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Abrogation of p53-induced Apoptosis by the Hepatitis B Virus X Gene[1]

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Abstract

The p53 tumor suppressor gene product is a transcriptional transactivator and a potent apoptotic inducer. The fact that many of the DNA tumor virus oncoproteins bind to p53 and affect these p53 functions indicates that this interaction is an important step in oncogenic transformation. We and others have recently demonstrated that the hepatitis B virus oncoprotein, HBx, can form a complex with p53 and inhibit its DNA consensus sequence binding and transcriptional transactivator activity. Using a microinjection technique, we report here that HBx efficiently blocks p53-mediated apoptosis and describe the results of studies exploring two possible mechanisms of HBx action. First, inhibition of apoptosis may be a consequence of the failure of p53, in the presence of HBx, to upregulate genes, such as *p21*^{WAF1}, Bax, or *Fas*, that are involved in the apoptotic pathway. Data consistent with this hypothesis include HBx reduction of p53-mediated p21^{WAF1} expression. Alternatively, HBx could affect p53 binding to the TFIIH transcription-nucleotide excision repair complex as HBx binds to the COOH terminus of p53 and inhibits its binding to XPB or XPD. Binding of p53 to these constituents of the core TFIIH is a process that may be involved in apoptosis. Because the *HBx* gene is frequently integrated into the genome of hepatocellular carcinoma cells, inhibition of p53-mediated apoptosis by HBx may provide a clonal selective advantage for hepatocytes expressing this integrated viral gene during the early stages of human liver carcinogenesis.

Introduction

The p53 tumor suppressor gene product is involved in the maintenance of genomic integrity (1). Various DNA tumor viruses encode transforming oncoproteins that interact with p53. These include T antigen from SV40 (2, 3), E6 from oncogenic HPV[3] (4), E1B M_r 55,000 protein from adenovirus (5), EBNA-5 from EBV (6), IE84 from human CMV (7), and HBx from HBV (8–10). Among them, T antigen from SV40 and E1B M_r 55,000 proteins have been shown to inhibit p53-dependent programmed cell death (apoptosis; Refs. 11 and 12), an intrinsic cellular process essential for maintaining tissue homeostasis (13, 14). In addition, IE84 was also shown to abolish p53 transcriptional activation, which may lead to coronary restenosis (7). *p53* is a potent inducer of apoptosis (15, 16), which contributes to its function as a tumor suppressor gene (17–19). Disruption of p53-mediated apoptosis by viral oncoproteins may be an important step in carcinogenesis.

HBV is a major risk factor associated with hepatocarcinogenesis (20). HBx, an oncogenic protein encoded by HBV, can transactivate cellular genes (21); activate the protein kinase C pathway (22, 23); activate the ras, raf, and MAP kinase signaling cascade (24); neoplastically transform rodent cells *in vitro* (25–27) and, as a transgene, induce hepatocellular carcinoma in mice (10, 28). The *HBx* gene is frequently integrated into the genome (29) and expressed (30) in hepatocellular carcinoma from the geographic areas of high cancer incidence. Human hepatocellular carcinoma, which accounts for over 90% of primary liver cancers, can progress through inactivation of the *p53* gene via mutations (31–33) and/or interaction with HBx (8–10). We, and others, have recently shown that HBx can bind to p53 *in vivo* and *in vitro* (8–10); inhibit p53 sequence-specific DNA binding and transcriptional activation; prevent p53 binding to the transcription-repair factor, XPB (9, 34); and prevent p53 entry into the nucleus (10). To further explore the oncogenic mechanism of HBx, including the hypothesis that HBx can block the induction of p53-mediated apoptosis, we examined the physical and functional interactions between p53 and HBx in the p53-mediated apoptotic pathway.

