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Transcriptional Activation by p53 of the Human Type IV Collagenase (Gelatinase A or Matrix Metalloproteinase 2) Promoter

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p53, a tumor suppressor and a transcription factor, has been shown to transcriptionally activate the expression of a number of important genes involved in the regulation of cell growth, DNA damage, angiogenesis, and apoptosis. In a computer search for other potential p53 target genes, we identified a perfect p53 binding site in the promoter of the human type IV collagenase (also called 72-kDa gelatinase or matrix metalloproteinase 2 [MMP-2]) gene. This p53 binding site was found to specifically bind to p53 protein in a gel shift assay. Transcription assays with luciferase reporters driven by the promoter or enhancer of the type IV collagenase gene revealed that (i) activation of the promoter activity is p53 binding site dependent in p53-positive cells but not in p53-negative cells and (ii) wild-type p53, but not p53 mutants commonly found in human cancers, transactivates luciferase expression driven by the type IV collagenase promoter as well as by a p53 site-containing enhancer element in the promoter. Significantly, expression of the endogenous type IV collagenase is also under the control of p53. Treatment of U2-OS cells, a wild-type p53-containing osteogenic sarcoma line, with a common p53 inducer, etoposide, induced p53 DNA binding and transactivation activities in a time-dependent manner. Induction of type IV collagenase expression followed the p53 activation pattern. No induction of type IV collagenase expression can be detected under the same experimental conditions in p53-negative Saos-2 cells. All these in vitro and in vivo assays strongly suggest that the type IV collagenase gene is a p53 target gene and that its expression is subject to p53 regulation. Our finding links p53 to a member of the MMP genes, a family of genes implicated in trophoblast implantation, wound healing, angiogenesis, arthritis, and tumor cell invasion. p53 may regulate these processes by upregulating expression of type IV collagenase.

p53, a 53-kDa nuclear protein, is one of the most commonly studied molecules in cancer research as well as molecular biology. Structurally, p53 consists of three distinct domains: a transactivation domain at the N terminus, a specific DNA binding domain at the center part, and an oligomerization domain at the C terminus of the molecule. Mutations at the specific DNA binding domain were identified in nearly 50% of all human cancers worldwide (15). Biochemically, p53 is a transcription factor which recognizes a specific consensus DNA sequence consisting of two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0 to 13 bp (10), and p53 efficiently binds to this sequence and transactivates expression of the target genes. Several biologically significant genes were found to contain this consensus sequence and to be subject to p53 regulation. Among the commonly studied genes in this group are those for Waf1/Cip1/p21 (11), Mdm2 (1), Gadd45 (22), Bax (30), proliferating cell nuclear antigen (32, 39), cyclin G (36), epidermal growth factor receptor (29), and thrombospondin (7). p53 also functions as transcription repressor to inhibit the in vivo transcription of several cellular and viral promoters which lack p53 binding sites (see reference 9 and references therein) as well as endogenous expression of genes encoding microtubule-associated protein (34) and DNA topoisomerase IIα (51). p53 may also be involved in DNA replication by inhibiting helicase activity and by preventing the complex formation between proliferating cell nuclear antigen and DNA polymerase δ through upregulation of p21 protein (see reference 38 and references therein). In addition, p53 can stimulate annealing of single-stranded DNA (see reference 38 and references therein) and act as an exonuclease (33). Biologically, p53 induces G_1 arrest and apoptosis following DNA damage, either to halt cell division until the damage is fully repaired or to eliminate the cells whose DNA is irreparably damaged (see reference 21 and references therein). As a common tumor suppressor, p53 suppresses tumor cell growth both in vitro and in vivo (see reference 24 and references therein). p53 also functions as a guardian to the genome to preserve genetic stability (see reference 26 and references therein). In addition, p53 may play a role in differentiation, senescence, and angiogenesis (5, 16, 24, 50).

The best-characterized p53 biochemical activity remains its activity as a transcription factor. Many p53 functions are mediated through the activation or repression of a series of genes whose expression is subjected to p53 regulation. It is therefore important to identify and characterize the p53-regulatory genes to achieve a better understanding of the functions and mechanism of action of p53. We have previously cloned the gene for mouse tissue inhibitor of metalloproteinase 3 (TIMP-3) (46, 47), a member of the family of genes mainly involved in tissue remodeling and metastasis prevention by inhibiting matrix metalloproteinase (MMP) activity (28, 42). Molecular cloning and characterization of the mouse TIMP-3 promoter reveal a putative p53 binding site, 5′-**GGGCTTGCTT** GACGTCCA **GAA CAGGGTC**-3′, at position −648 to −620 relative to the transcriptional initiation site. This site contains two copies of the p53 binding motif (in boldface) separated by 8 bp, with two mismatches (underlined) at the second motif (47). Characterization of this putative p53 binding site revealed that binding of p53 protein is largely prevented by the 8-bp spacer sequence and that the TIMP-3 gene is not a p53-regulatory gene (2). In a search for other potential p53 target genes, we identified a

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perfect p53 binding site in the promoter of the gene encoding human type IV collagenase (also called gelatinase A or MMP-2, a naturally occurring enzyme subject to TIMP inhibition), among other known p53 target genes. In this study we cloned and characterized the type IV collagenase gene promoter and found that it was indeed subject to p53 regulation both in vitro and in vivo. The results reported here provide the first linkage between a powerful tumor suppressor, p53, and a member of the MMPs, which have been implicated in many physiological as well as pathological processes. The linkage will extend our understanding of p53 functions and may lay groundwork for future studies of how p53 differentially regulates these processes under physiological as well as pathological conditions.

MATERIALS AND METHODS

Cell culture and drug treatment. Human HT1080 (a fibrosarcoma line), 293 (a transformed kidney line), and U2-OS and Saos-2 (osteogenic sarcoma lines) cells were grown in 10% Eagle minimal essential medium (for 293 cells), 10% Dulbecco modified Eagle medium (for HT1080 and Saos-2 cells), or 10% McCory medium (for U2-OS cells). The endogenous p53 statuses in these lines, as reported previously, are wild type for HT1080 (25a), 293 (6a), and U2-OS (22) and p53 null for Saos-2 (45). The doubling times are about 24 h for HT1080, 293, and U2-OS and 40 h for Saos-2 under these culture conditions. For drug treatment, U2-OS and Saos-2 cells were exposed to etoposide (10 μM) (Sigma) for various periods of time up to 48 h.

