

Differential regulation of plasminogen activator and inhibitor gene transcription by the tumor suppressor p53

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ABSTRACT

The ability of p53 to activate or repress transcription suggests that its biological function as tumor suppressor is in part accomplished by regulating a number of genes including such required for inhibition of cell growth. We here give evidence that p53 also may regulate genes responsible for the proteolytic degradation of the extracellular matrix, which is considered a crucial feature for local invasion and metastasis of neoplastic cells. An important and highly regulated cascade of such proteolytic events involves the plasminogen activator system. We show that wild-type p53 represses transcription from the enhancer and promoter of the human urokinase-type (u-PA) and the tissue-type plasminogen activator (t-PA) gene through a non-DNA binding mechanism. Oncogenic mutants lost the repressing activity. In contrast, wild-type but not mutant p53 specifically binds to and activates the promoter of the plasminogen activator inhibitor type-1 (PAI-1) gene. Interestingly, one of the p53 mutants (273his) inhibited PAI-1 promoter activity. Our results suggest that altered function of oncogenic forms of p53 may lead to altered expression of the plasminogen activators and their inhibitor(s) and thus to altered activation of the plasminogen/plasmin system during tumor progression.

INTRODUCTION

Tumor cells show two major features: excessive proliferation and the capacity to migrate and invade other tissues. Much attention has been focussed on the function of the tumor suppressor p53 (1,2) on the one hand and the role of matrix-degrading proteolytic enzymes such as metalloproteases and the plasmin/plasminogen activator system (3,4) on the other hand.

The p53 gene is mutated in the majority of human tumors (4,5). Conversely, introduction of the normal p53 gene into p53-deficient tumor cells suppresses transformation and tumorigenesis (6,7) and inhibits proliferation of many types of cells, causing them to arrest at G1/S (8,9). This is mediated, at least in

part, by p53 acting as a transcription factor (11–13) regulating genes required for the inhibition of cell growth. Genes *trans*-activated by p53 via binding to a specific recognition site within their promoter include p21/WAF (14–16), Gadd 45 (17), mdm2 (18,19), cyclin G (20) and Bax (25). Furthermore, p53 inhibits transcription from a number of other promoters that lack p53-binding sites (21–23), most likely through interaction with the TATA-binding protein (TBP) associated factors TAFII40 and TAFII60 (24). Loss of the *trans*-activating and/or repressing functions accounts for the tumorigenic potential of p53 mutations (26–28).

Migration of tumor cells and invasion into other tissues requires degradation of the extracellular matrix (ECM) by proteolytic enzymes. One protease extensively studied in this context is the serine protease plasmin, which is able to dissolve fibrin clots, digest extracellular matrix proteins and activate latent enzymes (29). The active enzyme is generated from the proenzyme plasminogen through proteolytic cleavage by two specific serine proteases, the plasminogen activators u-PA and t-PA (urokinase-type and tissue-type plasminogen activator, respectively; 30). These in turn are controlled by specific inhibitors, mainly the endothelial-type plasminogen activator inhibitor PAI-1 (30).

Cellular transformation often results in a dramatic increase in the production of the plasminogen activators, in particular of u-PA (29,31) and in an altered expression of the inhibitor PAI-1 (32). The balance between PAs and their inhibitors appears to be critical for the invasive phenotype of tumor cells (33,34), implying that altered expression slightly in favor of plasminogen activators (32) contributes to the malignant phenotype (31,32,35–37).

In this context, we investigated the regulation of the u-PA gene by oncogenic signal transduction pathways (C. Kunz and D. von der Ahe, unpublished results). Furthermore we speculated that PA and PAI gene promoters and enhancers might as well be a target for the tumor suppressor p53. Mutation of p53 in tumor cells would then provide a link between the deregulation of cell growth on the one hand and invasiveness due to increased proteolytic potential on the other hand. Therefore we examined a potential role of p53 in u-PA, t-PA and PAI-1 gene regulation by cotransfecting plasminogen activator or inhibitor enhancer/promoter-chloramphenicol acetyltransferase (CAT) reporter

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constructs together with an expression vector for wild-type (wt) or mutant p53 (27).

MATERIALS AND METHODS

Plasmids

The pCMVp53wt (pC53-SN3) and p53 mutant vectors have been described (27). The u-PA parental reporter plasmid -5.5/+60-CAT was constructed by inserting the *Bam*HI/*Nae*I(-5.5/+60) genomic fragment containing 5500 bp of the human u-PA upstream sequence (D. von der Ahe and C. Kunz, unpublished) into the promoterless pCAT-Basic vector (Promega). The deletion mutants -4300, -3000, -2500, -700 and -93 were derived from the parental plasmid. The +Enh -93/+60 reporter was constructed by inserting a PCR-generated 126 bp/*Hind*III fragment containing the human u-PA enhancer (-1986 to -1867; ref. 38) into the -93/+60-CAT vector immediately upstream of the u-PA basal promoter (C. Kunz and D.v.d. Ahe, unpublished). +Enh-Inr-CAT: the *Hind*III-*Xba*I linked *Bam*HI-*Sac*I fragment from plasmid VI (39) containing the TdT Inr element was cloned into pCAT-Basic; +Enh-(21 bp)₁-Inr-CAT: the *Xba*I fragment from plasmid VI (39) containing one SV40 21 bp repeat and TdT Inr was inserted into pCAT-Basic; +Enh -91/-31-Inr-CAT: oligos of the TdT Inr element (5'-CTAGA/GGCCCTCATTCTGGAGACTCTAGA/T-3') and the uPA GC-box region -91/-31 (38 and references herein) were inserted into the *Xba*I and the *Sal*I site of the pCAT-Basic. The 126 bp/*Hind*III u-PA enhancer fragment described above was cloned into the *Hind*III site of the latter three +Enh constructs. The (21 bp)₂-Inr-CAT reporter was constructed by inserting the *Hinc*II-*Bam*HI fragment of plasmid VI (39) into *Xba*I site of pCAT-Basic.