Materials and Methods

Cell Cultures and Microinjection. Cell strains, culture conditions, and microinjection were essentially as described (35). Primary human fibroblasts were grown in Ham's F-10 medium supplemented with 10% fetal bovine serum. In microinjection experiments, primary fused and unfused fibroblasts were used. In some experiments, cells were fused by β-propiolactone-inactivated Sendai virus, seeded onto a coverslip, and incubated for an additional 2–3 days before injection. Plasmid cDNA, at 100 μg/ml in PBS, was injected into the nuclei of cells by using a glass microcapillary. For each experiment, at least 50 cells were injected. Before fixation, cells were incubated for an additional 24 h to allow expression of introduced genes. In comparing the effects of p53 alone to HBx plus p53, microinjections were performed on the same slide to minimize variations due to differences in immunostaining or processing of the slides, etc.

Immunohistochemical Analysis of p53 and p21^{WAF1}. Cells were fixed in 2% paraformaldehyde (in PBS), followed by methanol treatment. p53 was visualized by staining with anti-p53 polyclonal CM-1 antibody (1:200 dilution; Signet Laboratories, Dedham, MA), followed by FITC-conjugated antirabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA). Nuclei were stained with 4′,6-diamidino-2-phenylindole. For simultaneous demonstration of p53 and p21^{WAF1} expression, cells were double-labeled with CM-1 antibody (rabbit IgG) and monoclonal anti-p21^{WAF1} antibody (mouse IgG; Oncogene Science, Uniondale, NY), followed by FITC-conjugated antirabbit immunoglobulin antibody and Texas red-conjugated antimouse immunoglobulin antibody (Vector Laboratories).

***In Vitro* Protein-binding Assay.** Expression and purification of recombinant proteins, as well as *in vitro* protein binding and analyses of protein complexes, have been described previously (9). GST fusion proteins were produced in *Escherichia coli* and purified on glutathione-Sepharose 4B beads according to the manufacturer's instructions (Pharmacia LKB, Piscataway, NJ). Protein concentrations were estimated by Coomassie blue staining of SDS-PAGE and by comparison to molecular weight standards (Bio-Rad, Hercules, CA) run on the same gel. pZAP10 encodes an XPB cDNA and was used for *in vitro* synthesis of XPB protein. pGEM3zf(+)T7XPD was used for

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[3] The abbreviations used are: HPV, human papillomavirus; CMV, cytomegalovirus; HBV, hepatitis B virus; wt, wild type.