Promoter cloning and DNA sequencing. A 1,716-bp DNA fragment (bp −1659 to +57) upstream from the transcription initiation site of the type IV collagenase gene (27) was PCR amplified with Expand High Fidelity DNA polymerase (Boehringer Mannheim). Human placenta DNA (Oncor) was used as the template. The primers used were T4.01 (5′-CACACCCACCAGACAAGCCT) and T4.02 (5′-AAGCCCCAGATGCGCAGCCT). The p53 binding site is located at nucleotides (nt) −1649 to −1630. The fragment was gel purified and subcloned into TA vector (Invitrogen). Both strands were sequenced with the DNA Sequenase kit (Amersham) as well as with an automatic DNA sequencer (Prism 377 DNA sequencer; ABI). Potential transcription factor binding sites were searched for in GenBank by using a Genetics Computer Group program.

Gel retardation assay. The assay was performed as described previously (2, 44). Briefly, a 42-bp synthetic oligonucleotide (r2) containing the putative p53 binding site in the type IV collagenase promoter and its complementary strand were annealed and labelled with ^{32}P by using T4 polynucleotide kinase and [γ-^{32}P]ATP. DNA binding reaction mixtures of 20 μl contained 20 mM Tris-HCl (pH 7.5), 4% Ficoll-400, 2 mM EDTA, 0.5 mM dithiothreitol, 0.2 μg of poly(dI-dC), ^{32}P-labelled oligonucleotide (20,000 cpm), and 1 μg of partially purified recombinant p53 or nuclear extract from U2-OS cells after etoposide treatment (2, 44). The recombinant p53 protein used was produced in insect cells infected with a baculovirus vector carrying human p53 cDNA and partially purified through DNA affinity chromatography. In certain cases, 0.2 μg of anti-p53 antibody pAb421 and 50-fold excesses of unlabelled corresponding oligonucleotides were also included. As a positive control, a 20-bp oligonucleotide (PC) (5′-AGACATGCCTAGACATGCCT-3′), consisting of the consensus p53 binding site, was also used in the assay. The mixture was incubated at room temperature for 45 min and then loaded onto a 3.5% polyacrylamide gel. The gel was run in 0.5× Tris-borate-EDTA buffer at 60 V, dried, and exposed to Kodak film.

Luciferase reporter construction. The following luciferase reporter constructs driven by the type IV collagenase promoter or enhancer sequence were made.

(i) T4 with p53 site. The entire 1,716-bp DNA fragment subcloned in the TA cloning vector was digested with restriction enzymes *Kpn*I and *Xho*I and ligated into a predigested pGL2-Basic luciferase reporter (Promega).

(ii) T4 without p53 site. The T4 collagenase promoter without the p53 binding site (nt −1629 to +57) was generated by PCR amplification with the primers T4.03 (5′-GAAGCCCACTGAGACCCAAG) and T4.02. The resulting fragment was subcloned first in the TA vector and then in pGL2-Basic as described above.

(iii) pGL-Pro-3Xr2F and pGL-Pro-3Xr2R. A previously identified 42-bp enhancer sequence (r2) (12), 5′-GATCCACACCCACCAGACAAGCCTGAACTTGTCTGAAGCCCG (the perfect p53 binding site is underlined), and its complementary strand were synthesized (Bethesda Research Laboratories), annealed, and blunt-end ligated into a pGL-promoter luciferase reporter (Promega) at the *Sma*I site. Several resulting clones were sequenced, and two clones containing three repeats of the r2 region in opposite orientations were selected and designated pGL-Pro-3Xr2F (forward) and pGL-Pro-3Xr2R (reverse), relative to luciferase reporter sequence.

DNA transfection and luciferase assay. Single suspended cells were seeded into 24-well plates at a cell concentration of 10^5 per well (or 10^6 for 293 cells) 16 to 24 h prior to transfection. The calcium phosphate method was used to transiently transfect HT1080, 293, and Saos-2 cells, as described previously (44), whereas the Lipofectamine method (Bethesda Research Laboratories) was used for U2-OS transfection according to the manufacturer's instruction. The luciferase reporters described above, along with the control plasmids, were cotransfected with a β-galactosidase construct in the presence or absence of constructs expressing wild-type or mutant p53 proteins. The ratio of the amounts of DNAs for the p53-expressing vector versus the luciferase reporter was 1:1 or 1:2. The transient transfection and luciferase assays were performed as detailed previously (44). The results are presented as the fold activation of the empty reporter after normalization with β-galactosidase activity.

Northern analysis. U2-OS cells, an osteogenic sarcoma line harboring endogenous wild-type p53 (22), and Saos-2 cells, a p53-negative osteogenic sarcoma line (45), were treated with etoposide (10 μM), a known p53 inducer, for various periods of time up to 48 h. Total RNA was isolated by using RNAzol solution (Tel-Test), and 15 μg of total RNA was subjected to Northern analysis as described previously (48). The probe used was a 300-bp human type IV collagenase cDNA obtained from the American Type Culture Collection. The human housekeeping gene product glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control for densitometric quantitation.

Nucleotide sequence accession number. The promoter sequence of the human type IV collagenase gene has been submitted to GenBank with the accession number U96098.

RESULTS

Identification of a perfect p53 binding site in the promoter of the type IV collagenase gene and molecular cloning and sequencing of the promoter. In an attempt to identify a potential p53 target gene(s), we searched GenBank with a p53 binding consensus motif, consisting of two copies of 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, and identified a perfect p53 binding site, AGACAAGCCTGAACTTGTCT (with no spacer between the two motifs), in the promoter of the type IV collagenase gene, among several other known p53 target genes (data not shown). A sequence of 42 nt containing this site was previously reported to be an AP-2 binding enhancer region (r2) and to be localized at nt −1635 relative to the transcription initiation start in the human type IV collagenase promoter (12). Surprisingly, we found through the computer search that only very limited 5′ flanking sequence of the human type IV collagenase gene has been reported (13, 19, 27). We therefore went on to clone by PCR (with Expand High Fidelity DNA polymerase; Boehringer Mannheim) a 1,716-bp DNA fragment upstream of the transcription initiation site of human type IV collagenase. The DNA sequence of the 1,716-bp fragment is shown in Fig. 1. The sequence has been confirmed by sequencing a mixture of six individual clones. The p53 binding site is localized at nt −1649 to −1630. Some of the many other common transcription factor binding sites are also shown in Fig. 1, including those for Ets-1, CREB, AP-1, AP-2, PEA3, c-myc, and GCN4. Computer comparison of the type IV collagenase promoters in human and rat (17) revealed 76% identity in a 240-bp region (nt +42 to −198). No significant homology can be found in the other regions. Importantly, however, the promoter of the rat type IV collagenase gene also contains a p53 binding site (5′-GGGCAAGTCTGAACTTGTCa) with one mismatch to the consensus sequence (lowercase letter) (17). The results indicate that the p53 binding site in the promoter of type IV collagenase is conserved between species, which implies its functional importance in regulation of type IV collagenase expression.

