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The promoter of human p22/PACAP response gene 1 (PRG1) contains functional binding sites for the p53 tumor suppressor and for NFκB

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Received 24 August 1998

Abstract **We describe functional binding sites for the tumor suppressor p53 and for NFκB residing in the promoter of the novel human early response gene p22/PRG1 (IEX-1/DIF-2). Gel shift and supershift assays demonstrate binding of p53 and NFκB to their corresponding sites in vitro. CAT-reporter gene assays show transactivation of the human p22/PRG1 promoter by p53 in Hep3B cells stably transfected with a temperature-sensitive mutant p53, but not in p53-deficient Hep3B cells. TNFα induced NFκB dependent transactivation was shown in HepG2 cells or in 818-4 pancreatic cancer cells. These data imply that human p22/PRG1 is a target gene for p53 and NFκB involved in growth regulation and stress response.**

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Key words: Transcription factor; Growth regulation; Tumor cell; Stress response

1. Introduction

The novel proliferation associated early response gene p22/*PACAP response gene1* (PRG1) is expressed in rats [1], mice (gly96) [2] and humans (IEX1/DIF2) [3,4]. Besides its early transcriptional induction during growth stimulation in various cell types [1,2], the induction of this gene has also been demonstrated in irradiated human breast cancer cells [3] or in human monocytes before their differentiation into macrophages [4]. In humans, p22/PRG1 represents a 22-kDa protein comprising 156 amino acids, yet the exact function and cellular localization is unclear. Current data strongly indicate that p22/PRG1 may be involved in distinct cellular processes like stimulation of proliferation, cellular adaptation and stress response. More recently, we could show that rat p22/PRG1 is a target gene for the tumor suppressor p53 presumably involved in p53-dependent growth regulation [5]. However, the detailed role of p22/PRG1 in this context is still under investigation. Meanwhile, we and others [6] have also cloned parts of the human p22/PRG1 promoter providing the opportunity to elucidate the signal transduction mechanisms leading to transactivation of p22/PRG1 in humans. In this study, we demonstrate that, as in rats, the human p22/PRG1 promoter contains also functional binding sites for p53 and for NFκB, both responsible for the induction of p22/PRG1 during regulation of cellular growth, stress response and cellular adaptation.

2. Material and methods

2.1. Materials

Recombinant wild-type and mutant p53 and pCMV-p53 and pCMV-C5 expression vectors were a generous gift from Prof. W. Deppert, Heinrich Pette Institute, Hamburg, Germany. Hep3B(p53^{Val135}) and Hep3B cells were kindly provided by Prof. P. Galle, 1st Dept. of Medicine, University of Mainz, Germany. Oligonucleotides were custom synthesized by Biometra (Göttingen, Germany), [γ-^{32}P]dATP was from Amersham (Braunschweig, Germany) and cell culture media were from Seromed (Hamburg, Germany).

2.2. Cell culture

Hep3B(p53^{Val135}) and Hep3B cells were grown in EMEM (0.1 mg/ml Na-pyruvate; 1% glutamine; 10% FCS) ± 500 μg/ml G418 (Clontech). HepG2 cells were cultured in DMEM (high glucose, 1% glutamine, 10% FCS) and 818-4 pancreatic carcinoma cells and HeLa cells were cultured in RPMI (1% glutamine; 10% FCS).

2.3. Gel shift and supershift assays

For p53 gel shifts, purified recombinant baculovirus expressed wt p53 or double mutated p53-C5 (80 ng) were incubated in incubation buffer (Stratagene) together with a γ-^{32}P-labelled oligonucleotide (*aggtgccacatgctccgacatgtgcctgca*) containing the p53 binding site from the human p22/PRG1 promotor. For NFκB gel shifts, nuclear extracts from TNFα-stimulated Jurkat cells were prepared as described recently [7] and incubated with a γ-^{32}P-labelled oligonucleotide (*aatcgtcggaatttccagcccg*) containing the NFκB binding site from the human p22/PRG1 promoter or a consensus NFκB-binding site. After 30 min incubation at room temperature, samples were submitted to native PAGE. Gels were dried and exposed to X-ray Hyperfilm (Amersham). For supershift assay, a monoclonal p53 antibody (Ab1, Calbiochem) or anti p65/p50 NFκB antibodies (Santa Cruz) were added for 1 h, 4°C.