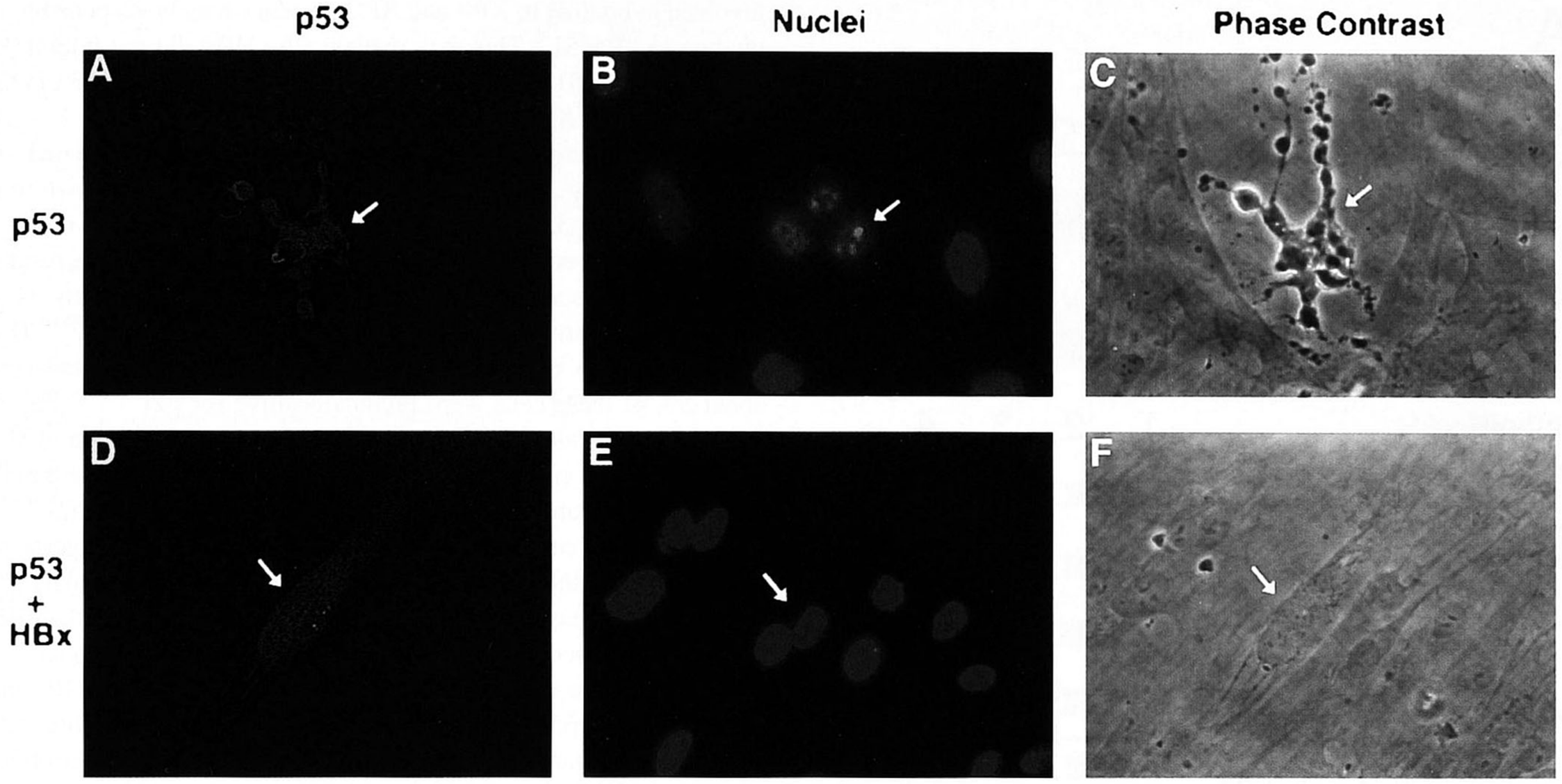

Fig. 1. Inhibition of p53-dependent apoptosis by the hepatitis B viral *X* gene. Induction of apoptosis in normal primary human fibroblasts was achieved by microinjection of the wt p53 expression vector. Cells were injected with wt p53 expression vector alone *(A–C)* or coinjected with wt p53 and the *HBx* gene *(D–F)*, followed by incubation for 24 h before fixation. The p53 protein was stained with CM-1 antibody (*A* and *D*). Nuclei were stained by 4′,6-diamidino-2-phenylindole (*B* and *E*). The apoptotic phenotype was monitored by morphology visualized under phase-contrast microscopy (*C* and *F*). *Arrows,* cells expressing p53 after microinjection.

in vitro synthesis of XPD protein. pCRII-HsRad51 was used for *in vitro* synthesis of hRad51 protein. pSPX46 was used for *in vitro* synthesis of HBx protein. To label the *in vitro* synthesized proteins, the corresponding plasmids were incubated at room temperature for 90 min with [^{35}S]cysteine (DuPont, Boston, MA) in a one-step *in vitro* transcription and translation system (Promega, Madison, WI). Binding assays were done in 500 μl IP buffer [50 mM Tris-HCl (pH 8.0)-120 mM NaCl-0.5% Nonidet P40] by incubating the glutathione-Sepharose 4B bead-bound GST fusion proteins with the ^{35}S-labeled, *in vitro* translated proteins for 60 min at room temperature. After washing with IP buffer, the bound proteins were released by boiling in Laemmli buffer for 5 min, separated by SDS-PAGE, and visualized by fluorography.

