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YB-1 Binds to the MMP-13 Promoter sequence and Represses MMP-13 Transactivation via the AP-1 site

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Abstract

Matrix metalloproteinases (MMPs) are key enzymes that implement degradation of the extracellular matrix during cellular invasion in development, tissue remodeling, and pathogenic disease states. MMP-13 has pivotal roles in the pathogenesis of invasive cancers and arthritis. Here we report the identification of Y-box binding protein-1 (YB-1) as a new repressor of MMP-13 transactivation. YB-1 binds in vitro in DNA affinity chromatography to the activator protein-1 (AP-1) DNA sequence within the MMP-13 promoter. Chromatin immunoprecipitation assays reveal that YB-1 binds in living cells to the MMP-13 gene promoter, to a region of the MMP-13 promoter containing the AP-1 site. YB-1 represses tumor promoter-induced MMP-13 promoter transactivation at the AP-1 site. This is the first report demonstrating YB-1 binding in vitro and in living cells to a mammalian AP-1 target gene, and the first report of YB-1 regulation of the MMP-13 promoter.

Keywords

Activator protein-1 (AP-1); chromatin immunoprecipitation (ChIP); matrix metalloproteinase-13 (MMP-13); NAPSTER; transactivation; Y-box binding protein-1 (YB-1)

Introduction

Matrix metalloproteinases (MMPs) comprise a large family of degradative enzymes that mediate tissue remodeling in wound healing, invasion, metastasis, and other events [1,2]. MMPs implement these processes by virtue of their capacities to degrade extracellular matrix (ECM) and basement membrane components. The expression of most MMPs is regulated primarily at the transcriptional level via exogenous and intracellular signals that trigger activation of transcription factor binding to specific DNA sequences in the promoter regions of these genes. A number of MMPs contain AP-1 DNA binding sites in their promoters, and are transcriptionally regulated via this site [2].

MMP-13 (alias collagenase-3) belongs to the collagenase subfamily of MMPs and has broad substrate specificity for type II collagen and other ECM macromolecules (see [3] for review).

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Aberrant expression of MMPs mediates malignant growth and invasion of tumor cells, and is pivotal in the pathogenesis of arthritis [4–6]. MMP-13 expression is regulated at the transcriptional level [2]. Transcriptional control of MMP-13 expression has been suggested to represent an effective target in cancer therapeutic design [7]. Although our knowledge in the field of MMP-13 biology is growing rapidly, the precise molecular pathways that control its expression remain to be elucidated.

Activator protein-1 (AP-1) proteins are transcription factors of the bZIP family of transcriptional regulators that bind to 5'-TGAGTCA-3' consensus DNA sequences (also termed "AP-1 DNA binding sites" or "AP-1 sites") that generally reside within transcriptional promoters of AP-1 target genes [8,9]. AP-1 is a dimer composed of subunits of the *jun* and *fos* proto-oncogene families. The MMP-13 promoter contains a single transcription factor binding site for AP-1 at position -50 with respect to the transcriptional initiation site [10]. Several studies have found the AP-1 site in the MMP-13 promoter to be critical for its activity [10–12].

AP-1 mediated transcription is dependent on a variety of factors including other proteins that interact with AP-1 proteins [13–15], and other proteins that bind at the AP-1 site [16–18]. In our effort to identify new proteins that bind and regulate gene expression at the AP-1 DNA binding site, we previously identified Y box binding protein-1 (YB-1) as a novel AP-1 DNA binding protein [19]. YB-1 is a member of the Y-box family of DNA binding proteins that are defined by the presence of a cold-shock domain in the N-terminal region [20]. Originally, YB-1 was identified by screening cDNA expression libraries for proteins that could bind to DNA sequences containing an inverted CCAAT element, also called the "Y-box" [21]. YB-1 protein has been implicated in transcriptional activation and repression of several genes [20,22–24]. In previous work we reported that YB-1 binds in vitro to AP-1 sequences. We also demonstrated that YB-1 represses AP1-dependent promoter transactivation of the gene encoding matrix metalloproteinase-12 (MMP-12), an AP-1 target gene that encodes a matrix metalloproteinase that degrades extracellular matrix elastase and participates in tissue remodeling events in inflammation, tumor inhibition, and other pathogenic processes [25]. Based on these data we hypothesized that YB-1 binds to AP-1 sites of other AP-1 target gene sequences and regulates their transcriptional activation activity. We now report that YB-1 interacts with the AP-1 site within the MMP-13 promoter in vitro and binds to a region of the MMP-13 promoter containing the AP-1 site in living cells. Overexpression of YB-1 potentially represses AP-1 dependent transactivation of the MMP-13 promoter.