The t-PA-CAT reporter plasmids were constructed by inserting the *Bam*HI/*Xho*II (-1442/+118) and the *Nco*I/*Xho*II (-438/+118) fragments isolated from plasmid pF17*Bam*2 (containing ~1400 bp of 5'-flanking region, the first exon and part of the first intron of the human t-PA gene; ref. 40) into the promoterless pCAT-Basic vector.

The human PAI-1 promoter region and the PAI-CAT reporter plasmids have been described (41). They all carry the indicated region upstream of the transcription start site and 72 bp of the 5'-untranslated region. 3× PAI-u-PA: three copies of the PAI-1 p53 footprint sequence were inserted in the *Hind*III site immediately upstream of the u-PA basal promoter. -189 PAI: intact PAI-1 promoter (-189/+72); -189 dpm R-PAI: point mutations introduced at positions 4 and 7 of the right monomer of the p53 binding site within the -189/+72 PAI-1 promoter. The PG13 control reporter plasmid has been described (27).

Oligonucleotides

PAI-1 wild-type sequence (PAI-1): 5'-AGCTT/ACACA-CATGCCTCAGCAAGTCC-CAG/A-3'; PAI-1 point mutations PAI-mut:dpmR: 5'AGCTT/ACACACATGCCTCAGAAAT-CCCAG/A-3' and PAI-mut:2dpmRL: 5'-AGCTT/ACACAAAT-TCCTCA-GAAAT/CCCAG/A3'; the p53 consensus site (42): 5'-AGCTT/AGACATGCCTAGACATGCCT/A-3'; an unrelated DNA sequence (url): 5'-AGCTT/CTAAGGGACCTGCGGTT-GGCAT/A-3'.

Transfections

Transfections of HT1080 (ATCC:CCL121), HeLa (ATCC:CCL2) and Saos-2 (ATCC:HTB85) cells were performed at 60–70% of confluency following a modified calcium phosphate method (43). Five µg of each reporter plasmid, 2.5 µg of wt or mutant p53 effector construct and 5 µg of a RSV-β-galactosidase expression plasmid (as internal control for transfection efficiency) were used. The total amount of transfected DNA was always adjusted to 20 µg per 100 mm plate with pCMV-Neo-*Bam*. Twenty-four to thirty hours after transfection, the cells were extracted and assayed for β-gal and CAT activity. All samples were normalized for β-gal activity and/or protein concentration. CAT activity was quantified using a PhosphoImager (Molecular Dynamics, SF, ImagerQuant Software). Expression efficiency of the different mutant p53 proteins was assessed by Western blot analysis with anti-p53 antibody PAb 1801 (Oncogene Sciences).

In vitro protein synthesis and whole cell extracts

For the production of recombinant wild-type p53 protein, *in vitro* transcription/translation of the pGEM-mouse-p53 plasmid (a gift from M. Oren) was performed using the TNT coupled reticulocyte lysate system (Promega) according to the suppliers instructions. Protein extracts from COS-7 cells and Saos-2 cells transfected with p53 expression plasmid were prepared as described (42).

EMSA

The 5'-end labeled PAI oligo was incubated for 10 min at 30°C with either 4 µl of *in vitro* synthesized wt p53 protein and 0.1 µg of the activating antibody PAb 421 (45; Oncogene Science) or with 10 µg of COS-7 whole cell protein in a volume of 20 µl containing 20 mM Tris-HCl, pH 7.5, 50 µg/ml poly(dI-dC), 100 mM KCl, 7% glycerol, 1 mM DTT, 0.1 mM PMSF, 10 U/ml aprotinin and 0.1 µg/ml SBTI. Then a 100-fold molar excess of competitor oligo or 0.1 µg of the p53 specific second antibody DO-1 (45; Oncogene Science) or of a control antibody (anti-*Jun*, Oncogene Science) was added and incubation was continued for 10 min at room temperature prior to loading the samples onto a 5% polyacrylamide gel.

Immunoprecipitation linked DNase I footprinting and methylation interference assays

A PCR fragment containing the -189/+72 PAI-1 promoter region subcloned into the *Eco*RV site of pBluescriptII⁺ was labeled on one end (*Bam*HI) by Klenow-fill-in reaction and subsequently gel-purified. 1 pmol (170 ng, 1.3×10^6 c.p.m.) of the labeled DNA was incubated with either 15 µl of *in vitro* translated p53 or 20 µl of COS-7 whole cell extract for 30 min at 4°C in 100 µl DNA-binding buffer [20 mM Tris-HCl, pH 7.5, 50 µg/ml poly(dI-dC), 100 mM NaCl, 10% glycerol, 5 mM DTT, 1% NP-40]. DNA bound to p53 was immunoprecipitated by the anti-p53 antibodies PAb 421 and PAb 1801 as previously described (42). The precipitated samples were incubated with 300 ng DNase I at 25°C in DNA binding buffer supplemented with 5 mM MgCl₂ and 3 mM CaCl₂ for 3 min, then the digest was stopped by addition of 3 mM EDTA, 0.1% SDS and 0.125 mg/ml Proteinase K. After phenol extraction and ethanol precipitation the samples were submitted to electrophoresis on an 8% sequencing gel.