Results and Discussion

Apoptosis was examined by using a microinjection technique to introduce CMV-driven p53 expression vector into the nucleus of normal human primary fibroblasts. Injection of the wt *p53* gene resulted in elevated p53 levels, accumulating predominantly in the nucleus (32%) or in both the nucleus and cytoplasm (68%; Fig. 1*A* and Table 1). No cytoplasm-only staining of p53 was observed (Table 1). p53 was virtually undetectable by immunohistochemistry in uninjected cells (Fig. 1*A*). Of the p53-immunopositive cells identified after a 24-h incubation, 19% exhibited the characteristic features of apoptosis (chromatin condensation, nuclear fragmentation, and apoptotic bodies; Fig. 1, *B* and *C*, and Table 1). p53-induced apoptosis of normal primary human fibroblasts was wt-specific because p53 mutants (143^{ala}, 175^{his}, 248^{trp}, and 249^{ser}) failed to induce apoptosis and, in fact, blocked wt-induced apoptosis, although the mutant protein levels were equal to or exceeding that obtained with wt p53.[4]

We then coinjected wt *p53* and *HBx* genes (both CMV promoter driven) into the nuclei of primary human fibroblasts. Elevated levels of p53 were observed 24 h after coinjection, comparable to the amount seen with wt alone, when both microinjections were performed on the same slide (Fig. 1*D*), indicating that HBx did not influence the expression of exogenous *p53* gene. However, the addition of HBx resulted in only 2% of the p53 immunopositive cells displaying the apoptotic phenotype (Table 1), in contrast to the 19% seen with wt p53 alone. It was of interest that coinjection of HBx and p53 resulted in an increased percentage of cells with p53 accumulation in the nucleus-only cells (81%), as compared to 32% when p53 alone was microinjected. Similar results were obtained in normal primary human fibroblasts from a different donor (GM07532; data not shown). Therefore, we conclude that HBx can efficiently block p53-mediated apoptosis in primary human fibroblasts.

We recently observed that p53-induced apoptosis involves the TFIIH-associated factors XPB and XPD (34).[4] Therefore, we examined whether HBx may prevent p53-induced apoptosis by inhibiting binding of p53 to the XP proteins. Our approach involved an *in vitro* binding/competition assay between a GST-p53 fusion protein and *in vitro* translated XPB, XPD, or HBx proteins (Fig. 2). Although HBx itself did not bind to XPB protein *in vitro* (data not shown), it prevented the binding of p53 to both XPB and XPD proteins (Fig. 2, *A* and *B*). This inhibition was specific to XPB and XPD because HBx did not inhibit p53 binding to hRAD51 (data not shown), a human RecA-like protein that may be involved in

Table 1 *Inhibition of the p53-dependent apoptosis by the hepatitis B viral X gene*

Microinjected expression vector	Percent apoptotic cells[a] (n[b])	p53 localization (% of cells)		
		Nucleus only	Cytoplasm only	Both
wt p53	19 (59)	32	0	68
wt p53 and HBx	2[c] (52)	81	0	19

[a] Cells with condensed and fragmented nuclei as well as cytoplasmic blebbing characteristic of cells undergoing apoptosis.

[b] n, number of p53 immunopositive cells scored following microinjection of the p53 expression vector.

[c] χ^2 test compared to wt p53 alone ($P < 0.002$).

[4] Submitted for publication.

