YB-1 represses AP1-dependent gene transactivation and interacts with an AP-1 DNA sequence

Shaija SAMUEL*, Jean-Claude TWIZERE*[1] and Lori R. BERNSTEIN*†[2]

*Department of Pathology and Laboratory Medicine, Texas A&M University System Health Science Center, College Station, TX 77843-1114, U.S.A., and †Center for Environmental and Rural Health, Texas A&M University, College Station, TX 77843, U.S.A.

Involvement of the AP-1 (activator protein-1) transcription factor has been demonstrated previously in the regulation of cell proliferation and cell-cycle progression, in the control of cell migration, invasion and metastasis, and in signal transduction, stress responsiveness, DNA replication and DNA repair. YB-1 (Y-box-binding protein-1) has also been implicated in many of these processes. However, the mechanism by which YB-1 mediates these processes is poorly understood. In the present study, we report that overexpression of a transfected gene encoding YB-1 in human HeLa cervical carcinoma cells significantly represses the transactivation of a minimal AP-1 reporter construct in response to the tumour promoter PMA. YB-1 also represses mRNA expression and PMA-induced promoter transactivation of the endogenous AP-1 target gene encoding matrix metalloproteinase-12 (metalloelastase). YB-1 transrepression of both the minimal and matrix metalloproteinase-12 promoter reporter constructs is dependent on the AP-1 sequence. To identify new nuclear proteins that bind specifically to the AP-1 DNA-binding site, we devised a DNA-affinity-chromatography-based assay termed NAPSTER (nucleotide-affinity preincubation specificity test of recognition) and discovered a 49 kDa protein from human cancer cells that binds in a sequence-specific manner to the AP-1 DNA sequence. By tandem MS fragmentation sequencing analyses we determined that p49 is a YB-1. Immunoblotting of the NAPSTER-purified p49 protein using anti-YB-1 antibodies confirmed YB-1 binding to the AP-1 DNA sequence, as did gel mobility-supershift assays using YB-1 antibodies. This is the first report of YB-1 transrepression and interaction at the AP-1 DNA-binding site.

Key words: AP-1, DNA binding, matrix metalloproteinase-12, NAPSTER, transactivation, YB-1.

INTRODUCTION

AP-1 (activator protein-1) proteins belong to the bZIP family of transcriptional activators and are key mediators in the pathogenesis of cancer and other disease states [1,2]. AP-1 co-ordinately regulates the expression of target genes that control a number of physiological processes within the cell, including cell-cycle progression, tissue remodelling and invasion, and angiogenesis, cell migration, cell differentiation and apoptosis. AP-1 proteins bind as dimers to a DNA sequence called the AP-1 DNA-binding site or AP-1 site, with the consensus sequence 5′-TGAc/gTCA-3′, which is usually located within the transcriptional promoter regions of its target genes. AP-1 dimers are composed of subunits from the *jun* and *fos* proto-oncogene families. The Jun family consists of c-Jun, JunB and JunD, and the Fos family consists of c-Fos, Fra-1, Fra-2 and FosB. The levels of *jun* and *fos* mRNAs and proteins, as well as the DNA binding and transactivation activities of AP-1 dimers, are regulated by tumour promoters, growth factors, hormones, genotoxic agents and other stimuli.

YB-1 (Y-box-binding protein-1) belongs to the YB protein family that is distinguished by the presence of a cold-shock domain [3]. YB-1 is expressed in multiple tissues. Its expression is up-regulated in many tumours and is highly correlated with adverse cancer prognosis and resistance to cancer drugs [4]. YB-1 is implicated in RNA processing, chaperoning, stabilization and packaging, in translational regulation and in chromatin modification. Similar to AP-1, YB-1 has been implicated in transcriptional activation and repression, cellular signalling, stress responsiveness, DNA replication and repair, and also in cell migration, invasion, metastasis, cell proliferation and the cell cycle [3,5–7]. YB-1 binds to an inverted CAAT-box sequence termed the 'Y-box' and to several other DNA sequences in transcriptional promoters that it regulates, both positively and negatively.

Because of the commonality of biological and biochemical functions exerted by AP-1 and YB-1, it is logical to postulate that YB-1 may execute some of its functions on an AP-1 pathway. We now report that YB-1 represses transactivation of a minimal AP-1 reporter construct in response to the tumour promoter PMA. YB-1 also represses mRNA expression and promoter activation of the AP-1 target gene MMP-12 (matrix metalloproteinase-12; also known as metalloelastase) in real-time PCR and promoter reporter assays. In the course of purifying new proteins that bind to the AP-1 site, we found that YB-1 binds with sequence specificity to the AP-1 DNA-binding site in a DNA-affinity-chromatography-based NAPSTER (nucleotide-affinity preincubation specificity test of recognition) assay [8,9] and in electrophoretic mobility-shift assays.

EXPERIMENTAL

Reagents and cell lines

Reagents and supplies not described herein were purchased from vendors cited in [10]. Human colon HT29 adenocarcinoma cells and adherent human HeLa cervical carcinoma cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and were cultured as described in [9].

Abbreviations used: AP-1, activator protein-1; β-gal, β-galactosidase; CMV, cytomegalovirus; GALV, gibbon ape leukaemia virus; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP-12, matrix metalloproteinase-12; NAPSTER, nucleotide-affinity preincubation specificity test of recognition; NE, nuclear extract; Oct-1, octamer-1; wt, wild-type; YB-1, Y-box-binding protein-1.

[1] Present address: Biologie Cellulaire et Moléculaire, Faculté Universitaire des Sciences Agronomiques, Gembloux, Belgium.

[2] To whom correspondence should be addressed (email lbernstein@tamhsc.edu).

