The balance between matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) is a key determinant in the homeostasis of the extracellular matrix. We have identified two cis-acting elements involved in the transcriptional regulation of TIMP-2. The first is an inverted CCAAT box located at position −73 to −69 in the TIMP-2 promoter that binds the transcription factor NF-Y. The second is a GAGGAGGGGG motif located at position −107 to −98, that binds the transcription factors Sp1 and Sp3. NF-Y and Sp1 cooperate for the basal transcription activity of the promoter. We then determined that TIMP-2 is transcriptionally up-regulated by cAMP analogs. Up-regulation of TIMP-2 by dibutyryl cAMP is a delayed response that requires de novo protein synthesis and does not affect RNA stability. The NF-Y and Sp1 binding site are both involved in cAMP-dependent up-regulation of TIMP-2. Whereas NF-Y is essential for cAMP mediated regulation, Sp1 alone is not sufficient but enhances the activity of NF-Y. Dibutyryl cAMP has no effect on the expression of MMP-2 and MMP-9 and switches the MMP-TIMP balance in favor of the inhibitor.

The molecular basis for the transcriptional regulation of MMP and TIMP expression has been investigated, and several cis-acting elements in the promoter of many MMPs and TIMPs responsible for transcription control have been identified. In particular, a PEA-3 motif and an AP-1 motif located in close proximity in the promoter of many MMPs and TIMPs have been shown to mediate their enhanced expression by phorbol esters and serum factors (15), and a transforming growth factor β inhibitory element involved in the down-regulation of MMP-3 by transforming growth factor-β1 has been identified (9). Signaling pathways involved in MMP and TIMP regulation have only recently begun to be explored. The mitogen-activated protein kinase and JAK/STAT (signal transducers and activators of transcription) pathways have been implicated in the regulation of MMP-1 expression by oncostatin M (13). TIMP-1 and TIMP-2 are up-regulated by 8-bromo-cAMP in HT1080 cells, but the signaling pathway involved has not been entirely defined (16).

In our laboratory we have previously isolated and characterized the human TIMP-2 gene and partially characterized its promoter (17). This promoter has many features classically observed in housekeeping genes including a typical CpG island and several Sp1 motifs, suggesting that in contrast to TIMP-1 or TIMP-3, which respond to a large variety of cytokines and growth factors, TIMP-2 functions predominantly in providing a basal level of inhibitory activity. We demonstrate here that TIMP-2 is transcriptionally up-regulated by cAMP in a manner that specifically alters the MMP/TIMP balance in favor of the TIMP. Moreover we show that up-regulation of TIMP-2 by cAMP involves a cooperative action between NF-Y and Sp1.

EXPERIMENTAL PROCEDURES

Cell Culture—Human breast epithelial cancer cells MDA-MB, human fibrosarcoma H1080 cells, and murine NIH3T3 cells were cultured in minimum Eagle’s medium (MDAMB, H1080) or Dulbecco’s modified Eagle’s medium (NIH3T3) containing 10% fetal bovine serum and supplemented with 200 μg/ml penicillin and streptomycin.

Plasmids and Mutagenesis—The full-length 2243 base pairs (pTIMP2–2.3K) and 276 base pairs (pTIMP2–276) TIMP-2 promoters...
cloned into the firefly luciferase reporter vector pGL-2 (Promega, Madison, WI) have been previously described (17). Both plasmids served as a template for deletion and site-directed mutagenesis studies in the TIMP-2 promoter. Mutational deletions were performed by PCR cloning, using a PCR core kit (Qiagen, Chatsworth, CA), 10 ng of DNA template, and 10 pmol of sense and antisense primers. The sequence of the sense primer for TIMP2–187 is 5'-CCGCGGCCCAGCCCACCGGGGCCCA- GGTGTGTGC-3', for TIMP2–131 5'-CCGCGGCCCAGCCCACCGGGGCCCAAGGGTGTGTGC-3', for TIMP2–112 5'-CCGCGGCCCAGCCCACCGGGGCCCAAGGGTGTGTGC-3', for TIMP2–100 5'-CCGCGGCCCAGCCCACCGGGGCCCAAGGGTGTGTGC-3', and for TIMP2–86 5'-CCGCGGCCCAGCCCACCGGGGCCCAAGGGTGTGTGC-3'. Each construct (4 μg) was transiently co-transfected into MDAMB cells with a pRL-SV40 plasmid (10 ng) containing a Renilla luciferase (LUC) reporter gene. After 63 h, the activities of cellular firefly and Renilla luciferases were individually measured, and the ratio of firefly over Renilla luciferase activities (FFL/RL) was calculated. The data (right panel) represent the mean (+ S.D.) of two separate experiments performed each in triplicate.