Enhancer region 2 in the type IV collagenase promoter specifically binds p53 protein. Since this 20-bp perfect p53 binding site was localized within the enhancer region 2 (r2) (a 42-bp oligonucleotide) in the promoter of the type IV collagenase gene and was previously characterized as an AP-2 binding site (12), we examined potential binding of the r2 oligonucleotide with purified AP-2 (Promega) and partially purified p53 (2). Under our experimental conditions, we did not see any significant binding of full-length AP-2 protein with the r2 oligonucleotide (data not shown). In contrast, p53 protein binds to r2 significantly and specifically. As shown in Fig. 2, p53 binds to the 20-bp p53 consensus sequence (PC) used as a positive

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-1659  cacacccacc agacaagcct gaacttgtct gaagcccact gagacccaag
                     p53       p53
-1609  ccgcagagac ttttctagct gtgatgatca agacataatc agtgacctcc

-1559  aatgcccccc acaagtatat tgttcctgat tctttcagcc cctgacctta

-1509  cttctcaaac tgttccctgc tgacccccag tcctatctgc ccccttccta

-1459  ggctggtcct tactgacccc tccagctcca tcccctcacc ctgtgcccca

-1409  ccttttt cag atagaaaaaa ctttcttctc cagtgcctct tgctgttttt

-1359  catctctggg ccattgtcaa tgttccctaa aacattcccc atattcccca

-1309  cccagcactc tacctcttta gctcttcagg tctcagctca gaagtcactt
                                                  AP-1/rev
-1259  cttccaggaa gccttccttg attgtcttta ctagtttagg ggctgaagtc
         Ets-1
-1209  aggcgttccc aacagcctgc tggagttccc catcacagct tatctctcaa
                                                        c-myc
-1159  ctgtctttcc tgagagaggg agaagacatt cctcagagac ggttgtcaca
       /rev
-1109  gggagaactt caaaattggg attcgacctg agaggccaca tggattcttg

-1059  gcttggcgca ggaaaggatt caagagtgag tggggaattc gtggaactga
              PEA3
-1009  gggctcctcc ccttttaga ccatataggg taaacctccc cacattgcca

 -959  tggcatttat aaactgccat ggcactggtg ggtgcttcct ttaacatgct

 -909  aatgcattat aattagcgta aaatgagcag tgaggatgac cagaggtcgc

 -859  tttctttgcc atcttggttt tggctggctt cttcactgca tactgtttta

 -809  tcagtggggt ctttgtgacc tctatcgtat taaaccagtc ttgcccaatt

 -759  tctatctcat cctgtgaccg agaatgcgga ccctcctggg agtgcagccc

 -709  agcaggtctc agcctcattt tacccagccc cctgttcaag atggagtcgc

 -659  tctggttcca acgtctctaa cgcggggccc ctgactgctc tatttcccaa

 -609  ggtgtatcta gcatctcgca ctatacgagg ccaagttaag gcttacacat

 -559  ttgcagaagg aaagaggtaa ggaagcaacc tgggaccttc cactgtctct
             PEA3          PEA3
 -509  gtttccatct ctctctttcc atctctgttc atcccagaat ctctctgtcc

 -459  ctatccctaa atatcgaaaa tttctgtctc tgaccatcta tcattgtggc

 -409  tgatcatctg tttctgacca ttccttcccg ttcctgaccc cagggagtgc

 -359  agggtgtcct agccaagccg gcgtccctcc tagtagtacc gctgctctct

 -309  aacctcagga cgtcaagggc ctagagcgac agatgtttcc cagcaggggg
              CREB
 -259  ttctgaggct gtgcgcccag atcgcgagag aggcaagtgg ggtgacgagg
                                                   GCN4-His
 -209  tcgtgcactg agggtggacg tagaggccag gagtagcagg cggccgggga

 -159  aaagaggtgg agaaaggaaa aaagaggaga aaagtggagg agggcgagta
                    PEA3
 -109  gggggggtgg gcagagaggg gcgggcccga gtgcgccccc cgcccccagc
                                              AP-2      AP-2
  -59  cccgctctgc cagctccctc ccagcccagc cggctacatc tggcggctgc
                                                      AP-2
   -9  cctcccttgt ttccgctgca tccagacttc ctcaggcggt ggctggaggc
          +1
   42  tgcgcatctg gggctt
                    +57
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FIG. 1. Molecular cloning and sequencing of the human type IV collagenase (gelatinase A or MMP-2) promoter. Human placenta DNA was used as a template for PCR amplification with Expand High Fidelity DNA polymerase and an upstream primer in the r2 region (12) and a downstream primer at nt 38 to 57 relative to the transcription initiation site (27). The resulting 1,716-bp fragment was subcloned and sequenced as detailed in Materials and Methods. The sequence has been confirmed by sequencing a mixture of six individual clones with an automatic DNA sequencer. The underlined consensus sequences include a p53 site and site for Ets-1, CREB, AP-1, AP-2, PEA3, c-myc, and GCN4; there are many other transcription factor binding sites.

control as well as to the r2 oligonucleotide (lanes 1 and 4, respectively). In the presence of the p53 antibody pAb421 (a monoclonal antibody raised against the C terminus of the p53 molecule [amino acids 370 to 378] which enhances and stabilizes p53 DNA binding [2]), the binding was enhanced and supershifted (Fig. 2, lanes 2 and 5). The binding was specific, since it can be completely blocked with a 50-fold excess of cold PC or r2 oligonucleotide (Fig. 2, lanes 3 and 6, respectively). Cross-blockage of the p53 binding can also be observed. As shown in Fig. 2, lane 8, the binding of p53 to ^{32}P-labelled r2 can be blocked by cold PC; that to hot PC can be blocked by cold r2 (not shown). However, the binding of p53 to r2 cannot be blocked by a cold oligonucleotide containing a consensus AP-2 binding sequence (5′-ACTGACCGCCCGCGGCCCGT) (Fig. 2, lane 7). It is noteworthy that (i) partially purified p53 expressed in baculovirus often showed two supershifted bands in the presence of pAb421 (2) and (ii) there is some nonspecific binding of the 42-bp r2 oligonucleotide, but not of the 20-bp PC oligonucleotide, with baculovirus proteins copurified with p53 (compare lanes 1 to 3 with lanes 4 to 8 in Fig. 2). This nonspecific binding cannot be blocked by the cold oligonucleotides (Fig. 2, lanes 6 and 8). The results clearly demonstrated that the r2 enhancer region contains a p53 binding site and that this site specifically binds to p53 protein. We therefore focused our study on the characterization of this p53 binding site as the basis for potential p53 regulation of the type IV collagenase gene.

