Combinatorial Interactions of p53, Activating Protein-2, and YB-1 with a Single Enhancer Element Regulate Gelatinase A Expression in Neoplastic Cells*

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Peter R. Mertens‡§¶, Karin Steinmann§¶, Maria A. Alfonso-Jaume|, Abdelaziz En-Niat‡, Yi Sun**, and David H. Lovett‡

From the ‡Department of Nephrology and Immunology, Medical Clinic II, RWTH Aachen, Pauwelsstraße 30, 52057 Aachen, Germany, the §Department of Medicine, San Francisco Veterans Affairs Medical Center/University of California, San Francisco, California, and **Cancer Molecular Sciences, Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, Michigan 48105

Gelatinase A, also denoted matrix metalloproteinase 2, plays multiple critical roles in the neoplastic process, including facilitation of neoangiogenesis and formation of distal metastases. The transcriptional regulation of the gelatinase A gene is under the control of strong, evolutionarily conserved cis-acting enhancer elements, designated the r2 (human) or RE-1 (rat), that harbor contiguous binding motifs for the transcription factors activating protein-2 (AP2), p53, and YB-1. Using recombinant transcription factors, complex patterns of RE-1 binding were observed by electrophoretic mobility shift assay. Increased complex formation was detected with the AP2/YB-1 and AP2/p53 combinations, while YB-1 competed with p53 for binding. The combination of AP2, p53, and YB-1 yielded novel ternary complexes, particularly when binding to single-stranded RE-1 probes. Transient transfection of hepatocellular carcinoma cell lines with a series of gelatinase A luciferase reporter constructs were in accordance with the binding patterns determined by electrophoretic mobility shift assay. Combined AP2 and p53 increased gelatinase A luciferase reporter activity significantly, and the inclusion of YB-1 yielded further increase in both reporter activity and secreted levels of gelatinase A protein. YB-1 and p53 expression are increased following multiple genotoxic stresses, including irradiation, and the synergistic interactions of these induced transcription factors with the widely expressed AP2 protein provide a probable pathophysiologic mechanism for the enhanced tumor cell synthesis of gelatinase A induced by radiation.

Considerable attention has been focused on the gelatinase A enzyme (matrix metalloproteinase-2), since histopathological studies indicate a close association of tumor or stromal cell gelatinase A expression with neoangiogenesis and distal metastasis formation (1–4). The underlying mechanism for these critical activities of gelatinase A resides in the ability of this enzyme to degrade multiple components of basement membranes, including type IV and V collagens and laminin. In some cases, the cleavage products generated by gelatinase A have intrinsic biologic activity. For example, incubation of laminin-5 with gelatinase A generates peptide products that facilitate breast epithelial cell attachment and motility (5).

Several in vivo studies have substantiated a critical role for gelatinase A in growth, angiogenesis, and extracellular matrix turnover. Gelatinase A-deficient mice show significant decreases in growth rate and also demonstrate diminished corneal and tumor-induced angiogenesis (6, 7). Recently, one form of inherited osteolysis, vanishing bone syndrome, was shown to be the result of a mutation inducing a stop codon within the coding region of the human gelatinase A gene (8). These gelatinase A-deficient individuals have severe defects in both bone and soft tissue structure associated with short stature.

In hepatic fibrosis and chronic glomerulosclerosis, high level expression of gelatinase A is spatially and temporally linked to disease activity (9–13). In vitro studies suggest that gelatinase A is not merely engaged in matrix turnover but rather has a direct influence on the cellular phenotype, with induction of an activated prosclerotic phenotype (14, 15). These prosclerotic effects of gelatinase A observed in inflammatory diseases may have their functional counterparts in the development of the desmoplastic stroma of neoplasia, in which enhanced gelatinase A synthesis has been reported (16).

Net activity of gelatinase A is regulated at four levels: (i) gene transcription (17, 18), (ii) mRNA stability (19, 20), (iii) activation of proenzyme (21–23), and (iv) inhibition/activation of enzyme through the tissue inhibitors of metalloproteinases (19, 24, 25). These highly complex regulatory mechanisms permit a high degree of control of gelatinolytic activity at discrete pericellular sites.