Materials and Methods

Reagents and cell lines

Reagents and supplies not described herein were purchased from vendors cited [19,26]. Adherent human HeLa cervical carcinoma cells were cultured as described [19].

Antibodies and Immunoblotting

Western immunoblotting was performed according to published procedures using 0.3 µg/ml anti YB-1 (αYB-1) antibody (custom prepared by Bethyl Laboratories) or 0.5 µg/ml αJunD antibody from Santa Cruz Biotechnologies (Santa-Cruz, CA, USA) [19,26].

Plasmid constructs

The pGL3-MMP-13-luciferase promoter reporter construct contains a MMP-13 promoter sequence spanning base pairs -405 to +1 (contains one AP-1 site at -50 with sequence TGACTCA) ligated upstream of the luciferase gene in the pGL3 reporter construct. The mutated pGL3-MMP-13- promoter luciferase reporter construct is comprised of the identical

sequence with three point mutations in the AP-1 site (**ACTCTCA**, mutations in bold). Both constructs were a gift of Dr. Constance Brinckerhoff (Dartmouth College; [7]). pSVβgal, pcDNAFlag-YB-1 (YB-1 overexpression construct), and pcDNA3.1(+) vector control plasmid are as described [19].

NAPSTER AP-1 DNA binding assays

Nucleotide Affinity Preincubation Specificity Test of Recognition (NAPSTER) was performed as described with minor modifications [27,28], using streptavidin beads (Pierce Biotechnology, Rockford, IL) conjugated to biotinylated double-stranded oligonucleotides (oligos) containing bases -65 to -39 within the human MMP-13 promoter sequence (5' CCTATCCATAAGTGAT**GACTC**ACCATT -3'; AP-1 sequence in bold). NAPSTER reactions were performed using 150 µg of HeLa nuclear extract and 10 µg of DNA on beads per sample in a final reaction volume of 45 µl. Prior to incubation of nuclear extract with the DNA beads, nuclear extract was preincubated on ice for 15 minutes with no unconjugated oligo (sample I), or with a 1.5 fold molar excess of the wild-type (wt) MMP-13 AP-1 oligos (sample II) or mutated MMP-13 oligos containing two inactivating mutations within the AP-1 site (sample III; "mut" AP-1 site: 5' CCTATCCATAAGTGAG**GACTC**TCCATT -3', AP-1 site in bold, mutations underlined). Beads were added after preincubation, and NAPSTER reactions were performed for 3 hours at 4° C.

Chromatin immunoprecipitation Assay

Preparation of chromatin and chromatin immunoprecipitation (ChIP) assays were performed using a ChIP-IT assay kit (Active Motif, Carlsbad, CA) according to the kit protocol, with modifications. Chromatin was prepared from HeLa cells that had been treated for 24 hours with 100 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA) prior to harvest. 15 µg of chromatin was used for each immunoselection. Pre-cleared chromatin was immunoselected, processed and subjected to PCR amplification with platinum Taq polymerase (Invitrogen, Carlsbad, CA) using sequence specific PCR primers to amplify the region of the MMP-13 promoter containing the AP-1 site (Table 1). Reaction products were subjected to Tris borate EDTA/Polyacrylamide gel Electrophoresis (TBE/PAGE) gel electrophoresis. Gels were stained for 5 minutes in 1 µg/ml ethidium bromide (BioRad, Hercules, CA) and visualized using a FluorChem 8900 Imaging System (Alpha Innotech, San Leandro, CA).

