Promoter characterization of the novel human matrix metalloproteinase-26 gene: regulation by the T-cell factor-4 implies specific expression of the gene in cancer cells of epithelial origin

George N. MARCHENKO*, Natalia D. MARCHENKO*, Jay LENG† and Alex Y. STRONGIN*[1]

*The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, U.S.A., and †Chemicon International, Inc., 28820 Single Oak Drive, Temecula, CA 92590, U.S.A.

A novel matrix metalloproteinase-26 (MMP-26) is known to be specifically expressed in epithelial carcinomas. To facilitate studies of MMP-26 transcriptional regulation, we have cloned and characterized a 1 kb 5′-flanking region of the human MMP-26 gene. Altogether, our findings indicate that the MMP-26 promoter has distinctive structural and functional features among MMP genes. An unusual polyadenylation site proximal to the transcription-factor-binding sites protects transcription of the MMP-26 gene from the upstream promoters and represents a part of the stringent transcriptional regulation of the gene. The MMP-26 gene has a consensus TATA-box and one transcriptional start site located 60 and 35 nucleotides upstream of the translational start site, respectively. The MMP-26 promoter was able to drive luciferase expression in human A549 lung carcinoma, HT1080 fibrosarcoma and HEK293 embryonic kidney cells. The basal transcription efficiency of the MMP-26 promoter is relatively low, thereby explaining the minute expression of the gene in most cells and tissues. When compared with other MMP genes, the MMP-26 promoter contains binding sites for a few transcription factors. Sequential deletion and mutation analysis, and electrophoretic mobility-shift assay have identified the T-cell factor-4 (Tcf-4) motif and the activator protein-1 site as the major regulatory elements of the MMP-26 promoter. Since previous studies have established that the Tcf-4 transcription factor is subjected exclusively to regulation through the β-catenin/E(epithelial)-cadherin pathway, this implies the specific expression of MMP-26 in cancer cells of epithelial origin.

Key words: β-catenin, carcinomas, E-cadherin, epithelial cancer, matrilysin-2, metalloproteinases, transcription.

INTRODUCTION

Matrix metalloproteinases (MMPs) comprise a family of at least 25–30 zinc endopeptidases with a critical role in tissue remodelling and cell motility [1]. MMPs are thought to be involved in a variety of normal and pathophysiological processes, including tumour progression, metastasis and angiogenesis [1–7]. Because of the destructive nature of these potent proteinases, the activity of MMPs is tightly controlled at the levels of gene expression, proenzyme activation and enzyme activity [1,6–9].

Identification of the expanding role of MMPs in complex regulatory and remodelling processes has stimulated the search for genes for encoding proteinases with a unique function, regulation and expression pattern. Recently, we identified a novel gene-encoding MMP tentatively called MMP-26 [12,14]. This confirms previous findings [10,11,13]. Since MMP-26 is related closely in its size and minimal domain structure to MMP-7 (matrilysin) [1], matrilysin-2 was a recommended trivial name for MMP-26. However, transcriptional regulation, substrate specificity and the functional role of MMP-26 and MMP-7 in cancer are likely to be distinct [10–13]. MMP-26 is primarily expressed in epithelial cancers such as lung, breast, endometrium and prostate carcinomas and in the corresponding tumour cell lines [10–13]. In normal adult tissues, the expression of MMP-26 is largely restricted to the kidney [12]. Specific expression of MMP-26 in epithelial carcinomas together with the relatively high proteolytic activity of this enzyme against fibronectin, vitronectin and fibrinogen [14] are intriguing, since it may indicate the existence of an important function for this proteinase. Additional studies are needed to identify the functional significance of the expression of the MMP-26 gene, and to elucidate the role of MMP-26 in tumours.

Recent studies have demonstrated that MMPs are regulated both negatively and positively at the transcriptional level by a variety of transcription factors [7]. So far, no information is available on the mechanisms involved in the transcriptional regulation of MMP-26. To elucidate these mechanisms, the present study attempted to determine the structure and function of the MMP-26 promoter. Here, we report a functional analysis of the MMP-26 promoter region and response elements that are likely to be important in mediating its unique expression in cancer cells of epithelial origin.

EXPERIMENTAL

Materials

Restriction endonucleases and other reagents used for molecular cloning were purchased from New England Biolabs (Beverly, MA, U.S.A.). *Pfx*-polymerase and media for cell culture were obtained from Gibco BRL (Rockville, MD, U.S.A.). Culture reagents for bacterial cultures were obtained from Difco Laboratories (Detroit, MI, U.S.A.). Chemiluminescence-digoxigenin

Abbreviations used: AP-1, activator protein-1; CMV, cytomegalovirus; DIG, digoxigenin; EMSA, electrophoretic mobility-shift assay; MMP, matrix metalloproteinase; PEA3, polyoma virus enhancer A-binding protein-3; Tcf-4, T-cell factor-4.

[1] To whom correspondence should be addressed (e-mail strongin@burnham.org).

The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF291665.

Table 1 Oligonucleotide primers used in the structural and functional characterization of the MMP-26 promoter

For cloning purposes the *Bgl*II restriction site was introduced in the sequence of the MMP26{-367} and MMP26{-72} primers. The modified nucleotides are shown in lower-case letters. The sequence of the AP1{cons} (direct) and AP1{cons-r} (reverse) primers corresponds to the known consensus sequence of the AP-1 site.

Name	Sequence	Orientation
MMP26{141}	5′-C^{141}ACCTACCTCAACAAAGTCCCATCCTTTATGG^{110}-3′	−
MMP26{-542}	5′-G^{-542}TCTaGATCTCCTGACTTGTG^{-522}-3′	+
MMP26{-367}	5′-aagaT^{-367}CTGGAGCAAGATTG^{-353}-3′	+
MMP26{-98}	5′-G^{-98}ACGCCACTCACAGATTCAAAGAAAGGGCAAAC^{-66}-3′	+
MMP26{-98-r}	5′-G^{-66}TTTGCCCTTTCTTTGAATCTGTGAGTGGCGTC^{-98}-3′	−
MMP26{-72}	5′-aagatctG^{-72}GCAAACTGGCAGAGTGAG^{-54}-3′	+
MMP26{AP1}	5′-C^{-66}TGGCAGAGTGAGTCATTGGATGT^{-43}-3′	+
MMP26{AP1-r}	5′-A^{-43}CATCCAATGACTCACTCTGCCAG^{-66}-3′	−
MMP26{TCFmut}	5′-G^{-98}ACGCCACTCACAGAgggAAAGAAAGGGCAAAC^{-66}-3′	+
MMP26{TCFmut-r}	5′-G^{-66}TTTGCCCTTTCTTTcccTCTGTGAGTGGCGTC^{-98}-3′	−
MMP26{AP1mut}	5′-C^{-66}TGGCAGAGTctGTCATTGGATGT^{-43}-3′	+
MMP26{AP1mut-r}	5′-A^{-43}CATCCAATGacAGACTCTGCCAG^{-66}-3′	−
MMP26{CCAATmut}	5′-G^{-72}GCAAACTGGCAGAGTGAGTCATTacATGTTGC^{-40}-3′	+
MMP26{CCAATmut-r}	5′-G^{-40}CAACATgtAATGACTCACTCTGCCAGTTTGCC^{-72}-3′	−
MMP26{AP4}	5′-C^{-126}ACTCTGCCCTCAGCAGGTATGGATGATG^{-98}-3′	+
MMP26{AP4-r}	5′-C^{-98}ATCATCCATACCTGCTGAGGGCAGAGTG^{-126}-3′	−
AP1{cons}	5′-CGCTTGATGAGTCAGCCGGAA-3′	+
AP1{cons-r}	5′-TTCCGGCTGACTCATCAAGCG-3′	−
MMP26{PAmut-r}	5′-G^{-150}CAGCCATGGAATTcgTTTTTAAGCTCAG^{-178}-3′	−