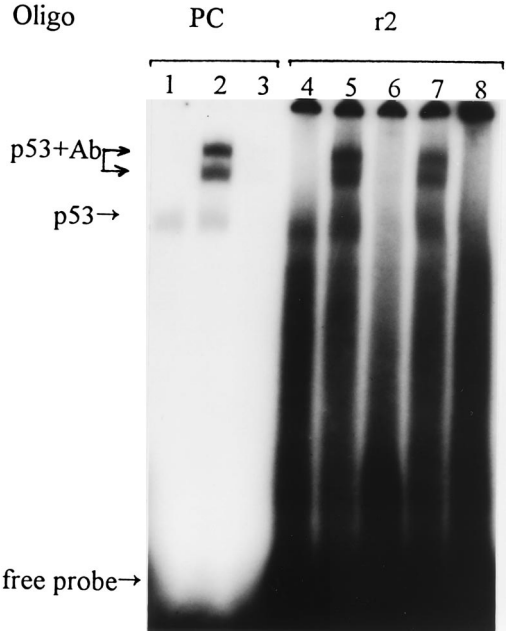

FIG. 2. Enhancer region 2 (r2) specifically binds to wild-type p53. Synthetic oligonucleotides (Oligo) of the p53 binding consensus sequence (PC) (5′-AG ACATGCCTAGACATGCCT-3′) and a 42-bp enhancer sequence (r2) (5′-GA TCCACACCCACCAGACAAGCCTGAACTTGTCTGAAGCCCG) were annealed and labelled with ^{32}P by using T4 polynucleotide kinase and [γ-^{32}P]ATP. The gel retardation assay was performed as detailed in Materials and Methods. Lanes 1 and 4, p53 plus ^{32}P-labelled oligonucleotides; lanes 2 and 5, same as lanes 1 and 4 but the p53 antibody pAb421 was included; lanes 3 and 6, a 50-fold excess of cold PC or r2 oligonucleotide, respectively, was included; lanes 7 and 8, cold AP-2 consensus oligonucleotide (5′-ACTGACCGCCCGCGGCCCGT) or cold PC oligonucleotide, respectively, was included. Lanes 3 and 6 to 8 contained the p53 antibody pAb421. The p53 (lower) and p53 antibody-supershifted (upper) bands are indicated by the arrows. The band on the bottom of gel is free labelled probe. Some nonspecific binding of the r2 with baculovirus proteins copurified with p53 can be seen in lanes 4 to 8.

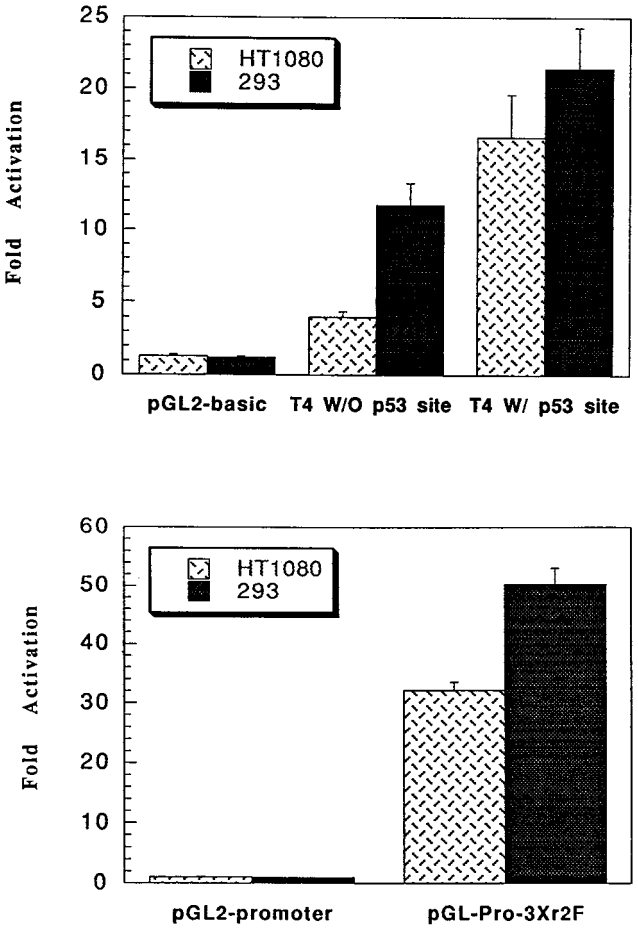

FIG. 3. p53 binding site-dependent activation of the human type IV collagenase promoter as well as enhancer. Two type IV collagenase promoter sequences with (T4 W/p53) or without (T4 W/O p53) p53 binding sites were constructed at the 5′ end of the luciferase reporter (pGL2-Basic) (top), and three repeats of the 42-bp enhancer sequence were subcloned into a promoter luciferase reporter (pGL2-Promoter) (bottom). They were transiently transfected into HT1080 and 293 cells (both harboring wild-type p53), respectively, and luciferase activity was assayed as described in Materials and Methods. The means ± standard errors of the means were derived from three independent transfections and assays, each run in duplicate, after normalization with β-galactosidase activity for transfection efficiency. The luciferase activity from the vector control was arbitrarily set as 1 for the calculation of fold activation. The ranges of the relative light units for HT1080 cells are 200 (vector control) to 3,600 (T4 W/p53) in the top panel and 1,200 to 38,000 in the bottom panel. For 293 cells, the ranges are 12,000 to 290,000 in the top panel and 220,000 (without dilution) to 1,400,000 (after 10-fold dilution) in the bottom panel, due to a higher transfection efficiency in 293 cells.

p53 binding site-dependent activation of the human type IV collagenase promoter as well as enhancer. We first examined the promoter activity of the cloned 1,716-bp 5′ flanking sequence of the type IV collagenase gene. Luciferase reporters (pGL2-Basic; Promega) driven by the promoter with or without the p53 binding site were constructed and transiently cotransfected (with a β-galactosidase-expressing plasmid) into two human cell lines, HT1080 and 293, both harboring wild-type p53 (6a, 25a), and luciferase activity was measured as detailed previously (44). Transfection efficiency was normalized with β-galactosidase activity. As shown in Fig. 3 (top), the 1,716-bp 5′ flanking sequence is a functional promoter. It activates luciferase expression up to 20-fold in both lines, compared to the promoterless vector control. The importance of the p53 binding site was revealed by the luciferase expression driven by the promoter without the p53 binding site. Without a p53 binding site, the promoter activity was decreased up to two- to fourfold (Fig. 3, top).

Since the p53 site-containing r2 region has been previously characterized as an enhancer sequence (12), we have examined the enhancer activity by placing three repeats of the r2 DNA fragment (3Xr2) into pGL2-promoter luciferase constructs (Promega), followed by transient transfection and luciferase assay. As shown in Fig. 3 (bottom), the enhancer activity was revealed by a 30- and 50-fold activation of luciferase activity in HT1080 and 293 cells, respectively. The results indicate that the p53-containing r2 region can increase the promoter activity as well as function as an enhancer.