Transient Transfections and Reporter Gene Assays

HeLa cells growing in 6-well tissue culture plates (Nalge Nunc International, Roskilde, Denmark) were co-transfected with 1 µg of the wild type or mutant MMP-13 promoter luciferase reporter plasmid along with 200ng pSVβGal plasmid with or without 1 µg of pcDNAFlag-YB-1 expression plasmid using Lipofectamine 2000 as per manufacturers instructions (Invitrogen, Carlsbad, CA). Cells were TPA treated and assayed for luciferase and β-galactosidase (β-gal) activities as described [19]. The ratio of luciferase to β-gal activity for each transfection was normalized to wild type luciferase reporter activity and expressed as relative luciferase activity. Samples for each test condition were independent transfections run in duplicate. The percentage of AP-1 specific repression by YB-1 was calculated as:

$$100 \times \frac{(\text{Activity of wt reporter in TPA - treated cells}) - (\text{Activity of wt reporter plasmid in TPA - treated cells with transfected YB - 1})}{(\text{Activity of wt reporter in TPA - treated cells}) - (\text{Activity of wt reporter plasmid in TPA - untreated cells})}$$

Results and Discussion

YB-1 binds specifically to the AP-1 site within the MMP-13 promoter in NAPSTER assays

Nucleotide Affinity Preincubation Specificity Test of Recognition (NAPSTER) is a rapid assay for specific binding of DNA binding proteins to DNA sequences. DNA binding proteins are

identified in NAPSTER by analytical scale DNA chromatography of nuclear extracts. Three samples are used in NAPSTER to assess binding specificity. Each sample uses streptavidin beads attached to a biotinylated DNA sequence harboring a wild-type AP-1 site. Sample I nuclear extract is incubated directly with DNA beads. Sample II and sample III nuclear extracts are preincubated with excess wild-type (for Sample II) or mutated (for Sample III) AP-1 oligo prior to addition of the beads.

NAPSTER was used to determine whether YB-1 binds specifically to the AP-1 site in the MMP-13 promoter. NAPSTER was performed using beads conjugated to MMP-13 oligos containing the AP-1 site. Samples I, II and III of NAPSTER purified nuclear extract material were separated on SDS-PAGE and immunoblotted with anti YB-1 ("αYB-1"). A signal of significantly greater intensity is detected by YB-1 antibody in samples I and III than in sample II (Figure 1), with samples I and III exhibiting 2.5-fold times the YB-1 protein binding detected in sample II (non-specific binding control). These data demonstrate that YB-1 binds specifically to the AP-1 site within the MMP-13 promoter sequence. αYB-1 antibody recognizes YB-1 protein with specificity, since the YB-1 protein signal is abrogated when the antibody is preincubated with the peptide that was used as the antigen to generate the antibody, whereas it is not abrogated by preincubation with a non-specific peptide sequence (not shown).

JunD protein is a component of the AP-1 transcription factor protein complex. NAPSTER studies were performed with αJunD as a positive control for AP-1 binding to the AP-1 site. Three protein species are detected by immunoblotting of HeLa nuclear extract with αJunD antibody (Fig. 1, bottom right panel). The three bands are JunD species since detection of all three proteins in immunoblotting is abrogated by preincubation with JunD peptide antigen, but not by preincubation with an unrelated peptide ([28] and data not shown). In positive control NAPSTER studies, sequence-specific DNA binding of the smaller two JunD protein species is detected at the MMP-13 DNA AP-1 sequence in samples I and III but not in sample II (Figure 1, lower left panel). The largest of the three JunD species binds non-specifically to the AP-1 site since it is not competed by wild-type or mutant oligos in samples II or III of NAPSTER.

YB-1 binds in living cells to the MMP-13 promoter

Since we observe YB-1 binding to the AP-1 site in the MMP-13 gene promoter, we predicted that we can also detect YB-1 binding to the MMP-13 promoter in living cells. The chromatin immunoprecipitation (ChIP) assay was performed to test whether YB-1 binds to a region of the MMP-13 promoter that contains the AP-1 site. Immunoselections were performed with chromatin isolated from human HeLa cervical carcinoma cells. After immunoselection of chromatin with αYB-1 antibody, enrichment of the endogenous MMP-13 promoter sequence bound to YB-1 was monitored by PCR amplification using primers specific for a region of the MMP-13 promoter that contains the AP-1 site. These primers flanked the AP-1 site within 65bp upstream and 79bp downstream of the AP-1 sequence.