(DIG) detection employing the DIG Nucleic Acid Detection Kit was performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). The EMBL-3SP6/T7 genomic library in the λEMBL3 phage vector and AdvanTaq polymerase were obtained from Clontech (Palo Alto, CA, U.S.A.). Reagents for DNA-cycle sequencing were purchased from Promega (Madison, WI, U.S.A.) and [γ-^{32}P]ATP was from Amersham (Piscataway, NJ, U.S.A.). Nuclear extracts from HeLa cells and purified c-Jun protein were from Promega. All oligonucleotides used in our studies were synthesized by MWG Biotech (High Point, NC, U.S.A.). Human A549 lung carcinoma, HT1080 fibrosarcoma and HEK293 embryonic kidney cell lines were obtained from ATCC (Manassas, VA, U.S.A.). The oligonucleotide primers used in the structural and functional characterization of the MMP-26 promoter are presented in Table 1.

Isolation of genomic clones

Employing the DIG-labelled primers derived from the sequence of the MMP-26 gene, we screened a human genomic DNA library according to standard screening protocols. Briefly, for isolation of the genomic 5′-non-translation region of the MMP-26 gene, approx. 10^6 pfu of the human placental genomic library in λEMBL3 phage were screened using a 5′-DIG-labelled oligonucleotide MMP26{141} as probe that was complementary to nucleotides 110/141 of the human MMP-26 cDNA (numbering starts from the transcription initiation site of the MMP-26 genomic gene). Recombinant phages from seven isolated positive plaques were grown on *Escherichia coli* Y1090r$^-$ strain. Characterization of the positive recombinant phage clones was performed by extensive restriction-endonuclease mapping, PCR analysis and sequencing. The 1.8-kb fragment of the MMP-26 gene was cut with *Ssp*I and *Sca*I endonucleases and cloned into *Hin*cII site of the pUC18 vector. Further, this plasmid was used as a template for generation of the chimeric reporter pLUC plasmids.

Primer-extension analysis

The transcription start was identified by primer-extension analysis. For these purposes, the MMP26{141} oligonucleotide primer was 5′-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Total poly(A)-rich mRNA was isolated from the lysate of A549 lung adenocarcinoma cells employing poly(dT) magnetic beads (Dynal, Lake Success, NY, U.S.A.). An aliquot (50 ng) of total mRNA was mixed with the labelled primer (10^5 cpm) and incubated for 3 min at 70 °C. Then the temperature was decreased slowly to 42 °C to allow the primer to hybridize with the target mRNA. The mRNA was reverse-transcribed for 1 h with AMV reverse transcriptase (Roche Molecular Biochemicals). DNA products generated by primer-extension reaction were electrophoretically analysed on 6% acrylamide denaturing gel. The stepwise-sequencing ladder was obtained by using the standard M13 forward primer and the pGEM-3Zf(+) plasmid (Promega) as template.

Construction of the chimeric reporter plasmids

The promoterless pLUC-null vector was used to subclone several PCR-generated MMP-26 promoter fragments upstream of the *Renilla* luciferase reporter gene. For constructing the pLUC-null vector, we used two commercially available plasmids pEGFP-N2 (Clontech) and pRL-null (Promega). For this purpose, the *Ase*I–*Nhe*I fragment of the pEGFP-N2 plasmid encoding the cytomegalovirus (CMV) promoter was deleted by restriction nuclease cleavage followed by ligation. Next, the GFP gene in this promoterless pEGFP-N2 plasmid was replaced with the *Renilla* luciferase gene derived from the pRL-null vector. The 683-, 508-, 239- and 213-bp DNA fragments were amplified by PCR using the 1.8-kb genomic MMP-26 fragment as template and the MMP26{-542}, MMP26{-367}, MMP26{-98} and MMP26{-72} oligonucleotides as direct primers, respectively. The MMP26{141} oligonucleotide was used as reverse primer in these reactions. For cloning purposes, the *Bgl*II restriction site was introduced in the sequence of the MMP26{-367}

and MMP26{-72} primers. The T-cell factor-4 (Tcf-4) and activator protein-1 (AP-1)-binding motifs were modified using the MMP26{141} reverse primer, and the MMP26{TCFmut} and MMP26{AP1mut} mutant oligonucleotides as direct primers in the PCR respectively. The construct exhibiting the mutant CCAAT-binding motif was obtained by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) and the MMP26{CCAATmut} and the MMP26{CCAATmut-r} oligonucleotides as direct and reverse primers, respectively.

The generated PCR products were cloned into the *Hin*cII restriction site of the pUC18 plasmid, cut with *Bgl*II–*Xba*I or *Bam*HI–*Xba*I and further recloned into the *Bgl*II–*Xba*I sites of the pLUC-null vector to yield the pLUC-542, -367, -99 and -72 reporter plasmids, respectively. The *Sma*I and *Nco*I fragments were further deleted from the sequence of the pLUC-367 plasmid to generate the shorter pLUC-209 and -159 reporter plasmids, respectively. In the pLUC-367CMV, the putative poly(A) 5′-AATAAA-3′ -167/-162 region was modified by site-directed mutagenesis employing the MMP26{-367} and MMP26{PAmut-r} oligonucleotides as direct and reverse primers, respectively.