Transcriptional Regulation of TIMP-2 by cAMP

Analysis of the TIMP-2 Promoter Activity—Cells were transiently transfected by lipofection using the DOTAP-N-[1-(2,3-dioleoyloxy)pro- pyl]-N,N,N-trimethylammonium salts) liposomal transfection kit...
(Roche Molecular Biochemicals). To normalize the data for transfection efficiency, the pRL-SV40 (Promega), which contains the Renilla luciferase reporter cDNA under the transcriptional control of the SV40 promoter, was co-transfected with each test plasmid. Cells were transfected at 80% confluency 24 h after being plated into 6-well tissue culture clusters. Four μg of test plasmid was mixed with 10 ng of pRL-SV40 and 20 μl of DOTAP liposomal transfection reagent, and the mixture was added to the cells in the presence of 4 ml of medium supplemented with 10% fetal bovine serum. When indicated, cultures were treated with dibutyryl cyclic AMP (Bt2cAMP, Sigma) 15 h after transfection. The cells were further maintained for 48 h before being washed and lysed in the presence of 100 μl of lysis buffer (Dual-Luciferase™ reporter assay system, Promega). The activity of firefly luciferase (test plasmid) and Renilla luciferase (control plasmid) was measured in 20-μl aliquots of cell lysate using the Dual-Luciferase™ reporter assay system and a Lumat LB950 luminometer (Berthold, Germany). Transfection experiments were done in triplicate dishes and repeated at least three times. When indicated, stably transfected clones were obtained by co-transfecting cells with 8 μg of Psf/Nee plasmid (a gift of Dr. W. E. Laug, Children’s Hospital Los Angeles, Los Angeles, CA) and selecting cells against G418 (600 μg/ml).

Electrophoretic Mobility Shift Assay—Nuclear extracts from MDAMB cells were prepared according to the method of Dignam et al. (18). The sense sequences of the DNA probes generated from hTIMP-2 are the following. The wild type probe containing NF-Y binding site is GCGCCCAGAGCCTGCT(A)TTA(G)GCCGCCAGCCA; the mutant probe is GCGCCCAGAGCCTGCTATTGGCCGCCAGCCA, and its mutant probe is GGGGGATG(GTG)GAGGGGGCTGCTGGGAGC, and its mutant probe is GGGGGATG(GTG)GAGGGGGCTGCTGGGAGC. Synthetic double-stranded DNA probes were labeled with [32P]dATP using the T4 polynucleotide kinase. Six μg of the nuclear extracts were mixed with these radiolabeled DNA probes (20,000 cpm) in the presence of 10 mM HEPES (pH 7.8), containing 5 mM MgCl2, 50 mM KCl, 9% glycerol, 0.5 μM leupeptin, 0.7 μl/ml pepstatin, 1 μg/ml aprotinin, 1 mM spermidine, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonfyl fluoride. The mixture was incubated at room temperature for 15 min before being loaded and electrophoresed in a 4% polyacrylamide gel under constant voltage (150 volts) in 0.4 x TBE buffer (36 mM Tris borate (pH 8.0) and 0.8x EDTA). When indicated, non-radiolabeled (cold) double-stranded nucleotides or monoclonal antibodies against NF-YA (Pharmingen, San Diego, CA) and against Sp1, Sp2, Sp3, and Sp4 (Santa Cruz Biotechnology, Santa Cruz, CA) were pre-incubated with the nuclear extracts for 15 min at room temperature before the addition of the radiolabeled probes. After electrophoresis, the gels were dried and autoradiographed at −80 °C.

