −56 (PR2), −109 to −85 (PR3); −137 to −110 (PR4), and −170 to −143 (PR5) regions. The promoterless CAT gene. We report functional characterization of the 3.5-kb MN 5′ upstream region and detailed analysis of the −173 to +31 fragment. Cis-regulatory elements critically affecting the expression of MN have been identified, and a novel protected region 4 (PR4) silencer element, located at −134 to −110, has been characterized.

RESULTS

Identification of MN Promoter—In order to determine the MN promoter, we first tested the activity of several MN reporter constructs in transient transfections into MN-positive tumorogenic CGL3 HeLa × normal fibroblast hybrid cells. Using the MN 3.5-kb upstream region progressively deleted from the 5′-end, we found that the CAT activity produced in these hybrid cells is generally low, some of the constructs yielding
least three individual experiments. The transient transfection format is expressed as a percentage of the CAT activity obtained with pBMN5 construct in MaTu cells (100%). Each of the bars represents the mean value ± S.D. of the CAT activity from at least three individual experiments.

In an attempt to increase the low activities generated by MN constructs, we decided to perform transient transfections in MaTu cells, which are known to express the MN protein at about 30 times higher levels than HeLa cells (6). Direct comparison of Bd3 and pBMN5 constructs in MaTu and HeLa cells (measured by a CAT enzyme-linked immunoassay kit) confirmed that MN fragments indeed produced higher activity in MaTu cells even at the level of the −173 to +31 fragment (Fig. 2). Apparently, transactivation or derepression by MaTu cells was generated from the −243 to +31 fragment (clone pBMN5). Further deletions again led to decreased activities (Fig. 1A). Because of the low reporter activity generated by the MN constructs in transient experiments, an alternative strategy based on selection of stable clones was employed; transcriptional activity is deduced from the number of clones produced with the tested fragment and the control in the presence of selective condition (21). CGL3 cells, transfected with MN fragment-driven neo constructs, were selected in G418 for 2 weeks, and Giemsa stained plates are shown in Fig. 1B. Under experimental conditions used, the −30 to +31 fragment (i) displayed virtually zero activity, and the −243 to +31 fragment (ii) exhibited considerably lower activity in comparison with the SV40 early promoter-driven positive control (iii).

In Vitro DNase I Footprinting Assay of the MN Promoter—Following identification of the MN promoter, a footprinting assay of this fragment was carried out to define regions binding nuclear proteins. As shown in Fig. 3, incubation of the labeled −173 to +31 fragment with NE prepared from CGL1 and CGL3 hybrid cells resulted in protection of five regions: PR1 to PR5. The only difference in protected patterns with NE from MN-positive CGL3 (lanes 6 and 12) and MN-negative CGL1 (lanes 3 and 9) cells was observed in PR4. Although NE from both hybrid cells prevents DNase I digestion, CGL1 NE offers more quantitative protection to PR4 and may thus be indicative of different levels of a nuclear factor binding to this cis-element in these two hybrid lines.
ences in their ability to bind to probes corresponding to individual PRs within the MN promoter. In agreement with the footprinting results, the only region recognized differently was PR4, which gave strong binding with extracts from MN-negative CGL1 hybrids, while binding with positive CGL3 hybrids is markedly reduced (Fig. 4A). Binding to PR2 is given in Fig. 4B to demonstrate equal amounts and integrity of NE used; the data for other PRs are not shown. EMSA results confirmed that a factor bound to PR4 could negligibly control expression of MN, since its level was considerably higher in MN-nonexpressing CGL1 hybrids. Testing NE from HeLa and MaTu cells with individual PRs revealed no differences in binding for any probe (data not shown). Compared with sparse culture, MN is markedly up-regulated in dense HeLa cells (6). When NE from dense and sparse HeLa cells were probed with PR4 to investigate whether down-regulation of the putative PR4 binding repressor is responsible for MN up-regulation in dense cultures, no difference in binding was observed (Fig. 4C). These results thus suggest that the mechanism(s) responsible for the MN up-regulation in MaTu cells or dense culture is/are different from the one functioning in CGL1/CGL3 cells.