Transient transfection and luciferase assay

A549 lung adenocarcinoma, HT1080 fibrosarcoma and HEK293 embryonic kidney cells were used in our transfection experiments. Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. Further, cells (1×10^4 cells per well) were placed into wells of the 24-multiwell plastic plate (Corning, Corning, NJ, U.S.A.) in fresh DMEM/10% foetal bovine serum and grown for 16–24 h to 80% confluence. Next, cells were transiently co-transfected with a mixture containing 2 μg of the individual luciferase reporter plasmid and 0.4 μg of the pβgal-control plasmid (an internal control; Clontech) by using LIPOFECTAMINE 2000 (Life Technologies, Rockville, MD, U.S.A.). Cells transfected with the promoterless pLUC-null vector were used as a negative control. Cells transfected with the pRL-CMV plasmid, where the *Renilla* luciferase gene was placed under the control of the CMV promoter (Promega) were used as a positive control in our transfection studies. PMA (40 nM) was added to the cells for the last 6 h of incubation. The pRSV-cJun plasmid bearing the full-length cDNA of the c-Jun transcription factor and the pZIP-Ras61L plasmid expressing the Ras oncogene cDNA (a gift from Dr Craig Hauser, The Burnham Institute) were each co-transfected with the *Renilla* luciferase reporter plasmids. After 48–60 h transfection, the cells were harvested, washed and lysed by three freeze–thaw cycles in a buffer containing 100 mM potassium phosphate and 1 mM dithiothreitol (DTT), pH 7.8. The lysates were centrifuged at 14000 ***g*** for 30 min. The pellet was discarded and each supernatant was measured in triplicate for both the luciferase and β-galactosidase activity. The luciferase activity was determined in potassium phosphate buffer, pH 7.8, containing 0.5 M NaCl, 1 mM EDTA and 1 mM coelenterazine (Biotium, Hayward, CA, U.S.A.). The β-galactosidase activity was assayed using the Luminescence β-Gal Assay kit according to the manufacturer's recommendations (Clontech). Chemiluminescence was measured in the MicroLumat EG&G LB96P luminometer according to the manufacturer's manual (Berthold, Bundora, Australia). The mean of three measurements was taken to obtain the relative luciferase activity that was expressed in relative units defined as the ratio of *Renilla* luciferase to β-galactosidase activity. Data are presented as mean ± S.E.M. of at least three independent transfection experiments.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared from A549 and HT1080 cells according to the standard protocols [15]. The direct and reverse 5′-DIG-labelled oligonucleotide primers that corresponded to the AP-1 promoter region (MMP26{AP1} and MMP26{AP1-r}, respectively), Tcf-4 region (MMP26{-98} and MMP26{-98-r}, respectively) and AP-4 region (MMP26{AP4} and MMP26{AP4-r}, respectively) were dissolved at a concentration of 10 pmol/μl in 20 mM Tris/HCl buffer, pH 7.9, containing 10 mM $MgCl_2$, 50 mM NaCl and 2 mM DTT at 92 °C and then slowly cooled to room temperature (20 °C). Nuclear extracts (2.5 μg total protein) or the purified c-Jun protein (1 μg) and DIG-labelled double-stranded primers (0.05 pmol each) were mixed in 10 μl of 10 mM Tris/HCl, pH 7.5, containing 4% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 10 μg/ml poly(dI-dC) and incubated for 30 min at 5 °C. Where indicated, 2.5 pmol (50-fold molar excess) of unlabelled specific (MMP26{AP1}/MMP26{AP1-r} and MMP26{-98}/MMP26{-98-r}) and mutant (MMP26{AP1mut}/MMP26{AP1mut-r} and MMP26{TCFmut}/MMP26{TCFmut-r}) double-stranded competitor oligonucleotides were added to the respective reactions. To detect the pre-formed complexes, the samples were run on 7% non-denaturing polyacrylamide gel in 0.5× Tris/borate/EDTA buffer and then transferred to a PVDF Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). Oligonucleotides were covalently attached to the membrane by UV-crosslinking. The DNA–protein complexes were detected on the membranes by the chemiluminescence detection method (Roche Molecular Biochemicals) followed by autography using an X-ray F-GX-57 film (Phenix Research Products, Hayward, CA, U.S.A.) for 3–12 h.

Nucleotide sequencing and computational analysis

Sequencing of both DNA strands was performed by the ABI Prism dye terminator method using an ABI377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide sequence of the 5′-flanking region of the MMP-26 genomic gene was deposited in the GenBank®. The Laser Gene (DNASTAR Inc., Madison, WI, U.S.A.), MatInspector (http://genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl) and Promoter Ver. 2.0 (http://www.cbs.dtu.dk/services/promoter/) software packages were employed for extensive structural analysis of the genomic gene and the identification of the putative transcription-factor-binding sites, respectively.

RESULTS

Molecular cloning of the 5′-flanking region of the human MMP-26 gene

According to our earlier observations, the human MMP-26 gene is expressed in epithelial carcinomas of various tissue/organ origin. Accordingly, expression of this MMP gene appears to be restricted and subjected to stringent regulation that is likely to be lost in epithelial breast, prostate, endometrium and lung tumours and the respective tumour cell lines. In our attempts to elucidate the molecular mechanisms governing the expression of the MMP-26 gene and to identify regulatory motifs and structural elements that could affect its transcription, we started a structural and functional analysis of the promoter region of the MMP-26 gene.

For these purposes, we identified the nucleotide sequence of a 1.0-kb genomic fragment containing the 5′-flanking region of the MMP-26 gene. To identify a genomic clone containing the 5′-flanking region of the genomic MMP-26 gene, we screened a

Figure 1 Sequence of the 5′-flanking region of the human MMP-26 gene

Numbering starts from the C nucleotide of the transcription initiation site and is indicated with a bent arrow. The TATA-box (TATA), the AP-1, Tcf-4, AP-4-like motif, HFH-3 motif and the poly(A) site (PA) are boxed. An arrow indicates the CCAAT motif. The translation initiation ATG codon is underlined. The amino-acid sequence of MMP-26 is shown above the DNA sequence. The predicted amino-acid sequence of the uncharacterized proximal protein is shown in italics and the termination codon is marked with a star. The sequence of the first intron of the MMP-26 gene is italicized and given in lower-case letters. The putative HFH-3 motif was identified but not studied since it is located upstream of the poly(A) site.

human placental genomic library with the DIG-labelled specific MMP26{141} probe. Seven positive phage clones were isolated from the library. These clones were analysed by restriction mapping and PCR. The 1.8-kb *Ssp*I–*Sca*I fragment comprising a 5′-untranslated region, the coding sequence of the first exon and a part of the first intron of the MMP-26 gene was cloned into *Hin*cII site of the pUC18 plasmid. The sequence of this fragment was determined in both directions to confirm the sequence of the MMP-26 chromosomal gene that we have previously deposited in the GenBank®. Further, this DNA fragment (Figure 1) was used as template to generate the shorter chimeric luciferase constructs used in our transient-transfection assays.

Mapping of the transcription start site

Previously, we have reported the chromosome localization and the intron–exon structure of the human MMP-26 gene [12]. According to our data, the genomic gene of MMP-26 is localized at the 11p15.3 loci, thereby being distant from the 11q21-q23 cluster of at least eight MMP genes (MMP-1, -3, -7, -8, -10, -12, -13, -20 and -27) [16]. The chromosomal gene of MMP-26 consists of six exons. The exon–intron boundaries conform to the GT/AT rule for splice sites. The splice sites are at positions conserved among most MMP genes. The exon–intron structure of MMP-26 is strikingly similar to that of MMP-7, thus indicating close relationships between these two MMPs. So far, no alternative splicing of the MMP-26 mRNA has been identified [12].