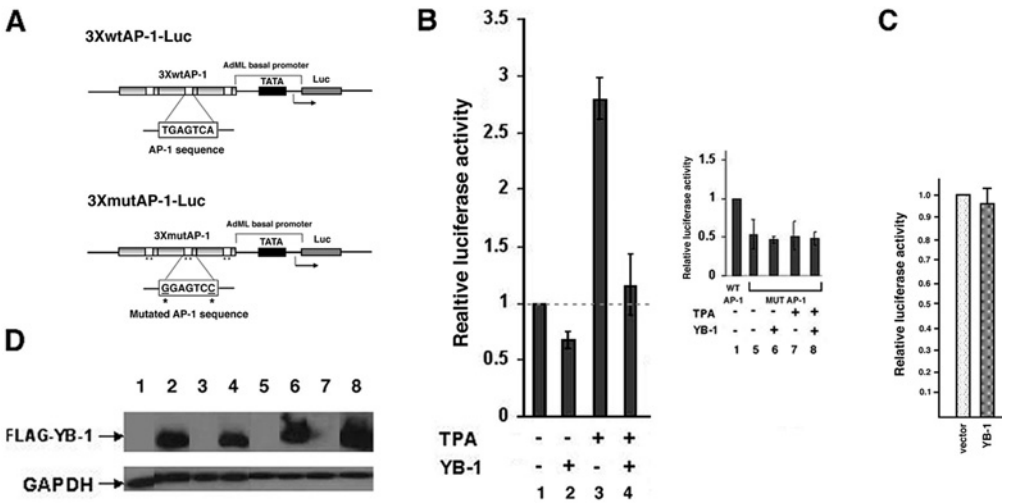

Figure 1 YB-1 represses PMA-induced AP-1 transactivation in HeLa cells

(**A**) AP-1 luciferase reporter constructs. Upper diagram: wt 3 × wtAP1-luc luciferase reporter construct; lower diagram: mutant 3 × mutAP1-luc reporter construct. 'AdML', adenovirus major late basal promoter; 'luc', luciferase reporter gene. (**B**) AP-1 transactivation studies. HeLa cells were transiently co-transfected with either pcDNAFlagYB-1 or pcDNA3.1(+) vector control plasmid plus wt or mutant (inset) AP-1 luciferase reporter constructs and pSVBgal plasmid. Cells were treated with PMA (TPA; 100 ng/ml) or DMSO solvent control for 24 h. All data are normalized to β-gal activity. The percentage repression of induced activity is calculated as: 100× {(relative PMA-treated luc activity) − (relative PMA-treated, YB-1 transfected activity)}/{(relative PMA-treated activity) − (relative PMA untreated activity)}. Error bars represent the S.E.M. for five independent experiments, each performed in duplicate. (**C**) YB-1 does not repress the transactivation of a constitutive CMV-luc construct. HeLa cells were co-transfected with 2 μg of CMV-luc in the presence of 1 μg of pcDNAFlagYB-1 or vector control plasmid. Error bars represent the S.E.M. for three independent experiments, each performed in duplicate. (**D**) FLAG–YB-1 protein expression. Expression of transfected YB-1 was confirmed by immunoblotting 10 μg of total protein from control (pcDNA) and YB-1-transfected cells with anti-FLAG antibody. Numbering of the samples is the same as in (**B**). Equal protein loading was verified by immunoblotting with anti-GAPDH antibody.

Antibodies and immunoblotting

All anti-AP-1 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Anti-YB-1 antibody was a 'C1' immunoaffinity-purified peptide antibody custom prepared by Bethyl Laboratories (Woodlands, TX, U.S.A.; [11]). Anti-FLAG M2 was obtained from Sigma (St. Louis, MO, U.S.A.) and anti-GAPDH (where GAPDH stands for glyceraldehyde-3-phosphate dehydrogenase) was from Research Diagnostics (Flanders, NJ, U.S.A.). Western immunoblotting was performed as described in [10] using antibodies at the following concentrations: anti-YB-1 (0.5 μg/ml), anti-FLAG (0.49 μg/ml) and anti-GAPDH (0.4 ng/ml).

Plasmid constructs

pcDNAFlag-YB1 plasmid harbouring a full-length human FLAG–YB-1 gene was a gift from Dr K. Kohno (University of Occupational and Environmental Health, Kitakyushu, Japan). A constitutive CMV (cytomegalovirus) overexpression construct CMV-luc (CMV-luciferase) was constructed by subcloning the firefly luciferase gene into the pcDNA3.1(+) plasmid at HindIII–BamHI restrictions sites. The full-length constitutive pSVBgal β-gal (β-galactosidase) overexpression construct was obtained from Promega (Madison, WI, U.S.A.).

Minimal AP-1 reporter constructs

Oligonucleotides ('oligos') harbouring three tandem copies of the wt (wild-type) (5′-agccagagaaatagatgagtcaacagc-3′; the AP-1 sequence underlined) or mutant (5′ agccagagaaatagaggagtctacagc-3′; mutations in boldface) AP-1 DNA-binding site from the GALV-LTR {GALV (gibbon ape leukaemia virus) long terminal repeat [9]}, flanked by 5′-XhoI and 3′-BamHI restriction sites, were custom synthesized (Integrated DNA Technologies, Coralville, IN, U.S.A.) and subcloned into the pGL3AdML-Luc at XhoI and BamHI sites by replacing 5× UASG sequences to generate wt 3 × wtAP-1-luc and mut (mutant) 3 × mutAP-1-luc luciferase reporter constructs, as depicted in Figure 1(A).

Promoter reporter constructs

Wt and mutant promoter reporter constructs pGL3-MMP-12-luciferase and pGL3-MMP-12(AP-1mut)luciferase for the MMP-12 gene were gifts from Dr M. Feinberg (Brigham and Women's Hospital, Boston, MA, U.S.A. [12]).

Luciferase reporter transactivation assay

HeLa cells were plated and transiently co-transfected with 2 μg (for minimal reporter assays) or 1 μg (for promoter reporter assays) of luciferase reporter plasmid, along with 1 μg of pcDNA-FlagYB1 plus 200 ng of pSVBgal, using Lipofectamine™ 2000 (1 μl/μg DNA; Invitrogen, Carlsbad, CA, U.S.A.). Cells that received no transfected pcDNAFlagYB1 were transfected with 1 μg of pcDNA3.1(+) vector control plasmid (Invitrogen). Cells were treated with PMA (100 ng/ml), 5 h after transfection. Cells were assayed 24 h post-transfection for luciferase activity (Roche Luciferase reporter gene assay kit; Roche, Indianapolis, IN, U.S.A.) using a Packard Lumicount luminometer (Packard Instruments, Downers Grove, IL, U.S.A.). β-Gal activity assays were performed using a β-gal enzyme assay kit obtained from Promega (Madison, WI, U.S.A.).

Preparation of whole cell lysates and NEs (nuclear extracts)

Whole cell lysates from HeLa cells and NEs from HT29 cells were prepared as described in [8,9,13]. HeLa cells were plated at 3×10^6 cells/150 mm dish, treated with 100 ng/ml PMA for 24 h and NE was prepared as described in [8,9].

NAPSTER AP-1 DNA-binding assays

Streptavidin beads (Pierce Biotechnology, Rockford, IL, U.S.A.). were conjugated with biotinylated double-stranded oligos

Table 1 Sequences of GALV wild-type and mutant oligos

AP-1 sites are underlined. Boldface letters denote mutated sequences.

Name of oligo	Sequence of oligo
GALV WT	AGCCAGAGAAATAGATGAGTCAACAGC
GALV DM	AGCCAGAGAAATAGA**G**GAGTC**T**ACAGC
GALV 5′-MUT	AGCCAGAGAAATAGA**G**GAGTCAACAGC
GALV 3′-MUT	AGCCAGAGAAATAGATGAGTC**T**ACAGC
GALV MUT 1	AGC**ACTC**GAAATAGATGAGTCAACAGC
GALV MUT 2	AGCCAGA**TCCC**TAGATGAGTCAACAGC
GALV MUT 3	AGCCAGAGAAA**GCTC**TGAGTCAACAGC
GALV MUT 4	AGCCAGAGAAATAGA**GTC**GTCAACAGC
GALV MUT 5	AGCCAGAGAAATAGATGA**TGAC**ACAGC
GALV MUT 6	AGCCAGAGAAATAGATGAGTCA**CACT**C

containing the 1× wt AP-1 GALV AP-1 sequence (same sequence as used in the reporter construct mentioned above), or with a 28 bp oligo containing four tandem 7 bp core AP-1 sites, as described in [8,9]. Batchwise microscale AP-1 DNA affinity chromatographic NAPSTER assays were performed using published methods [8,9]. Block mutants of the GALV AP-1 DNA sequences were prepared as described by Powers et al. [14]. Sequences of the block mutants are listed in Table 1.

DNA affinity purification and identification of p47 and p49 proteins

HT29 NE protein (60 mg) was dialysed against buffer Z and then subjected to DNA affinity column chromatography with 2.5 mg of mutant AP-1 DNA beads at 4 °C. The flowthrough was incubated with 120 μg of poly(dI-dC)·(dI-dC) for 10 min on ice and split into three portions: sample I, the starting material for purification (54 mg of NE protein) and NAPSTER control samples II and III (3 mg each of NE protein). Samples II and III were preincubated with 375 μg of wt or mut competitor oligo in solution as described in [9]. Streptavidin beads containing 2.7 mg of bound wt AP-1 DNA were added to sample I and beads containing 150 μg of AP-1 DNA were added to samples II and III. Binding reactions were performed as described in [9].