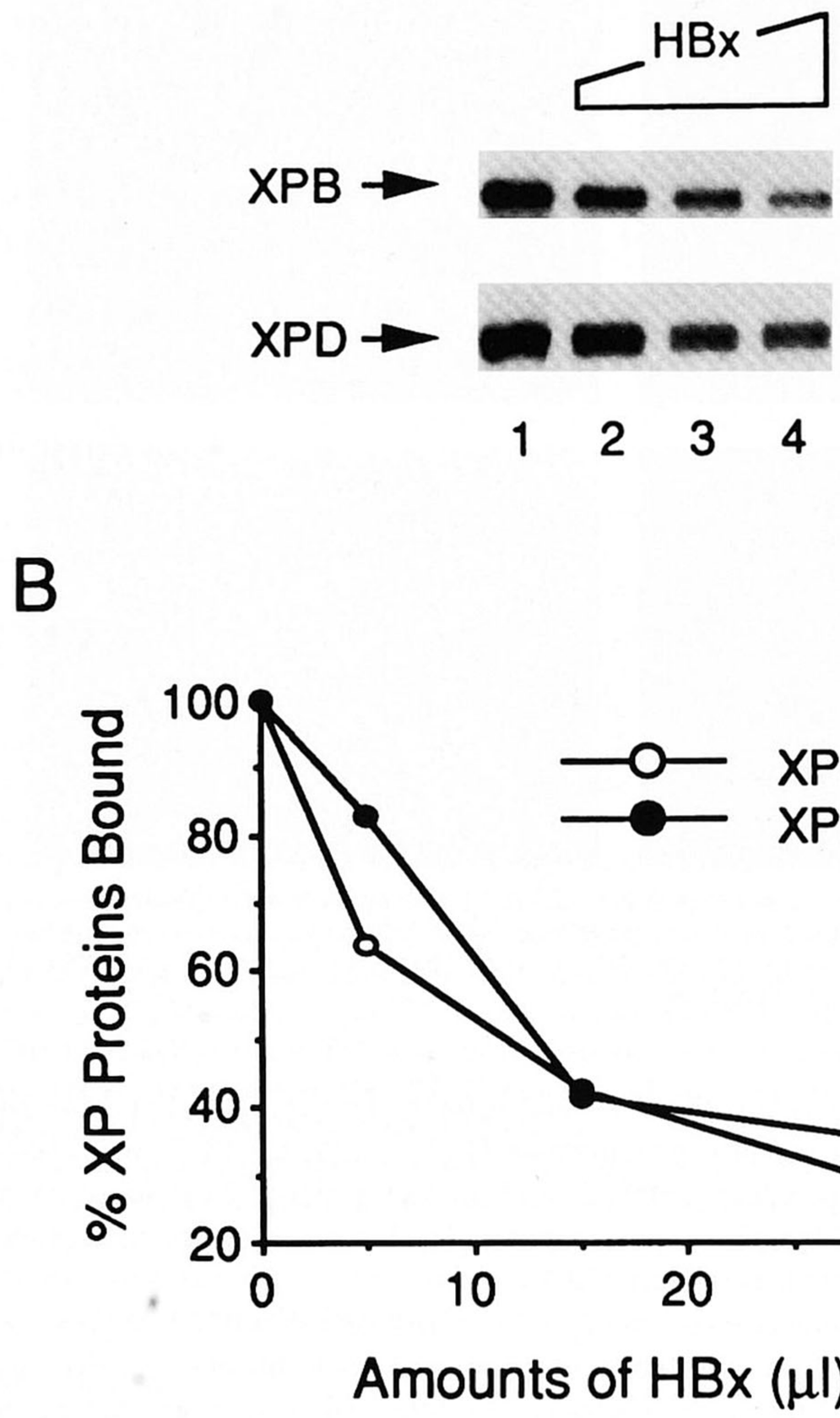

Fig. 2. HBx inhibits p53 binding to XPB and XPD. *In vitro* binding between p53 and XPB or XPD protein was measured using *in vitro* translation and *E. coli*-produced GST-fusion protein systems. *A*, 5 μl of ^{35}S-labeled XPB or XPD was mixed with glutathione-Sepharose beads loaded with 2 μg of the GSTp53 fusion protein in the absence *(Lane 1)* or presence of increasing amounts of *in vitro* translated HBx protein *(Lanes 2–4)*. Beads were washed with IP buffer, and XPB or XPD proteins that remained bound were analyzed by SDS/PAGE. *B*, densitometric analysis of data from *A*.