Here, we have attempted to define the site of transcription initiation of the MMP-26 gene by using primer-extension analysis with poly(A)-rich mRNA isolated from A549 lung carcinoma cells. We specifically used A549 cells for our studies since this cell type has been demonstrated to express the MMP-26 gene previously [12,14]. A 32-mer oligonucleotide MMP26{141} complementary to the 141/110 region of the genomic gene (numbering starts from the C nucleotide of the transcription initiation site of the MMP-26 genomic gene) was used as a primer. The primer extension resulted in a single 141-bp band indicating the existence of a single transcription initiation start of the MMP-26 gene located 35 nucleotides upstream of the ATG translation initiation codon (Figure 2). On the contrary, the mRNA samples from HT1080 cells, which according to our earlier findings failed to express the MMP-26 gene, generated no specific bands in primer-extension reactions (results not shown).

Promoter region and the putative transcription-binding sites

Computer analysis (Figure 1) of the 5′-flanking region revealed that coding part of the MMP-26 gene is located 587 bp downstream of a stop codon of a putative uncharacterized protein sequence that is highly similar to the Alu-like sequence recently deposited in GenBank® (accession no. P39190). Most probably, the promoter and the transcription-factor-binding motifs of MMP-26 are located within this 587-bp genomic region.

The sequence of the promoter region of the MMP-26 gene is a putative recognition site for several regulatory proteins. There is a canonical 6-bp 5′-TATAAA-3′ TATA-box at position -30 relative to the transcription initiation site. Other putative TATA-box-like sequences existing at the positions -251/-254 and -328/-331 are too distant from the transcription initiation site to be functionally important.

The putative binding sites of the HFH-3 (hepatocyte nuclear factor-3; 5′-TCTTTATT-3′), AP-4 (5′-CTCAGCAGG-3′), Tcf-4 (5′-TTCAAAG-3′), AP-1 (5′-TGAGTCA-3′) and CCAAT-binding protein (5′-ATTGG-3′) recognition sequences [17–20] were found at positions -428/-421, -117/-109, -83/-77, -57/-51 and -51/-47 upstream of the transcription initiation site, respectively. The respective positions of the TATA-box and the AP-1 site of the MMP-26 promoter are similar to those found in promoter regions of most other MMPs (Figure 3) [7,21–24]. These results provide the basis for future characterization of the regulation of the MMP-26 gene expression in cancer.

Thus, to perform a functional characterization of the regulatory structural elements identified in the 5′-flanking region of the MMP-26 genomic gene, DNA constructs containing various lengths of the promoter inserted in front of the *Renilla* luciferase reporter gene [25–27] were transiently transfected into A549 lung

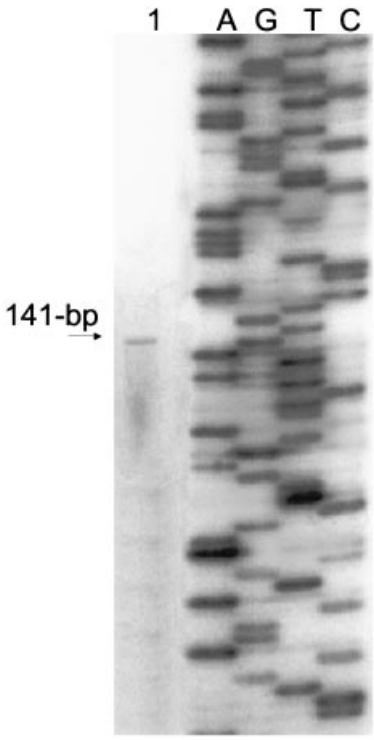

Figure 2 Determination of the transcription initiation site of the human MMP-26 gene

Lane 1: primer extension with mRNA isolated from A549 cells. Lanes A, G, T and C represent sequencing reactions of the pGEM-3Zf(+) plasmid (Promega) using the standard M13 forward primer and are used to determine the exact position of the 141-bp extended product (indicated with an arrow).

carcinoma and HT1080 fibrosarcoma cells. The transcriptional activity corresponding to each promoter fragment was determined in a dual luciferase/β-galactosidase assay. We examined six fragments with 5′-flanking sequences of the MMP-26 gene that ranged from 72 to 542 nucleotides (Figure 4A). The highest luciferase activity was observed for pLUC-98. Thus this construct demonstrated a 3-fold higher transcriptional activity compared with the shorter pLUC-72. This may be due to the positively acting AP-1- and CCAAT-binding sites and, especially, Tcf-4 transcription-factor-binding site located between positions -83 and -77 (Figure 1). According to the luciferase activity of the other four longer constructs (pLUC-542, -367, -208 and -159), no significant difference could be observed between these promoter fragments.

Interestingly, the chimeric pLUC-542, -367, -208, -159 and -98 were approx. twice as efficient in A549 cells when compared with HT1080 cells whereas the pLUC-72 lacking the Tcf-4 element was equally efficient in both cell types. These findings which indicated a possible role of the Tcf-4 binding motif in the regulation of transcriptional activity of the MMP-26 gene in A549 cells were further supported by our EMSA studies. In addition, these data are in agreement with the expression of the MMP-26 gene observed earlier in A549 cells but not in HT1080 cells [12].

Recently, the important role of the Tcf-4 transcriptional regulator has been demonstrated in regulating the transcriptional activity of the human and murine MMP-7 gene [28,29]. To evaluate further the significance of the AP-1, Tcf-4 and CCAAT-binding sites, these sites were modified by introducing mutations in the respective sequence regions of the pLUC reporter plasmids. The resulting mutant pLUC-98mut, -72mut and -72mut2 plasmids bearing the inactivated Tcf-4-, AP-1- and CCAAT-binding sites, respectively, were each transfected into A549 and HT1080 cells. According to our results, modifications of the Tcf-4- or AP-1-binding site strongly inhibited (at least, 2.5–3-fold in both cases) the transcriptional activity of the chimeric constructs. On the contrary, the inactivation of the CCAAT-binding site failed to affect strongly the transcriptional activity of the MMP-26 promoter.

The AP-1 sequence motif is known to be recognized by a transcriptional complex composed of members of the c-Fos and c-Jun families and to mediate phorbol-ester induction of a variety of genes. Oncoproteins such as Ras were also known to upregulate the expression of MMP genes. To this end, we transfected the reporter pLUC-98 and -72 plasmids into cells and tested inducibility of the MMP-26 promoter by PMA. In addition, to elucidate the effects of c-Jun and Ras, we co-transfected pLUC plasmids with pRSV-cJun and pZIP-RAS61L plasmids encoding for the full-length c-Jun and Ras cDNA, respectively. PMA or c-Jun alone did not cause any significant effect on the transcriptional activity of the MMP-26 promoter in both tumour cell types, whereas co-transfection of cells with Ras elevated it relative to the luciferase activity by approx. 30%.

