p53 binds and represses the HBV enhancer: an adjacent enhancer element can reverse the transcription effect of p53

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The transcription program of the hepatitis B virus (HBV) genome is regulated by an enhancer element that binds multiple ubiquitous and liver-enriched transcription activators. HBV transcription and replication are repressed in the presence of p53. Here we describe a novel molecular mechanism that is responsible for this repression. The p53 protein binds to a defined region within the HBV enhancer in a sequence-specific manner, and this, surprisingly, results in p53-dependent transcriptional repression in the context of the whole HBV enhancer. This unusual behavior of the HBV enhancer can be reconstituted by replacing its p53-binding region with the p53-binding domain of the *mdm2* promoter. Remarkably, mutation of the EP element of the enhancer reversed the effect of p53 from repression to transcriptional stimulation. Furthermore, EP-dependent modulation of p53 activity can be demonstrated in the context of the *mdm2* promoter, suggesting that EP is not only required but is also sufficient to convert p53 activity from positive to negative. Our results imply that the transcriptional effect of DNA-bound p53 can be dramatically modulated by the DNA context and by adjacent DNA–protein interactions.

Keywords: DNA damage/enhancer/HBV/*mdm2* promoter/p53 repression/transcription regulation

Introduction

Hepatitis B virus (HBV) is the prototype of the hepadnaviruses. It is a primarily hepatotropic, enveloped DNA virus with a very small genome that is replicated by reverse transcription. It has a unique mode of gene expression: the synthesis of all viral transcripts is regulated at the level of transcription, not by RNA processing. The viral genome contains an enhancer element that regulates viral gene expression in liver cells (Shaul *et al.*, 1985; Jameel and Siddiqui, 1986; Honigwachs *et al.*, 1989). Since RNA synthesis precedes viral replication, this enhancer has a key role in viral replication and life cycle. Hepadnaviruses are tightly associated with development of hepatocellular carcinoma (Beasley *et al.*, 1981) and at least in animals the homologous enhancer has a role in tumor formation (Wei *et al.*, 1992; Etienne *et al.*, 1994).

The HBV enhancer is divided into distinct functional elements (Dikstein *et al.*, 1990a). The E element has

intrinsic enhancer activity which works in isolation in a number of different cell lines (Faktor *et al.*, 1990). This element binds multiple factors of the basic leucine zipper family, including C/EBP (Dikstein *et al.*, 1990b), the AP-1 complex (Faktor *et al.*, 1990) and ATFs (Maguire *et al.*, 1991). A second important enhancer element, termed EF-C (Ostapchuk *et al.*, 1989) or EP (Ben-Levy *et al.*, 1989), has no intrinsic enhancer activity and in isolation is not functional as a positive element (Dikstein *et al.*, 1990a). Its exact role in the context of the enhancer is not yet known, but it appears to cooperate with either the E element (Dikstein *et al.*, 1990a) or the RXR/HNF4-binding element (Garcia *et al.*, 1993). Two distinct proteins bind the EP element: RFX1 (Siegrist *et al.*, 1993) and the proto-oncoprotein c-Abl, which has tyrosine kinase activity (Dikstein *et al.*, 1992). EP-associated c-Abl has strong kinase activity and EP-bound proteins are heavily tyrosine phosphorylated (Dikstein *et al.*, 1996). An interesting and open question is whether the ability of the EP element to cooperate in transcription activation is due to the kinase activity of c-Abl. NF1 is another ubiquitous cellular factor that binds the HBV enhancer at three sites (Ben-Levy *et al.*, 1989). One of these NF1 sites has an important role in enhancer activity, and mutation of this site decreases the production of the 3.5 kb viral pregenomic transcript (Ori and Shaul, 1995).

The p53 tumor suppressor gene product has a variety of transcription-regulatory activities (Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996). In a large body of independent studies, p53 has been shown to repress the activity of numerous viral and cellular promoters (Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991; Kley *et al.*, 1992; Moberg *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992). The emerging notion is that p53, a strong activator of transcription, has a general repressive effect on promoters that lack a specific p53-binding site, probably by sequestering components of the basal transcription machinery through protein–protein interactions (Seto *et al.*, 1992; Agoff *et al.*, 1993; Liu *et al.*, 1993; Martin *et al.*, 1993; Ragimov *et al.*, 1993; Lu and Levine, 1995; Thut *et al.*, 1995). It has been reported that p53 binds specifically viral DNA elements such as the GC boxes of the SV40 early promoter (Bargonetti *et al.*, 1991) and the Tax-responsive element of the HTLV I enhancer (Aoyama *et al.*, 1992). In the latter case, this interaction leads to activation of the viral promoter. The Tax-responsive element is similar in sequence to the E element of the HBV enhancer and these two elements respond to the Tax and X proteins in a similar manner (Faktor and Shaul, 1990). Therefore, we have investigated the effect of p53 on the HBV enhancer. We show here that p53 binds, sequence-specifically, to the 5' portion of the HBV enhancer and represses its activity. This unexpected p53-mediated repression activity is an intrinsic property