Bound proteins were eluted by incubating on a rotating clip wheel twice for 30 min at 4 °C in 1 ml of buffer Z containing 1 M KCl. They were then precipitated in 10 % (v/v) trichloroacetic acid, reprecipitated in absolute acetone (EM Science, Darmstadt, Germany) and loaded on to SDS/polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue. Bands corresponding to p49 and p47 were excised from sample I, as judged by their presence in samples I and III but not in sample II. p47 and p49 were analysed by nanospray MS/MS fragmentation sequencing (tandem MS fragmentation) at the Yale HHMI-Keck Laboratory as described in http://info.med.yale.edu/wmkeck/prochem. Spectra were searched manually and by using the Mascot peptide mass 'fingerprint' algorithm for protein identification (http://www.matrix-science.com/search_form_select.html).

Gel-shift and supershift analyses

NE from MCF-7 breast cancer cells purchased from Santa Cruz Biotechnology were used in gel-shift and supershift assays as described by Phillips et al. [15] using 1× wt AP-1 oligos. Anti-c-Jun and anti-YB-1 antibodies, and competitor peptide antigens employed for supershift specificity controls, were obtained from Santa Cruz Biotechnology.

Real-time PCR analyses

Real-time PCR was performed as described in [16] using HeLa cells that had been treated with 100 ng of PMA for 24 h before harvesting mRNA. Primer sets used in real-time PCR are listed in Table 2.

Table 2 Primers for real-time PCR analyses

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
YB-1	GTACCGCCGCAACTTCAATT	TCTTTGCCATCTTGTGGTTTAGG
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
MMP-12	GCACTTCTTGGGTCTGAAAGTGA	CGAGGTGCGTGCATCATCT

RESULTS

YB-1 transrepresses PMA-induced transactivation of AP-1-dependent gene expression

To determine whether YB-1 may function on an AP-1 pathway, we performed transactivation assays using minimal AP-1 reporter constructs in PMA-stimulated HeLa cells that overexpressed a transiently transfected YB-1 gene. Effects of YB-1 overexpression on PMA-induced transactivation were compared between cells transfected with wt reporter constructs versus reporter constructs harbouring inactivating mutations in the AP-1 sites (see Figure 1A for diagrams of wt and mutant AP-1 reporter constructs). PMA treatment enhanced AP-1 transactivation of the wt AP-1 construct in HeLa cells by 2.8-fold (Figure 1B), but had no effect on the mutant AP-1 reporter construct. YB-1 overexpression caused 91 % repression of PMA-induced AP-1 transactivation of the wt AP-1 reporter. Repression of basal expression of the wt AP-1 reporter construct was also statistically significant, exhibiting 32 % inhibition. YB-1 did not repress the transactivation of the mutant AP-1 reporter, demonstrating that YB-1 represses AP-1 gene transactivation in a manner that is dependent on the AP-1 DNA sequence. Overexpression of transfected YB-1 caused only 5 % repression of transactivation from a constitutive CMV-luc construct (Figure 1C). Overexpression of FLAG–YB-1 was confirmed by immunoblotting with anti-FLAG-tagged antibodies (Figure 1D). Immunoblotting with an N-terminal antibody against YB-1 (a gift from Dr C. Nelson, University of British Columbia, British Columbia, Canada; [17]) demonstrated at least a 1.8-fold increase in the levels of YB-1 protein after FLAG–YB-1 expression.

To investigate the possibility that overexpression of transfected YB-1 represses transactivation by causing toxicity, we measured the viability of the cells before and after YB-1 gene transfection. Little change in cell number was observed after transfection with YB-1 as measured by counting the cells in a haemocytometer and by immunoblotting equal volumes of the extract used in transactivation assays with anti-GAPDH antibodies (Figure 1D and results not shown). Approximately 97–98.5 % of the cells remained viable in Trypan Blue exclusion assays after transfection of YB-1. These results rule out the possibility that YB-1 transrepression of AP-1-dependent gene expression occurs through a non-specific toxic mechanism.

Transrepression of AP-1-dependent gene expression does not occur by YB-1 down-regulation of AP-1 subunit protein levels

It is possible that YB-1 could repress AP-1 transactivation by a pathway that ultimately decreases the availability of AP-1 proteins for binding the AP-1 site. One pathway to achieve this could be to reduce the steady-state intracellular concentrations of one or more AP-1 subunits. HeLa cells were transiently transfected with YB-1 and the intracellular levels of all seven AP-1 subunit proteins were measured (Figure 2A). Expression of transfected

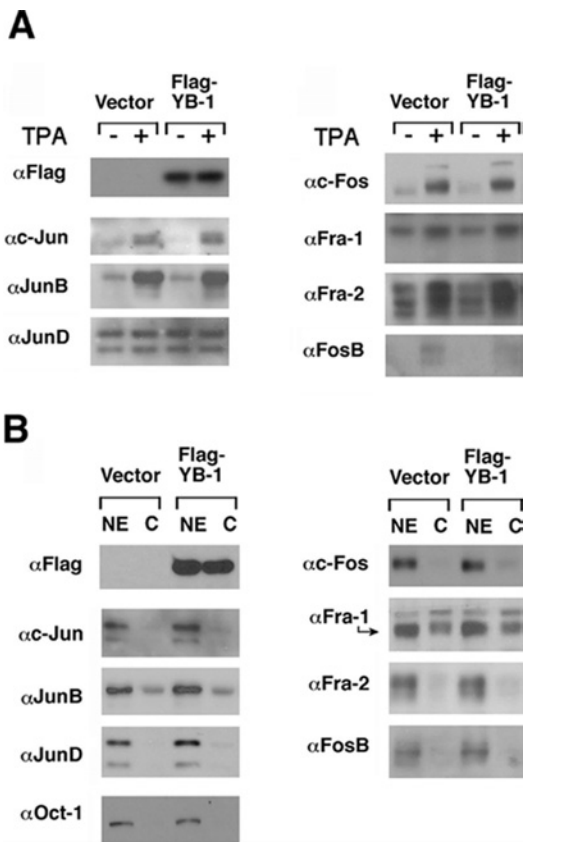

Figure 2 No effect of YB-1 on intracellular levels or nuclear localization of AP-1 subunits

(**A**) Overexpression of transfected YB-1 does not affect the intracellular levels of AP-1 subunits; 4.5×10^5 HeLa cells plated on 150 mm tissue culture dishes were transiently transfected with 20 μg of pcDNA3.1 or pcDNAFlag-YB-1 constructs. Whole cell lysates (10 μg/sample) were prepared after 24 h of PMA treatment (100 ng/ml, '+') or with DMSO control solvent ('−') and immunoblotted with antibodies raised against the seven AP-1 subunits and with anti-FLAG antibody. (**B**) Overexpression of transfected YB-1 does not alter nuclear concentrations of AP-1 subunits. HeLa cells were transfected, treated with PMA (TPA) and fractionated to separate the cytosolic and nuclear compartments. NE or cytosol were loaded in SDS/PAGE using NE and cytoplasmic samples derived from 4×10^5 cells in each lane to equalize the number of cellular equivalents per test sample. Samples were immunoblotted with antibodies raised against the seven AP-1 subunits, with anti-Oct-1 and with anti-FLAG. C, cytosol.