To examine further the effects of c-Jun on the transcriptional efficiency of the MMP-26 promoter, we employed human HEK293 embryonic kidney cells. Transfection of these cells with the reporter pLUC-98 plasmid alone induced a relatively low luciferase activity. Co-transfection of cells with the pLUC-98 and the pRSV-cJun failed to stimulate the transcriptional efficiency of the hybrid construct. However, treatment with PMA of the cells transfected with the pLUC-98 strongly upregulated the luciferase activity. Co-transfection of pLUC-98 with pRSV-cJun and treatment with PMA further increased the transcriptional efficiency of the promoter (Figure 4B). These observations suggest that the AP-1 site may contribute significantly to promoter activity of the MMP-26 gene.

The putative 5′-AATAAA-3′ polyadenylation site (positions -167/-162) was found between the translation stop codon of the Alu-3-like sequence and the translation initiation codon of the MMP-26 gene. To elucidate the role of this polyadenylation site we modified the pLUC-367 and -98 plasmids by inserting the potent CMV promoter upstream of the 5′-flanking region of the MMP-26 gene. The resulting pLUC-367CMV and -98CMV did and did not exhibit the polyadenylation site of the MMP-26 gene, respectively. The chimeric constructs were transiently transfected into A549 and HT1080 cells and the activity of luciferase was evaluated. The transcriptional efficiency of the pLUC-367CMV was relatively lower when compared with that of the original pLUC-367 plasmid (Figure 4A). However, a deletion of the polyadenylation site (pLUC-98CMV) increased the luciferase activity of the CMV construct by approx. 60-fold. The efficiency of the pLUC-98CMV was about 200-fold higher relative to that of the original pLUC-98 that exhibits the regulatory elements of the MMP-26 gene. Thus, the 5′-AATAAA-3′ -167/-162 polyadenylation site may represent a strong transcription termination signal. Furthermore, there is a (G+T)-rich region downstream of the -167/-162 site in the 5′-sequence of the MMP-26 gene. The (G+T)-rich region has been shown in many systems to be important for poly(A) site cleavage [30], lending more credence to our model.

In order to provide more convincing evidence for our hypothesis and to demonstrate the functionality of the 5′-AATAAA-3′ -167/-162 region as a poly(A) site, we mutated this

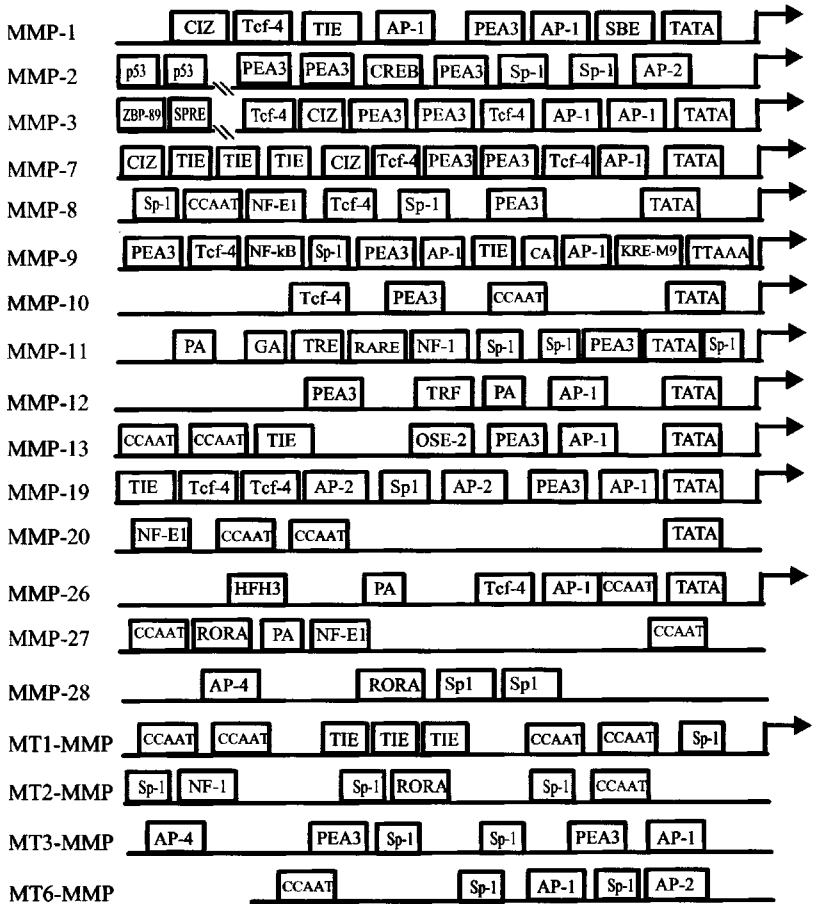

Figure 3 Regulatory elements of promoter regions of MMP

Putative transcription-factor-binding sites localized within an approx. 1-kb region (except the shorter sequence available for MT6-MMP) upstream of the transcription initiation site are indicated within boxes. Transcription start is indicated with a bent arrow except for MMP-20, -27, -28, MT2-, MT3- and MT6-MMP, where the respective transcription initiation sites remain unidentified. The relative positions of the binding sites were identified by using the available data from the literature. In addition, MatInspector (http://genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl) software package was employed for the identification of the putative transcription-factor-binding sites in the promoter regions of MMP-20 and MMP-27 (both in GenBank® accession no. AP000851), MMP-28 ([14]; and G. N. Marchenko and A. Y. Strongin, unpublished work), MT2-MMP (accession no. AC012182), MT3-MMP (accession no. AC090797), and MT6-MMP (accession no. AC083846). Contradictory data indicate that the unconventional TTAAA sequence may function as the TATA-box in MMP-9 [42]. No TATA-box was found in MMP-2, -27, -28, MT1-MMP, MT2-, MT3- and MT6-MMP. Note the Tcf-4 site and the highly unusual poly(A) site in MMP-26. TATA, TATA-box; TIE, TGF-β inhibitory element; Sp1, transcription factor Sp-1; OSE-2, osteoblast-specific element-2; TRF, octamer-binding protein; LBP-1, leader-binding protein; HFH3, hepatocyte nuclear factor-3; RORA, retinoic acid receptor-related orphan receptor alpha; CCAAT, CCAAT-binding proteins; CIZ, Cas-interacting zinc-finger protein; SBE, STAT-binding element; p53, p53-binding element; CREB, cAMP response element-binding protein; ZBP-89, the 89-kDa zinc-binding protein; SPRE, stromelysin-1 platelet-derived growth factor B PDGF-responsive element; NF-1, nuclear factor-1; NF-E1, nuclear factor-E1 (p45); NF-κB, nuclear factor-kappaB (p65); TRE, thyroid hormone-responsive element; RARE, retinoic acid-responsive element; GA and CA, GA- and CA-repeats, respectively; KRE-M9, keratinocyte differentiation factor-1 responsible element-4; PA, polyadenylation site.