Fig. 1. p53 binds the HBV enhancer. (A) Autoradiograms of the 32 P-labeled DNA fragments separated on a polyacrylamide gel. The total end-labeled plasmid restriction digest, containing the vector DNA and the HBV insert, is shown in lanes 3 and 4. The DNA recovered after incubation with p53-containing nuclear extracts (lane 2) or recombinant p53 (lane 6) immune selection with the p53-specific antibody PAb421. M, DNA size marker (*MspI*-digested pBR322 DNA). (B) Schematic representation of the HBV enhancer region with the indicated functional motifs, and the sequence of *EcoRV*–*StuI* fragment. The TGCCT repeats found in certain p53-binding sites (Kern *et al.*, 1991) are indicated by horizontal bars. Stretches with homology to the 10 bp half-consensus (RRRCWWGYYY; El-Deiry *et al.*, 1992) for p53 binding are also indicated.

of the HBV enhancer and depends on an intact enhancer EP element. Furthermore, the EP element is sufficient to block the p53-mediated activation of the *mdm2* promoter. Collectively, our results demonstrate a previously unknown transcription regulatory activity of p53 and raise the interesting possibility that the transcriptional effect of p53 may be modulated through interaction with an adjacent enhancer element.

Results

p53 binds specifically to the HBV enhancer

To study whether there was any interaction between p53 and the HBV enhancer, an immune selection assay was performed (Zauberman *et al.*, 1993). A plasmid containing the complete HBV enhancer was digested with appropriate restriction enzymes, radiolabeled and incubated with a nuclear extract containing wild-type p53 activity. The extract was prepared from Clone 6 cells overexpressing a temperature-sensitive p53 mutant (Michalovitz *et al.*, 1990). The cells were maintained at 32°C before preparing the extracts, to induce the accumulation of p53 in a conformation like that of the wild-type. Resultant p53–DNA complexes were immunoprecipitated with the p53-specific monoclonal antibody PAb421 (Figure 1A). The HBV enhancer DNA fragment, but not the vector DNA, was specifically immunoprecipitated in complex with p53 (lane 2). A similar experiment was done with purified recombinant baculovirus-expressed p53 and a shorter HBV DNA fragment. Only the fragment containing the HBV enhancer sequence was immunoprecipitated (lane 6). No precipitated DNA fragment was obtained in the absence of recombinant baculovirus-expressed p53, suggesting that

the antibody itself has no DNA-binding activity. Thus, the enhancer binds p53 in a sequence-specific manner. Interestingly, the sequence near the 5' end of the HBV enhancer, between the *EcoRV* and *StuI* sites (Figure 1B), has a similar structure to the p53-binding region of the muscle creatine kinase promoter (Weintraub *et al.*, 1991), as it contains stretches of partial homology to the 10 bp half-consensus (El-Deiry *et al.*, 1992), as well as adjacent repeats of the TGCCT sequence (Kern *et al.*, 1991).

p53 binds the 5' portion of the HBV enhancer

An EMSA was used to map the p53-binding site within the enhancer. Binding was performed with recombinant baculovirus-expressed p53 in the presence of the p53-specific monoclonal antibody PAb421, which is known to improve and to supershift the binding of p53 to its cognate sites (Hupp *et al.*, 1993). The results (Figure 2) reveal that the complete HBV enhancer (nt 1043–1235) and the 5' portion of it (nt 1043–1115) bind p53 specifically, as confirmed by inclusion of specific and nonspecific competitors in the reactions (Figure 2A). The antibody itself has no DNA-binding activity (lane 9). The 3' *StuI*–*SphI* fragment (nt 1115–1235) did not generate a specific complex with p53 whereas the *EcoRV*–*StuI* fragment (nt 1043–1115) bound efficiently (2B). Thus, the HBV enhancer binds p53 specifically within the *EcoRV*–*StuI* region.