2.4. Generation of CAT-reporter gene constructs

Using appropriate composite *Hin*dIII/*Xba*I and *Sal*I/*Xba*I PCR-primers, PCR-fragments of the human p22/PRG1 promoter were generated containing deletions in positions −226/−244 and −106/−111 (Δ^{p53}, $\Delta^{-NF\kappa B}$) or shortened at the 5′-end (pos. +1 to −439, −335, −285, −225, −140, and −110). Products were cloned into the CAT-basic vector (Promega) and checked by nucleotide sequencing.

2.5. Cell transfection and CAT-reporter gene assay

Hep3B(p53^{Val135}), Hep3B, HepG2 and 818-4 cells cultured in 6-well plates were transiently transfected (Lipofectamine; Gibco-BRL) with pCAT vectors (1.5 μg) containing human p22/PRG1 promoter fragments and 0.5 μg pCMV-lacZ. For cotransfection of Hep3B cells, 0.1 μg of CMV early enhancer promoter driven expression plasmids for wild-type p53 (pCMV-p53) or mutant p53 (pCMV-c5p53) were added. After 24 h culture, CAT expression (ng) was determined by ELISA (Boehringer) and normalized to β-galactosidase expression (β-gal ELISA, Boehringer).

3. Results

By means of *promoter finder* PCR, we cloned a 1864-bp genomic fragment of human p22/PRG1 identical to a recently published genomic sequence of the human DIF-2 gene [6]. This fragment consists of a 112-bp intron and a 633-bp

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[1]This work is part of a M.D. thesis.

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PII: S0014-5793(98)01109-0

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-633  ..........................tcctcacttataa
-620  gtgggaaccaaacattgggaactcgtggacgtaaagatgg
-580  caacaatagcaacttgagactactagaagaaggacggagg
                                 Ets-1
-540  gagcgagggagatcacgaggtcaggaaatcgagaccaccc
-500  tggccaaaagggtgaaaccccgtctctactaaaaataaaa
                  Sp1
-460  aaattagctgggcgtggtggcgcgtgcctgtattcccagc
           Ets-1
-420  tactcaggaagctgaggcaggaaaatcgcttgaaccagtg
       AP1
-380  agtcaggggttgcagtgagccgagatcgcgcctctggatt
-340  ccagcctggcgaccgaacgagactgctccatctccaaaaa
-300  aaaaaaaaaaggcctgtgagggatcctgtggctaaagtga
                            p53
-260  gcccctctccaggtgccacatgcctcgacatgtgcctgca
-220  gcccgggatctcacccacccccactcacgactcacacact
-180  cacaacgtgcagttgggcgcctaggattgtgcatgtcaag
                                      NFκB
-140  tctccacccactcccttgttaatcgtcggaatttccagc
-100  ccgctgctgccaaccgctccccagctgcgggaggaggagt
-60   tagaaggacccgcccaattttcaggagcacataaattacc
-20   tctgccggcagccgaccctc +1acttggccttacac...
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Fig. 1. Partial nucleotide sequence of the human p22/PRG1 promoter (pos. −633 to −1). Sequence of the 633-bp 5′-flanking region of the human p22/PRG1 gene harboring the putative promoter. Binding sites for transcription factors are double-underlined and indicated above. This sequence has been deposited in the EMBL data base, accession no. X96438.

5′-flanking sequence harboring the putative p22/PRG1 promoter (Fig. 1). Several potential binding sites were identified including an NFκB binding site (pos. −113 to −104) that is conserved between promoters of human and rat p22/PRG1 [1] or murine gly96 [2]. A potential p53 binding site is located in the human gene (pos. −226 to −245) at a quite similar position and with almost complete sequence identity compared to the functional p53 binding site of the rat p22/PRG1 promoter [5]. This site, ccACATGCCT cGACATGTgC, strongly matches the tandem consensus sequence $(RRRCWWGYYY)_2$ [8,9] common to established p53 target genes like p21/Waf1, Bax, mdm2 or GADD45 [10–13].

To elucidate whether these two conserved and prominent binding sites bind to NFκB and p53 in vitro, gel shift assays were performed. For gel shift assays on NFκB, nuclear extracts from TNFα-stimulated Jurkat cells were used. As shown in Fig. 2A, an intensively labelled protein/DNA complex could be detected when coincubating the nuclear extract and the NFκB probe derived from the p22/PRG1 promoter. The addition of an excess of the homologous unlabelled oligo completely reduced labelling of the protein/DNA complex, whereas an unrelated oligo – like AP1 – did not compete with the probe. The specificity of the identified protein/DNA complex was underlined by the appearance of a supershifted ternary complex with monoclonal antibodies against p65 and p50 (Fig. 2A).