YB-1 was confirmed by immunoblotting with anti-FLAG-tagged antibodies. Transfection efficiency was estimated to be in the range 75–90 % on the basis of the percentage of HeLa cells that incorporated green fluorescent protein plasmid, as detected by indirect immunofluorescence microscopy. Intracellular levels of the seven AP-1 subunits were not altered by overexpression of the transfected YB-1. These results rule out the possibility that YB-1 down-regulates steady-state intracellular levels of any of the AP-1 subunits by any of the following means: (i) down-regulating the transcription of genes encoding AP-1 subunits, (ii) up-regulating the rate of degradation of *jun* and *fos* mRNAs, (iii) inhibiting the cytoplasmic transport of *jun* and *fos* hnRNAs, (iv) inhibiting AP-1 protein translation and (v) up-regulating AP-1 protein degradation.

YB-1 does not block the entry of AP-1 into the nucleus

YB-1 could also limit the availability of AP-1 subunits for binding the AP-1 site by inhibiting the entry of AP-1 proteins into the nucleus. To test this hypothesis, we transfected HeLa cells with YB-1 and then measured the levels of all seven AP-1 subunits in the nuclear and cytoplasmic fractions (Figure 2B). No change in subcellular distribution or total intranuclear levels of any of the AP-1 subunits was observed on overexpression of the transfected YB-1. Immunoblotting with an antibody raised against the Oct-1 (octamer-1) transcription factor, a marker for nuclear localization, demonstrated exclusive localization of Oct-1 to the nucleus, confirming that intact subcellular fractions were isolated. These results rule out the hypothesis that YB-1 represses transactivation by decreasing the intranuclear concentrations of AP-1 proteins available to implement AP1-dependent transactivation.

NAPSTER DNA affinity chromatography: purification of YB-1 as a protein that interacts with the AP-1 sequence

While performing transactivation studies, we were engaged in a project whose purpose was to isolate and identify new proteins that bind to the AP-1 DNA sequence. We performed AP-1 DNA affinity chromatography to identify such proteins. To distinguish proteins that bind specifically to the AP-1 DNA sequence from proteins that bind non-specifically to DNA or to chromatography beads, we used the NAPSTER assay. NAPSTER consists of three samples. Nuclear extracts either directly undergo DNA affinity chromatography with an AP-1 DNA sequence conjugated with streptavidin beads (sample 'I' in all NAPSTER experiments) or are first preincubated with excess unconjugated wt (sample 'II') or mutant AP-1 oligonucleotide (oligo; sample 'III') before chromatography. Batchwise microscale DNA affinity chromatography is then performed, by methods adapted from Lee et al., with numerous modifications [8,9,18]. Samples from NAPSTER isolation are subsequently loaded in SDS/PAGE and subjected to immunoblotting with antibodies raised against the proteins of interest. When performing NAPSTER using streptavidin affinity beads conjugated with a wt GALV AP-1 oligo, we observed the specific binding of all seven Jun and Fos family AP-1 subunits to the AP-1 site. The results were reproducible, thus demonstrating the efficacy of the method (Figure 3A).

When purification by NAPSTER DNA affinity chromatography is performed followed by Coomassie Blue staining, two proteins of 47 and 49 kDa are reproducibly observed that bind the GALV AP-1 beads in samples I and III, but at much decreased levels in sample II (Figure 3B). Initially, we hypothesized that p47 and p49 were AP-1 subunits since they are in the molecular-mass ranges of these proteins. To test this hypothesis, Coomassie Blue-stained p47 and p49 gel bands were purified and immunoblotted with a mixture of antibodies raised against all seven Jun/Fos AP-1 proteins. Despite immunoblotting of Coomassie Blue-stained quantities of p47 and p49, no AP-1 signal was detected, indicating that p47 and p49 are not AP-1 proteins (Figure 3C).

Coomassie Blue-stained p47 and p49 from sample I purified from HT29 NE protein then underwent tandem MS fragmentation sequencing analyses. For p49, two peptides were isolated, with the sequences AEAANVTGPGGVPVQHSK and SVGDGETVEFDVVEGEK, both of which matched YB-1 in Mascot peptide mass 'fingerprint' analyses of the tandem MS fragmentation data. Peptides with the sequences SVGDGETVEFDVVEGEK and NGYGFINR, matching YB-1 in the Mascot procedure, were identified from analyses of p47.

Confirmation of the tandem MS fragmentation data was then sought by performing immunoblotting of NAPSTER-purified NE material using anti-YB-1 antibody. YB-1 antibody specifically recognized the YB-1 protein in affinity chromatographically purified samples I and III of the NAPSTER, but not in sample II in both HT29 and HeLa NE (Figure 4A). Specific YB-1 binding to the AP-1 site was observed in HT29 and HeLa NEs in 81 independent experiments. Preincubation of YB-1 antibody with YB-1 peptide

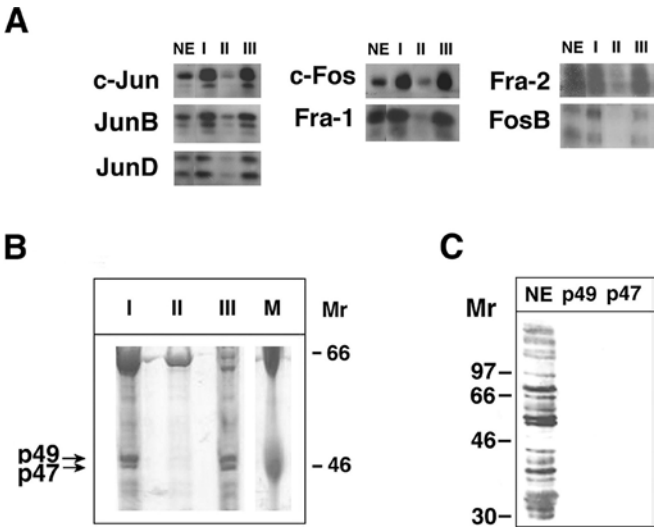

Figure 3 NAPSTER analyses of AP-1, p47 and p49 proteins

(**A**) Jun family and Fos family AP-1 subunit proteins bind specifically to the AP-1 DNA in NAPSTER. NAPSTER samples I, II and III originating from 700 μg of HT29 NE protein were generated, each split into seven individual fractions, run on an SDS/polyacrylamide gel alongside 30 μg of NE protein. Samples were then immunoblotted with each of the seven anti-AP-1 antibodies. Names to the left of autoradiograms indicate identities of Jun and Fos family antibodies used in immunoblotting analyses. (**B**) p47 and p49 proteins bind specifically to AP-1 DNA in NAPSTER. NAPSTER samples I, II and III were generated as described in the Experimental section and subjected to SDS/PAGE. The gel was stained with Coomassie Brilliant Blue. (**C**) p47 and p49 are not AP-1 proteins. p47 and p49 proteins were purified from sample I by NAPSTER and SDS/PAGE starting with 15 mg of NE protein, as described in the Experimental section. p47 and p49 were excised from the gel and loaded on to a second gel alongside 30 μg of HT29 NE extract protein (NE, first lane). Samples were immunoblotted with a mixture of all seven AP-1 antibodies.

antigen abrogated YB-1 antibody recognition of YB-1 protein, whereas preincubation with a non-specific peptide did not (results not shown), indicating that YB-1 antibody recognition of YB-1 protein is specific. YB-1 binding to the AP-1 site was competed by increasing the titrated concentrations of wt AP-1 oligo but not doubly mutated AP-1 oligo (Figure 4B).

The p47 protein was reproducibly recovered along with p49 protein and detected by Coomassie Blue staining when NAPSTER purifications were performed that involved several tandem purification steps. In contrast, in small-scale single-step NAPSTERs, we visualized the p49 band in every experiment, and the p47 band in just three out of 81 experiments (results not shown). The procedure employed to isolate YB-1 for Coomassie Blue staining and sequencing involves more steps and sample handling time compared with analytical NAPSTER assays, which consist of a single purification step. Since more extensive sample handling creates increased opportunity for sample degradation, we hypothesize that p47 may be a proteolytic digestion product or a hypophosphorylated form of YB-1 that is generated during the purification of p49 protein.