For methylation interference, 1 pmol of the DNA fragment described above was incubated with 1 µl DMS and 2 µg of calf

thymus DNA in 200 μ l 50 mM Na-cacodylate, pH 8.0 for 5 min, then the DNA was ethanol precipitated and resuspended in 10 μ l of 10 mM Tris-HCl, pH 7.5. p53 containing extracts were added and the complexes immunoprecipitated as described above. Subsequently, the DNA was cleaved by incubation at 90°C for 30 min in 100 μ l of 1 M piperidine, ethanol precipitated and electrophoresed on an 8% sequencing gel.

Northern and Western blots

Clone 6 cells derived from rat embryo fibroblasts carrying the ts p53 mutant p53^{val135} (7) were incubated at 37.5 or 32.5°C, respectively for 16 h. Control rat embryo fibroblasts carrying the wt p53 were cultured at 37.5°C for the same time period. Total RNA was extracted according to the method of Chomczynski and Sacchi (46) and 20 μ g of each sample were electrophoresed on a formaldehyde-agarose gel. Following transfer to a Hybond N⁺ membrane (Amersham), the blot was hybridized with a human PAI-1 and a rat GAPDH cDNA probe. Western blot analysis was performed with 0.5 ml lyophilized supernatant of clone 6 or normal rat embryo fibroblasts. Twelve μ g protein of each sample were electrophoresed on a 10% SDS-polyacrylamide gel (SDS-PAGE). Following transfer to nitrocellulose membrane, the blot was probed with the monoclonal anti-PAI-1 antibody CLB IIC8 (62).

RESULTS

p53 represses the u-PA and the t-PA promoter, but activates the PAI-1 promoter

Experiments concerning the influence of p53 on the constitutive u-PA promoter and enhancer activity were performed in HT1080 cells (human fibrosarcoma) because of their relatively high basal u-PA expression, whereas 12-*O*-tetradecanoylphorbol-13-acetate (TPA) inducibility was investigated in HeLa cells (human cervix carcinoma), which show a relatively low constitutive u-PA expression but are efficiently induced by TPA. Both HT1080 and HeLa cell lines express low amounts of wild-type p53 (not shown). Furthermore, the human osteosarcoma cell line Saos-2 constituted a useful system to study p53 effects due to its complete deficiency of endogenous p53 (47). As summarized in Figure 1a and b, constitutive and TPA-inducible activity of the u-PA promoter/enhancer (38,39) are strongly repressed by wt p53. A reporter construct containing the u-PA enhancer linked to the heterologous thymidine kinase promoter was also suppressed by p53 (data not shown). By comparison, mutant forms of p53 (27) exhibited no (mutant 143ala, 175his and 248trp) or significantly less repression (mutant 273his) (Fig. 1c). We obtained similar results with the t-PA promoter (40): cotransfection of wt p53 caused a strong reduction in t-PA-promoter driven CAT expression (Fig. 1e), whereas mutant forms of p53 exhibited no or substantially less suppression (data not shown).

In contrast, wt p53 efficiently activated (~4.5-fold in HeLa cells) transcription from the PAI-1 promoter (41) containing 216 base pairs upstream of the transcription initiation site (Fig. 1f). An analogous experiment in the p53-deficient cell line Saos-2 revealed a ~10-fold activation (Fig. 1g) arising from a considerably lower basal promoter activity. When the PAI-1 promoter was deleted beyond base pair -139 the basal promoter activity was significantly repressed (~3-4-fold; Fig. 1e). This suggests the existence of a p53 binding site within the -216/-139 region. Interestingly, the 273his mutant of p53 repressed the -216PAI-

CAT reporter as well as the further promoter deletion mutants -139 and -53 (Fig. 1f). For comparison, three other p53 mutants did neither significantly activate nor repress the -216PAI-promoter (Fig. 1g).

Repression of the u-PA gene depends on the promoter and the enhancer structure

p53 has been shown to suppress a number of promoters containing TATA elements (48,49). It has been suggested that this suppression is independent of p53 binding to its specific target DNA sequence, but may involve interaction with the TATA-binding protein (21,50,51) or associated factors (24). Such repression acting on the -93/+60 u-PA basal promoter containing a TATA-box and four SP-1 dependent GC-boxes (C. Kunz and D.v.d. Ahe, unpublished) could also prevent a full activation under control of the constitutive and inducible enhancer. Alternatively, p53 could independently act on the enhancer and abolish its activity.

In order to discriminate between enhancer and promoter interference, we compared different u-PA promoter/enhancer constructs containing initiator (Inr) instead of TATA-elements which, according to the report of Mack *et al.* (48), are relatively unaffected by wt p53. As seen in Figure 2, in HT1080 cells cotransfected with wt p53 expression plasmids, the SP1-dependent initiator construct (21 bp)₂-Inr-CAT (48) and the u-PA GC-box driven initiator-construct (-93/-31-Inr) are equally resistant to the repressive effects of wt p53 (lines 6 and 7). In contrast, all u-PA enhancer containing reporter plasmids directing transcription from the initiator element were efficiently inhibited by p53 (Fig. 2, lines 2-4), in particular the +Enh-(21 bp)₁-Inr-CAT reporter containing part of the solo unrepresable SV40-21 bp repeat/initiator-dependent promoter (40) (Fig. 2, line 3). Similar results were obtained under TPA stimulated conditions in Saos-2 and HeLa cells (data not shown). These experiments demonstrate that the repression of the u-PA enhancer by p53 is independent of TATA or initiator-directed promoters. This suggests that p53 may either directly interfere with the action of u-PA enhancer binding factor(s) or repress their gene transcription.