Studies of the signal transduction mechanisms that regulate gelatinase A expression have shown cell-specific induction of gelatinase A synthesis by the proinflammatory cytokines tumor necrosis factor-α and interleukin-1 (13). In addition, transforming growth factor-β stimulates gelatinase A synthesis at both the transcriptional and translational levels (13, 20). A similar response was observed with elevated endogenous cyclic adenosine monophosphate levels (13, 20), a pathway stimulated by prostaglandin E2 (26, 27).

Several groups have addressed the transcriptional regulation of gelatinase A within both the human and rat genomic contexts (18, 27–30). Within both genes, highly conserved enhancer elements were mapped between bp –1657 and –1619 in
The abbreviations used are: AP2, activating protein-2; EMSA, electrophoretic mobility shift assay.

The human 5′-flanking region (denoted r2) and between bp −1048 and −1008 bp in the rat (denoted RE-1). The importance of these elements is underscored by the finding that they represent evolutionarily conserved analogs that bind the same set of transcription factors (31). Among these, activating protein-2 (AP2)3 and one member of the Y-box transcription factor family, YB-1, were isolated and characterized with respect to their respective evolutionarily conserved analogs that bind the same set.

Specific YB-1 binding to this element has previously been demonstrated on estranged DNA templates (27). Although the individual binding motifs overlap, simultaneous AP2 and YB-1 binding to the RE-1 and r2 elements occurs, with evidence for direct protein-protein interaction (27, 31). In addition, a unique property of YB-1 was observed upon binding to the RE-1, involving DNA conformational changes, opening of the DNA double helix, and stabilization of single-stranded DNA templates (27).

Bian and Sun (29) demonstrated specific binding and trans-activation of the human gelatinase A enhancer element by the tumor suppressor protein p53. The p53 DNA consensus recognition sequence consists of two copies of a 10-bp motif, 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, that are separated by 0–13 bp. In the human gelatinase A enhancer element r2, a homologous sequence to this motif is present. Within the rat RE-1 sequence, the p53 recognition motif shows a perfect match with the exception of one base at the 3′-end. Alignment of the p53, AP2, and YB-1 binding motifs within the rat gelatinase A RE-1 sequence reveals that they are contiguous (Fig. 1), raising the question of whether more complex patterns of cooperative/competitive nucleoprotein binding exist. In this regard, DNA binding studies were performed with the respective recombinant proteins using both double-stranded and single-stranded RE-1 templates. The results provide evidence for complex DNA binding patterns of the three transcription factors. The results from transient transfection of hepatocellular carcinoma cell lines with a series of gelatinase A luciferase reporter constructs were in accordance with these binding patterns. Less than additive trans-activation of reporter gene expression through p53 and YB-1 was found, whereas AP2 and p53 enhanced gelatinase A gene transcription synergistically. A major increase in RE-1-dependent gene transcription and protein synthesis was observed with the combination of all three transcription factors. Both YB-1 and p53 synthesis are induced by multiple genotoxic stresses, including exposure to ultraviolet light and ionizing radiation (34–37). Several recent reports have demonstrated increased tumor cell synthesis of gelatinase A following exposure to sublethal irradiation (38–42). Increased gelatinase A activity was demonstrated in intestinal mucosa following preoperative radiotherapy for rectal carcinoma (41), whereas sublethal irradiation of human glioblastoma cells was associated with elevated levels of gelatinase A synthesis and increased invasiveness (40).

Thus, the cooperative interactions of YB-1, p53, and AP2 that regulate gelatinase A transcriptional activity described in this report may have direct clinical relevance for radiation therapy treatment protocols.

MATERIALS AND METHODS

Cells and Culture Conditions—HepG2 and Hep3B hepatoma cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s H-18 medium containing 100 μg of streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum. HEK 293, HepG2, and Hep3B cells and culture conditions were obtained from ATCC and grown in Dulbecco’s modified Eagle’s H-18 medium containing 100 μg of streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum.