Northern Blot—Total RNA was isolated from MDAMB cells using the method of Chomczynski and Sacchi (19). The cells were lysed in Trizol® (Life Technologies, Inc.) and centrifuged at 2,000 rpm to remove nuclei. The RNA in the supernatant was precipitated in isopropanol, washed at 75% ethanol, and resuspended in diethyl pyrocarbonate-treated water. Twenty μg of RNA for each lane was electrophoresed on a 1% formaldehyde agarose gel and blotted onto Zeta-probe membranes (Bio-Rad). Blots were sequentially hybridized with 32P-labeled DNA probes for 16 h at 65 °C in 0.5 M NaH2PO4/Na2HPO4 (pH 7.2), 7% SDS, 1% bovine serum albumin, 1 mM EDTA, and 15.4% formamide. Human TIMP-2 cDNA was available in our laboratory, and the human RNA 28 S cDNA (control) was purchased from Ambion Inc. (Austin, TX). After hybridization, the blots were washed 2 times in 40 mM NaH2PO4/Na2HPO4 (pH 7.2), 1 mM EDTA, and 1% SDS at 65 °C before autoradiography at −80 °C.

Western Blot—Western blot analysis was performed according to the method of Burnette (20) using a rabbit polyclonal antibody against human TIMP-2. Immune complexes were detected by enhanced chemiluminescence with luminol (Amersham Pharmacia Biotech) using a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase as a secondary antibody.

TIMP and MMP Analysis in Culture Medium—Analysis of MMP expression in serum-free culture medium of cell lines was performed using SDS-polyacrylamide gel zymography as described previously (21). When needed, samples were concentrated by ultrafiltration using Microcon filters (Amicon, Beverly, MA; molecular weight cutoff, 10,000). In these gels, a clear zone indicated the presence of a protein with gelatinolytic activity. Similar gels were used to detect the presence of TIMP with the exception that p-aminophenylmercuric acetate (Sigma)-activated crude gelatinase from cultured rabbit fibroblasts was incorporated into the gelatin-polyacrylamide mixture before polymerization. In these conditions, the presence of a stained zone of undigested gelatin indicated the presence of an inhibitor of gelatinases.

Invasion and Migration Assays—For invasion assays we used transwells (Corning Inc., Corning, NY) in which the 8-μm pore filter of the upper chamber had been precoated with 100 ng of Matrigel® (Becton Dickinson Labware, Bedford, MA). After drying overnight at room temperature, 100 μl of serum free medium was added to keep the filter wet for 2 h at room temperature. The upper chamber, to which 50,000 cells suspended in 200 μl of serum free medium were added, was then inserted into a well containing 500 μl of medium supplemented with 10% fetal bovine serum. After 36 h at 37 °C in the presence or absence of Bt2cAMP, the cells in the upper chamber were fixed and stained with
An Inverted CCAAT Box in the Human TIMP-2 Promoter Is Responsible for Basal Activity—We had previously shown that the basal TIMP-2 promoter was located within 276 nucleotides upstream of the transcription initiation site (17). To further locate element(s) responsible for basal expression in this promoter, we created a series of deletion mutants by PCR cloning of the pTIMP2–276 plasmid. These constructs were transiently transfected into MDAMB cells and tested for luciferase activity. The data (Fig. 1) indicated an almost complete loss of basal activity when a segment of the promoter located between positions –63 and –73 was deleted, whereas deletions of DNA segments located upstream of position –73 had no significant effect on basal activity. This 10-nucleotide-long DNA fragment (ATTGGCCGCC) appears essential for transcription activity and is located 39 nucleotide upstream of the TATA box. It (ATTGG) motif is responsible for basal promoter activity was then obtained by directed site mutagenesis. These experiments (Fig. 2) indicated a progressive loss of activity as the number of mutations in the motif increased from one (GTTGG) to two (TTTAG) and five (CCCCC) nucleotides. Thus the data demonstrate that the inverted CCAAT box is an essential motif in maintaining the basal expression of the TIMP-2 promoter.