Since YB-1 binding to single-stranded DNA sequences has been described in [19], NAPSTER was performed using AP-1 DNA beads that had been prepared by incubating a 5-fold molar excess of unbiotinylated AP-1 oligo with the biotinylated complementary strand before conjugating the double-stranded AP-1 oligo with streptavidin beads. The intensity and specificity of the YB-1-binding signal in NAPSTER assays employing these beads were equal to those of beads conjugated with AP-1 DNA that had been annealed using a 1:1 ratio of the two strands (results not shown), showing that YB-1 binds to a double-stranded AP-1 site.

YB-1 binds to AP-1 oligonucleotides in gel-supershift assays

We then sought to confirm the binding of YB-1 observed in NAPSTER by performing gel-shift analyses with anti-YB-1 antibodies (Figure 4C). A specific AP-1 shift was identified by preincubation of excess wt or mut AP-1 oligos with NE before the addition of labelled wt AP-1 oligo. Supershift assays were performed by preincubating NE with anti-c-Jun or anti-YB-1 before the addition of labelled oligo. Both anti-c-Jun and anti-YB-1 gave rise to supershifted protein–DNA complexes. In samples incubated with anti-c-Jun or anti-YB-1 antibodies, the sum of the intensities of the supershifted band and the shifted band is significantly less than the intensity of the shifted band in control samples incubated without antibodies. This suggests that anti-c-Jun and anti-YB-1 each concomitantly cause a supershift and

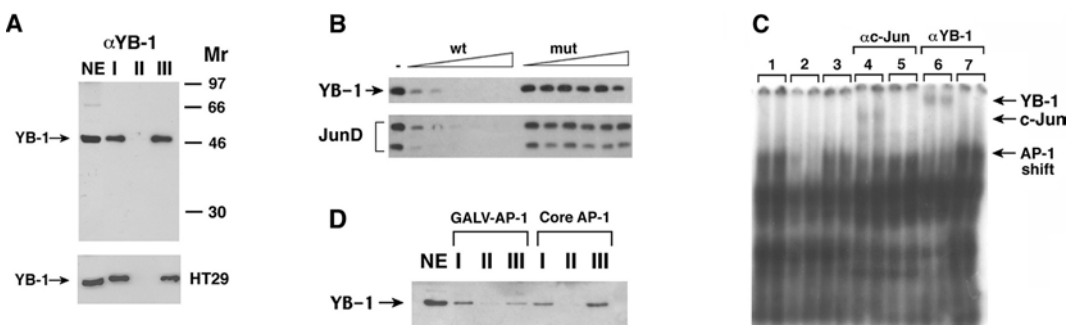

Figure 4 YB-1 binds specifically to an AP-1 site in NAPSTER and gel-shift assays

(**A**) Confirmation that p49 is YB-1 in NAPSTER; 300 μg of either HeLa (upper panel) NE protein or HT29 (lower panel) NE protein for each of the samples I, II and III was subjected to NAPSTER and immunoblotted with anti-YB-1. (**B**) Wt but not mutant AP-1 DNA competes for YB-1 binding as a function of increasing concentration; 150 μg of HT29 NE per sample was preincubated for 15 min with increasing fold molar excess of unconjugated wt or mut AP-1 oligo before the addition of 10 μg of wt AP-1 oligo on beads. Triangles above autoradiograms indicate increasing molar quantities of unconjugated competitor preincubation oligos relative to oligo on beads. 'wt', wt unconjugated competitor oligo3; 'mut', mutant unconjugated competitor oligo. '–', no competitor oligo. Consecutive lanes for wt and mut lanes contained 0.1-, 0.2-, 0.5-, 0.8-, 1.5- and 2.5-fold molar excess of oligo respectively. (**C**) YB-1 binds the AP-1 site in gel-shift assays. Shifted and supershifted bands are indicated by arrows. Duplicate gel-shift samples are shown in brackets. #1, AP-1 shift, no competitor oligo preincubation; #2, wt competitor oligo preincubation; #3, mutant competitor oligo preincubation; #4, anti-c-Jun; #5, anti-c-Jun + c-Jun peptide; #6, anti-YB-1; #7, anti-YB-1 + YB-1 peptide. (**D**) The core AP-1 element is sufficient for YB-1 binding; 300 μg of HeLa NE protein for each of samples I, II, and III were subjected to NAPSTER using streptavidin beads conjugated with the 3 × wtAP-1 oligo (the 3 × GALV-AP-1 sequence, left bracket) or with a 28 bp oligo containing four multimerized 7 bp core AP-1 sites (5′-TGAGTCA-3′; 'core AP-1', right bracket). Competitions for samples II and III were performed with 2.5-fold molar excess of unconjugated wt or mutant 3 × wtAP-1 oligo (left bracket) or with wt or mutant core AP-1 (right bracket). Mutated core AP-1 sequences contain four tandem doubly mutated AP-1 sites (5′-**G**GAGTC**T**-3′; mutations underlined and shown in boldface).

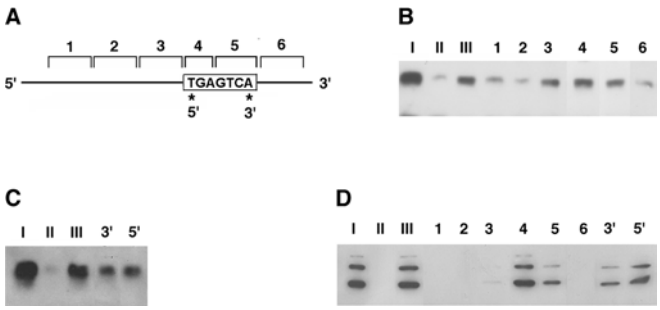

Figure 5 Mutational mapping of the YB-1-binding site

(**A**) Schematic representation of block mutations in the GALV 1 × AP-1 oligo. The line indicates GALV AP-1 oligo and the box shows the AP-1 sequence. 5′ and 3′ with asterisks demonstrate the locations of point mutations introduced in the AP-1 site. Numbered brackets indicate the locations of block-mutated sequences introduced along the length of the oligo. (**B**) YB-1 binding to the GALV AP-1 oligo involves sequences 3, 4 and 5 but not sequences 1, 2 and 6. NAPSTER analyses were performed for samples I, II and III using no competitor (lane I) or a 2.5-fold molar excess of wt (II) or doubly mutated (III) competitor oligo (lanes II and III); or using competitor oligos containing mutations in blocks 1–6 as indicated by the numbered lanes. (**C**) 5′- and 3′-nucleotides in the core AP-1 sequence are important for YB-1 binding to the core AP-1 site in the GALV AP-1 oligo. NAPSTERs were performed for wt (lane II) or doubly mutated competition oligos (lane III) or AP-1 oligos mutated in the 5′- or 3′-AP-1 nucleotide as indicated above the lanes. (**D**) JunD binding to the GALV AP-1 sequence involves only the AP-1 site. NAPSTER was performed using competitor oligos harbouring block mutations or point mutations as described above for (**B**, **C**), after which immunoblotting was performed with anti-JunD antibodies.

a partially blocked AP-1 shift. Controls for antibody specificity were performed by preincubating anti-c-Jun and anti-YB-1 with excess cognate peptide antigen competitor, which abrogated the c-Jun and YB-1 supershifts and caused the return of the AP-1 shifts to their original intensities. Preincubation of αYB-1 with heterologous peptide antigens from the ERK3 protein did not inhibit the supershift or blocked the shift caused by anti-c-Jun and anti-YB-1 antibodies. YB-1 binding to a Y-box motif in the human multidrug resistance-1 promoter was also observed in gel-shift assays (results not shown). Results of gel-shift assays confirm the NAPSTER data, demonstrating that YB-1 binds to oligos containing the AP-1 DNA sequence.