Promoter as well as enhancer activities of the p53-specific binding site are dependent on p53. We next examined whether the type IV collagenase promoter is regulated in a p53-dependent manner. A human osteogenic sarcoma line, Saos-2 was chosen as the recipient since this line contains no endogenous p53 due to p53 gene deletion (45). Luciferase activities of the reporters driven by the T4 collagenase promoter with or without the p53 binding site were not significantly different in the absence of p53. The fold activation was 9 and 10.9, respectively, compared to the vector control (Fig. 4, top). In the presence of the p53 (by cotransfection of a p53-expressing plasmid), however, a threefold increase of luciferase activity was observed in the construct containing the p53 binding site but not in the construct with the p53 site deleted. Interestingly, cotransfection of the p53-expressing plasmid decreased promoter activity of the construct lacking the p53 site by 2.5-fold, suggesting a negative effect of p53 on the promoter sequence downstream of the p53 binding site. The experiment demonstrated a p53-dependent transactivation of the type IV collagenase promoter. This p53 dependence was further confirmed in a transient transfection and luciferase assay when the three repeats of the r2 region were cloned into a pGL2-promoter construct, functioning as an enhancer element. In the absence of p53, no increased luciferase activity can be detected in cells transfected with pGL-promoter-3Xr2 as compared to those transfected with pGL-promoter vector (Fig. 4, bottom). This is in contrast to the observation made for HT1080 and 293 cells (both having endogenous wild-type p53), where 30- to 50-fold activation of luciferase activity was achieved (Fig. 3, bottom), further confirming the lack of p53 in Saos-2 cells. However, in the presence of p53 obtained by cotransfection of a p53-expressing plasmid, there was a more-than-300-fold induction of luciferase activity (Fig. 4, bottom, pGL-Pro-3Xr2F). The enhancer activity of the r2 region works in an orientation-independent manner. When 3Xr2 was placed in the reverse orientation, to give pGL-Pro-3Xr2R (there is also a perfect p53 binding site in this orientation), a more-than-600-fold induction was observed after normalization with β-galactosidase for transfection efficiency (Fig. 4, bottom). The results indicated that the promoter as well as enhancer activity of a p53 site-containing fragment is dependent upon the presence of p53. p53 functions as a transactivator as well as a "trans-enhancer" to drive the transcription by direct binding to its specific consensus site (Fig. 2) in the promoter of the type IV collagenase gene.

Tumor-derived p53 mutants fail to transactivate luciferase reporters driven by the type IV collagenase enhancer and promoter. Mutations in the p53 gene usually abolish p53 sequence-specific DNA binding and transactivation. We next examined whether p53 mutants have lost activity in activating the luciferase reporter driven by the r2 region (containing a p53 binding site), which has been shown to be a p53-dependent enhancer element (Fig. 4, bottom). The mutants used are p53-

143A, p53-175H, p53-248W, p53-273H, and p53-281G, which contain the most common p53 mutations found in human cancers (18) and have lost p53 consensus binding and transactivation activities (36a, 58, 59). The constructs used here are pGL2-promoter driven by three repeats of the r2 sequence in a forward orientation, functioning as an enhancer. p53-negative human Saos-2 cells were used as recipients. As shown in Fig. 5 (top) only wild-type p53 induces significant activation (358-fold). All five tumor-derived p53 mutants gave rise to a maximum activation of twofold.

We have further examined potential transactivation of the type IV collagenase promoter by p53 mutants, again using Saos-2 cells as recipients. As shown in Fig. 5 (bottom), compared to the vector control, all p53 mutants except 281G fail to transactivate luciferase expression driven by the type IV collagenase promoter. p53 mutant 281G showed a less-than-two-fold-higher activity than the vector control. Similar results were observed with p53 mutants when the luciferase reporter driven by the promoter without the p53 site was used (data not shown). To exclude the possibility that lack of transactivation activity by p53 mutants was due to a lack of protein expression, we performed Western analysis and found that the expression

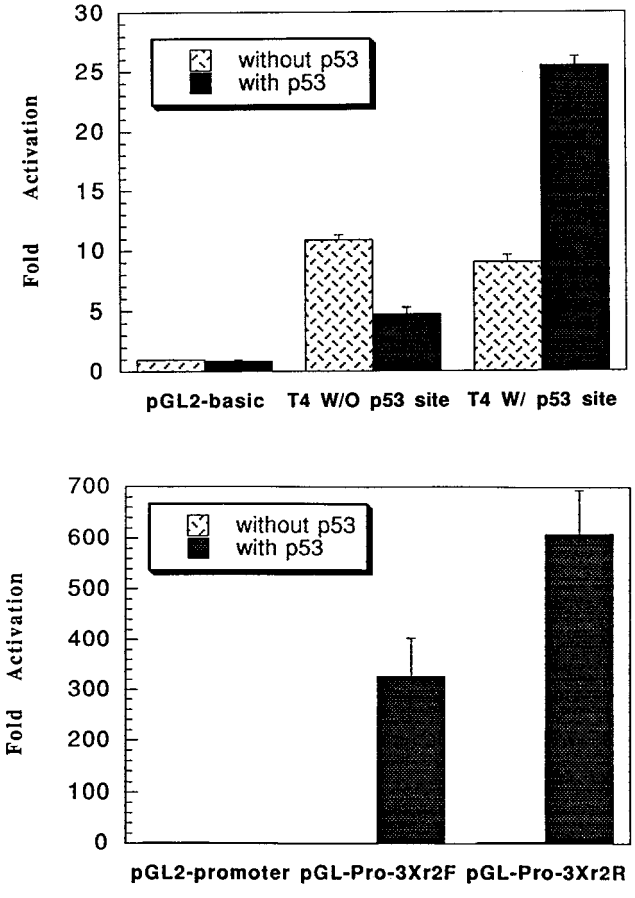

FIG. 4. Activation of the human type IV collagenase promoter as well as enhancer is dependent on wild-type p53. The constructs for measurement of promoter (top) as well as enhancer activity (bottom) were prepared as described in the legend to Fig. 3 and transiently transfected into human Saos-2 cells containing no endogenous p53. The results are expressed as means ± standard errors of the means derived from three independent transfections and assays, each run in duplicate, after normalization with β-galactosidase activity for transfection efficiency. The activities of the vector controls in the absence of p53 were set arbitrarily as 1 to calculate the fold activation. The ranges of relative light units are 200 to 5,200 in the top panel and 2,000 to 1,200,000 in the bottom panel.