DNA recombination (36). hRAD51 was recently shown to specifically associate with p53 *in vitro*.[5,6]

Since the COOH-terminal domain of p53 (between residues 293 and 393) is responsible for the interaction with XPB and XPD (34), we mapped the region of p53 required for binding to HBx. Mutant p53 proteins with deletions at the COOH terminus (residues 293–393) completely lost the ability to bind to HBx, whereas p53 mutants with deletions at the NH_2 terminus (residues 1–94) retained this ability (Fig. 3). Further deletion of up to 154 N-terminal residues containing part of the DNA binding domain exhibited reduced binding to HBx. These mapping data indicate that the C-terminal domain (CTD) of p53, which is required for binding to the XP proteins, is also involved in the interaction with HBx. However, the N-terminal domain of p53 (1–94), a region possessing the transactivation domain and targeted by the viral oncoproteins E1B and E6 (4,5,37), does not interact with HBx. Additional data showed that p53 domains, other than the CTD involved in binding to XPB and XPD proteins, may be responsible for binding to hRad51.[6] This may explain why HBx did not inhibit p53 binding to hRad51. It is likely that HBx protein prevents p53 binding to the XPB and XPD proteins by masking the p53 CTD.

We then examined whether HBx can block p53-mediated up-regulation of target genes, such as *p21^{WAF1}*, which was shown to be elevated during p53-mediated apoptosis (38). Microinjection of the wt p53 expression vector into normal primary human fibroblasts resulted in increase of both p53 and p21^{WAF1} protein levels. Sixty % of p53-immunopositive cells also exhibited expression of p21^{WAF1} (Fig. 4). Whereas the p53 protein was undetectable in uninjected cells, about 5% of these cells were immunopositive for p21^{WAF1} (Fig. 4), indicating p53-independent p21^{WAF1} expression. Coinjection of HBx and wt p53 into cells on the same slide that was injected in parallel with wt p53 alone resulted in a moderate decrease in p21^{WAF1}-immunopositive cells (about 40%; $P < 0.05$; Fig. 4). This suggests that HBx can reduce the expression of endogenous genes regulated by p53. This finding is consistent with our previous observation that HBx blocks p53 transcriptional activation of the PG13CAT reporter construct (9). Failure of p53 to transactivate in the presence of HBx may be the result of p53-DNA interactions by HBx (9). Therefore, HBx appears to abrogate two p53 functions, apoptosis and transcriptional transactivation, both of which may be important for its tumor suppressor function (17–19).

HBx has a less pronounced effect on p53-mediated transactivation than on p53 binding to the XP proteins tested and p53-mediated apoptosis (compare Table 1 to Figs. 2 and 4). Furthermore, microinjection of a CMV promoter-driven *p21^{WAF1}* gene into normal human fibroblasts did not result in apoptosis (data not shown). Although these results do not rule out the possibility that HBx inhibits p53-induced apoptosis by abrogating transactivation of other p53-regulated genes other than *p21^{WAF1}*, such as *GADD45*, *Bax*, and *Fas* (39–41), our data are consistent with recent findings that p53-mediated apoptosis does not require activation of downstream genes (42, 43),[4] and that p53-mediated transactivation is not required for its tumor suppressor function (44). Thus, we favor the model that HBx blocks p53-mediated apoptosis by disrupting its binding to the XPB/XPD complex, which may be important in p53-dependent apoptosis.

A recent report (45) indicated that the p53-mediated cellular response to DNA damage is normal in a hepatoblastoma cell line (HepG2) genetically engineered to produce replicative hepatitis B virus. Because HBx is likely to be expressed during HBV replication, the authors suggested that p53 may not be inactivated at the "physiological conditions" of their experiments. The explanation for the apparent discrepancy between this report and other recent data is not clear at the present time. One possibility could be the level of HBx expression at different conditions. In fact, HBV-related hepatocellular carcinoma cells do not have replicating form of the virus and instead have integrated DNA sequences including HBx (30, 46), which probably express a subset and different level of viral proteins when compared to the intact virus.

p53-mediated apoptosis is an important factor in maintaining tissue homeostasis and preventing neoplastic transformation (17–19). Inactivation of p53-mediated apoptosis by HBx could lead to the disruption of the normal cellular surveillance mechanism for removing damaged cells, which could result in the accumulation of genetically abnormal cells, from which clones with a survival advantage would be selected. Thus, reduction of p53-mediated apoptosis by HBx may contribute to the development of human hepatocellular carcinoma.