region in the pLUC-367CMV plasmid by site-directed mutagenesis employing the MMP26{PAmut-r} oligonucleotide. The resulting pLUC-367CMV-mut mutant plasmid bearing the inactivated $-167/-162$ site with the sequence 5′-AAcgAA-3′ was generated after certain routine manipulations (modified nucleotides are shown in lower-case letters). The mutations of the poly(A) region increased the luciferase activity of the CMV construct in A549 cells approx. 3-fold relative to that of the original pLUC-367CMV plasmid. Since the post-transcriptional cleavage of the chimeric mRNA exhibiting the modified poly(A) motif was not affected, the increase in the luciferase activity of the mutant indicates that the 5′-AATAAA-3′ -167/-162 region may indeed function as a poly(A) site. It is likely that the existence of this polyadenylation site aborts putative transcription of the MMP-26 gene from the upstream promoters and represents an additional control element that is involved in maintaining the minute levels of the MMP-26 mRNA in most cell types.

Results from EMSA analysis

To investigate further the role of AP-1 and Tcf-4 motifs in the regulation of the MMP-26 gene, we used the respective wild- type and mutant oligonucleotides in an EMSA. For these purposes, DIG-labelled double-stranded oligonucleotides containing the potential AP-1- and Tcf-4-binding sites were incubated with HeLa, A549 and HT1080 nuclear extracts or purified recombinant c-Jun protein. Specific or non-specific competition of AP-1 and Tcf-4 was performed by the addition of 50-fold molar excess of the unlabelled competitor (Figure 5). One specific protein–DNA complex was observed in the AP-1 DIG-labelled oligonucleotides incubated with the c-Jun. Excess of non-

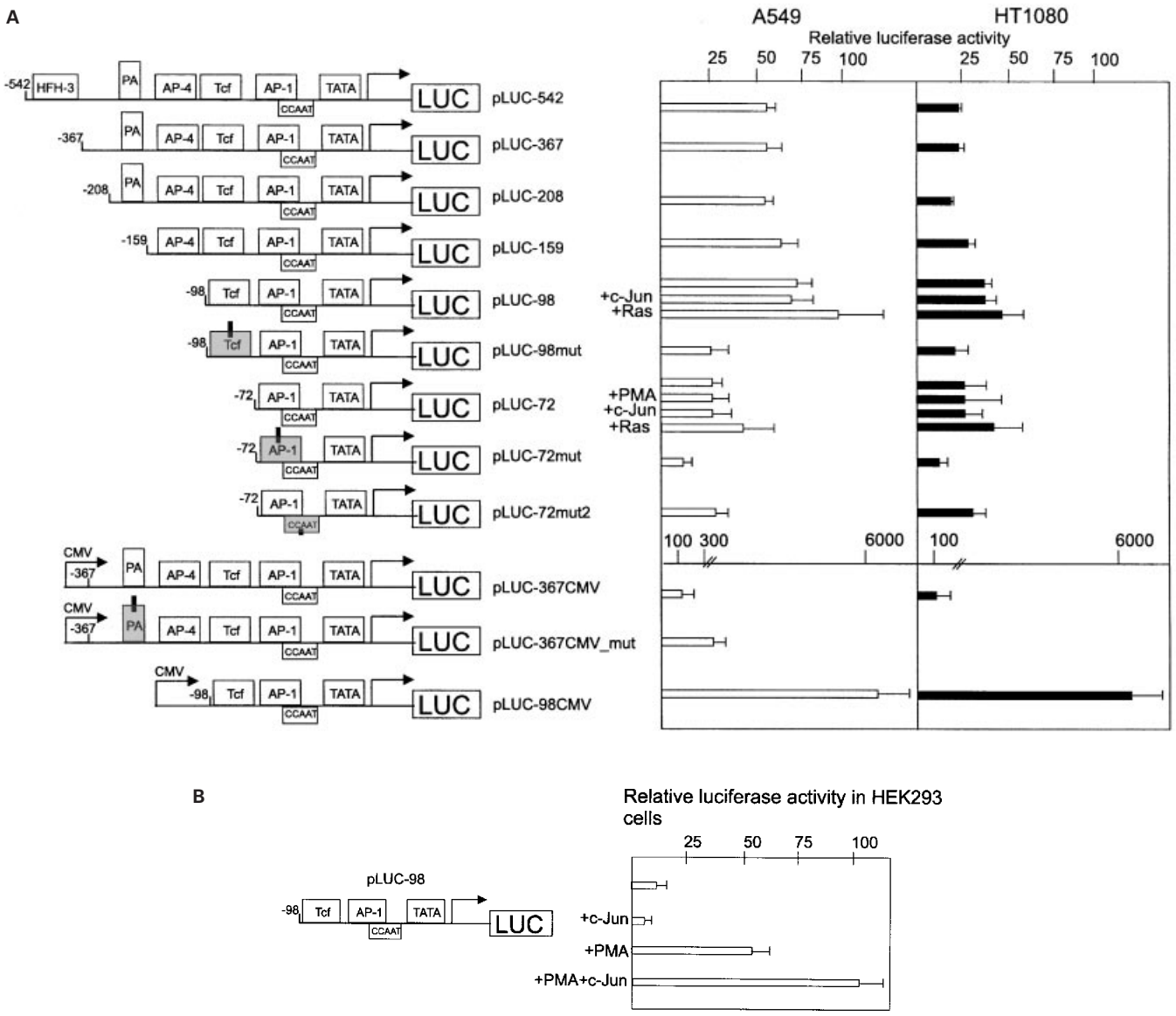

Figure 4 Functional analysis of the MMP-26 promoter–luciferase chimeric genes

The individual chimeric pLUC plasmids (schematically shown on the left-hand-side panel) were co-transfected into A549 and HT1080 cells (**A**) and HEK293 cells (**B**) with the pβgal control plasmid, which was used as an internal control for transfection efficiency. Luciferase activity was determined using coelenterazine as a substrate. The background luciferase activity of the control promoterless pLUC-null plasmid was subtracted from the activity of the chimeric pLUC constructs. The luciferase activity was expressed in relative units defined as the ratio of *Renilla* luciferase to β-galactosidase activity. The relative luciferase/βgal activity in (**A**) and (**B**) is shown on the right-hand-side panel and as percentage relative to the activity of the pLUC-98 construct in A549 cells co-transfected with the pZIP-RAS61L plasmid (Ras) and as percentage relative to the activity of the pLUC-98 construct in the PMA-treated HEK293 cells co-transfected with the pRSV-cJun plasmid (c-Jun). The regulatory motifs are shown with boxes. Mutations inactivating the Tcf-4, AP-1, PA and CCAAT motifs are shown as shaded boxes pierced with a thick line. Following transient transfection, PMA (40 nM) was added, where indicated, to cells during the last 6 h of incubation. The pLUC-98 and -72 constructs were each co-transfected with the pRSV-cJun and pZIP-RAS61L plasmids in A549 and HT1080 cells. Where indicated, the pLUC-98 plasmid was co-transfected with the pRSV-cJun plasmid in HEK293 cells. Data are presented as mean $\pm$ S.E.M. of three measurements of at least three independent transfection experiments.

labelled specific double-stranded MMP26{AP1} competitor inhibited this complex. A similar DNA–c-Jun complex was observed in the experiments employing the AP-1 consensus double-stranded DIG-labelled AP1{cons} oligonucleotide, thereby indicating a potentially important role of c-Jun in regulating the transcriptional activity of the MMP-26 gene. As expected, the mutant version of the AP-1 motif when used as a competitor failed to affect the DNA–c-Jun complex formation.