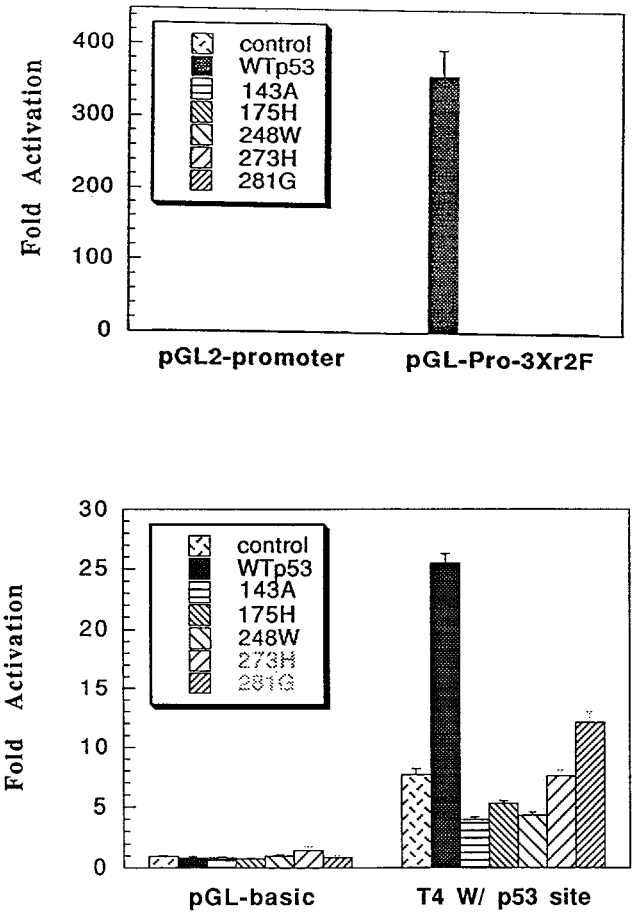

FIG. 5. Tumor-derived p53 mutants do not transactivate the type IV collagenase enhancer and promoter. The type IV collagenase enhancer/luciferase reporter (top) and the type IV collagenase promoter/luciferase reporter (bottom) were constructed and cotransfected into Saos-2 cells with constructs encoding tumor-derived p53 mutants, and luciferase activity was assayed as detailed in Materials and Methods. Three independent transfections (six transfections for the vector control of the T4 W/p53 site construct), each run in duplicate, were performed, and the results were expressed as the means ± standard errors of mean after normalization with β-galactosidase activity for transfection efficiency. Fold activation was calculated by arbitrarily setting the activities of the vector controls in the absence of p53 as 1. Relative light unit ranges for wild-type p53 were similar to those given in the legend to Fig. 4.

levels of the p53 mutant proteins were comparable to that of the wild-type p53 (within a range of ±15%) (data not shown). The results demonstrated that the type IV collagenase enhancer as well as the promoter is regulated by wild-type p53 but not by its mutants. If a p53 mutant (e.g., p53-281G) does have some transactivation activity, it should be mediated through binding to a sequence element(s) other than the p53 binding site in the type IV collagenase promoter (Fig. 5, top).

Induction of endogenous type IV collagenase is dependent on wild-type p53. To assess the in vivo regulation of type IV collagenase expression by p53, we examined type IV collagenase mRNA levels following exposure of intact cells to known p53 inducers. We reasoned that if type IV collagenase is a true p53 target gene, expression of the gene should increase following p53 induction in cells containing wild-type p53 but not in p53-negative cells. U2-OS, a human osteogenic sarcoma line harboring endogenous wild-type p53, was used. Several DNA-damaging reagents and cell cycle blockers were tested by a gel shift assay for their ability to induce p53 (data not shown), and

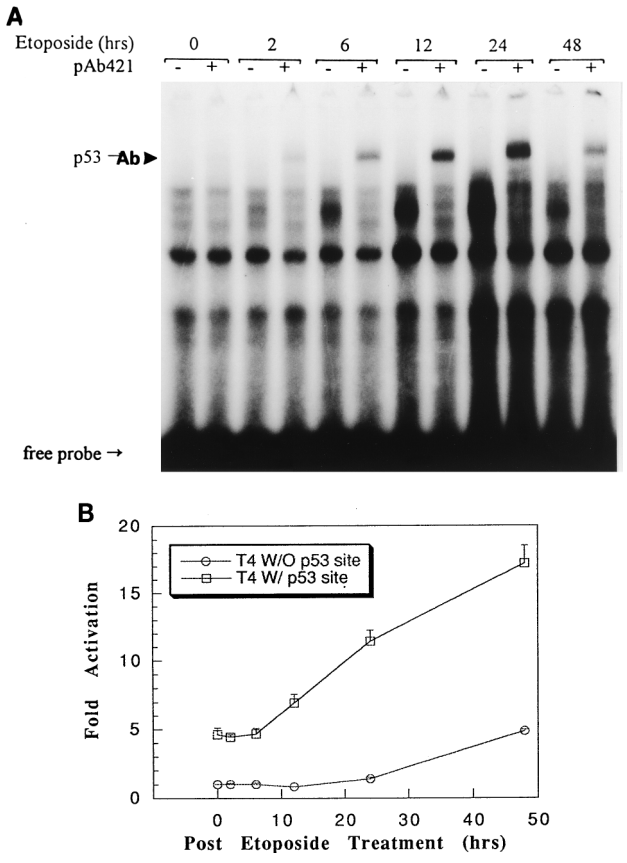

FIG. 6. (A) Induction of p53 DNA binding activity by etoposide. Subconfluent U2-OS cells were subjected to etoposide (10 μM) treatment for various times up to 48 h as indicated. The nuclear extract was prepared and subjected to a gel shift assay with the PC oligonucleotide as the probe (see the legend to Fig. 2) in the absence or presence of p53 antibody pAb421, as detailed in Materials and Methods. The bands corresponding to the p53-pAb421 complex and free probe are indicated. (B) Induction of p53 transactivation activity by etoposide. Subconfluent U2-OS cells were transfected with a luciferase reporter with or without the p53 site, along with a β-galactosidase-expressing construct, by the Lipofectamine method. Cells were treated with etoposide (10 μM) at 24 h posttransfection for 0, 2, 6, 12, 24, or 48 h and subjected to a luciferase assay as detailed in Materials and Methods. Three independent transfections and luciferase assays, each run in duplicate, were performed, and the results are expressed as the means ± standard errors of means after normalization with β-galactosidase activity for transfection efficiency. To calculate the fold activation, the luciferase activity from the construct without the p53 site without etoposide treatment was arbitrarily set as 1. The range of relative light units is from 2,500 to 50,000.

etoposide, a topoisomerase II inhibitor and a known p53 inducer in several cell models (49, 52), was chosen for its remarkable induction of p53 DNA binding activity in U2-OS cells. We first examined the time course of p53 induction following etoposide treatment by a gel shift assay. As shown in Fig. 6A, DNA binding activity of p53 from a nuclear extract of treated cells started to increase after 2 h of exposure to etoposide and reached peak at 24 to 48 h. It is noteworthy that p53 protein from the nuclear extract showed one supershifted band in the presence of pAb421 and that again there was some nonspecific binding.