p53 binds to the PAI-1 promoter

Our further studies aimed for the definition of the presumed p53 binding sequence within the PAI-1 promoter. For this purpose we performed an immunoprecipitation linked DNase I footprinting and methylation interference analysis (42) of the -189/+72 promoter fragment containing the p53 responsive element. The DNase footprints in Figure 3a and b show that in the presence of either *in vitro* translated wt p53 (Fig. 3a and b, lanes 2), wt p53 expressed in Saos-2 cells (Fig. 3a and b, lanes 4) or COS-7 cell extract proteins (Fig. 3a, lanes 5 and Fig. 3b, lanes 6) a 22 bp stretch of DNA was protected from digestion. The sequence of this region ranging from position -160 to -139 matches at 18 out of 20 positions the p53 consensus binding site proposed by El-Deiry *et al.* (42) (Fig. 3d). The methylation interference assay of the upper strand shown in Figure 3b demonstrates that methylation of Gs at positions -151, -145 and -141 interfered with binding of p53.

To support further that the protected region in the PAI-1 promoter is a target for specific binding of the p53 protein we performed electrophoretic mobility shift assays (EMSA) with *in vitro* translated p53 and COS-7 cell extract (Fig. 4a). The *in vitro* synthesized protein had to be activated with the antibody PAb421

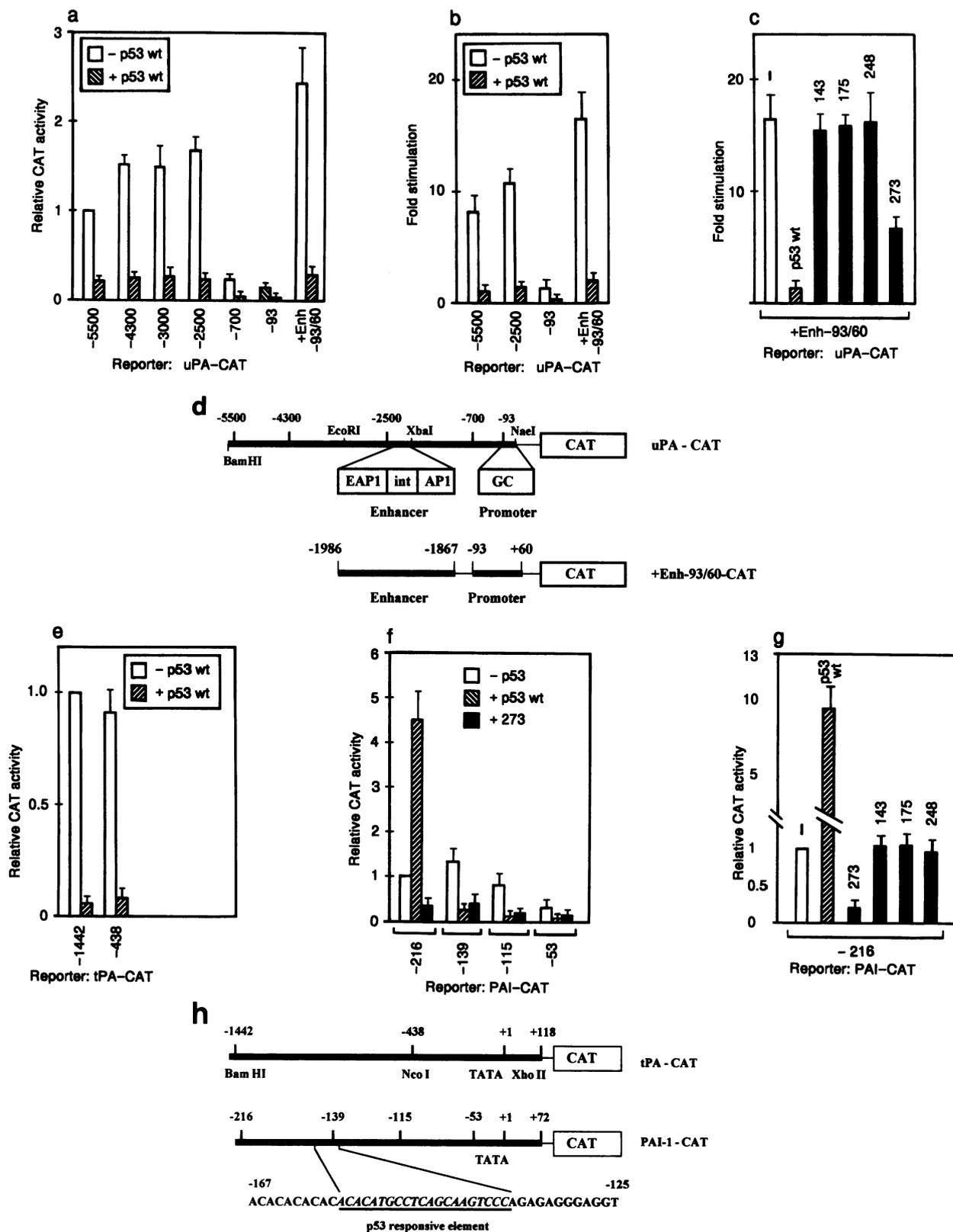


Figure 1. Effects of p53 on transcription from the u-PA enhancer/promoter and the t-PA and PAI-1 promoters. HT 1080 (a) HeLa (b, e and f) and Saos-2 cells (c and g) were transfected with u-PA-CAT, t-PA-CAT and PAI-1-CAT reporter plasmids as indicated, together with expression vectors encoding wt p53 or mutant forms (273his, 248trp, 175his or 143ala) of p53 as indicated. (d and h) Diagrammatic representations of the uPA-, tPA- and PAI-1-CAT reporter constructs. The ordinate displays relative chloramphenicol acetyltransferase (CAT) activities or the factor of their stimulation by 100 ng/ml TPA, depending on the cotransfection of p53 expression plasmids as given on the abscissa. The results shown are the averaged values from at least three different experiments.