The Transcription Factor NF-Y Binds to the Inverted CCAAT Box in the TIMP-2 Promoter—We used electrophoretic mobility shift assays (EMSA) to identify in MDAMB cells nuclear protein(s) binding to the inverted CCAAT motif (Fig. 3). In a first set of experiments, nuclear extracts from MDAMB cells were incubated in the presence of a 30-mer radiolabeled double-stranded oligonucleotide probe corresponding to the TIMP-2 promoter region extending from position –89 to –59 and containing the ATTGG motif. These experiments demonstrated the presence of 2 shifted radioactive bands (Fig. 3, left panel). The specificity of the upper band was demonstrated in the presence of increased amounts of a non-radiolabeled (cold) double-stranded oligonucleotide. To determine that the binding involved the inverted CCAAT box, we used a two-point mutation in the ATTGG motif (TTTAG) and demonstrated an absence of competition in the presence of this non-radiolabeled mutated oligonucleotide. NF-Y proteins have been identified as the major proteins binding to the inverted CCAAT motif (22, 23). The NF-Y complex is composed of three subunits (A, B, and C) of transcription factors. The subunit A is the subunit interacting with DNA, and subunits B and C form a binary complex that interacts with subunit A to promote DNA binding (23, 24). To determine whether NF-Y binds to the inverted CCAAT motif of the TIMP-2 promoter, we performed a second set of EMSA in the presence of an anti-NF-YA monoclonal antibody (Chemicon, Temecula, CA). The data (Fig. 3, right panel) showed the presence of a super-shift in the presence of the antibody at the level of the upper band, which was not observed in the presence of nonspecific murine IgG. The data indicate that the NF-Y complex binds to the inverted CCAAT box in the TIMP-2 promoter and controls basal expression.

Transcriptional Up-regulation of TIMP-2 by cAMP—The CCAAT motif is a bidirectional cis-acting element present in
many eukaryotic promoters (22, 25, 26). This motif has been recently reported to mediate the response of several genes that are up-regulated by an increase in cAMP (27–29). CAMP-mediated regulation of these genes however differs from the rapid (within minutes) cAMP response seen in genes that contain the typical cAMP-responsive elements (CREs) by a delayed (within hours) response to changes in cAMP (30). In view of a previous report indicating that cAMP up-regulates TIMP-2 expression in HT1080 cells (16), we explored the possibility that the inverted CCAAT motif in the TIMP-2 promoter is involved in cAMP response. We first examined the effect of an increase in cAMP concentration on TIMP-2 expression in MDAMB cells by testing the effect of Bt2cAMP treatment on TIMP-2 mRNA and protein expression. The data (Fig. 4A) indicated a dose-dependent increase in TIMP-2 mRNA and TIMP-2 protein expression upon treatment of these cells with 2 and 10 mM Bt2cAMP. To determine whether the increase in TIMP-2 mRNA represents a net increase in transcriptional activity or a change in RNA stability, we performed similar experiments in the presence of the transcriptional inhibitor 5,6-dichlorobenzimidazole riboside (DBR), which was added to the culture medium 40 h after exposure to Bt2cAMP. The data (Fig. 4B) revealed a similar decay of the 3.8- and 1.7-kb TIMP-2 mRNAs in the presence or absence of Bt2cAMP. These observations indicate that cAMP up-regulates TIMP-2 expression at a transcriptional level by affecting de novo RNA synthesis rather than RNA stability. We next conducted a time course analysis of TIMP-2 mRNA expression on Bt2cAMP-treated MDAMB cells to evaluate the timing of the response of TIMP-2 to an increase in cAMP. The data (Fig. 5A) indicated a progressive increase in mRNA expression starting 5 h after treatment and lasting beyond 48 h. There was therefore a 5-h delay between Bt2cAMP treatment and transcriptional up-regulation of TIMP-2, suggesting the presence of an intermediate regulatory mechanism. We therefore examined whether transcriptional up-regulation of TIMP-2 required de novo protein synthesis by testing the effect of cycloheximide on endogenous TIMP-2 mRNA expression in Bt2cAMP-treated MDAMB cells. The data (Fig. 5B) revealed that cycloheximide at a concentration of 2.5 μM inhibited the up-regulation of TIMP-2 mRNA induced by Bt2cAMP by 94% and 100% (3.8-kb and 1.7-kb mRNA, respectively). These observations and the absence of a typical CRE motif in the TIMP-2 promoter (17) are consistent with the concept that the up-regulation of TIMP-2 by cAMP involves a CRE-independent mechanism recently described for several cAMP-responsive genes such as the fatty acid synthase gene or the cystic fibrosis transmembrane conductance regulator gene (28, 29). Typically,
these genes have delayed response to cAMP mediated by NF-Y binding to the inverted CCAAT box.