[5] Stürzbecher *et al.*, submitted for publication.
[6] M. K. Gibson *et al.*, manuscript in preparation.

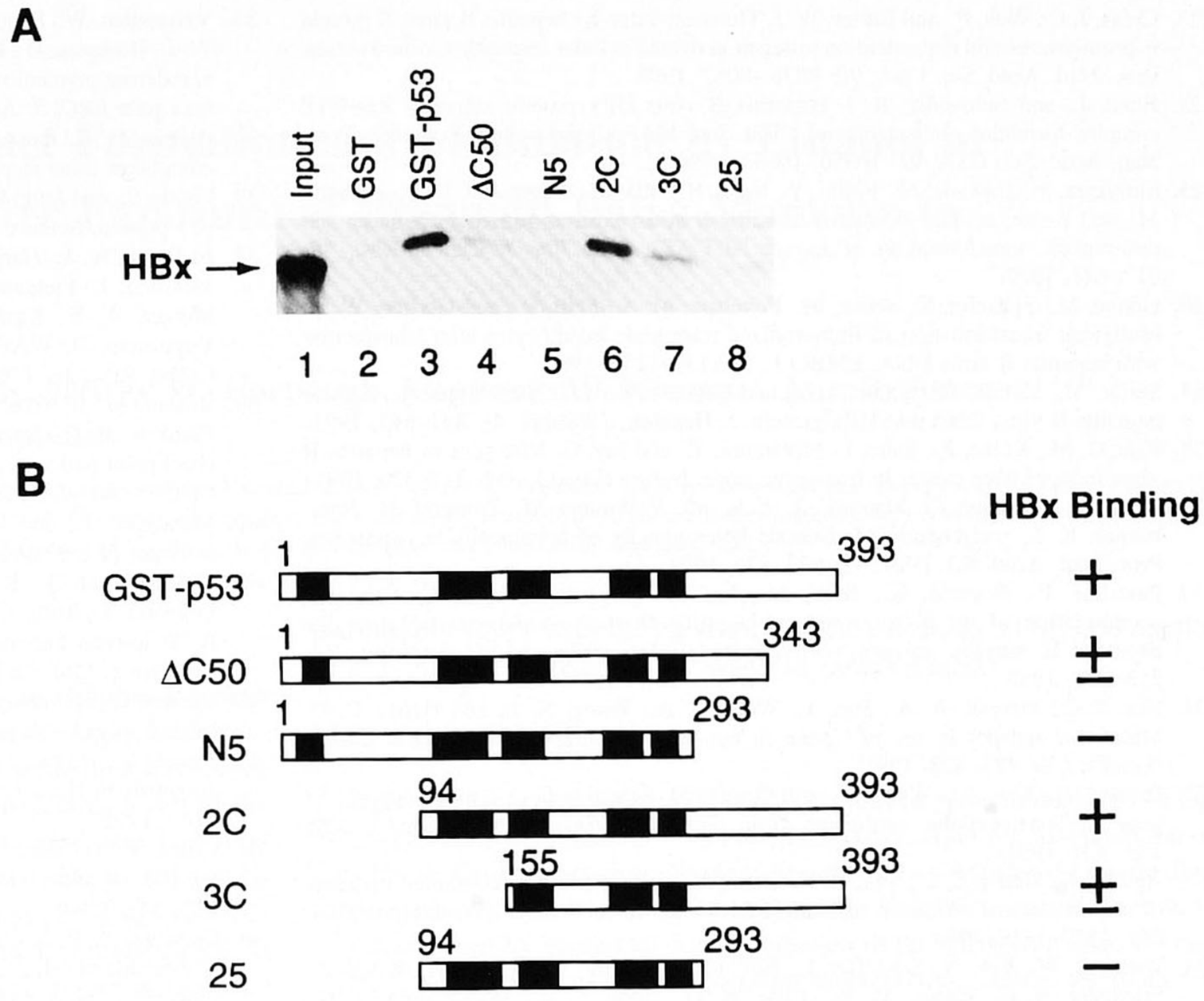

Fig. 3. HBx binds to carboxyl terminal domain of p53. *A*, *In vitro* translated HBx protein was incubated with glutathione-Sepharose beads loaded with either 4 μg of GST *(Lane 2)*, 2 μg each of GST-p53-wt *(Lane 3)*, or various GST-p53 deletion mutants *(Lanes 4–8)*. *Lane 1*, *in vitro* translated HBx, immunoprecipitated by anti-HBx antibody, was used as a reference. *B*, schematic representation of wt and deletion mutants of human p53 and a summary of their binding to the HBx protein. *ΔC50*, deletion of 50 residues at the COOH terminus of p53; *N5*, deletion of 100 residues at the COOH terminus of p53; *2C*, deletion of 94 residues at the NH_2 terminus of p53; *3C*, deletion of 155 residues at the NH_2 terminus of p53; *25*, deletion of 94 residues at the NH_2 terminus and 100 residues at the COOH terminus.