Using a radiolabelled oligo containing the p53 binding site from the human p22/PRG1 promoter as probe, coincubation with recombinant wt-p53 similarly produced an intensive radiolabelled protein/DNA complex (Fig. 2B). Labelling of this complex was fully abolished by the addition of an excess of the homologous unlabelled oligonucleotide and no band was observed when incubating the p53 probe with recombinant double mutant p53 [14]. Furthermore, the addition of an anti wt-p53 monoclonal antibody led to the formation of a supershifted ternary complex.

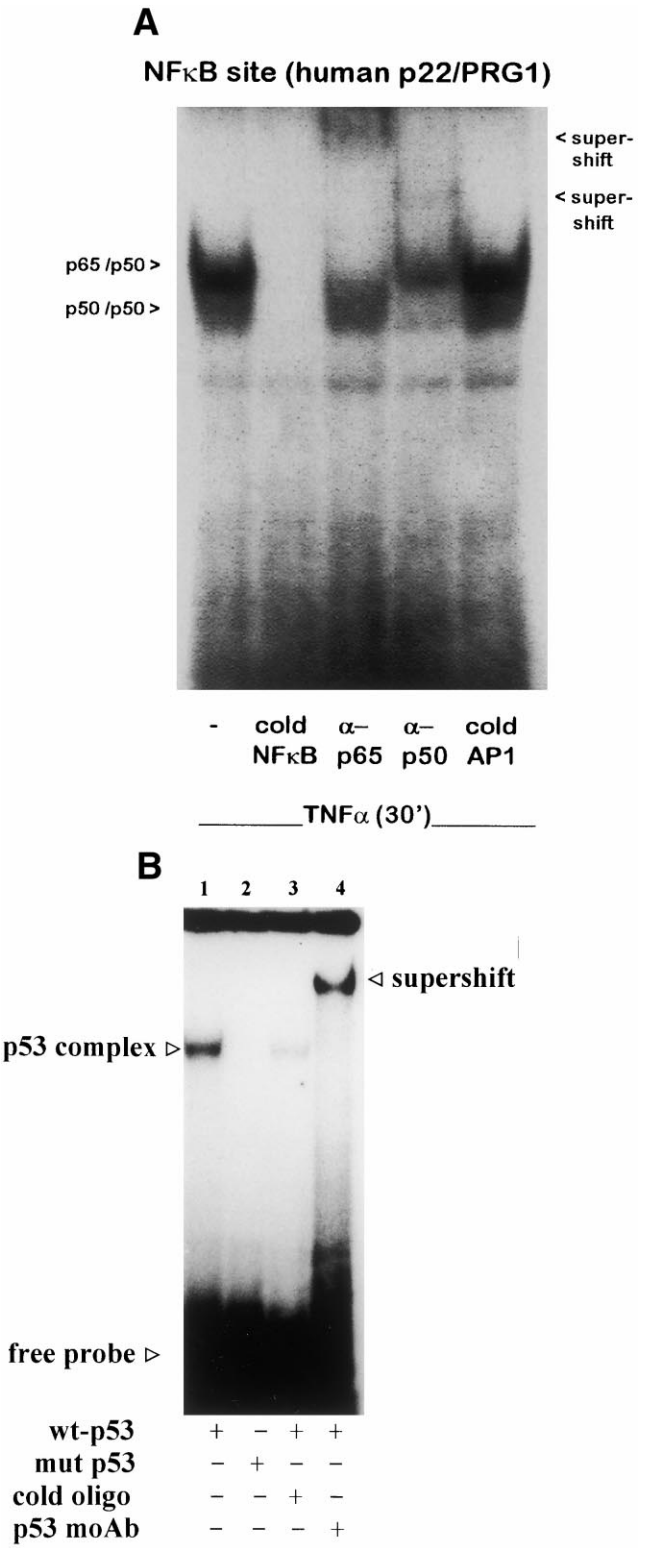

Fig. 2. Gel shift and supershift assay. A: Using nuclear extracts from TNFα-stimulated Jurkat cells, gel shift assays were performed with equal amounts of the γ-^{32}P-labelled oligonucleotide containing the NFκB binding site of the human p22/PRG1 promoter. B: Using baculovirus recombinant wild-type p53 (lanes 1, 3, 4) or recombinant mutant p53-C5 (lane 2), gel shift assays were performed with equal amounts of the γ-^{32}P-labelled oligonucleotide containing p53 binding site of the human p22/PRG1 promoter. Assays were conducted in the absence or presence of a 50-fold molar excess of unlabeled oligo, or in the presence of monoclonal antibody against p65/p50 (A) or the C-terminal domain of wild-type p53 (B).