Plasmids Used in This Study—A 40-bp sequence, 5′-AGGCTGCT-GGGCAAGTCGAGGTCGACACACATG-3′, with higher affinities for the single-stranded as opposed to the double-stranded element. YB-1 contact points to the sense and antisense strands are indicated by stars. All respective binding motifs are contiguous.

The plasmid pCos-53, expressing wild-type p53, and the plasmid p53-143A, expressing a transactivation-inactive mutant p53 protein, have been described previously (29).

Electrophoretic Mobility Shift Assays—Synthetic oligonucleotides were end-labeled with [γ-32P]ATP and T4 polynucleotide kinase for single-stranded binding assays. Double-stranded probes were prepared by digesting pT4-Luc RE-1 with Asp718 or BglII; the resultant overhangs in both elements were dephosphorylated with calf intestine alkaline phosphatase and end-labeled by means of T4 polynucleotide kinase using [γ-32P]ATP. Single- and double-stranded probes were purified on acrylamide gels and eluted, and 2 × 106 cpm of labeled probe was used in each binding reaction. Concentrations and combinations of transcription factors AP2, p53, and YB-1, as detailed in the figure legends, were incubated at 22 °C for 30 min in binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM NaCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), containing 300 μg/ml acetylated bovine serum albumin and 2 μg of poly(dI-dC) as carrier, in a total volume of 25 μl. The samples were electroforesed on 4% polyacrylamide, 5% glycerol gels, in a buffer containing 1× Tris borate/EDTA, followed by autoradiography.

Recombinant Proteins—Recombinant AP2 protein (Promega) employed for the DNA-binding experiments was renatured by step dialysis through urea and purified by DNA affinity chromatography. A rSET vector (In vitrogen) containing an insert coding for a hexahistidine-T7 epitope-YB-1 fusion protein was a kind gift from Dr. K. Chien (University of California, San Diego, CA). For expression in Escherichia coli, bacteria were induced with isopropyl-β-D-thiogalactoside, followed by the addition of M13/T7 helper phage encoding T7 polymerase. Expressed protein was released by sonication, and the purity of the expressed YB-1 fusion product was ascertained by analytical SDS-PAGE. The recombinant p53 wild-type protein used was produced in insect cells infected with a baculovirus vector carrying human p53 cDNA and purified through DNA affinity chromatography (29). Protein concentrations used are as indicated in the figure legends.

Transient Transfections—HepG2 and Hep3B cells were transfected using CaPO4 as described (43). For each quadruplicate transfection, 3 μg of the corresponding luciferase construct (pGL2-prom, pT4-Luc RE-1, or pT4-Luc1807) and 3 μg of pSV40-β-gal were used. Co-transfection experiments were performed by inclusion of expression plasmid pSG5-AP2, pCos-p53, or pSG5-YB-1, as indicated. Titration experiments were performed with variable amounts of expression plasmid pSG5-p53, as detailed in the figure legends. The amount of total DNA was equalized in each reaction by inclusion of pSG5 plasmid DNA. The luciferase activity was normalized to β-galactosidase activity to account for variability in transfections and variations in the efficiency of transfection, as described (18). Cells at 50–60% confluence in six-well culture plates were washed twice with 2 ml of phosphate-buffered saline, and calcium phosphate precipitates were added. Medium was removed and replaced with normal culture medium after 6 h. Cells were grown for another 42 h, washed twice with ice-cold phosphate-buffered saline, and extracted with 400 μl of Triton X-100.
buffer (1% Triton X-100, 1 mM dithiothreitol, 25 mM glycyglycine, pH 7.8, 15 mM MgSO4) on ice for 30 min, followed by measurement of luciferase and β-galactosidase activity. Luciferase assays were performed with 100 μl of the lysates as previously described (31). β-Galactosidase activity was measured using a commercial chemiluminescence assay (Promega). All transfections were performed in triplicate to quadruplicate and were repeated at least three times. Transfection results were averaged and are expressed as the mean ± S.D.