A representative ChIP assay is shown in Figure 2A. PCR products are of the expected sizes (142 bp). The signal intensity of the PCR product of chromatin immunoselected with αYB-1 is 5-fold greater than that selected by non-immune αIgG. Similar results were obtained in six independent experiments with three independent chromatin preparations. As a positive control, immunoselection with αJunD yields 4.3-fold more PCR product than the control IgG.

In positive control studies, YB-1 was tested for its ability to bind in ChIP to a previously identified YB-1 binding site termed the Y-box that lies within the promoter for the gene encoding human MHC class II histocompatibility antigen HLA-DRA [29]. YB-1 bound to the HLA-DRA sequence is enriched in the αYB-1 immunoselections compared to IgG control immunoselections, by 2.3-fold (Figure 2B middle left panel). In a negative control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter sequence, which lacks the

AP-1 site, is not enriched in α YB-1 immunoselections (Figure 2C). HeLa nuclear extract samples immunoprecipitated with α YB-1 display enrichment of YB-1 compared to samples immunoprecipitated with IgG, as detected by immunoblotting with α YB-1 (Figure 2D). This confirms that YB-1 is successfully immunoselected in ChIP assays. These control experiments along with the ChIP data showing enrichment of YB-1 binding to the MMP-13 sequence demonstrate that YB-1 is recruited in living cells to the MMP-13 promoter in a region of the MMP-13 promoter that contains the AP-1 site.

In previous studies we found that YB-1 binds to the gibbon ape leukemia virus long terminal repeat (GALV-LTR) sequence in NAPSTER and gel shift studies [19]. YB-1 binding occurs to the GALV AP-1 site and to an imperfect Y-box that lies immediately adjacent to the 5' end of the AP-1 site, since mutation of either site partially abrogates binding, and mutation of both sites abolishes binding completely. YB-1 also binds with sequence specificity to a 28 base pair sequence containing 4X multimerized 7 base pair AP-1 sites and no flanking sequences [19]. In the current investigation, YB-1 binding to the AP-1 site in the MMP-13 oligo is readily detected in NAPSTER but not in gel shift assays, whereas binding of AP-1 proteins to the GALV AP-1 site is detectable by both assays. The NAPSTER assay detects interactions of YB-1 with the MMP-13 promoter that are missed by gel shift and then validated in living cells by chromatin immunoprecipitation.

Why can we detect AP-1 and YB-1 binding to the MMP-13 AP-1 site in NASPTER and gel shift, but only AP-1 binding to the MMP-13 sequence in gel shift? Why do we detect binding of both YB-1 and AP-1 to the GALV binding site in both assays? We hypothesize that YB-1 binds to the AP-1 site with lower affinity than AP-1 binds to the site. Interactions of lower affinity are more readily detectable in NAPSTER than in gel shift, since the protein and DNA concentrations of the reactants are much higher in NAPSTER than gel shift [28]. Additionally, protein-DNA complexes are rapidly isolated in NAPSTER after steady-state binding is achieved, whereas in gel shift they have a much longer opportunity for dissociation while protein-DNA complexes encounter low concentrations of complexes during electrophoresis. We speculate that binding of YB-1 to AP-1 oligos such as the GALV or 4XAP-1 would be more readily detectable in gel shift than binding to the MMP-13 1X AP-1 site because the tandem YB-1 binding sites that are present in GALV or 4XAP-1 afford them higher affinity for YB-1 binding. Multimerized tandem binding sites have been shown in early seminal studies by Winship Herr's laboratory to markedly augment activity of transcription factors at their DNA binding sites [30,31]. YB-1 is a very abundant nuclear protein in a number of malignant cell types [20]. If the affinity of YB-1 for the AP-1 site were to be comparable to that of AP-1, then AP-1 might be denied access to its own binding sites. A lower affinity for YB-1 for a single AP-1 binding site in endogenous promoters such as MMP-13 may have utility by permitting access of AP-1 to its own DNA binding site. Future studies will be required to test this hypothesis by comparing the affinities of AP-1 and YB-1 for the AP-1 site.