To delineate the site of interaction of p53 with the enhancer, DNase I footprinting either with extracts enriched for wild-type-like p53 DNA-binding activity or with recombinant baculovirus-expressed p53 was performed. A conspicuous protected region was visible in each case (Figure 3). The footprint is extended and is

Fig. 2. p53 specifically binds the 5' portion of the HBV enhancer. (A) The complete enhancer and the *EcoRV*–*StuI* fragments were 32 P-end-labeled and used as probes for EMSA with 50 ng recombinant baculovirus-expressed p53. Reactions were supplemented with the monoclonal antibody PAb421 to improve binding. When indicated, the following oligonucleotide competitors ('comp DNA') were included: TGT3, which binds the hepatocyte nuclear factor 3 protein; EP, which binds the RFX1–c-Abl complex; GLN LTR, a high affinity p53 binding element derived from an endogenous retrovirus-like element (Zauberman *et al.*, 1993). (B) Fragments of HBV enhancer were subjected to EMSA with or without the addition of PAb421 monoclonal antibody, to show that only the *EcoRV*–*StuI* fragment binds p53. The antibody that was used failed to supershift the complex effectively, because cognate peptide epitope was present in the recombinant p53 protein samples (Zauberman *et al.*, 1993). The probes used are shown in (C).

centered around the second stretch of homology to the 10 bp half-consensus (AAACAGGCTT; Figure 1B) which exhibits the highest match (9/10) to the half-consensus (El-Deiry *et al.*, 1992). The region at the 5' end containing the TGCCT repeats was only poorly protected (lanes 4 and 5, and data not shown).

p53 represses the transcriptional activity of the HBV enhancer/X promoter

To test whether the specific binding of p53 to the HBV enhancer has functional significance, the effect of p53 on the HBV enhancer and the X promoter was examined by cotransfection into p53-null cells. Since the HBV enhancer is active mostly in liver cells, the hepatoma cell line Hep3B was used. Unexpectedly, wild-type p53 significantly reduced the luciferase activity of the reporter containing HBV enhancer and X promoter (Figure 4A, columns 1–3 and 5–7). A mutant p53 had only a marginal effect (columns 4 and 8). A very similar dose-dependent repression pattern was observed in another p53-null cell line, Saos2 (columns 5–8). In contrast, the *mdm2* promoter (Juven *et al.*, 1993; Wu *et al.*, 1993; Barak *et al.*, 1994) was strongly activated by wild-type but not mutant p53 (Figure 4B). The activation of the *mdm2* reporter indicates that p53 was efficiently produced in the transfected cells. As often observed at high p53 concentrations, the activation of the *mdm2* reporter plasmid was reduced. Previously, p53 has been shown to repress an array of different promoters (Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991; Kley *et al.*, 1992; Moberg *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992). To check whether in the HBV context the negative effect of p53 is mediated by the X promoter, a reporter plasmid containing only that promoter (XpLuc; reporter II) was used. p53 had no significant effect on this

promoter under these experimental conditions (Figure 4A, columns 9–12). The HBV enhancer had a similar effect in the context of a heterologous thymidine kinase (TK) promoter, in both enhancer orientations (Figure 4C), suggesting that the X promoter is not essential for the transcriptional repression activity of p53. Interestingly, no repression by p53 was seen in the presence of a fragment of the enhancer retaining functional enhancer activity but lacking the *EcoRV*–*StuI* fragment (columns 13–16). This implies that p53 represses the HBV enhancer activity through the *EcoRV*–*StuI* fragment, presumably by a mechanism requiring specific binding of p53 to a cognate site within this fragment. This is an unexpected finding, since sequence-specific DNA binding by p53 has so far always been reported to activate transcription (Kastan *et al.*, 1991; Weintraub *et al.*, 1991; El-Deiry *et al.*, 1992; Zambetti *et al.*, 1992; Juven *et al.*, 1993; Wu *et al.*, 1993; Okamoto and Beach, 1994; Miyashita and Reed, 1995; Zauberman *et al.*, 1995).

The p53-binding regions of the HBV enhancer can confer transcriptional activation when uncoupled from the rest of the enhancer

To test whether the unexpected repression of the HBV enhancer by p53 resulted from an intrinsic inhibitory activity of the p53-binding *EcoRV*–*StuI* fragment, four tandem repeats of this fragment were cloned upstream of the X promoter to yield 4(R-S)XpLuc (Figure 5B, reporter I). Surprisingly, luciferase activity was now induced rather than repressed by the cotransfected wild-type p53 (Figure 5A). The X promoter itself was refractory to the effect of p53 (columns 9–12). Since by itself the HBV *EcoRV*–*StuI* fragment conferred transcriptional activation upon interaction with wild-type p53, the repression activity

Fig. 3. Definition of the p53-binding site within the HBV enhancer by DNase I footprinting. An end-labeled *EcoRV*–*HpaI* fragment from the HBV enhancer (nt 1043–1196; the *HpaI* site was artificially inserted) was incubated with either cellular extract of Clone 6 cells (32°C) or recombinant p53 and then subjected to DNase I footprinting followed by polyacrylamide–urea gel electrophoresis. The amount of protein per reaction is indicated. The protected sequence is indicated; two Maxam–Gilbert reactions are shown in lanes 1 and 2.

must be mediated by the minimal HBV enhancer (nt 1115–1235). To examine this possibility, a construct containing four *EcoRV*–*StuI* (R-S) repeats upstream of the minimal enhancer [4(R-S)EXpLuc] was used (Figure 5B, construct II). The behavior of this reporter was exactly opposite that of 4(R-S)XpLuc: it was repressed dose-dependently by cotransfected wild-type p53 (Figure 5A, columns 5–7). Thus, the minimal HBV enhancer region can convert p53 from a positive regulator of transcription into a specific negative one.