To determine the effects of endogenous p53 stimulation by etoposide, cells were transiently transfected with construct pTF4-Luc1807, pTF-Luc RE-1, or the promoterless pGL2-basic vector (Promega) and expression plasmids as detailed above. At 24 h prior to harvest, the controls were given fresh medium, and the experimental p53 induction group was given fresh medium containing 10 μM etoposide. Harvest and quantitative determination of luciferase and β-galactosidase activities were as described above.

Quantitative Zymography—Cells were transfected with the various expression plasmids as above. After 24 h, cells were washed twice with phosphate-buffered saline, and serum-free medium supplemented with 0.2% bovine serum albumin was added. The media were conditioned for 24 h and cleared by centrifugation for 3 min at 14,000 rpm. Aliquots (15 μl) were loaded on 7.5% SDS-polyacylamide gels containing 2 mg/ml gelatin and separated by electrophoresis. Processing of gels was as described (44). Experiments were performed in triplicate and repeated at least three times. The major gelatinolytic activity with approximate size of 66 kDa was quantified by laser densitometry and standardized using serial dilutions of known quantities of purified gelatinase A.

Statistical Analysis—Statistical significances were determined for paired comparisons using Student’s t test or by analysis of variance for multiple comparisons where appropriate.

RESULTS

Patterns of Transcription Factor Binding to the Gelatinase A RE-1—Prior efforts have focused on the characterization of potent enhancer elements (denoted RE-1 and r2, respectively) located in the near 5′ regions of the rat and human gelatinase A genes and have demonstrated that specific interactions of AP2 and YB-1 drive high level gelatinase A transcription (27, 30). The observations by Bian and Sun (29) concerning the specific interaction of the p53 protein with the human r2 element, we set out to determine whether more complex interactions of these three transcription factors occur and whether such interactions affect gelatinase A gene transcription and protein synthesis in defined hepatocarcinoma cell lines.

The first set of experiments used electrophoretic mobility shift assays (EMSA) to examine the binding properties of various combinations of purified transcription factors AP2, p53, and YB-1 to the RE-1. With the double-stranded RE-1 probe, individual binding of all three transcription factors, AP2, p53, and YB-1, was detected, although with significantly different affinities (Fig. 2). Recombinant YB-1 was used in a higher concentration (16 ng) than that chosen for the AP2 and p53 proteins (2 ng). The higher concentration of YB-1 used for the double-stranded binding studies is based on our prior demonstration of a relatively low binding affinity of this protein for double-stranded as opposed to single-stranded templates (30).

With the combination of AP2 and YB-1 (Fig. 2, lane 7), an additional band (labeled 7→) with lower mobility appeared, which represents AP2/YB-1 heterodimers, as previously demonstrated (27). Furthermore, AP2 facilitated YB-1 binding to the double-stranded RE-1 template, as reported (27) (Fig. 2, cf. lanes 6 and 7).

The combination of AP2 and p53 yielded two bands with the same mobilities as AP2 alone (cf. lanes 1 and 8). However, the binding affinity was increased by greater than 20-fold as determined by densitometry (cf. lanes 4 and 8). Antibody supershift studies indicated that the prominent bands in lane 8 consisted of AP2/p53 heterodimers (data not shown).

The inclusion of p53 protein in the binding reaction significantly decreased YB-1 binding to the double-stranded RE-1 template (cf. lanes 6 and 9). The combination of all three transcription factors resulted in a major change in the binding pattern (lane 10). The band with the lowest mobility observed with the AP2 and YB-1 combination (labeled 7→ in lane 7) was diminished, as were all bands corresponding to YB-1 mono- and multimers (lanes 3 and 7). In contrast, the high affinity binding pattern observed with AP2 and p53 was conserved. These changes indicate that transcription factor binding patterns to the RE-1 are complex and can be modified by the presence or absence of any one of the three proteins examined. In general, the EMSA studies indicate that p53 decreases YB-1 binding to the double-stranded template and augments AP2 binding.