Interestingly, in similar EMSA studies employing the wild-type DIG-labelled Tcf-4 double-stranded oligonucleotide MMP-26{-98}, one specific similar DNA–protein complex was observed in HeLa and HT1080 nuclear extracts (Figure 5). In contrast, an additional protein–DNA complex was observed in A549 cells. This complex may represent a heterodimer involving Tcf-4 and, probably, other regulatory proteins. The protein–DNA complexes were competitively inhibited by 50-fold molar excess of the respective specific unlabelled oligonucleotides but not mutant oligonucleotides, where the Tcf-4-binding site (5′-TTCAAAG-3′) was inactivated by mutations (5′-gggAAAG-3′; mutant nucleotides are shown in lower-case letters).

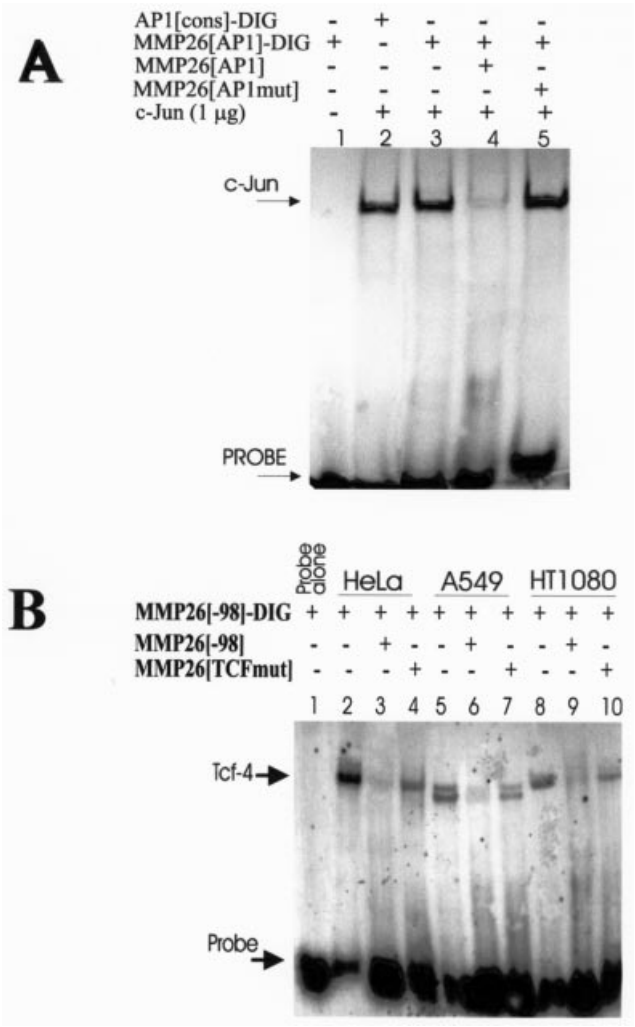

Figure 5 EMSA of the AP-1 (A) and Tcf-4 (B) binding motifs of the MMP-26 promoter

(**A**) Double-stranded, DIG-labelled MMP26{AP1} oligonucleotide corresponding to the AP-1 site was incubated alone (lane 1) or with bacterially derived and purified c-Jun (1 μg) (lanes 3–5). Unlabelled oligonucleotides MMP26{AP1} and MMP26{AP1mut} used as specific and non-specific competitors were added in 50-fold molar excess (lanes 4 and 5, respectively). Double-stranded, DIG-labelled AP1{cons} corresponding to the consensus sequence of the AP-1 motif was used as control (lane 2). (**B**) Double-stranded, DIG-labelled oligonucleotide MMP26{-98} corresponding to the sequence of the Tcf-4-binding site was incubated alone (lane 1) or with nuclear extract from HeLa (lanes 2–4), A549 (lanes 5–7) or HT1080 (lanes 8–10) cells. Unlabelled oligonucleotides MMP26{-98} and MMP26{TCFmut} used as specific (lanes 3, 6, 9) and non-specific (lanes 4, 7, 10) competitors were added in 50-fold molar excess.

EMSA employing oligonucleotides that contained the potential AP-4 binding site failed to detect any specific protein–DNA complex in nuclear extracts of either HeLa, A549 or HT1080 cells (results not shown).

DISCUSSION

Recent studies have identified the human genes coding for MMP-26, MMP-28 and MMP-21 [10–14,31,32]. One of the three newly isolated genes encoded MMP-26. MMP-26 is distinguished by the unique PHCGVPD cysteine-switch motif. The latency of zymogens of all known MMPs is maintained by co-ordination of the active-site zinc by an unpaired cysteine sulphydryl group within a PRCG(V/N)PD conserved sequence motif of the propeptide domain. The presence of a His-81 in this motif distinguishes MMP-26 from all MMPs characterized so far [1]. In our efforts to identify the role of MMP-26 in cancer, we have already elucidated the nucleotide sequence, the chromosomal location in the genome, expression pattern in normal and tumour tissues and the genomic organization of the gene-encoding MMP-26 [12]. The protein sequence, minimal modular domain structure, exon–intron map and computer modelling demonstrate relatively close relations of MMP-26 with MMP-7 (matrilysin) [1,33]. However, substrate specificity, transcription regulation and a functional role of MMP-26 and MMP-7 in cancer are likely to be distinct. Surprisingly, MMP-26 is specifically expressed in carcinomas of epithelial origin [10–13]. The restricted expression pattern suggests that MMP-26 is subjected to a tight regulation that has been lost in epithelial carcinomas. In the present study, we characterized both structurally and functionally the promoter of the MMP-26 gene. We identified a single transcript of the MMP-26 gene with the transcription-initiation site located 35 nucleotides upstream of the ATG translation-initiation codon of the MMP-26 gene.