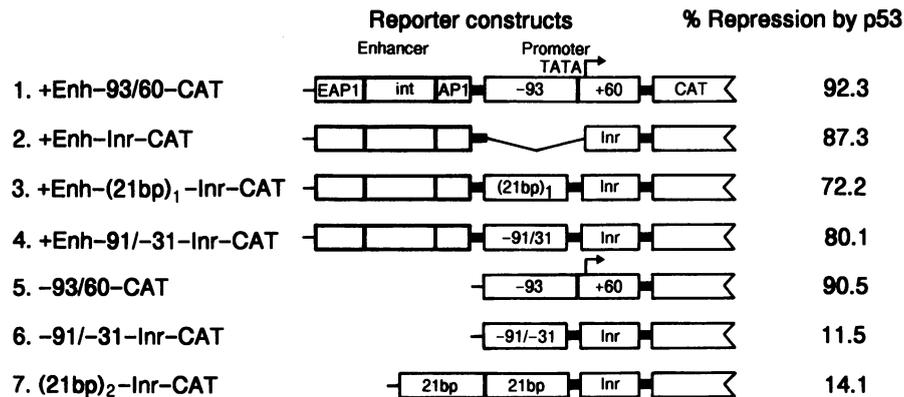


Figure 2. p53 specifically represses the u-PA enhancer independent of the promoter structure. The u-PA enhancer is composed of three major domains as shown: the promoter-distal Ets-AP-1 site (EAP1), the non-inducible inter-region (int) and the promoter-proximal AP-1 site (AP1) (details in ref. 38 and C. Kunz and D.v.d. Ahe, published elsewhere). The promoters of the reporter constructs differ in that they either initiate transcription from the terminal transferase (TdT) initiator (Inr) element (56; lines 2, 3, 4, 6 and 7) or from the u-PA-TATA-directed initiation site (lines 1 and 5). Repression due to cotransfection of p53 wt expression plasmid is expressed as the per cent reduction compared with CAT activity in the absence of p53. The given values are the average ($\pm 20\%$) from three independent experiments.

to obtain significant binding (45), whereas in COS-7 extract, which in Western blot analysis proved to contain high levels of p53, binding was independent of activation. A 100-fold molar excess of unlabeled PAI-wt and p53 consensus but not of an unrelated oligonucleotide abolished the complex formation (lanes 3–5 and 9–11). Specificity of the binding could be further confirmed by a supershift through a second p53 specific antibody, DO-1 (45) (Fig. 4a, lanes 6 and 7 and 12 and 13).

As the G:C base pairs in positions 4 and 7 are highly conserved in most of the known genomic p53 binding sites (42), we were interested in the effects of changing these positions in the p53 binding site of the PAI-1 promoter into A:T base pairs. The respective double point mutation in the right monomer (dpmR) or in both monomers (2dpmRL) abolished the ability to compete efficiently for p53 binding, even when increasing the molar excess up to 200-fold (Fig. 4b). Correspondingly, the mutant sequences did not bind p53 (data not shown).

Next we attempted to correlate binding of p53 to the PAI-1 promoter with *trans*-activation of transcriptional activity by testing the heterologous u-PA minimal promoter -93/60-CAT driven by three copies of the PAI-1 promoter p53 binding site (Fig. 5a). As shown in Figure 5b, the 3 \times PAI-u-PA-CAT reporter was significantly stimulated by co-expressed wt p53, although the u-PA promoter alone is repressed by p53 (Fig. 1a). The strongly inducible (30-fold) PG13 reporter which contains 13 copies of the p53 consensus binding sequence (27) was used as a positive control. Furthermore, the double point mutation of the right monomer within the intact PAI-1 promoter (-189/+72) interfered completely with induction by wt p53 (Fig. 5b), as did the 2dpmRL mutant (data not shown).

The chromosomal PAI-1 gene is activated by p53

Finally we turned our attention to the endogenous chromosomal PAI-1 gene as a putative p53 target gene. Total RNA from clone 6 rat embryo fibroblasts (REFs) transformed with an activated Ras and the temperature sensitive human p53 mutant val135 (7) was subjected to Northern hybridization with a PAI-1 cDNA. In cells maintained at 37.5°C, where the ts p53 is in its mutant inactive conformation (7), the levels of the PAI-1 specific transcripts were not detectable. A temperature shift down to

32.5°C, where the ts p53 is in an active conformation, resulted in a considerable increase in the amounts of PAI-1 transcripts (Fig. 6a, lanes 1 and 2) whereas control cells carrying the wt p53 did not express significant amounts of PAI-1 specific transcripts at either temperature (Fig. 6a, lanes 3 and 4). These results suggest that wt p53 might be able to *trans*-activate the PAI-1 promoter also within its natural chromosomal context. This was further supported by the transient *trans*-activation of the PAI-1 promoter-CAT reporter through the p53 val135 ts mutant at the permissive temperature in Saos-2 cells (data not shown). Additionally, we performed a Western blot analysis for PAI-1 protein using a monoclonal anti-PAI-1 antibody (62) and supernatants of temperature-induced and uninduced REFs. The results in Figure 6b show that the temperature shift resulted in a considerable increase in the amount of PAI-1 protein whereas control cells did not express significant amounts of PAI-1 protein. These results confirm that the induced PAI-1 mRNA is translated into PAI-1 protein.