Mapping of Transcriptional Activity within the MN Promoter—Previous experiments revealed a low activity of the MN promoter in most of the cell lines tested, frequently generating background reporter activities. In order to preserve as much of the promoter activity as possible for further detailed characterization of the −173 to +31 region, a set of internally deleted constructs was prepared by inverse polymerase chain reaction. A combination of an antisense primer from the upstream PR and a sense primer from the downstream PR was used to generate a construct lacking a single PR and its flanking sequences, while the rest of the promoter was maintained. CAT activities generated from these constructs upon transfection into MaTu cells are shown in Fig. 5. From the significantly decreased CAT activities produced by pBMN5(−PR3) and pBMN5(−PR5) constructs, it can be concluded that 1) PR1 and PR2 bind strongly activating transcription factors and 2) the presence of both cis-acting elements is crucial for the activity of the MN promoter. Preliminary analysis of the nucleotide sequences with Signal Scan (13) indicated the presence of putative binding sites for the AP2 (CCCGMNSSS) and AP1 (TGAGT-CAG) transcription factors in PR1 and PR2, respectively. Competition EMSA with probes containing consensus AP2 and AP1 binding sites also supported the involvement of both AP2 and AP1 (or similar) trans-factors (Fig. 6). However, in the case of PR1, multiple complexes were generated, and among these only one complex could be competed out with the AP2 oligonucleotide (Fig. 6A). This indicated the complex binding propensity of PR1; additional work will be required to analyze the trans-factors present in other complexes and their contribution to the MN promoter activity. Decreased activities of pBMN5(−PR3) and pBMN5(−PR5) constructs suggest that PR3 and PR5 also bind trans-acting factors, contributing to the transcriptional activity of MN promoter, but to a lesser extent than PR1 and PR2. Deletion of PR4 yielded CAT activities almost 3 times higher than the control pBMN5 (Fig. 5). Consistent with the results of footprinting and EMSA, deletion analysis confirmed the negative role of PR4 on transcription from the MN promoter.

UV Cross-linking of the PR4 Repressor Complex—In order to estimate the number of repressor components directly in contact with the DNA and to determine their molecular weight, we performed UV cross-linking of CGL1 NE to the labeled PR4 probe. A SDS-polyacrylamide gel electrophoresis separation identified two cross-linked complexes (Fig. 7), generated by
proteins of 35 and 42 kDa. The repressor complex thus appears to consist of at least two subunits.

**Effect of PR4 on a Heterologous Promoter—**Previous results of footprinting and EMSA pointed out that PR4 binds a factor negatively affecting transcription, a conclusion further supported by increased reporter activity produced by a variant with deletion of this element and its flanking regions. In theory, the increased activity of the pBMN5(−PR4) construct could be the result of altered spacing between the remaining PRs in the promoter. In order to rule out this possibility and demonstrate at the same time that the effect of PR4 is not restricted to its position in the MN promoter, we decided to test it in conjunction with a different promoter. The MN promoter is TATA-less; therefore, it was interesting to examine the effect of PR4 on a more disparate TATA-box-containing SV40 promoter. Initially, a single copy of PR4 was tested both upstream and downstream of the transcription unit in pCAT promoter vector. In neither case had the element any effect on transcription (Fig. 8A). Then we decided to test whether multiple copies of PR4 are required to silence the relatively powerful SV40 promoter. The double-stranded PR4 oligonucleotide was multimerized in a head-to-tail fashion, and fragments containing four and eight copies of PR4 were inserted upstream of the SV40 promoter in the same vector. Multiple PR4 copies in both orientations upstream of the promoter significantly reduced the reporter activity in MaTu and HeLa (Fig. 8A) cells, confirming that PR4 can silence heterologous promoters. It is noteworthy that while a single copy did not have any effect, four copies already inhibited by 76% and eight copies inhibited by 84%. The effect of an additional four copies is thus limited to 8%, and inhibition per copy in the four-copy-containing construct is 19%, while in the eight-copy-containing construct it is less than 10%. When placed downstream of the CAT gene in the pCAT promoter, multiple copies of PR4 had similar effects (Fig. 8B). These results clearly demonstrated that the PR4 sequence exhibits a position- and orientation-independent silencing activity on heterologous promoter both in HeLa and MaTu cells, albeit multiple copies may be required. The silencing activity is contained within the 25 bp of PR4 sequence; moreover, the repressing activity of PR4 does not seem to be restricted to TATA-less promoters.