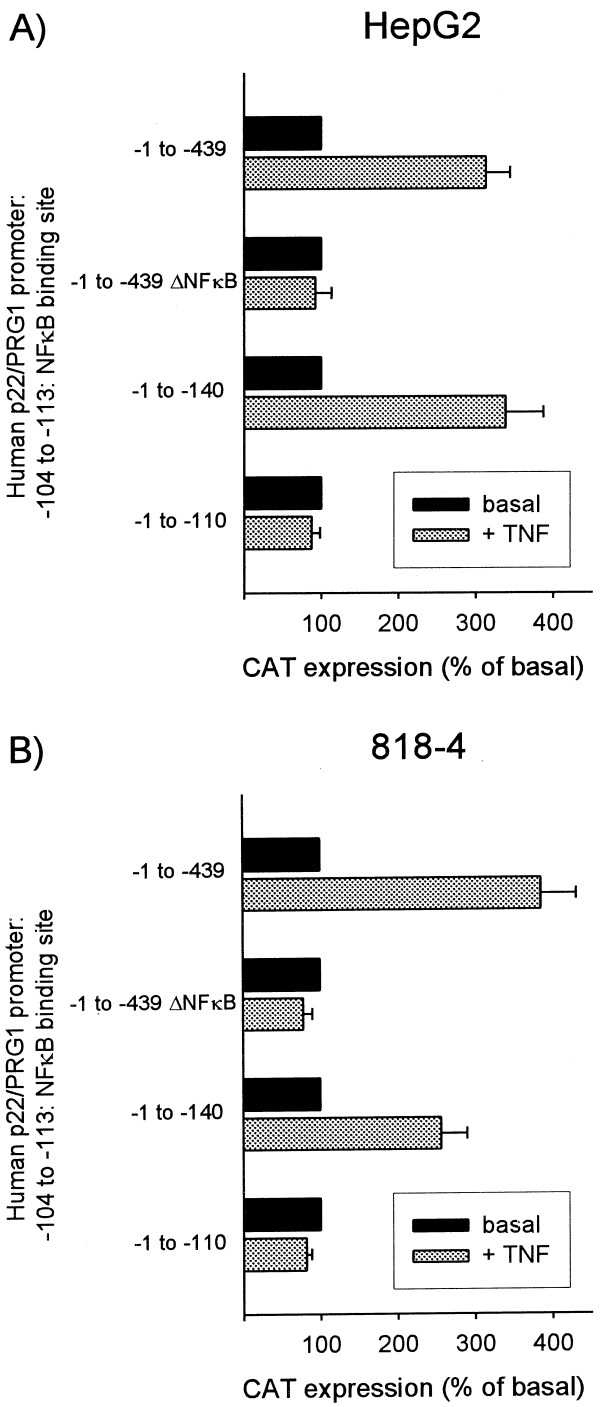

Fig. 3. Effect of TNFα on transcriptional activity of the p22/PRG1 promoter. HepG2 (A) and 818-4 (B) cells were transiently co-transfected with CAT reporter-gene vectors containing the depicted fragments of the human p22/PRG1 promoter and pCMVlacZ, as transfection control. Upon subsequent stimulation with 0.2 nM TNFα for 6–8 h, CAT expression was quantified by a CAT-ELISA and normalized to β-galactosidase activity. Basal activity was determined in cells treated with PBS (mean ± S.D., $n = 4$).

To test the potential of the NFκB and p53 binding sites to drive transactivation of the human p22/PRG1 promoter, CAT reporter gene assays with various modified promoter fragments of human p22/PRG1 were performed. As shown in Fig. 3A and B, fragments of the human p22/PRG1 promoter containing the NFκB binding site mediate strong transcriptional induction (>300%) of the CAT gene in transiently transfected HepG2 or 818-4 cells in response to TNFα. This TNFα-dependent increase of CAT expression was not observed in cells transfected with those CAT-reporter gene constructs lacking the NFκB binding site. Instead, in these transfectants CAT expression was slightly decreased (up to 20%) by TNFα.