YB-1 represses TPA induced transactivation of MMP-13 gene promoter

Since YB-1 binds to the AP-1 site in the MMP-13 promoter, we hypothesized that YB-1 regulates MMP-13 transactivation via the AP-1 site. Reporter constructs containing the MMP-13 promoter spanning the AP-1 site cloned upstream of the luciferase gene were transiently transfected into HeLa cells. Treatment with TPA causes 3.1-fold transactivation of the MMP-13 promoter (Figure 3). Overexpression of YB-1 upon transient transfection of 1 μ g of constitutively expressed pcDNAFlagYB-1 represses 100% of TPA-induced MMP-13 promoter activity. Lower doses of pcDNAFlagYB-1 cause partial inhibition of TPA-induced transactivation, with 10 ng causing 6% inhibition, and 100 ng causing 65% inhibition (not shown). 1 μ g of transfected YB-1 overexpression construct also partially represses MMP-13 transactivation in TPA-untreated cells, by 47% (Figure 3). In other controlled studies, YB-1

does not repress transactivation of CMVluc, a constitutively expressed luciferase construct whose expression is driven by the cytomegalovirus (CMV) promoter ([19], not shown). YB-1 overexpression after transient YB-1 transfection was confirmed by immunoblotting [19]. No toxicity was associated with overexpression of YB-1 as judged by trypan blue exclusion assays ([19]; not shown).

The MMP-13 promoter luciferase reporter construct used in this study comprises the promoter region spanning from -405 to +1, and contains a number of transcription factor binding sites in addition to the AP-1 site. These include sites for Ets, Runx, HSF, Nkx-2, and Cdx A transcription factor binding (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The activity of an MMP-13 promoter construct with a mutated AP-1 site is not stimulated by TPA, demonstrating that TPA induction of the MMP13 promoter occurs specifically through the AP-1 site and not the other sites. Luciferase activity of the mutated reporter is significantly lower than that of the wt MMP-13 reporter, suggesting that the -50 AP-1 site is also responsive to stimulatory factors present in the serum and culture medium in which the cells are grown. YB-1 repression of MMP-13 transactivation also occurs primarily via the AP-1 site and not the other transcription factor binding sites, since significant repression by YB-1 is not observed in the mutated AP-1 constructs. Repression of the mutated reporter construct would have been readily detectable had it occurred, since the values for raw luciferase activity for samples transfected with the mutated reporter significantly exceed those of untransfected control samples (1,000–2,000 units of activity for mutant transfectants versus 50–100 units for untransfected controls). In other studies, YB-1 also represses TPA-induced MMP-13 mRNA expression in reverse transcriptase real time PCR studies (Samuel, Beifuss, and Bernstein, unpublished results).

In non-malignant cells such as primary cell lines, YB-1 is predominantly localized in the cytoplasm, not in the nucleus ([32], and references therein). Preferential cytoplasmic YB-1 localization in malignant cell types is also commonly observed [33]. High levels of nuclear YB-1 expression occur in many types of tumor cells and are highly correlated with tumor aggressiveness and size, invasiveness, metastatic capacity, poor prognosis, and multi-drug resistance [20]. Therefore we expect that YB-1 DNA binding at promoter regions and gene expression regulation at the level of transcription will be restricted to those cell types with nuclear YB-1 localization. HeLa cervical carcinoma cells were chosen for these studies since we found that they have high levels of YB-1 in the nucleus (Twizere, Samuel and Bernstein, unpublished data), and have a good transfection efficiency suitable for transactivation studies. Future studies will determine the degree to which the findings reported here for HeLa cells can be generalized to other cell types and tissues.