NF-Y and Sp1 Cooperate in cAMP-dependent Up-regulation of the TIMP-2 Promoter—We therefore determined whether the 276-nucleotide-long TIMP-2 promoter, which contains the inverted CCAAT motif, could mediate a cAMP response. MDAMB cells were transfected with the plasmid pTIMP-276, and stably transfected cells were selected in the presence of G-418. These cells were tested for the effect of Bt2cAMP on luciferase expression. These experiments (Fig. 6) indicated that Bt2cAMP stimulated the expression of luciferase in a dose-dependent manner at concentrations ranging between 0.05 mM and 10 mM with a 2-fold increase in luciferase expression achieved in the presence of 0.5 mM Bt2cAMP (inset) and a 4-fold increase in the presence of 10 mM Bt2cAMP. To further localize the cAMP-responsive element in this segment of the promoter, we tested the effect of Bt2cAMP on MDAMB cells transiently transfected with TIMP-2 promoter deletion mutants. These experiments indicated the presence of three specific mobility shifts of the promoter fragment containing the GAGGAGGGGG motif in the presence of MDAMB nuclear extracts. These specific complexes could be super-shifted with antibodies against Sp1 and Sp3 but not against Sp2 or Sp4. Thus the data identified NF-Y and Sp1 as key transcriptional factors involved in cAMP response.

Bt2cAMP Treatment of MDAMB Cells Inhibits Invasion in Vitro—To determine whether the up-regulation of TIMP-2 expression by cAMP in MDAMB cells was affecting the balance between MMPs and TIMPs and the invasive potential of these cells, we examined the effect of Bt2cAMP treatment on the expression of MMP-2 and MMP-9 and TIMP-1 and TIMP-2 and on the ability of these cells to invade a Matrigel®-coated filter. The data indicated that the expression of MMP-2 and MMP-9 in these cells was unaffected by Bt2cAMP, whereas both TIMP-1 and TIMP-2 were up-regulated (Fig. 9A). This change in the MMPs/TIMPs ratio in favor of the TIMPs was associated with a marked inhibition of cell invasion through Matrigel®. In the presence of 2 mM and 10 mM of Bt2cAMP, a 43% and 50% inhibition of invasion was observed, respectively. Consistent with a specific effect
Transcriptional Regulation of TIMP-2 by cAMP

In this manuscript we describe a novel transcriptional regulatory mechanism that controls TIMP-2 expression. We demonstrate that TIMP-2 belongs to a family of genes that have a delayed response to cAMP treatment and that this response involves the cooperative action of two transcription factors, NF-Y and Sp1.