A temperature-dependent increase of CAT expression was observed in Hep3B cells stably transfected with the (Val^{135})p53, an inactive temperature-sensitive mutant p53. At the permissive temperature of 32°C promoting formation of a wild-type like configuration of (Val^{135})p53, CAT expression was elevated (3–4-fold) in cells transfected with p22/PRG1-promoter constructs containing the p53-binding site (Fig. 4A), but not in cells transfected with constructs lacking this site by either deletion or by 5′-truncation. In contrast, no temperature-dependent and site-restricted increase of CAT expression occurred in p53-deficient Hep3B cells. In these cells, CAT levels decreased at the permissive temperature. Transient cotransfection of Hep3B cells with CAT promoter constructs and a CMV promoter driven expression vector for wild-type p53 [15] led to a p53-dependent increase of CAT expression (6–10-fold) only in cells receiving promoter constructs containing the p53 binding site, but not in transfectants lacking this site (Fig. 4B). In contrast, cotransfection with an expression plasmid for mutant p53 (pCMV-c5p53) resulted in no site-restricted increase of CAT expression.

4. Discussion

Similar to the rat p22/PRG1 gene, the promoter of human p22/PRG1 contains highly conserved binding sites for NFκB and p53 at almost identical positions. This high degree of conservation that can be extended to the murine homologue gly96 [2] indicates an essential role of these promoter elements. As expected from the high identity with the corresponding consensus sequences [8,9,16], we could demonstrate that NFκB and p53 both specifically interact with the human p22/PRG1 promoter in vitro and in vivo. In accordance with our recent finding that p22/PRG1 expression is induced by TNFα in pancreatic carcinoma cells (Schäfer H. et al., manuscript in preparation), recruitment of NFκB by TNFα in HepG2 and 818-4 cells was followed by activation of the human p22/PRG1 promoter. This activation clearly depends on the presence of the NFκB binding site since no induction of CAT expression in response to TNFα was observed when this site was deleted from the p22/PRG1 promoter. Therefore, it can be assumed that p22/PRG1 is involved in biological actions of TNFα mediated via NFκB. These actions include anti-apoptotic survival functions that initiate at the TNFR-1 receptor and its interaction with TRAF-2 [17]. Similar to the stress response (UV-light, γ-irradiation) [3] that also involves NFκB activation, recruitment of p22/PRG1 by TNFα may contribute to a cellular survival and regeneration program.

Another even more meaningful finding of this study is the presence of a functional binding site for the tumor suppressor p53 in the promoter of human p22/PRG1. By demonstrating binding of wild-type p53 to the p53 binding site in vitro as well as p53-dependent transactivation via this site, we provide now first evidence that p22/PRG1 may be a target gene for p53, as already shown for rat p22/PRG1 [5]. In its predom-