Repression of AP1-dependent transactivation by YB-1 suggests that YB-1 may be a new repressor of AP-1 target genes. Inappropriate overexpression of AP-1 target genes occurs in the pathogenesis of many diseases including cancer, atherosclerosis, cardiovascular disease and stroke, inflammation, arthritis, neurodegenerative diseases, asthma, and chronic obstructive pulmonary disease [34–37] and references therein]. Inappropriate AP-1 activity leads to activation of AP-1 target genes that mediate pathological processes in these diseases. Since there are not many repressors of AP-1 activity, identification of YB-1 as a new AP-1 repressor may provide a new means in future work to prevent overactive AP-1 activity in some of these disease states.

For example, MMP-13 plays important roles in tumor invasion and metastasis, and in connective tissue destruction in arthritis [3,37]. This study describes a new function for YB-1 as a repressor of MMP-13 promoter transactivation. Engineered nuclear targeted YB-1 repression of MMP-13 may be specifically applicable to targeted therapeutic interventions for cancer and arthritis in future studies.

This paper is the first to report binding in vitro of YB-1 to an AP-1 site within a mammalian promoter. It is also the first paper to report binding of YB-1 in living cells to an AP-1 target gene, to a region of the promoter that contains the AP-1 binding site. These findings may turn out to be generalizable to other AP-1 target genes, since in our previously published work we found that YB-1 represses transactivation of the MMP-12 gene promoter via the AP-1 site, and now we find that YB-1 represses transactivation of the MMP-13 promoter, again via the AP-1 site. Taken together these data suggest that YB-1 may inhibit transactivation of MMP-12 and MMP-13 promoter sequences by binding to the AP-1 sites within these sequences. Additional studies are ongoing to test this hypothesis further, and to determine the extent to which it may be generally applicable to other AP-1 target gene promoter sequences.

YB-1 is a multifunctional protein with roles in cellular proliferation, multidrug resistance, neoplastic transformation, DNA repair, stress responsiveness, tumor invasion, and other biological processes [20,38–43]. YB-1 mediates mRNA stability, RNA processing, chaperoning, packaging, translation, and transcription by binding to RNA and DNA sequences [20,44]. Previous studies by other labs have demonstrated YB-1 binding and regulation at a variety of promoter sequences [19,20].

YB-1 binds to Y-box and other promoter sequences and transcriptionally represses expression of human $\alpha 2$ collagen, $\alpha 1$ collagen, vascular endothelial growth factor (VEGF), and other genes (see [20] for review). Other reports have demonstrated that YB-1 binds to Y-box and other sequences in the promoter regions of matrix metalloproteinase-2 (MMP-2) p21, Fas, GM-CSF, and several other genes, and *induces* transcriptional activation. Remarkably, YB-1 can bind and induce *or* repress transcriptional activity of the gene promoters encoding MMP-2 and multidrug resistance gene-1 (MDR1), depending upon the cellular context. These data indicate paradoxical functions for YB-1.

Up-regulatory and downregulatory transcriptional activities of YB-1 are mediated by various mechanisms. In some cellular and promoter contexts, YB-1 behaves as a conventional transcription factor, binding directly to the DNA at a double-stranded Y-box sequence, activating (e.g., for MDR-1) or repressing (e.g., for MDR-1, $\alpha 2$ collagen) gene expression. In other contexts YB-1 functions as a co-activator (e.g., for GM-CSF, p21) or co-repressor (e.g., for grp78) of promoter activity. Yet still in other cellular and promoter contexts, YB-1 binds to the single-stranded regions of the promoter, either enhancing (e.g., for MMP-2, Fas, $\alpha 1$ collagen) or inhibiting (e.g., for $\alpha 1$ collagen, GM-CSF, vascular endothelial growth factor, MMP-2) DNA binding and activation by other transcription factors. Whether YB-1 represses MMP-12 and MMP-13 by these or other mechanisms is currently unknown.

It is evident that YB-1 plays an emerging role in regulating transactivation of several collagens and MMPs, at least including $\alpha 1$ and $\alpha 2$ collagens, MMP-2, MMP-12, and MMP-13. This suggests the possibility of an important role for YB-1 in dynamic control of extracellular matrix structure and function. ECM remodeling is a dynamic process defined by an orchestrated interplay of synthetic and degradative processes. It is logical to postulate that YB-1 may be at a nexus of regulatory control, activating and repressing transactivation of MMP and collagen genes in various cellular contexts in a concerted fashion to orchestrate cellular ECM remodeling in response to various physiological stimuli. Future studies will investigate this notion, and will further characterize the mechanisms by which YB-1 regulates MMP transactivation activity.