DNA Binding Affinities as Determined by Protein Titration/EMSA—Protein titration experiments were performed to further characterize the cooperative AP2/p53 and competitive p53/YB-1 binding interactions. As shown in Fig. 3A, AP2 binding alone to the double-stranded RE-1 oligonucleotide was not detectable at the chosen concentration and exposure time (8 h, lane 1), whereas there was a faint band apparent with recombinant p53 alone (labeled 1→ in lane 2). The addition of p53 protein ranging from 0.5 to 4.0 ng to a fixed, 2-ng amount of AP2 yielded a major increase in total shifted RE-1 oligonucleotide consisting of three discrete shifted complexes of diminishing mobilities (2→, 3→, and 4→, respectively). Complex formation was maximal at an AP2/p53 protein ratio of 2:1 and was virtually absent at an AP2/p53 ratio of 1:2. These experiments indicate that p53 increases the binding affinity of AP2 for the RE-1 oligonucleotide in a concentration-dependent manner.

The interactions of p53 and YB-1 within the context of the
RE-1 were examined in a similar series of experiments. YB-1 multimerization was evidenced by a ladder (labeled through ) of bands of decreasing mobilities, as determined previously (30) (lane 2). The addition of increasing amounts of p53 decreased YB-1 binding affinity, as assessed by the diminished bands 1–4 in lane 6, with the concomitant appearance of a high mobility band (labeled ).

Combinatorial Interactions of AP2, p53, and YB-1 with Single-stranded RE-1 DNA Templates—We have reported that AP2 and YB-1 can bind to the single-stranded components of the RE-1 (27). There is considerable evidence that the ability of YB-1 to promote DNA strand separation is coupled to its function as a transcription factor (27, 45). To extend these observations to potential combinatorial interactions of AP2, YB-1, and p53, additional electrophoretic mobility shifts analyses were performed using the sense and antisense RE-1 strands as probes. In these experiments (Fig. 4), high affinity binding to the sense and antisense strands was only observed with recombinant YB-1 (Fig. 4, A and B, lanes 2). AP2 binding to the single-stranded components of the RE-1 was minimally detectable at the chosen exposure time (Fig. 4, A and B, lanes 3). No p53 binding to the single-stranded templates could be detected under the conditions used (Fig. 4, A and B, lanes 4).

The extent of YB-1 binding to the single-stranded templates was unaffected by the addition of either AP2 or p53 (Fig. 4, A and B, lanes 5 and 6). The combination of the AP2 and p53 proteins did not enhance the DNA complex formation seen with AP2 protein alone (cf. Fig. 4, A and B, lanes 3 and 7). In contrast, the combination of all three transcription factors dramatically changed the binding pattern with the appearance of a new low mobility band ( in lanes 8 of A and B).
reactions, where YB-1 was combined with AP2 or p53 alone.

**p53-dependent Transactivation of the Rat Gelatinase A Promoter in Hepatocellular Carcinoma Cell Lines**—Two model cell lines with differences in their AP2 and p53 background were chosen. HepG2 hepatoma cells harbor the wild type (WT) p53 but are AP2-deficient (confirmed by reverse transcriptase-PCR). Hep3B hepatoma cells are p53-negative and express AP2 (confirmed by reverse transcriptase-PCR (46)). For both cell lines, constitutive YB-1 expression was detected by Western blotting (not shown). In the first series of experiments, p53 was transiently expressed in HepG2 cells with luciferase reporter constructs harboring either the 5′ regulatory sequence of the rat gelatinase A gene up to −1807 bp relative to the transcription start site (pT4 Luc1807) or the isolated RE-1 subcloned into a reporter construct containing the SV40 early promoter (pT4 Luc RE-1). As shown in Fig. 5, A and B, transfection of the

**Fig. 5. Transient transfection study I: p53 trans-activates rat gelatinase A transcription via the RE-1.** Transient transfection studies were performed with HepG2 hepatoma cells (p53 WT/WT, AP2 −/−). A, luciferase activity of construct pT4 Luc1807 was stimulated up to 3-fold by p53 in a concentration-dependent manner. B, to confirm that WT p53 trans-activates gene transcription via binding to the RE-1, similar transient transfection experiments were performed with the RE-1 reporter construct pT4 Luc RE-1. An even more pronounced concentration-dependent stimulatory effect, up to 12.5-fold, was observed with pCOS-53. Results represent the mean ± S.D. of three independent transfections performed in quadruplicate.