DISCUSSION

A primary event in tumorigenesis is the cellular transformation as a result of changes in two sets of genes, the proto-oncogenes and the tumor-suppressor genes (52). Subsequent steps in tumor progression are vascularization of the growing cell masses and metastasis of detaching cells. Both processes require proteolytic degradation of the extracellular matrix (4,53). The role of plasminogen activators and their inhibitors for ECM degradation and tissue invasion has been demonstrated in numerous experiments (54–56); yet, the molecular mechanisms leading to a disturbance in the balance of the system and thus to the enhanced proteolytic capacity of transformed cells are so far not clear.

Here, we present evidence that the expression of the u-PA, t-PA and PAI-1 genes might be differentially regulated by the tumor suppressor p53. We found, that the promoter of the t-PA gene and the promoter and enhancer of the u-PA gene were repressed, whereas the PAI-1 promoter was activated by wt p53 protein. A specific p53 binding site was identified within the PAI-1 promoter (Fig. 3), which functions as a p53 dependent *cis*-activating element in transient transfection assays (Fig. 5b). Furthermore, by using a temperature sensitive form of p53, it

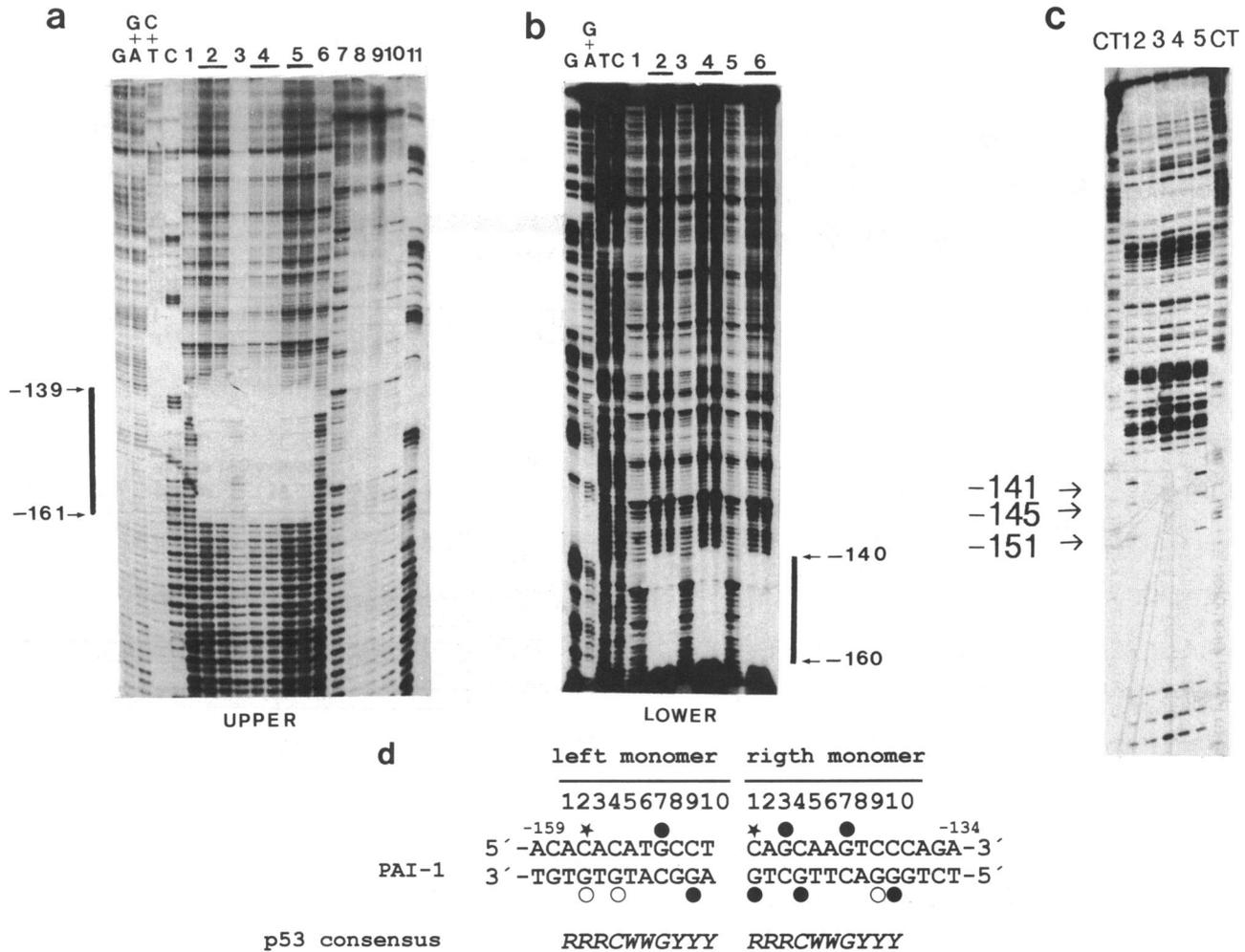


Figure 3. DNase I protection and methylation interference of the p53 responsive PAI-1 promoter region. The indicated numbers refer to the distance from the transcription initiation site. (a) DNase I footprinting of the PAI-1 promoter fragment -189/+72 (upper strand). Lanes 1, 3, 6 and 7: no protein; lane 11: COS-7 cell extract deprived of p53 by immunoprecipitation with antibodies PAb421 and 1801; lanes 2 (double lane): *in vitro* translated p53; lanes 4 (double lane): cell extract from p53 transfected Saos-2 cells; lanes 5 (double lane): COS-7 cell extract. (b) DNase I footprinting of the PAI-1 promoter fragment -189/+72 (lower strand). Lanes 1, 3 and 5: no protein; lanes 2 (double lane): *in vitro* translated p53; lanes 4 (double lane): cell extract from p53 transfected Saos-2 cells; lanes 6 (double lane): cell extract from COS-7 cells. All lanes contain an equivalent amount of DNA. (c) Methylation interference of the PAI-1 promoter fragment -189/+72 (upper strand). Lanes 1 and 5: no protein; lane 2: *in vitro* translated p53; lane 3: cell extract from p53 transfected Saos-2 cells; lane 4: COS-7 cell extract. (d) DNA sequence of the PAI-1 promoter region bound by p53. Filled and open circles indicate full and partial interference with p53 binding by methylated G residues. The stars in positions 1 and 2 of the right and left monomer of the PAI-1 promoter binding site indicate mismatches compared to the p53 consensus sequence (42) aligned below.

could be shown that the expression of the endogenous PAI-1 gene is inducible by the presence of functional p53 (Fig. 6a) and this increases significantly the amount of translated PAI-1 protein (Fig. 6b).