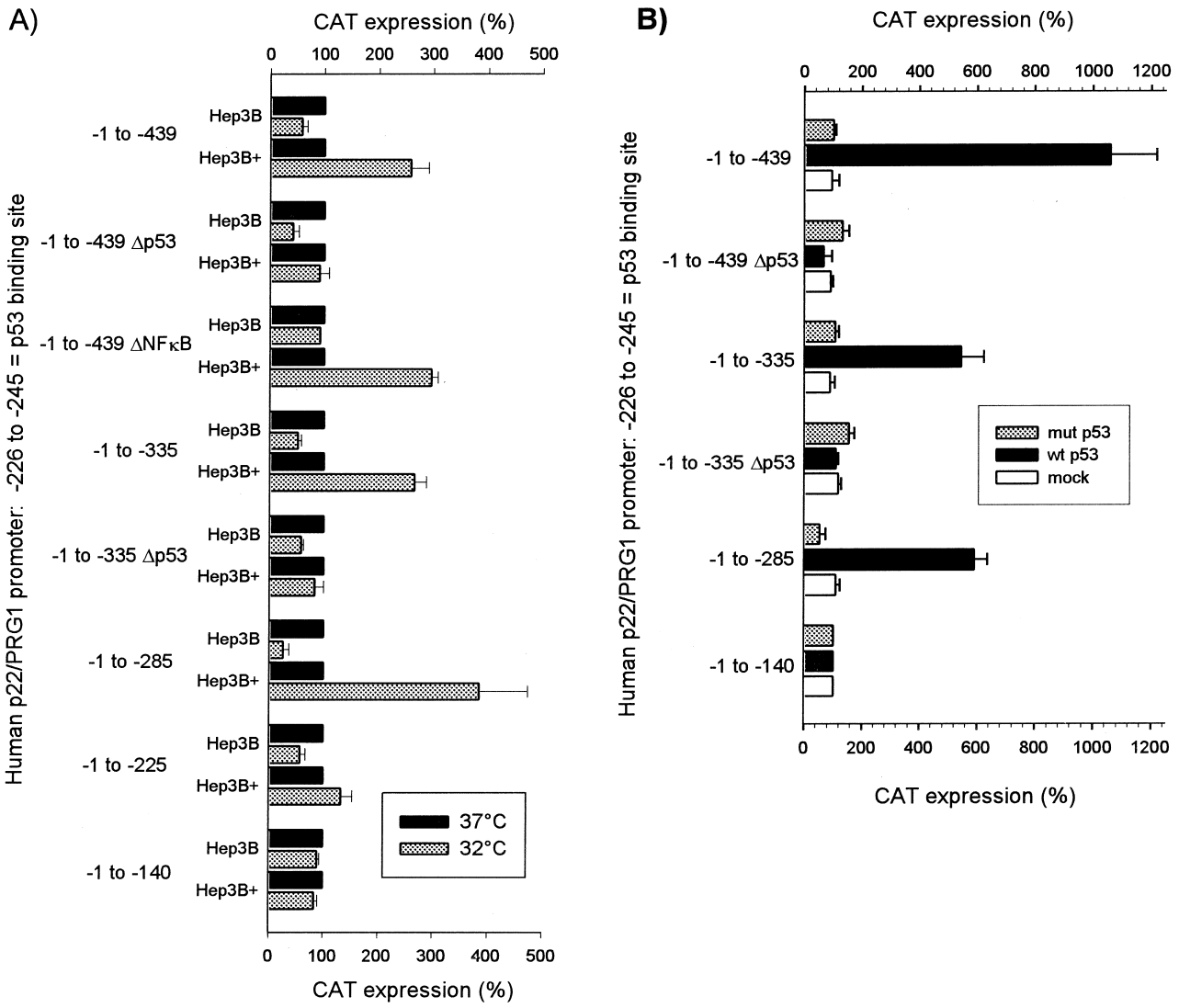

Fig. 4. Effect of p53 on transcriptional activity of the p22/PRG1 promoter. A: The depicted CAT reporter gene constructs were transiently co-transfected with pCMV-lacZ in Hep3B cells or in Hep3B+ cells expressing (Val^{135})p53. After overnight culture at 37°C, cells were further incubated at 37°C or at 32°C for 24 h. CAT expression was determined as described in B (mean ± S.D., $n = 4$). B: Hep3B cells were transiently co-transfected with 0.1 μg of pCMV-p53 or pCMV-C5p53 plus 1.5 μg of various CAT reporter gene constructs of the human p22/PRG1 promoter and 0.5 μg pCMV-lacZ as control. Basal activity was determined by cotransfection without p53 expression vectors, CAT expression was quantified by a CAT-ELISA and normalized to β-galactosidase activity (mean ± S.D., $n = 4$).

inant function as transcription factor [18], p53 recruits genes that are involved in growth arrest and DNA-repair or in apoptosis [19]. The actions of these genes interrupt division of DNA damaged cells or even eliminate them by initiation of the cellular suicide program. This is to prevent propagation of potentially mutated cells and the lack of such a p53-dependent growth control is a major cause of cancer. The recruitment of p22/PRG1 by p53 may be related to another mode of p53-dependent growth regulation [20,21] in that cells recovered from DNA damage are allowed to reenter the cell cycle and thus to survive.

Acknowledgements: The authors wish to thank Prof. Wolfgang Deppert and Prof. Peter Galle for kindly providing cell lines, recombinant protein and expression vectors. The authors also thank Anke Janssen for excellent technical assistance. This work is supported by a grant of the Deutsche Forschungsgemeinschaft (DFG Schm 805/4-2) and the IZKF in Kiel.

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