**Characterization of the Core Protein Binding Sequence within PR4—**Next we wanted to determine the nucleotides within the 25 bp of PR4 sequence involved in the interaction with the putative repressor. The binding sites of the majority of transcription factors characterized to date appear to consist of sequences with either dyad symmetry (inverted repeats) or direct repeats (22). Since PR4 contains an AGGGCACAGGGC direct repeat, we designed three double-stranded probes with the following mutations in the putative core sequence (mutated nucleotides are underlined): M1 ATTTTcacAGGGC; M2, AGGGCacATTTC; and M3, CTTTAcCTTTA. These three mutated probes were tested for their binding to HeLa NE and in competitive EMSA against the wild type PR4. While the M2 mutant competed partially against the wild type PR4, the M1 and M3 probes failed to do so (Fig. 9A), probably indicating that the repeats are not equally important for establishing contact with the repressor complex and that the first AGGGC sequence, closer to the 5’-end, is dominant. M1 and M3 probes failed to do so (Fig. 9A), probably indicating that the repeats are not equally important for establishing contact with the repressor complex and that the first AGGGC sequence, closer to the 5’-end, is dominant. M1 and M2 mutants retained some affinity to HeLa nuclear proteins, but these complexes seemed largely nonspecific, since they migrated to different positions (Fig. 9B). The binding pattern of M1 and M2 is virtually identical, except for stronger signals of M2 complexes. Competition experiments with the wild type PR4 or corresponding mutant oligonucleotide also indicate predominantly nonspecific binding because of very limited competition (Fig. 9B). The M3 mutant lost the binding capacity altogether.

**DISCUSSION**

One of the most striking features of the MN protein is its almost exquisite tumor-associated expression. In the absence of
any proven role for MN in the tumorigenic process, investigation of transcriptional regulation could provide important information about mechanisms leading to MN expression. The 3.5-kb upstream region of the MN gene was tested for the transcriptional activity of reporter gene constructs in MN-expressing cell lines. Transient transfection experiments demonstrated that this fragment was transcriptionally competent in CGL3, HeLa, and MaTu cells. In agreement with previous reports (6), reporter activities generated in MaTu cells were considerably higher than those in HeLa cells.

Deletion analysis of the 3.5-kb upstream region revealed the presence of several positive and negative regulatory regions, confirming thus the complex regulation of MN expression on transcriptional level. An enhancer element was located roughly around −1600, and two distal negative elements were located in the regions around −2000 and −900. The −173 to +31 region was designated as the MN promoter and was found transcriptionally active in CGL3, MaTu, and HeLa cells. The ratio of MaTu- and HeLa-produced reporter activity was similar to the one obtained with the 3.5-kb region, narrowing down the location of the cis-element(s) through which MN is up-regulated in MaTu cells to a relatively small region.

On the basis of deletion analysis, the −173 to +31 region seemed to contain most of the cis-elements critical for MN expression. Therefore, this region was investigated in detail by DNase I footprinting assay, EMSA, and transfection experiments using internally deleted promoter constructs. For footprinting, NE from CGL1 (MN-negative) and CGL3 (MN-positive) hybrids were used with the intent of revealing regions recognized differently, since these might be responsible for activation/repression of the MN promoter in CGL3/CGL1 cells. Footprinting revealed the presence of five PRs within the −173 to +31 fragment with no differences in patterns protected by NE from CGL1 and CGL3 cells. However, there was a quantitative difference in protection of PR4; while NE from CGL3 cells offered only partial protection, CGL1 extract protected the same region completely. Analogous results were obtained in EMSA with probes corresponding to each of the PR. NE from both cell lines produced the same binding pattern with the exception of PR4 where CGL1 NE produced much more PR4-specific complex. The results of footprinting and EMSA thus demonstrated a higher level of PR4 binding trans-factor in CGL1 cells, suggesting that this factor has a negative role in MN expression and functions as a repressor.

Next we investigated whether changed levels of PR4-specific complex are also associated with MN up-regulation in dense cultures and MaTu cells. However, comparative EMSA showed that NE from dense and sparse HeLa cultures as well as HeLa and MaTu cells produced quantitatively comparable PR4 binding. These results indicated that the mechanism leading to MN up-regulation in dense cultures and MaTu cells is distinct from the one in CGL1/CGL3 hybrid cells.