**Synergistic Transactivation of the RE-1 through Transcription Factors AP2, p53, and YB-1**—Transient transfection experiments were set up in HepG2 cells with expression plasmid concentrations that elicited submaximal transactivation of the pT4 Luc RE-1 reporter construct to prevent squelching. For the p53 expression plasmid, a concentration of 0.5 μg/well was chosen, since this concentration demonstrated a submaximal response. For transcription factors AP2 and YB-1 alone, the

**Fig. 6. Transient transfection study II: WT p53-dependent trans-activation of the RE-1 in Hep3B cells.** Transient transfections were repeated as described in the legend to Fig. 5, A and B, with Hep3B cells (p53 −/−, AP2 WT/WT) and reporter construct pT4 Luc1807 (A). A concentration-dependent increase of luciferase activity (up to 17.5-fold) was induced by co-transfection with pCOS-53. In contrast, p53-143A had no effect on transcription rates. B, in similar experiments using the isolated enhancer element RE-1 in the context of a heterologous SV40 promoter, luciferase activity was stimulated by about 85-fold through co-expressed pCOS-53 but not p53-143A. Results represent the mean ± S.D. of three independent transfections performed in quadruplicate.
same concentration led to a similar 3–5-fold induction of luciferase activity (Fig. 7A). When co-transfections were performed with a combination of AP2 and YB-1 or AP2 and p53, 14- and 25-fold increases, respectively, in luciferase activity were detected. In contrast, the combination of p53 and YB-1 led to a less than additive effect in luciferase reporter activity. These results are most consistent with cooperative interactions of AP2/YB-1 and AP2/p53 in terms of RE-1-dependent gelatinase A transcription, whereas there was no evidence for cooperative interactions between YB-1 and p53. In contrast, when all three expression plasmids were combined, a major, 220-fold increase in RE-1-dependent luciferase activity was detected. The patterns exhibited in the transient transfection studies with expression plasmids correlate well with the interaction analyses detailed in the EMSA experiments. Thus, the partnering of AP2 with YB-1 or with p53 was evidenced by enhanced complex formation, and the combination of all three transcription factors yielded major increases in RE-1 binding activity (compare Figs. 2 and 4).

HepG2 transfections were repeated with the pT4 Luc1807 construct to determine whether the superinduction of gelatinase A transcription by the combined three transcription factors also occurred within the context of the intrinsic gelatinase A promoter. In these experiments, etoposide was used to stimulate endogenous p53 expression (Fig. 7B). With etoposide addition alone, reporter luciferase activity was stimulated about 4-fold in HepG2 cells, a value similar to the one observed with the isolated RE-1 and expression plasmid pCOS-53. Individual transfection of AP2 and YB-1 or with p53 was evidenced by enhanced complex formation, and the combination of all three transcription factors yielded major increases in RE-1 binding activity (compare Figs. 2 and 4). HepG2 transfections were repeated with the pT4 Luc1807 construct to test for combinatorial effects of transcription factors AP2, p53, and YB-1 on RE-1-dependent gene transcription (A). At submaximal concentrations of the respective expression plasmids (0.5 μg/well), a stimulatory effect could be detected for p53 and YB-1, whereas AP2 did not stimulate transcription. Combinations of p53 or YB-1 with AP2 led to an additional increase in reporter activity, whereas p53 and YB-1 combined led to a less than additive effect. A superinduction of gene transcription, 220-fold, was observed when all three transcription factors combined were expressed. B, the effect of stimulated endogenous WT p53 expression by etoposide treatment (10 μmol) was tested in HepG2 cells transiently transfected with AP2, YB-1, and AP2/YB-1. A similar pattern of trans-activation of reporter construct pT4 Luc1807 compared with overexpressed p53 protein (cf. A) was seen with a major, 330-fold induction of luciferase activity with combined expression of AP2/YB-1 and stimulated endogenous WT p53. *, p < 0.05; **, p < 0.005 compared with etoposide treatment alone.