cAMP-regulated genes have been classified in two categories: immediate responsive genes whose expression is increased by cAMP within minutes and unconventional responsive genes whose expression is delayed within hours after an increase in intracellular cAMP and requires a stimulation of cAMP analogs at a millimolar concentration range. The former category includes a large variety of genes controlled by hormones and growth factors (30, 32). The rapid up-regulation of these genes involves activation of protein kinase A followed by the release and subsequent nuclear localization of its catalytic subunit. This catalytic subunit phosphorylates a family of activators that bind to CRE motifs including the transcription factors CRE-binding protein, CREM, and ATF-1 (33). In the case of unconventional responsive genes, the signaling pathways are not as well understood, but the observation that the increase in gene transcription begins only after a delay of several hours indicates that the response does require de novo protein synthesis (34). Our data clearly indicate that TIMP-2 belongs to the second category of cAMP-responsive genes because up-regulation of mRNA expression was observed more than 5 h after treatment with Bt2cAMP, and the response was abolished in the presence of cycloheximide. The absence of any CRE consensus or variants thereof in the segment of the promoter that responds to cAMP is also consistent with this conclusion (17). As an alternative for an indirect effect of cAMP on de novo RNA transcription was the possibility that cAMP may affect TIMP-2 mRNA stability. Our experiment performed in the presence of a transcriptional inhibitor, DBR, eliminated this possibility because we observed an absence of effect of Bt2cAMP on decay of TIMP-2 mRNAs. The observation that the 3.8- and 1.7-kb TIMP-2 mRNA species responded similarly to cycloheximide and DBR is consistent with our published data indicating that they differ by the positions of the polyadenylation signals and share the same transcription initiation site (17).

We demonstrate that the inverted CCAAT motif located at position −73 to −69 in the TIMP-2 promoter is not only responsible for basal activity but is also involved in the delayed response to cAMP. Although this element was initially believed to contribute only to basal transcription (26, 35, 36), it has been implicated in the cAMP response of several genes such as the fatty acid synthase gene (28), the tryptophan hydroxylase gene (27, 34), the cystic fibrosis transmembrane regulator gene (29), and the hexokinase II gene (26). Our mutation analysis indicated that the inverted CCAAT motif acts as a transcriptionalenhancer.

**DISCUSSION**

on the MMP/TIMP balance and ECM degradation, Bt2cAMP had no effect on cell migration when the filters were not coated with Matrigel (Fig. 9B).

**FIG. 8.** Analysis of Sp1-binding proteins by electrophoretic mobility shift assay. EMSA was performed in the presence of nuclear extracts (6 μg) from MDAMB cells incubated with a 32P-labeled double-stranded synthetic oligonucleotide corresponding to the −87 to −111 sequence of the TIMP-2 promoter and containing the GAGGAGGGGGG motif. When indicated, an unlabeled (cold) wild type and mutated (GATTAGGGGG) double-stranded oligonucleotide (25 molar excess) was added to the reaction mixture as a competitor. To identify the specific DNA-binding proteins, monoclonal antibodies (1 μg) against Sp1, Sp2, Sp3, Sp4, or murine IgG (1 μg) were added to the reaction before the addition of the radiolabeled DNA probe. The specific DNA-protein complexes are indicated on the left, and the super-shifted complexes generated in the presence of antibodies are shown on the right.

**FIG. 9.** Effect of Bt2cAMP on MMP and TIMP expression and on MDAMB cells invasion and migration. Panel A, gelatin zymographic analysis for MMPs (upper panel) and TIMPs (lower panel). MDAMB cells were treated with the indicated concentrations of Bt2cAMP for 48 h at 37 °C in serum-free medium. The medium was then collected and analyzed for gelatinases (MMP-2 and MMP-9) and TIMP (TIMP-1 and TIMP-2) expression by gelatin polyacrylamide zymography as indicated under “Experimental Procedures.” The positions of MMP-9, MMP-2, TIMP-1, and TIMP-2 are indicated by arrows on the left. Panel B, MDAMB cells were treated with indicated concentrations of Bt2cAMP and transferred into the upper compartment of a transwell. The data represent the mean number of cells (+ S.D.) that migrated to the lower side of the filter from triplicate samples. Top figure, filters precoated with Matrigel (invasion). Bottom figure, filters not coated with Matrigel (migration).
icates that in the case of TIMP-2 this element does both, mediating cAMP-dependent up-regulation and maintaining basal expression. By EMSA we demonstrate that NF-Y binds to the inverted CCAAT box of the TIMP-2 promoter. NF-Y is a ubiquitous transcription factor complex consisting of three subunits (A, B, and C) that has been implicated in the basal expression of several ECM-related genes such as fibronectin and interstitial collagen I (25, 26, 37). Regulation of TIMP-2 expression by this factor may therefore be part of a broader regulatory process that controls the expression of several genes affecting the composition and homeostasis of the ECM. Interestingly, a CCAAT motif is present in the murine TIMP-1 promoter (38). It is therefore conceivable that this motif is similarly involved in the cAMP response of TIMP-1, since our data in MDAMB cells indicate an increase in both TIMP-1 and TIMP-2 expression by Bt2cAMP.