The negative effect of PR4 cis-element on MN transcription was demonstrated with a promoter construct in which PR4 and its flanking regions were internally deleted. Internally deleted construct instead of progressive deletion was employed for two reasons: 1) in this way, the contribution of the deleted PR to the transcriptional activity can be seen directly, and 2) internally deleted construct should generally produce higher reporter activity than the progressively deleted one as a result of retaining most of the promoter sequences. The construct lacking PR4 generated a 3 times higher reporter activity than the control pBMN5 (intact −173 to +31 construct) upon transfection into MaTu and HeLa cells. This was in good agreement with the previous results of footprinting and EMSA and strongly supported the theory that PR4 binds a repressor. A computer search of the Transfac data base with the PR4 sequence using Signal Scan identified putative binding sites for LF-A1 (GGGCA) and nuclear factor 1 (GCCA). Apparently, LF-A1 is a hepatocyte-specific factor (23); rather ubiquitous nuclear factor 1 was found to regulate negatively the PIT1/GHF1 (24) and P450A1 (25) promotors.

Next we carried out some preliminary characterization of the PR4 cis- and trans-acting elements. Although a single PR4 copy did not have any effect on an unrelated TATA-box containing SV40 early promoter, multiple tandemly arrayed copies of PR4 were capable of virtually abrogating transcription from this promoter regardless of the orientation or position. Functionally defined cis-acting elements that down-regulate transcription were recently classified as silencers and negative regulatory elements. Generally, while the former exhibit position-independent activity and direct active repression, the latter are position-dependent, inducing passive repression (26). Both categories can function in either orientation, may or may not affect heterologous promoters, and may be constitutive or inducible (27–29). Despite the growing number of genes controlled by silencers or negative regulatory elements, the significance of negative regulation of transcription remains to be established (30, 31). Apparently, PR4 element functions in a promoter-, orientation-, and position-independent manner and belongs to the group of “classical” silencers. Significant PR4 binding activity present in cells capable of MN expression suggests that PR4 may belong to the same category of silencer elements as those of B29 (32), bcl-2 (27), ETS-1 (33), and AP-1 (34) genes. Interestingly, all of these genes possess TATA-less promoters and initiate transcription at multiple start sites. Another feature common to many silencers seems to be GC-rich motifs (35). The fact that these silencers are active in cell types where their genes are expressed led to the postulation that they may serve to modulate the level of transcription of their respective genes rather than to control cell type-specific gene expression. Such silencers would restrict fluctuations in gene activity, thereby preventing deleterious consequences of expression (32). Constitutive silencers have also been demonstrated in promoters of such diverse genes as c-myc (36), c-fos (37), insulin (38), and growth hormone (39). An interesting feature of the PR4 si-
lencer is its cooperative mode of action that clearly distinguishes it from B29 gene silencer, for which the activity of two copies was essentially the same as that of a single copy (32). The experiments with heterologous promoter revealed that while a single copy did not have any observable effect, four copies of PR4 had already a marked effect on transcription. The effect of an additional four copies (eight total) was minor and may be either the result of saturation or limited availability of PR4 binding repressor. Also, we can conclude that the silencing information is contained within the PR4 25 bp.

EMSA experiments with mutant PR4 probes indicated that the repressor protein complex requires the direct AGGGCaGGGTC repeat for efficient binding. Mutation of any of the two repeat halves virtually abrogated the ability to compete against the wild type PR4 and severely compromised the DNA binding capacity. UV cross-linking of the PR4 binding repressor-DNA complex revealed that there are two repressor subunits in direct contact with the DNA with estimated molecular masses of 35 and 42 kDa. This makes involvement of the nuclear factor 1 in negative regulation of MN unlikely, since its estimated molecular mass is 74 kDa (40). Repressor complexes in general are composed of two integral components, a specific DNA targeting subunit and a second component mediating the repression (41). At present, we are unable to judge to what extent composition of PR4 binding repressor complies with this rule.