Sequence requirements for YB-1 binding to AP-1 oligonucleotides

In addition to observing YB-1 binding to the GALV AP-1 oligos in NAPSTER, we also observe specific YB-1 binding to the GCN4 AP-1 site, which has flanking sequences that differ from those of the GALV AP-1 DNA (results not shown). YB-1 binding is also observed in NAPSTER using a synthetic 28 bp sequence containing four multimerized copies of the core 7 bp 5′-TGAGTCA-3′ sequence, indicating that the core AP-1 sequence lacking flanking sequences is sufficient for YB-1 binding (Figure 4D). Binding of JunD to the GCN4 and core AP-1 sequences was also observed (results not shown).

Although these results indicate that flanking sequences are not required for YB-1 binding to the AP-1 site, it is still possible that they may facilitate YB-1 binding. To investigate this notion, a set of six GALV oligos mutated in six consecutive blocks from the 5′-end to the 3′-end was generated by substituting inverse complementary bases in adjacent consecutive sets of 4 bp (Figure 5A and Table 1). Each of these block mutants was used as competition DNA in NAPSTER assays using GALV beads harbouring the wt AP-1 site. Importance of each of the sequences in the block mutants for YB-1 binding was compared by assessing the relative intensities of bands visualized after using the mutated oligos in NAPSTER, where a band of dark intensity indicates that the sequence in question is important for protein binding, and a light band indicates that the sequence is not important. As shown in Figure 5(B), three block mutations in the GALV-flanking region, namely mutants 1, 2 and 6 in the 5′-end, middle and 3′-end of the GALV sequence, do not decrease their effectiveness as competitor oligos in NAPSTER. This indicates that these sequences do not bind YB-1. However, mutagenesis of block 3, the TAGA sequence immediately adjacent to the 5′-end of the core AP-1 site, significantly impairs the potency of the GALV AP-1 sequence as a NAPSTER competitor oligo, indicating that this sequence does associate with YB-1. Block mutagenesis of core AP-1 blocks 4 and 5 located in the 5′-half and the 3′-half of the AP-1 site significantly decreases their potencies as competitors in NAPSTER, indicating that both sequences are important for YB-1 binding. Furthermore, oligos harbouring mutations in only the 5′-base of the core AP-1 site (GGAGTCA, the mutated base is underlined) and oligos harbouring a mutation in the 3′-base (TGACTCT, mutated base underlined) each exhibit partial competition relative to doubly mutated AP-1 oligos (TGACTCT; compare lanes labelled 5′ and 3′ with lane III of Figure 5C), suggesting that both bases are required for optimum YB-1 binding. Intensity of the band for the 5′-mutant competition was somewhat higher than that of the 3′-mutant competition in some experiments. Similar to YB-1, JunD does not bind to blocks 1, 2 and 6 and binds to blocks 4 and 5, but unlike YB-1, JunD does not bind to block 3 (Figure 5D). Taken together with the data in Figure 4(D) demonstrating that the core AP-1 site is sufficient for specific YB-1 binding, the data in Figure 5 indicate that YB-1 binds both to the core AP-1 site and to the adjacent 5′-flanking sequence.

YB-1 represses PMA transactivation of the MMP-12 gene

Since YB-1 represses transactivation at an AP-1 site and binds to an AP-1 DNA sequence, we hypothesized that it would down-regulate promoter activity and mRNA expression of an endogenous AP-1 target gene. MMP-12 is an AP-1 target gene that encodes a major extracellular matrix MMP that degrades elastase in the extracellular matrix. Promoter reporter constructs from the MMP-12 gene containing one wt or one mutated AP-1 site were transiently co-transfected into HeLa cells with FLAG–YB-1 or with pcDNA vector control construct. Treatment with PMA resulted in 4.2-fold activation of the MMP-12 promoter reporter (Figure 6A). Mutation of the AP-1 site blocks PMA activation. YB-1 represses PMA-induced wt MMP-12 promoter activity by 61 %. Activity of the mutated MMP-12 promoter construct is not inhibited by YB-1. These results show that YB-1 mediates the repression of the MMP-12 promoter through the AP-1 site.

Real-time PCR analyses were performed to quantify steady-state mRNA expression levels of MMP-12 in the presence and absence of YB-1 overexpression (Figure 6B). Transfected FLAG–YB-1 caused a 30-fold increase in YB-1 mRNA expression. YB-1 overexpression caused 2.5-fold repression of MMP-12 expression. Control studies demonstrated that YB-1 did not affect the mRNA expression of GAPDH, which is not an AP-1 target gene.

DISCUSSION

YB-1 has several biochemical and biological functions that overlap with AP-1, since both proteins have nucleic acid binding and transcription-regulatory activities and have been implicated in mediating DNA repair, cell cycle, cell proliferation and neoplastic transformation [3,20]. Overlapping biological functions of YB-1 and AP-1 may be attributable in part to DNA binding and trans-repression activities of YB-1 at the AP-1 site. YB-1 is also implicated in mediating RNA processing, stabilization, chaperoning and packaging, and in RNA translation. Roles for YB-1

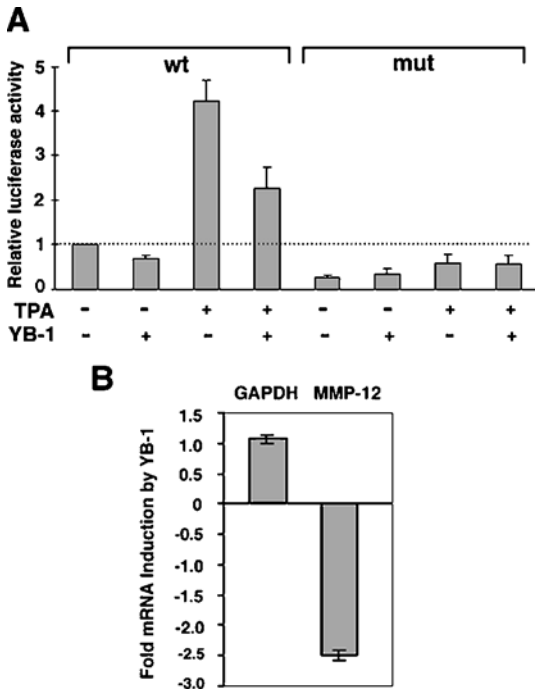

Figure 6 YB-1 represses the AP-1-dependent transactivation and mRNA expression of the MMP-12 gene

(**A**) YB-1 represses PMA transactivation of the MMP-12 gene promoter at the AP-1 site. HeLa cells were transiently co-transfected with 1 μg of wt or AP-1 mutant MMP-12 promoter reporter constructs plus pcDNAFlagYB-1 or pcDNA 3.1(+) vector control along with pSVBgal plasmid. Transfection conditions, PMA (TPA) treatment and data analyses were as described in the Experimental section and in the legend to Figure 1. Error bars represent the S.E.M. for four independent experiments, each performed in duplicate. (**B**) YB-1 represses mRNA expression of the MMP-12 gene in real-time PCR analyses. Error bars represent the S.E.M. for three independent experiments.

in such a broad range of functions implies that an important activity for YB-1 may be the co-ordinated integration of transcription, replication and translation, such that it may orchestrate complex biological processes such as cell proliferation.