In contrast, the repression of the t-PA and the u-PA promoters by wt p53 could not be ascribed to a distinct p53 binding element. This parallels the findings of others, that p53 negatively regulates a variety of genes lacking a p53 responsive element (49,57,58). The mechanistic basis of this negative effect appears to be a direct interaction with the TATA-binding protein or TBP-associated factors (21,24). Our results with the u-PA enhancer/promoter constructs suggest a bipartite model for the repression by p53: (i) downmodulation of the u-PA promoter by a TBP-dependent mechanism or by interaction with TBP-associated factors (24) and; independently (ii) negative regulation of the remote u-PA enhancer by interaction with enhancer binding factors. This idea is consistent with observations in other

systems, where p53 negatively regulates promoters by interaction with activating transcription factors (18,49).

When testing different mutant forms of p53 for their influence on the PAI-1 promoter we found that they lost the DNA binding and *trans*-activating capacity or even gained a dominant negative function (273his; Fig. 1g), a phenomenon already observed in previously published studies on the regulation of the multidrug resistance (*mdr*) gene promoter/enhancer (59). The u-PA promoter/enhancer and the t-PA promoter were no longer or to a lesser extent repressed by the respective mutants (Fig. 1c, and data not shown).

In the light of these findings it is tempting to speculate that mutations in p53 might cause a shift in the balance of PA and PAI-1 activity leading to an enhanced proteolytic capacity of the affected cells. It has been shown that only a minimum of PA activity is required for the invasive property of tumor cells (32,33,60,61). Under normal circumstances, the u-PA gene

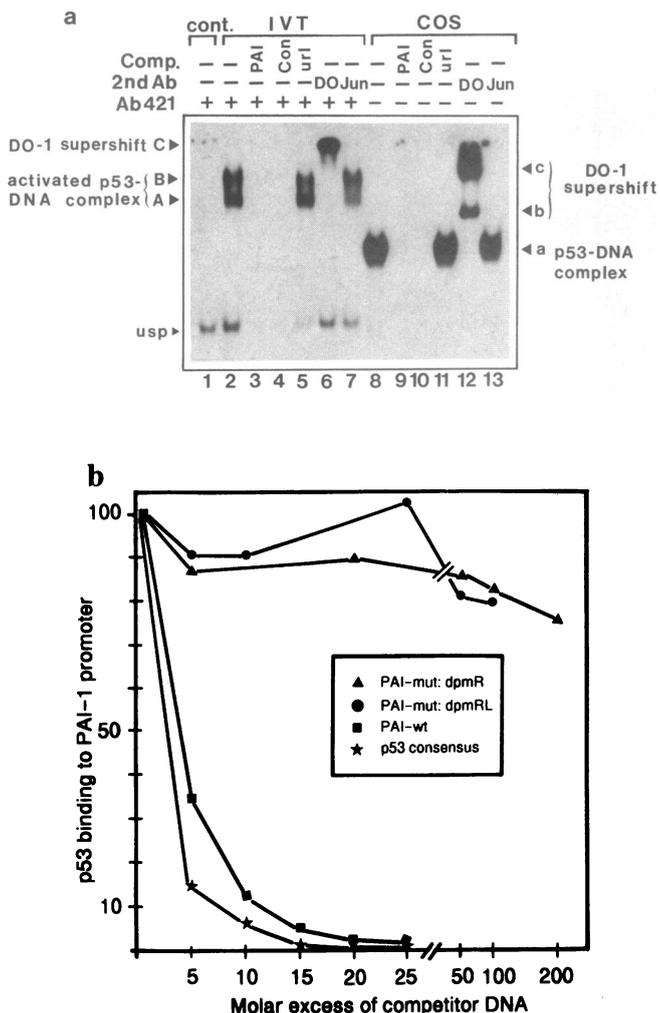


Figure 4. p53 specifically binds to the PAI-1 promoter. (a) EMSA with an oligonucleotide (PAI) containing the p53 footprint region (-159/-134) of the PAI-1 promoter and *in vitro* translated p53 wt protein (IVT) or COS-7 whole cell protein (COS). The indicated competitor oligos representing the PAI-1 wt sequence (PAI), the p53 consensus sequence (42; con) and an unrelated DNA sequence (url) were included in 100-fold excess. The specific p53-DNA complexes are indicated (A, B and C for IVT, and a, b and c for COS); usp indicates an unspecific protein-DNA complex. (b) Concentration-dependent competition of p53 binding to the PAI-1 promoter binding site by the PAI wt oligo (PAI), an oligo mutated in positions 4 and 7 of the right monomer (PAI-mut:dpmR), an oligo mutated in positions 4 and 7 of the left and right monomer (PAI-mut:2dpmRL) and the p53 consensus oligo (con). Quantitative analysis was performed with a PhosphoImager (Molecular Dynamics, SF, ImagerQuant Software).

appears not to be constitutively expressed in most cell types, although transcription can be induced by a number of agents, including TPA, cAMP, growth factors and oncogene products (C. Kunz and D.v.d. Ahe, unpublished results). Loss of functional p53 might lead to a slight increase in constitutive u-PA expression, which, combined with a reduction of PAI-1 levels, might be sufficient to result in an enhanced proteolytic activity.