The most striking feature of the MMP-26 promoter is its relatively simple organization and tight regulation limited to a very few structural elements such as the canonical TATA-box, the AP-1-binding element, the TCF-4-binding sequence and the polyadenylation site at a position closely upstream of the transcription initiation site. Our experimental results suggest that the putative CCAAP- and AP-4-binding sites are not critical for the induction of basal transcription of the MMP-26 gene, whereas the TATA-box, the AP-1 and, especially, the Tcf-4 and the poly(A) sites are involved in the transcriptional regulation of the gene. Many MMP promoters share the 5′-(C/G)AGGAAG(T/C) consensus element that was observed in conjunction with the AP-1-binding site. This binding element, designated as PEA3 (polyoma virus enhancer A-binding protein-3), is located upstream of the AP-1 element in many MMPs including MMP-1, -3, -7, -9, -12, -13 and, probably, -19 [7,22]. On the contrary, the MMP-26 promoter exhibits no sequence elements that are structurally related to the PEA3 consensus motif, nor the Sp-1 element, which is also common for many MMPs or any other regulatory motifs. The structural organization of the MMP promoters is summarized in Figure 3.

The positional arrangement of the TATA-box and the AP-1 site is similar to that found in most other MMP promoter regions, with the exception of MMP-11 lacking a consensus AP-1 site, and MMP-2, MMP-28, MT1-MMP and MT2-MMP all lacking both the TATA and AP-1 motifs [7,14]. In EMSA, the oligonucleotide that represented the AP-1 site of MMP-26 was capable of binding to purified c-Jun, thereby suggesting the functionality of the AP-1 motif in the MMP-26 promoter. Inactivation of the AP-1 site of the reporter luciferase plasmid by mutations decreased the efficiency of the MMP-26 promoter. In transfection experiments employing the luciferase reporter plasmids and HEK293 embryonic kidney cells, stimulation of cells with PMA or co-transfection with c-Jun significantly activated the minimal MMP-26 promoter. Co-transfection with Ras also enhanced the transcriptional activity of the minimal promoter that included the AP-1 and TATA-elements. These findings indicate that c-Jun is possibly a potent activator of MMP-26 gene expression. However, transactivation of the MMP-26 promoter may be dependent on the interactions with other transcriptional factors binding to additional regulatory *cis*-elements in the 5′-flanking region of the MMP-26 gene. The most probable candidate is the Tcf-4 element. Thus, deletion or inactivation by mutations of the putative Tcf-4-binding site significantly reduced

the transcriptional efficiency of the respective MMP-26 promoter constructs in both A549 and HT1080 cells. The ability of the Tcf-4 motif to bind directly specific transcription factor(s) in nuclear extracts from HeLa, A-549 and HT1080 cells was confirmed in EMSA.

The unexpected feature of the 5′-flanking region of the MMP-26 gene is the presence of the polyadenylation site located within the promoter region. In the respective chimeric reporter plasmids, this site efficiently terminated the transcription initiated by the CMV promoter. In agreement, inactivation of the poly(A) site by mutations promoted the activity of the CMV promoter by approx. 3-fold. Furthermore, deletion of the sequence fragment that included both the poly(A) motif and the putative (G+T)-rich post-transcriptional cleavage site caused a 60-fold increase in the efficiency of the CMV promoter. No putative poly(A) sites were identified in similar positions in other MMPs except MMP-12 and -27, where the function of this site is unknown (Figure 3). However, by supporting a functional role for the poly(A) site our data do not exclude the presence of a classical DNA-binding transcriptional repressor site in the -367/-98 region of the MMP-26 gene. The nature of this site remains yet to be identified.

Thus our studies demonstrated that the limited but stringent regulation of the MMP-26 gene is likely to involve the AP-1 and, especially, Tcf-4 elements and the poly(A) site. Our observations suggest that there is an evident difference between the regulation of MMP-26 and structurally similar MMP-7. It is likely that the presence of the poly(A) site inhibits transcription of the MMP-26 gene from the upstream promoters and represents a part of the stringent transcriptional regulation of this gene. The basal transcription efficiency of the MMP-26 promoter is relatively low, thereby explaining the minute expression of the gene.

Interestingly, in contrast with most other MMP genes, Tcf-4 transcription factor appears to be a potent transcription regulator of MMP-26. Tcf-4 is a member of the high mobility group of architectural transcription factors [34], which introduce sharp bends in the DNA and facilitate interactions with other regulatory factors. Tcf factors, including Tcf-4, constitute a downstream component of the Wnt signal transduction pathway [35]. In the absence of the Wnt signalling, β-catenin is constitutively degraded in proteasomes, whereas in the presence of Wnts, β-catenin is stabilized and can heterodimerize with the TCF proteins to form a transcriptional activator complex [36]. The loss of functional adenomatous polyposis coli protein results in the stabilization of cytosolic β-catenin and activation of genes that contribute to Tcf family transcription factors. In tumours, β-catenin degradation is blocked by mutations either in β-catenin or adenomatous polyposis coli. As the activating subunit of the Tcf-4–β-catenin nuclear complex, β-catenin performs dual tasks: it alleviates repression of target gene promoters and subsequently activates them. The β-catenin–Tcf-4 transcription complex was demonstrated to upregulate strongly the transcription of MMP-7 [28,37], c-Myc, cyclin D1, Tcf-4 and fibronectin [38–40]. In addition, it has been well established that β-catenin when complexed with the cytoplasmic domain of E(epithelial)-cadherin, the major cadherin molecule specifically expressed by epithelial cells, mediates Ca^{2+}-dependent cell–cell adhesion [41]. Generally, a loss of expression or function of E-cadherin facilitates a reciprocal increase in the Tcf-4–β-catenin complexes. Since E-cadherin is expressed uniquely in epithelial cells, the axis involving Tcf-4, β-catenin and E-cadherin is likely to function efficiently only in epithelial tumours. Since the structure of the MMP-26 promoter suggests that there are no transcription factors except Tcf-4 and AP-1 capable of effectively regulating the expression of the MMP-26 gene, it explains why the expression of the MMP-26 gene has been restricted largely to cancer cells of epithelial origin. Thus our data correlate and extend the observations that Tcf-4 plays an important role in the transcriptional regulation of the MMP genes.

Probably, a co-ordinated, combined contribution of several transcriptional and regulatory factors including c-Jun, c-Fos and Ras (through the AP-1 site) and, primarily, Tcf-4, β-catenin and E-cadherin (through the Tcf-4 element) is necessary to upregulate the transcriptional efficiency of the MMP-26 gene in epithelial carcinomas of various organ/tissue origin. Our latest results strongly support this hypothesis. Thus co-expression of the MMP-26 promoter – luciferase chimeric gene with Tcf-4 and the non-phosphorylatable β-catenin-S33Y mutant dramatically increased the transcriptional efficiency of the MMP-26 promoter in HEK293 cells (N. D. Marchenko, G. N. Marchenko and A. Y. Strongin, unpublished work). Altogether, our findings support the hypothesis that the regulation via the Tcf-4 pathway is a distinction of the MMP-26 promoter, which may lead to the unique expression pattern of this MMP in epithelial carcinomas.

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