Since, in many contexts, AP-1 transactivation mediates neoplastic transformation, YB-1 transrepression of AP-1-dependent gene expression appears paradoxical in the light of results from many groups correlating YB-1 expression with malignant progression. This paradox is addressed in a recent study by Bader et al. [21], who found that expression of transfected YB-1 inhibits neoplastic transformation of chick-embryo fibroblasts, indicating a tumour suppressor function for YB-1. A tumour suppressor function for YB-1 is consistent with its transrepressing activity. Several other genes that display tumour suppressor function also exhibit increased expression as a function of malignant progression [22,23].

YB-1 has been shown to mediate both transcriptional activation and repression of a number of genes [3]. YB-1 has been found to transactivate promoters of the genes encoding the P-glycoprotein MDR-1 multidrug-resistance gene and the myosin light chain 2v, both through the Y-box sequence. YB-1 has been shown to bind to a Y-box and repress transactivation of the procollagen α1(I) and α2(I) genes [5]. The present study is the first to demonstrate that YB-1 represses the transactivation of gene expression at the AP-1 site. It is also the first to report that YB-1 can repress the expression and transactivation of the MMP-12 gene.

The present study is the first to report physical association of YB-1 with the AP-1 DNA-binding site. In addition to the Y box, YB-1 is capable of binding to and regulating transcription at a number of other sequences, including several nucleotide sequences in RNA, single-stranded DNA and double-stranded DNA. YB-1 recognizes duplex DNA with strong purine/pyrimidine asymmetry and binds preferentially to the pyrimidine-rich strand in several genes [24]. The mechanism by which YB-1 binds to diverse sequence motifs is not known. One possible mechanism is that YB-1 dimerizes or forms higher-order multimers with many different proteins and that these different complexes have distinct DNA-binding specificities.

AP-1 was originally purified in groundbreaking studies performed in 1987 employing purification schemes that involved DNA affinity chromatography [18,25]. Whereas the predominant species identified in those purifications was AP-1, YB-1 binds in much greater abundance compared with AP-1 in NAPSTER. HeLa NEs were used in one of the original purification studies, and YB-1 from HeLa NE has AP-1 DNA-binding activity in NAPSTER. It is possible that YB-1 was co-purified along with AP-1 components in the original purifications. In the original studies, multiple silver-stained bands were visualized with molecular masses in the same range as YB-1. One of them could have been YB-1. The original work on AP-1 antedated protein microsequencing technology, so the actual cloning of Jun proto-oncogenes occurred through indirect studies in which mammalian cDNA homologues of the v-*jun* oncogene were cloned because v-Jun, similar to purified AP-1 proteins, was found to bind the AP-1 DNA sequence [26]. By these means, YB-1 binding to the AP-1 site may have been visualized in silver-stained gels, but not pursued or identified. It may also be that YB-1 was not recovered in the original purifications. Whereas the NAPSTER purification used to isolate YB-1 consists only of several steps, the original methods used to isolate AP-1 had multiple steps, including precipitation with ammonium sulphate, molecular sieve chromatography, heparin affinity chromatography and stepwise KCl elution after DNA affinity chromatography. These additional steps may have removed YB-1 from these preparations.

A number of bZIP proteins of the AP-1 superfamily bind with sequence specificity to the minimal 5′-TGAg/cTCA-3′ AP-1 consensus sequence, either in the presence or absence of AP-1 proteins [27,28]. These include members of the Nrf, Maf and CREB/ATF families. SFA-2 and SNFT bZIP proteins bind the AP-1 site when heterodimerized with c-Jun [29,30]. BZLF1 (also known as ZEBRA), a divergent member of the bZIP family, also binds to the minimal consensus AP-1 site, either unaccompanied or in a complex with c-Fos and transcriptionally activates gene expression [31]. SNFT, JDP2, CREB and CREM bZIP AP-1 superfamily members bind to the AP-1 site and repress gene transactivation. A variety of non-bZIP transcription-regulatory factors can also repress AP-1 transactivation. Included among these are YY1, PE1, p300, Bcl-6, MyoD, menin, p202, Ets and several members of the nuclear receptor superfamily (e.g. peroxisome proliferator receptor, glucocorticoid receptor and retinoic acid receptors RAR and RXR; see [32,33] for reviews). Interaction of these molecules with the AP-1 site has not been demonstrated and, in many cases, has been ruled out. To our knowledge, YB-1 is the first non-bZIP protein outside the AP-1 superfamily that associates with the AP-1 site and represses AP-1 transactivation.

NAPSTER DNA-binding data indicate that the core AP-1 consensus sequence is sufficient for specific YB-1 binding to the AP-1 site. Sequences in flanking regions of the DNA are not required for YB-1 to bind to the AP-1 site. Nevertheless, we also found that bases within the TAGA sequence in the 5′-flanking region immediately adjacent to the AP-1 site mediate YB-1's association with the GALV oligo. Taken together, these results suggest that the GALV oligo contains two adjacent sequences involved in YB-1 binding, comprising the AP-1 site and the adjoining 5′-flanking

TAGA sequence. YB-1 repression of transactivation at the GALV core AP-1 site is abrogated 100 % by the introduction of mutations into the AP-1 site, indicating that the AP-1 site is required to mediate YB-1 transregulation of the GALV sequence. Efficient YB-1 transrepression can occur without GALV flanking sequences, since we also observe 80 % transrepression of a GCN AP-1 reporter, whose sequences flanking the AP-1 site are unrelated to those of the GALV sequence (results not shown).

It is probable that more YB-1 than AP-1 binds in NAPSTER since YB-1 is much more abundant compared with AP-1. Abundant proteins such as YB-1 may interact with specific sequences with lower affinity and provide a strategy for basal transcriptional repression. Several AP-1 transcriptional repressors have been the subjects of intense interest, partly due to their potential clinical applicability [32–34]. For example, glucocorticoids and retinoids are widely studied for their abilities to bind their respective receptors, repress AP-1 transactivation and exert powerful antiproliferative and chemopreventative effects.

Because de-regulated AP-1 transcriptional activity has been shown in many instances during the pathogenesis of cancerous, cardiovascular and neurological disease states, YB-1 repression of AP-1 transactivation may be important for its potential ability to mediate these processes. Similar to AP-1, both YB-1 and MMP-12 have been found to be mediators of invasion and metastasis [1–3,5,7]. Regulation of MMP-12 expression by YB-1 through the AP-1 site suggests that an interplay may exist between AP-1, YB-1 and MMP-12 to regulate these important biological processes.

This work was supported by NIH grant CA73783 to L. R. B., a grant from the Elsa U. Pardee Foundation to L. R. B., and a NIEHS grant (5 P30 ES09106-03) to L. R. B. We thank Dr L. Dangott and other staff of the Protein Technologies Facility Core at Texas A&M University for expert technical assistance through the NIEHS Center grant no. P030ES09106 to the Center for Environmental and Rural Health at Texas A&M University. We are grateful to Ms D. Caldwell and Ms K. Beifuss for helpful experimental assistance and to Dr D. Peterson, Dr G. Kunkel and Dr S. Maxwell for a critical evaluation of this paper. We apologize to authors whose works have not been cited due to space limitations.