The mechanism by which cAMP increases the transcriptional activity through NF-Y has remained unknown. It is conceivable that cAMP might transcriptionally up-regulate NF-Y or increase its binding affinity to DNA. By Western blot analysis and EMSA we have not observed a change in protein expression or DNA binding affinity upon cAMP treatment (data not shown). It is also possible that cAMP increases the expression or the binding affinity of other proteins interacting with NF-Y. Several co-activator proteins such as p300, GCN5, P/CAF, and the high mobility group protein HMG-I(Y) have been reported to bind to different NF-Y subunits (39–41), and it is plausible that cAMP may activate the TIMP-2 promoter by enhancing the recruitment of p300 by NF-Y. The acetyltransferase activity of p300 could modify NF-Y and TFIIIE and enable NF-Y to make more active contacts with other transcription factors. Acetylation of TFIIIE and TFIIIF might also facilitate their function within chromatin (42, 43). As an alternative, post-transcriptional modification of NF-YA such as phosphorylation could be influenced by cAMP and affect DNA binding and transcriptional activity. These possibilities are currently investigated in our laboratory.

Our study also shows that Sp1 binding to a GAGGAGGGG (-107/-98) motif in the TIMP-2 promoter is involved in basal activity and cAMP up-regulation. That Sp1 can compensate for NF-Y activity is demonstrated by the fact that whereas mutations of the inverted CCAAT motif suppress basal promoter activity in the absence of the Sp1 binding motif (Fig. 1), they have no effect in the presence of the Sp1 binding motif (Fig. 7B). That Sp1 cooperates with NF-Y for cAMP response is shown by the observation that mutation of the Sp1 binding motif alone decreases (but does not suppress) the cAMP response by 50% (Fig. 7). Cooperation between NF-Y and Sp1 has been demonstrated in the regulation of several genes including the major histocompatibility complex class II-associated invariant chain (44), the p27Kip1 gene (45), the hamster thymidine kinase gene (46), and the rat fatty acid synthase gene (47). The promoters of these genes all have in common one or several Sp1 binding sites located in close proximity (20 to 30 nucleotides) with an inverted CCAAT motif. Cooperation between these two transcription factors is involved in the insulin response of the fatty acid synthase gene, the vitamin D3 response of the p27Kip1 promoter, and the serum response of the thymidine kinase promoter. The molecular mechanism responsible for this cooperative activity has been recently partially elucidated by the demonstration of the cooperative DNA binding of NF-YA and Sp1 and the presence of specific protein-protein interaction domains in NF-YA and Sp1 (47, 48). In the human TIMP-2 promoter, there is a Sp1 binding site located 34 nucleotides upstream of the NF-Y binding site, and our mutational analysis indicates that although Sp1 alone does not mediate cAMP response, it synergizes the activity of NF-Y upon cAMP stimulation. To our knowledge, this is the first demonstration of such cooperation between Sp1 and NF-Y in mediating cAMP response.

Finally, it is noteworthy to point out in contrast to most regulatory agents that regulate simultaneously MMP and TIMP expression, cAMP up-regulates the expression of TIMP-2 and TIMP-1 without affecting the expression of MMP-2 and MMP-9. Thus, cAMP alters the balance between MMPs and TIMPs in favor of the TIMPs and inhibits ECM degradation and cell invasion.

In summary, we demonstrate that two ubiquitously expressed transcription factors, NF-Y and Sp1, cooperate in the transcription of the human TIMP-2 gene and its up-regulation by cAMP in a manner that alters the MMP-TIMP balance in favor of the inhibitor. These observations suggest that alteration of ECM homeostasis must be considered in the pharmacological manipulation of cAMP.

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NF-Y and Sp1 Cooperate for the Transcriptional Activation and cAMP Response of Human Tissue Inhibitor of Metalloproteinases-2
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