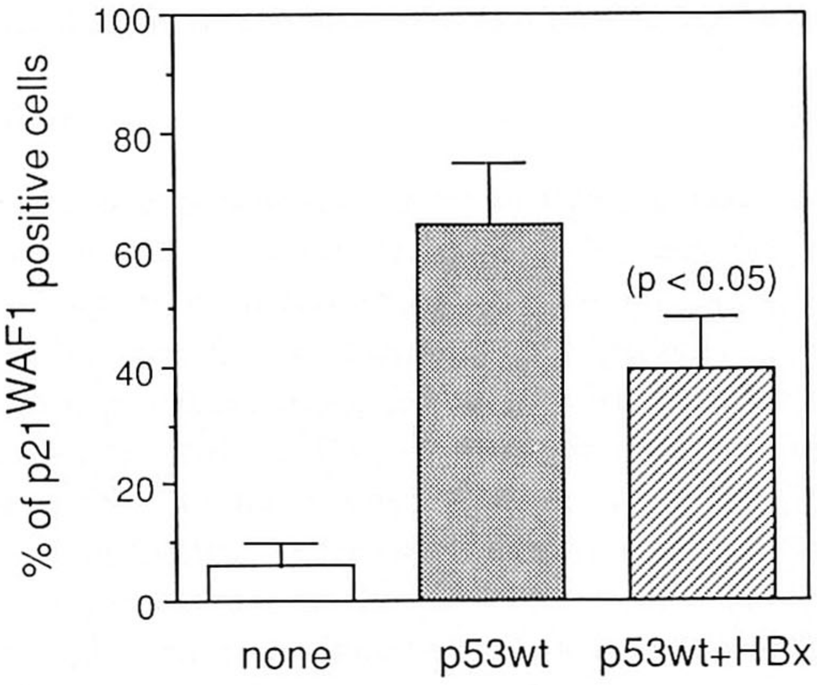

Fig. 4. Induction of endogenous $p21^{WAF1}$ gene expression in normal primary human fibroblasts given injections of wt p53 and HBx. Cells were given injections of the p53 expression vector alone (▒) or a combination of p53 plus HBx expression vectors (▨) and incubated for 24 h before fixation. $p21^{WAF1}$-immunopositive cells were expressed as a percentage of the $p21^{WAF1}$ staining cells over the p53-positive cells. At least 20 p53-positive cells were obtained from each microinjection experiment. *Columns*, average of three independent experiments; bars, SD. The p53 protein was normally undetectable in uninjected cells, and the $p21^{WAF1}$ protein was occasionally detected in uninjected cells; these were expressed as the background $p21^{WAF1}$ staining (□). A Student's *t* test was used to compare wt p53 plus HBx group to the wt p53 alone group, and a *P* value < 0.05 was obtained.

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