REFERENCES

1 Eferl, R. and Wagner, E. F. (2003) AP-1: a double-edged sword in tumorigenesis. Nat. Rev. Cancer **3**, 859–868
2 Shaulian, E. and Karin, M. (2002) AP-1 as a regulator of cell life and death. Nat. Cell Biol. **4**, E131–E136
3 Kohno, K., Izumi, H., Uchiumi, T., Ashizuka, M. and Kuwano, M. (2003) The pleiotropic functions of the Y-box-binding protein, YB-1. Bioessays **25**, 691–698
4 Jurchott, K., Bergmann, S., Stein, U., Walther, W., Janz, M., Manni, I., Piaggio, G., Fietze, E., Dietel, M. and Royer, H. D. (2003) YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. J. Biol. Chem. **278**, 27988–27996
5 Kuwano, M., Uchiumi, T., Hayakawa, H., Ono, M., Wada, M., Izumi, H. and Kohno, K. (2003) The basic and clinical implications of ABC transporters, Y-box-binding protein-1 (YB-1) and angiogenesis-related factors in human malignancies. Cancer Sci. **94**, 9–14
6 Huber, E., Vlasny, D., Jeckel, S., Stubenrauch, F. and Iftner, T. (2004) Gene profiling of cottontail rabbit papillomavirus-induced carcinomas identifies upregulated genes directly Involved in stroma invasion as shown by small interfering RNA-mediated gene silencing. J. Virol. **78**, 7478–7489
7 Zucker, S. and Vacirca, J. (2004) Role of matrix metalloproteinases (MMPs) in colorectal cancer. Cancer Metastasis Rev. **23**, 101–117
8 Kumar, N. V. and Bernstein, L. R. (2001) A new analytical scale DNA affinity binding assay for analyses of specific protein-DNA interactions. Anal. Biochem. **299**, 203–210
9 Kumar, N. V. and Bernstein, L. R. (2001) Ten ERK-related proteins in three distinct classes associate with AP-1 proteins and/or AP-1 DNA. J. Biol. Chem. **276**, 32362–32372
10 Bernstein, L. R. and Walker, S. E. (1999) Tumor promotion resistant cells are deficient in AP-1 DNA binding, JunD DNA binding, and JunD expression and form different AP-1 DNA complexes than promotion sensitive cells. Biochim. Biophys. Acta **1489**, 263–280
11 Ohga, T., Koike, K., Ono, M., Makino, Y., Itagaki, Y., Tanimoto, M., Kuwano, M. and Kohno, K. (1996) Role of the human Y box-binding protein YB-1 in cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and ultraviolet light. Cancer Res. **56**, 4224–4228
12 Feinberg, M. W., Jain, M. K., Werner, F., Sibinga, N. E., Wiesel, P., Wang, H., Topper, J. N., Perrella, M. A. and Lee, M. E. (2000) Transforming growth factor-beta 1 inhibits cytokine-mediated induction of human metalloelastase in macrophages. J. Biol. Chem. **275**, 25766–25773
13 Young, M. R., Nair, R., Bucheimer, N., Tulsian, P., Brown, N., Chapp, C., Hsu, T. and Colburn, N. H. (2002) Transactivation of Fra-1 and consequent activation of AP-1 occur extracellular signal-regulated kinase dependently. Mol. Cell. Biol. **22**, 587–598
14 Powers, C., Krutzsch, H. and Gardner, K. (1996) Modulation of JunD.AP-1 DNA binding activity by AP-1-associated factor 1 (AF-1). J. Biol. Chem. **271**, 30089–30095
15 Philips, A., Teyssier, C., Galtier, F., Rivier-Covas, C., Rey, J. M., Rochefort, H. and Chalbos, D. (1998) FRA-1 expression level modulates regulation of activator protein-1 activity by estradiol in breast cancer cells. Mol. Endocrinol. **12**, 973–985
16 Samuel, S. and Bernstein, L. R. (2004) Adhesion, migration, transcriptional, interferon-inducible, and other signaling molecules newly implicated in cancer susceptibility and resistance of JB6 cells by cDNA microarray analyses. Mol. Carcinog. **39**, 34–60
17 Gimenez-Bonafe, P., Fedoruk, M. N., Whitmore, T. G., Akbari, M., Ralph, J. L., Ettinger, S., Gleave, M. E. and Nelson, C. C. (2004) YB-1 is upregulated during prostate cancer tumor progression and increases P-glycoprotein activity. Prostate **59**, 337–349
18 Lee, W., Mitchell, P. and Tjian, R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell (Cambridge, Mass.) **49**, 741–752
19 Izumi, H., Imamura, T., Nagatani, G., Ise, T., Murakami, T., Uramoto, H., Torigoe, T., Ishiguchi, H., Yoshida, Y., Nomoto, M. et al. (2001) Y box-binding protein-1 binds preferentially to single-stranded nucleic acids and exhibits 3′−>5′ exonuclease activity. Nucleic Acids Res. **29**, 1200–1207
20 Wisdom, R. (1999) AP-1: one switch for many signals. Exp. Cell Res. **253**, 180–185
21 Bader, A. G., Felts, K. A., Jiang, N., Chang, H. W. and Vogt, P. K. (2003) Y box-binding protein 1 induces resistance to oncogenic transformation by the phosphatidylinositol 3-kinase pathway. Proc. Natl. Acad. Sci. U.S.A. **100**, 12384–12389
22 Binder, C., Marx, D., Binder, L., Schauer, A. and Hiddemann, W. (1996) Expression of Bax in relation to Bcl-2 and other predictive parameters in breast cancer. Ann. Oncol. **7**, 129–133
23 Zeng, L., Rowland, R. G., Lele, S. M. and Kyprianou, N. (2004) Apoptosis incidence and protein expression of p53, TGF-beta receptor II, p27Kip1, and Smad4 in benign, premalignant, and malignant human prostate. Hum. Pathol. **35**, 290–297
24 Higashi, K., Inagaki, Y., Fujimori, K., Nakao, A., Kaneko, H. and Nakatsuka, I. (2003) Interferon-g interferes with transforming growth factor-b signaling through direct interaction of YB-1 with Smad3. J. Biol. Chem. **278**, 43470–43479
25 Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987) Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans acting factor. Cell (Cambridge, Mass.) **49**, 729–739
26 Bos, T. J. (1988) v-jun encodes a nuclear protein with enhancer binding properties of AP-1. Cell (Cambridge, Mass.) **52**, 705–712
27 Chinekov, Y. and Kerppola, T. K. (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. Oncogene **20**, 2438–2452
28 Motohashi, H., O'Connor, T., Katsuoka, F., Engel, J. D. and Yamamoto, M. (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. Gene **294**, 1–12
29 Hasegawa, H., Utsunomiya, Y., Kishimoto, K., Tange, Y., Yasukawa, M. and Fujita, S. (1996) SFA-2, a novel bZIP transcription factor induced by human T-cell leukemia virus type I, is highly expressed in mature lymphocytes. Biochem. Biophys. Res. Commun. **222**, 164–170
30 Bower, K. E., Zeller, R. W., Wachsman, W., Martinez, T. and McGuire, K. L. (2002) Correlation of transcriptional repression by p21(SNFT) with changes in DNA.NF-AT complex interactions. J. Biol. Chem. **277**, 34967–34977
31 Farrell, P. J., Rowe, D. T., Rooney, C. M. and Kouzarides, T. (1989) Epstein-Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. **8**, 127–132
32 Almawi, W. Y. and Melemedjian, O. K. (2002) Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. J. Leukoc. Biol. **71**, 9–15
33 Altucci, L. and Gronemeyer, H. (2001) The promise of retinoids to fight against cancer. Nat. Rev. Cancer. **1**, 181–193
34 Bode, A. M. and Dong, Z. (2000) Signal transduction pathways: targets for chemoprevention of skin cancer. Lancet Oncol. **1**, 181–188

Received 2 September 2004/31 January 2005; accepted 9 February 2005
Published as BJ Immediate Publication 9 February 2005, DOI 10.1042/BJ20041497