We next examined whether etoposide-induced p53 DNA binding can be translated into increased transactivation and whether the p53 binding site found in the type IV collagenase promoter functions in the context of the native promoter. U2-OS cells were transiently transfected with luciferase reporters driven by either the entire promoter or the promoter with the p53 binding site deleted, followed by etoposide (10 μM) treatment for 0, 2, 6, 12, 24, and 48 h. The transfection efficiency was normalized with β-galactosidase activity. As shown in Fig. 6B, a fivefold, p53-site-dependent activation was seen (compare fold activations of the two constructs at time zero) in U2-OS cells. This was consistent with the observation made for HT1080 and 293 cells. Like for the time course of etoposide-activated p53 DNA binding (Fig. 6A), transactivation as reflected by luciferase activity started to increase at 12 h posttreatment and reached a peak at 48 h in the wild-type-promoter-driven construct. In the construct lacking the p53 binding site, no induction of luciferase activity was seen up to 24 h. Induction by severalfold was observed at 48 h, which appeared to be independent of p53 (Fig. 6B). The results indicated that (i) etoposide-induced DNA binding did translate into increased transactivation and (ii) the p53 binding element is functional in the context of the native promoter.

Having established p53 activation (both DNA binding and transactivation) by etoposide in U2-OS cells, we finally measured endogenous expression of type IV collagenase after etoposide treatment by Northern analysis. As shown in Fig. 7, induction of expression followed the p53 induction time course. Expression started to increase at 12 h posttreatment and reached peak at 24 to 48 h. The fold induction of type IV collagenase mRNA was densitometrically quantitated by using GAPDH as a loading control, and results after normalization are presented in Fig. 7. In general, two- to threefold induction was observed after 24 h of exposure to etoposide. Importantly, when p53-negative Saos-2 cells were exposed to etoposide under the same experimental conditions, no induction of type IV collagenase expression could be detected up to 48 h after normalization with GAPDH for equal loading (Fig. 8). These experiments demonstrated that endogenous expression of type IV collagenase is also subject to p53 upregulation. Thus, the type IV collagenase gene is likely a p53 downstream effector gene in vivo.

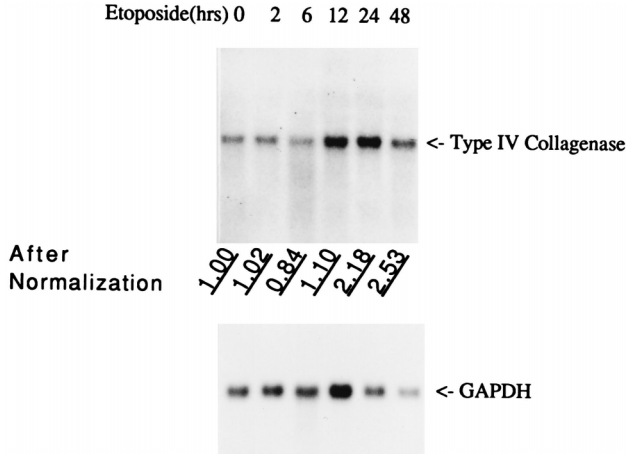

FIG. 7. Induction of endogenous type IV collagenase expression by etoposide in p53-positive cells. Subconfluent U2-OS cells were treated with etoposide (10 μM) for various times up to 48 h, followed by total RNA isolation and Northern analysis (with 15 μg), using a 300-bp human type IV collagenase cDNA as a probe. The housekeeping gene product GAPDH was used as a loading control. The level of induction of type IV collagenase expression was densitometrically quantitated and fold induction after normalization was expressed by arbitrarily setting the value for control cells as 1, as indicated.

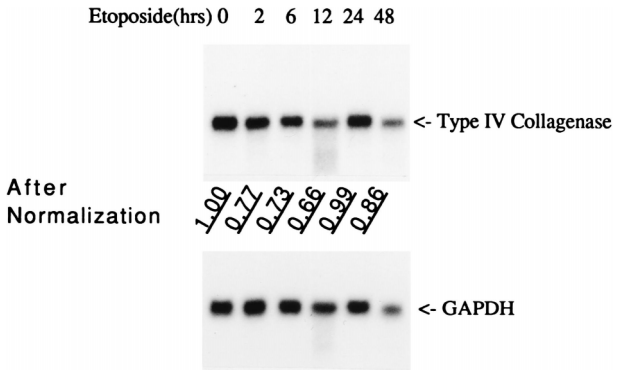

FIG. 8. Lack of induction of type IV collagenase expression by etoposide in p53-negative cells. Subconfluent p53-negative Saos-2 cells were treated with etoposide (10 μM) for various times up to 48 h and subjected to total RNA isolation and Northern analysis (with 15 μg), using a 300-bp human type IV collagenase cDNA as a probe. GAPDH served as a loading control. The level of induction of type IV collagenase expression was densitometrically quantitated, and fold induction after normalization was expressed by arbitrarily setting the value for control cells as 1, as indicated.

DISCUSSION

We have demonstrated here that type IV collagenase is a downstream p53 target gene, subject to p53 regulation. This is evident by (i) the presence of a perfect p53 consensus binding site in the promoter of the gene and specific binding of p53 to the site, (ii) wild-type p53-dependent activation of the type IV collagenase promoter and of a p53 binding-site-containing enhancer element, and (iii) p53-dependent activation of endogenous type IV collagenase gene expression.

The type IV collagenase, also called MMP-2 or 72-kDa gelatinase (gelatinase A), is a member of the large family of the MMPs (54). The enzyme is synthesized and secreted in an inactive proenzyme form and becomes activated by proteolytic degradation (8). The enzyme mainly degrades type IV collagen and fibronectins and has been implicated in both physiological and pathological conditions, such as trophoblast implantation, wound healing, angiogenesis, arthritis, and tumor cell invasion (3, 4, 8, 28, 31, 37, 42, 54). The type IV collagenase is subject to three levels of regulation, including transcriptional induction, proenzyme activation, and binding to and inhibition by TIMPs (8). It has been previously shown that expression of type IV collagenase can be induced by transforming growth factor β1 (6), UVB/interleukin-8 (40), concanavalin A (56), short-term exposure to alpha and gamma interferons (20), and transfection of c-Ha-ras (14) but is inhibited by retinoic acid (35), TPA (6), long-term exposure to alpha and gamma interferons (20), and the calcium influx inhibitor carboxy amidotriazole (25). Plasma membrane association (43, 55) and binding with membrane-type 1 MMP (23) facilitate proenzyme activation. We have reported here the cloning and sequencing of the 5′ upstream sequence of the type IV collagenase gene and have demonstrated its promoter activity and its transcriptional activation by wild-type p53. The other *cis* elements identified in the type IV collagenase promoter include Ets-1, CREB, AP-1, AP-2, PEA3, c-myc, and GCN4, among many others. The cloning and sequencing of the promoter would provide a molecular basis for understanding how type IV collagenase is regulated by multiple factors and would facilitate the identification of other transcription factors which may also control its expression. It is likely that expression of type IV collagenase is subject to regulation by many transcription factors. The outcome of net expression should result from the cross-talk among these factors in response to external stimuli.