In an attempt to clone any of the two DNA binding components of the repressor, we used the MATCHMAKER one-hybrid system (CLONTECH Laboratories) with eight randomly arrayed copies of PR4 as a bait. However, upon transformation and selection on appropriate media, all YM4271 transformants generated very high levels of reporter expression (both HIS and LacZ). This may indicate the existence of an endogenous yeast transcription factor interacting with PR4 or a part of it and at the same time positively affecting transcription.

Internally deleted promoter constructs were employed also for characterization of the remaining PRs within the −173 to +31 region. With the exception of PR4, all other PRs were found to have a positive effect on transcription. The most pronounced effect was observed with PR1 and PR2; deletion of either of these dramatically decreased the reporter activity and led to identification of these PRs and their trans-acting factors as the major positive regulators of the MN transcription. Based on the computer search, PR1 and PR2 showed homology to the consensus binding sites for AP2 (CCCMNSSS) and AP1 (TGAGTCAG) transcription factors, respectively. Competition EMSA with unrelated probes containing AP2 and AP1 binding sites was used for preliminary verification of involvement of the respective factors. While AP1 probe completely abolished the PR2 binding, AP2 probe competed out just a single band generated by PR1. Although the documented positive effect of AP2 (42) and AP1 (43) on transcription would be in good agreement with the involvement of these transcription factors in MN regulation, identification of factors in other PR1 complexes and substantiation of their role will be the subject of further investigation. PR3 and PR5 seem to have a minor effect on transcription, since their deletion led to a small decrease of the reporter activity. The protection of PR3 and PR5 in a footprinting assay, compared with other PRs, was limited, and so was the affinity of these PRs in EMSA.

Experiments with internally deleted −173 to +31 constructs also suggested that there is a significant synergistic effect among trans-acting factors binding to cis-elements in the MN promoter. Synergistic cooperation among transcriptional activators in eukaryotic systems appears to be a general rule rather than an exception (44, 45). Deletion of PR1 or PR2 and their flanking regions resulted in a much more pronounced decrease in reporter activity than expected, and this may indicate potent interactions between PR1 and PR2 binding factors. In an enhancer mode, AP1 was found to stimulate activity of NF1, CP1, ATF/cAMP-response element-binding protein, and GC-box element in proximal position (46). There are several examples of promoters that require both an AP1 element and another element(s) for the transcriptional response characteristic of endogenous genes; e.g. the AP1 site was demonstrated to functionally cooperate with a neighboring upstream regulatory sequence in the stromelysin promoter (47), polyoma virus enhancer α-domain element in the collagenase promoter (48), and nuclear inhibitory protein in the interleukin-3 promoter (49). Again, to understand the synergism between PR1 and PR2 binding factors, it will be necessary to identify critical factor(s) that recognize PR1.

Detailed analysis of the MN promoter led to the identification of a novel type of silencer element in the −135 to −110 region of the MN promoter. Although the repressor binding to this region seems to be expressed in most cell lines tested, a significantly lower level of repressor is linked to MN expression in CGL1/CGL3 hybrid cells. At present, we are unable to establish a link between the levels of repressor and tumorigenic potential. Molecular cloning of a repressor subunit would help to further clarify these important issues.

On the other hand, conditions known to induce MN expression in other systems (increased cell density, MaTu cells) did not change the level of binding to the proximal silencer element. This points to the existence of a positively acting mechanism(s) that is necessary for overriding the silencing activity of this region. In the case of vascular endothelial growth factor, also positively regulated by high cell density, involvement of mitogen-activated protein kinases was demonstrated (50). If this correlation between cell density and mitogen-activated protein kinase activity is confirmed in HeLa cells, it would open up a possibility of MN regulation via AP1 and its synergistic activity.

The second positive mechanism seems to be in operation in MaTu cells, the only in vitro system where it can be observed. Increased cell density still leads to MN up-regulation, but the basal expression is considerably higher. Further work is required for establishing whether activation of MN expression in dense cultures and MaTu cells is indeed mediated by two independent pathways or just one.

On the basis of our work on the MN transcriptional regulation, we propose the following model of MN expression. A repressor binding to a proximal silencer element under normal circumstances tightly controls MN transcription. For activation of MN expression in vitro or in tumors in vivo, specific activation mechanisms are then required for overriding this repression.

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