However, the physiological and pathophysiological relevance of a regulation of PA and the PAI-1 genes by p53 remains to be demonstrated, as the results so far were obtained under *in vitro* conditions with high amounts of overexpressed p53. Furthermore, it can not be excluded, that other *cis*-acting elements within

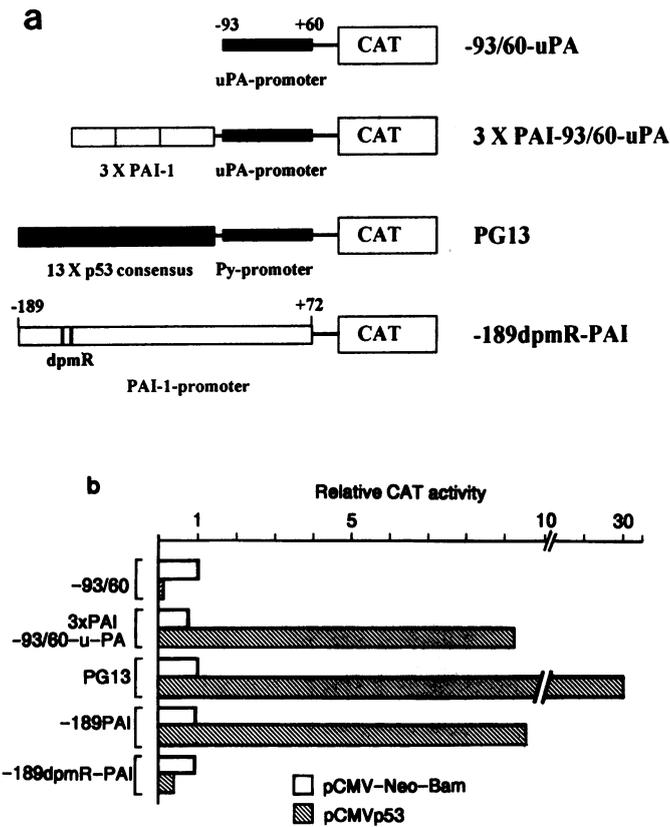


Figure 5. The p53 responsive element of the PAI-1 promoter. (a) Diagrammatic representations of the promoters of the reporter genes. (b) Induction of CAT activity by wt p53. Multimerized PAI-1 promoter p53 binding site confers p53 inducibility to the heterologous u-PA promoter. Double point mutation in one half-site of the binding motif (dpmR, as indicated) within the intact (-189) PAI-1 promoter completely abolishes stimulation by p53. Results represent the average values of two independent transfection experiments.

the regulatory region of the genes may modulate or override the p53 effects *in vivo*. So, the influence of physiological p53 levels on the endogenous genes will have to be investigated. Also, it would be interesting to study the influence of an accumulation of cellular p53, for example after UV induced DNA damage, on PA and PAI-1 expression.

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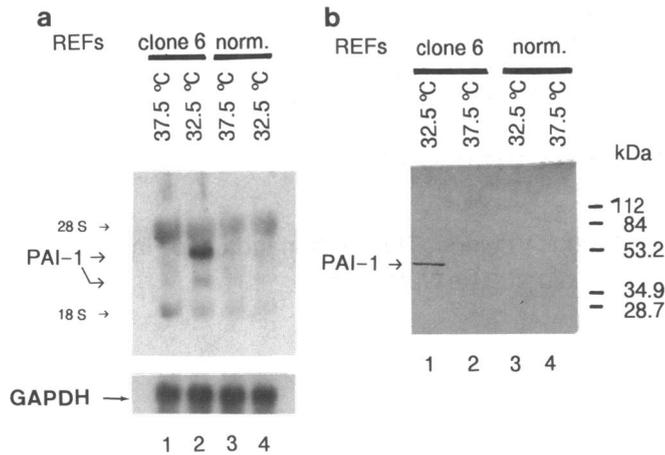


Figure 6. PAI-1 expression as determined by Northern and Western blot analysis. (a) Northern blot showing induction of PAI-1 transcripts by p53 in clone 6 cells derived from rat embryo fibroblasts (REFs) carrying the ts p53 mutant p53val135 (7). (b) Western blot showing induction of PAI-1 protein by p53 in clone 6 cells. Proteins of 0.5 ml supernatant was loaded per lane. Analysis was performed with the monoclonal anti-PAI-1 antibody CLB IIC8 (62). At a temperature of 37.5°C, where these cells produce only inactive p53, no PAI-1 mRNA and protein are detected, whereas at 32.5°C, when active p53 is present, the PAI-1 mRNA and protein increase to significant levels. Lanes 1 and 2: clone 6 rat embryo fibroblasts; lanes 3 and 4: normal (wild-type) rat embryo fibroblasts. Aliquots of 20µg total RNA from the cells indicated were sequentially probed with the PAI-1 and the GAPDH cDNAs. 28S and 18S ribosomal RNAs were used as size markers.

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