The human type IV collagenase promoter has been partially characterized previously, although the entire promoter sequence was not published (12). An enhancer sequence located at nt −1635 and a downstream inhibitory sequence have been identified. Enhancer region 2 (r2), previously identified as an AP-2-regulatory sequence, allowing few mismatches and gaps (12), was found in this study to be a p53-regulatory region as well. This region has been recently shown to bind with a partial AP-2 protein (amino acids 165 to 437, containing the DNA binding and dimerization domains) and to be subject to AP-2 upregulation and E1A repression (41). Using the full-length AP-2 protein purchased from Promega, we were unable to show AP-2 binding to the r2 sequence, although strong binding could be seen when a typical AP-2 consensus sequence was used under gel shift assay conditions described by Williams and Tjian (53) (data not shown). The discrepancy could be derived from the use of different AP-2 proteins (full length versus N-terminal truncation mutant) or different gel shift assay conditions. Nevertheless, it is very interesting that the same region, which is important for type IV gene expression, is subject to both p53 and AP-2 upregulation and E1A repression (by targeting AP-2) (41). It is therefore of great importance to elucidate potential cross-talk between p53 and AP-2 in controlling type IV collagenase expression under normal and pathological conditions. Both p53 and AP-2 have been shown to upregulate Waf-1/p21 expression through binding to their own consensus binding sites (11, 57). p53 binding and transactivation of Waf-1/p21, a universal cell cycle inhibitor, are believed to contribute to p53 induced G_1 arrest, while activation of Waf-1/p21 by AP-2 leads to differentiation and tumor cell growth inhibition (57).

The promoter of type IV collagenase (MMP-2) in rat has been recently cloned and characterized (17). An 80-bp strong MMP-2 enhancer element which contained a consensus p53 binding site with one nucleotide mismatch (5′ GGGCAAGTC TGAACTTGTCa) was identified. Although the authors did not identify the nuclear protein which binds to this region, it is most likely to be p53. This assumption is supported by the facts that (i) there is a p53 binding site in the sequence; (ii) DNA footprinting analysis revealed protection at the p53 binding site; (iii) binding to this nuclear protein is abolished when the deletion involves the p53 binding site, while it is retained if the p53 site is not affected; and (iv) transient transfection coupled with luciferase assay revealed 10-fold activation when the intact p53 binding site is retained and a basal level of luciferase activity when the site is partially deleted. p53, therefore, regulates both human and rat type IV collagenase promoters. The conservation between species further suggests the importance of p53 in regulation of type IV collagenase expression.

Although p53 specifically binds to the r2 enhancer region (containing a perfect p53 binding site) and induces a 30- to 300-fold activation of luciferase activity in a wild-type p53-dependent manner (Fig. 3 and 4, bottom; Fig. 5, top), p53 causes only up to fivefold activation of luciferase activity when the 1,716-bp real promoter is used in a transient transfection and a two- to threefold induction of type IV collagenase mRNA when a p53 inducer is used. We have also observed that although etoposide caused a dose-dependent (from 1 to 100 μM) activation of p53 DNA binding in U2-OS cells as demonstrated in a gel shift assay, induction of mRNA expression did not occur in a dose-dependent manner (data not shown). All of these observations suggest that regulation of type IV collagenase expression is rather complex. Indeed, a transcriptional silencer located downstream of p53 binding site which negatively regulates the type IV collagenase promoter has

been previously described (12). Furthermore, p53, as a multiple gene activator or repressor (see the introduction), could also cause the up- or downregulation of the other genes in addition to activating type IV collagenase. Those p53-regulatory gene products may act on the type IV collagenase promoter, and net activation of the promoter may be the result of cross-interactions among multiple factors.

p53 has previously been shown to transrepress several cellular and viral promoters which lack its binding site (see reference 9 and references therein) as well as endogenous expression of the genes encoding microtubule-associated protein (34) and DNA topoisomerase IIα (51). One interesting finding in this study is that p53 represses the type IV collagenase promoter when the p53 binding site is deleted (Fig. 4). This suggests that p53 also functions as a negative regulator of the promoter. p53 may therefore play a dual role in regulation of the type IV collagenase promoter. On the one hand, p53 binds to its consensus binding site and transactivates the expression, while on the other hand, it works with the elements downstream from the binding site or with other transcription factors and transrepresses the expression. Thus, p53-induced transactivation (by sequence-specific DNA binding) of the type IV collagenase promoter can be partly counteracted by its negative effect. This may explain why there is no substantial induction of luciferase activity when the full-length promoter, compared to the p53 binding site alone, is used. It may also explain why there is no dose-dependent induction of type IV collagenase mRNA expression even though the p53 DNA binding occurs in a dose-dependent manner after etoposide treatment (data not shown). It will be of interest to identify and characterize this potential p53 negative regulatory element or factor(s) in the promoter of the type IV collagenase gene.

p53 has been shown to transactivate a number of genes involved in cellular response to DNA damage, cell cycle regulation, cell growth regulation, and angiogenesis (1, 7, 11, 21, 28, 29, 31, 35, 38). Here we have added the type IV collagenase gene to this list. This finding links p53, a common tumor suppressor and cell cycle regulator, to MMPs, a group of enzymes with a normal function of degradation of extracellular matrix components. The biological significance of this finding can be inferred from the biological activities of type IV collagenase in numerous physiological processes as well as in the pathogenesis of diseases, including trophoblast implantation, wound healing, angiogenesis, arthritis, and tumor cell invasion. It seems paradoxical that p53, a tumor suppressor, on the one hand upregulates thrombospondin (7), an inhibitor of angiogenesis that acts to prevent metastasis, and on the other hand upregulates type IV collagenase, which promotes angiogenesis and tumor invasion. The fact that only wild-type p53, and not p53 mutants, induces type IV collagenase expression suggests that p53 may play a role in regulating type IV collagenase under physiological conditions such as trophoblast implantation, wound healing, and angiogenesis. During human carcinogenesis, mutations occur in the p53 gene which abolish its activity in regulation of the type IV collagenase gene. Thus, it is possible that under pathological conditions, other factors, rather than p53, play the major role in regulation of type IV collagenase expression. The availability of p53 knockout mice will provide invaluable in vivo tools to address this issue.

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