FIG. 8. Gelatin zymography. Gelatin zymography was performed with conditioned media from HepG2 hepatoma cells transfected with expression plasmids for AP2 and YB-1 and treated with etoposide (10 μmol), as detailed under “Materials and Methods.” Gelatinolytic activities were determined by densitometry of the predominant 66-kDa band. Results are expressed as mean ± S.D. of three independent experiments performed as triplicates. The gelatinolytic activity of control cells was arbitrarily set as 1. YB-1 and etoposide stimulated gelatinase A synthesis about 2-fold, whereas AP2 had no effect on gelatinase synthesis at the given concentration. AP2 expression in combination with either YB-1 or etoposide induced significant increases in gelatinase A synthesis, whereas the combination of YB-1 and etoposide had a less than additive effect (3-fold induction). The combination of AP2/ YB-1 and p53 induction yielded a 5-fold increase in gelatinase A synthesis of AP2/YB-1 and AP2/p53 in terms of RE-1-dependent gelatinase A transcription, whereas there was no evidence for cooperative interactions between YB-1 and p53. In contrast, when all three expression plasmids were combined, a major, 220-fold increase in RE-1-dependent luciferase activity was detected. The patterns exhibited in the transient transfection studies with expression plasmids correlate well with the interaction analyses detailed in the EMSA experiments. Thus, the partnering of AP2 with YB-1 or with p53 was evidenced by enhanced complex formation, and the combination of all three transcription factors yielded major increases in RE-1 binding activity (compare Figs. 2 and 4).
AP2, p53, and YB-1 take place in the context of the endogenous gelatinase A promoter sequence. As expected, when Hep3B cells (p53/−/) were used for these experiments, no synergistic effect could be detected (not shown).

Gelatinase A Protein Synthesis Is Maximally Induced by the Combination of AP2, p53, and YB-1—A final series of experiments was performed to determine whether the changes in transcription rates of introduced gelatinase A luciferase reporter plasmids correspond with actual increases in gelatinase A protein synthesis. Transient transfections of HepG2 cells with the AP2 and YB-1 expression plasmids were combined with etoposide treatment as detailed above. Secreted gelatinase A enzymatic activities in serum-free conditioned media were determined using quantitative zymography. As can be seen in Fig. 8, transfection with the YB-1 expression plasmid or etoposide treatment alone led to significant increases in gelatinolytic activity, whereas AP2 alone at the chosen concentration (0.5 μg/well) was without effect. Combinations of expression plasmids and/or etoposide treatment had a stimulatory influence on gelatinase A expression. Most importantly, the combination of AP2 and YB-1 in conjunction with etoposide treatment provoked the most significant change, with a 5-fold stimulation of gelatinolytic activity. This response is less pronounced than that obtained with gelatinase A luciferase reporter constructs. However, it is likely that the measured transcription rates of multiple copies of introduced reporter plasmids are significantly higher than those derived from the intrinsic promoter within a genomic context.

**DISCUSSION**

In this paper, a previously characterized enhancer element (RE-1) within the 5′-flanking region of the rat gelatinase A gene was analyzed for potential combinatorial interactions with three binding proteins: AP2, p53, and YB-1. The importance of this element for gelatinase A transcriptional regulation has been underscored by the recent demonstration of conservation between species with similar patterns of expression on gelatinase A promoter sequence. As expected, when Hep3B cells (p53/−/) were used for these experiments, no synergistic effect could be detected (not shown).

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