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Figure 1. YB-1 binds to the AP-1 site within the human MMP-13 promoter in NAPSTER analyses NAPSTER samples I, II and III were generated by co-incubation of 150 μ g of HeLa cell nuclear extract and 10 μ g of wild-type MMP-13 DNA oligos on beads per sample. Sample I consisted of direct batchwise AP-1 DNA affinity chromatography of nuclear extract proteins, Sample II and sample III were preincubated with 1.5-fold molar excess of unconjugated wild-type (sample II) or mutant (sample III) MMP-13 AP-1 competitor oligos prior to batchwise AP-1 DNA affinity chromatography. Upper panel immunoblotted with α YB-1; lower panel with α JunD. NE, 5 μ l of HeLa nuclear extract, loaded directly. Bracket indicates JunD bands. “S” indicates forms of JunD that binds specifically to that AP-1 site. “NS” indicates a form of JunD that binds non-specifically to the AP-1 site.

Figure 2. YB-1 binds in living cells to the MMP-13 promoter

A. *YB-1 and JunD bind in living cells to the MMP-13 promoter in the vicinity of the AP-1 site.* Immunoselections of HeLa chromatin were performed with α JunD, with α YB-1, with non-immune IgG. Mock immunoselections were performed with protein G beads in the absence of IgG. PCR amplifications of immunoselected samples were performed with primers that flank the MMP-13 promoter within 65 bp upstream and 79 bp downstream of the AP-1 site. Positive control PCR amplifications were performed with 5 μ l of a 1:10 dilution of unselected chromatin ("input"), and negative control PCR amplifications were performed with water in the absence of chromatin. Duplicate samples are indicated by brackets. M, DNA size markers bp, base pairs. **B.** *YB-1 binds the HLA-DRA promoter region in the vicinity of the Y-box YB-1 binding sequence.* HLA-DRA PCR primers were within 68bp upstream and 52 bp downstream flanking the Y-box sequence. **C.** *YB-1 does not bind sequences within the GAPDH promoter.* PCR primers amplified a region of the GAPDH promoter spanning within 120bp upstream and 46 bp downstream of the TATA TFIIB binding site. Left panel, α YB-1 versus IgG; right panel, α TFIIB versus IgG. **D.** α YB-1 antibody immunoprecipitates YB-1 under ChIP selection conditions. Left lane, non-immune IgG; right lane, α YB-1.

Figure 3. YB-1 represses transactivation of MMP-13 promoter reporter construct

HeLa cells were transiently co-transfected with 1 μ g of wild-type or AP-1 mutant MMP-13 promoter luciferase reporter construct and 1 μ g pcDNA Flag YB-1 expression construct (+), or with empty pcDNA3.1 vector (-). Cells were also co-transfected with 0.2 μ g pSV β gal beta-galactosidase expression construct as an internal control for transfection efficiency. Cells were treated with TPA (+) or DMSO solvent control (-), and harvested 24 hours thereafter as described [19]. Data are normalized to β -galactosidase activity. Error bars represent standard error of the mean.

Table 1**PCR primers for ChIP analyses**

Primer pair indicates the identity of the promoter sequence subjected to amplification. For, Forward; Rev, Reverse

Primer Pair	Primer Sequence	Annealing Temperature	PCR Product
MMP-13	For 5'-CCACGTAAGCATGTTTACCTTCAAGTGAC -3' Rev 5'-GATGCATCTTGAATGGTGATGCCTGG -3'	63° C	142 bp
GAPDH	For 5'-TACTAGCGGTTTTACGGGCG -3' Rev 5'-TCGAACAGGAGGAGCAGAGAGCGA -3'	59° C	166 bp
HLA-DRA	For 5'-CCTGGACCCTTTGCAAGAACCCTT -3' Rev 5'-TGTGGGGTGTAATAGAGTCTGACC -3'	62° C	120bp