The p53-binding region of the *mdm2* gene represses the HBV enhancer in a p53-dependent manner

If the observed negative effect of p53 is indeed an intrinsic property of the HBV minimal enhancer, the enhancer may be expected to act similarly in the context of other p53-binding sites. To test this possibility, the well characterized p53 response element of the mouse *mdm2* gene (MPRE) was used. When inserted directly upstream of the X promoter, MPRE conferred a strong positive response to cotransfected wild-type p53 (Figure 6, columns 5–8), in line with its behavior in other contexts (Juven *et al.*, 1993; Wu *et al.*, 1993; Friedlander *et al.*, 1996). However, exactly the opposite behavior was observed in the presence of the HBV enhancer, where MPRE now mediated strong

transcriptional repression (columns 1–4). Repression was not seen in the context of the X promoter, either alone (columns 9–12) or together with the HBV minimal enhancer (column 13–16). These data strongly suggest that the HBV minimal enhancer converts the outcome of sequence-specific p53 binding from positive transactivation into transcriptional repression.

The EP element of the HBV enhancer is required to convert p53 into a transcriptional repressor

The HBV enhancer binds multiple cellular activators and has been divided into distinct functional elements (Shaul and Ben-Levy, 1987; Ben-Levy *et al.*, 1989; Patel *et al.*, 1989; Dikstein *et al.*, 1990a; Trujillo *et al.*, 1991; Ori and Shaul, 1995). To investigate which of these might be responsible for converting p53 into a transcriptional repressor, reporter plasmids carrying enhancer mutants under the regulation of the MPRE were used. Since this effect of the HBV enhancer is not cell-type-specific (Figure 4A, columns 1–8) the responsible element must interact with ubiquitous factors. The HBV enhancer contains two known elements which bind ubiquitous factors and have an important role in enhancer activity. One element binds the NF1 activator and the other generates a large DNA–protein complex termed EP (Ben-Levy *et al.*, 1989) or EF-C (Ostapchuk *et al.*, 1989). Two proteins are known to associate with the EP elements: RFX1, an evolutionarily conserved DNA-binding protein (Reith *et al.*, 1994), and c-Abl, a tyrosine kinase proto-oncogene (Dikstein *et al.*, 1992). Enhancer mutants that do not bind the corresponding EP and NF1 proteins were assayed for their p53 response. Remarkably, mutation of the EP site converted p53 into a positive activator, whereas mutation of the NF1 site had no effect on the p53-mediated, HBV enhancer-dependent transcriptional repression (Figure 7A, compare columns 9–12 and 13–16). These results indicate that the intact EP element of the HBV enhancer is required for converting p53 into a sequence-specific transcriptional repressor.

The EP element is sufficient to block p53-mediated transcriptional activation

To test whether the EP element by itself can block transcriptional activation by p53, we introduced four tandem copies of the EP element into the mouse *mdm2* promoter between the MPRE and the core promoter region (Juven *et al.*, 1993). Remarkably, in the presence of the EP element, p53 almost completely lost its capacity to stimulate the *mdm2* promoter (Figure 8, columns 1–4). This effect is specific, as a mutated EP sequence and an unrelated 5×Gal4 element did not behave in this way (Figure 8, columns 5–8 and 9–12 respectively). These data suggest that the HBV-enhancer EP element can antagonize the transcriptional activation function of p53, and is sufficient to render a p53-inducible promoter unresponsive to p53. In this particular context the presence of the four EP repeats did not turn p53 into a specific transcriptional repressor. This observation is addressed in the Discussion.

Repression of HBV gene expression by a p53 inducer DNA-damaging agent

The HBV enhancer regulates the production of all the viral transcripts. The finding that p53 represses enhancer

Fig. 4. Wild-type p53 specifically represses HBV enhancer activity in p53-null cell lines. Various combinations of luciferase reporters and p53 expression plasmids were transiently transfected into Hep3B or Saos2 cells. (A) The HBV enhancer in context of the X promoter. (B) Effect of p53 on the *mdm2* promoter. (C) Effect of p53 on the HBV enhancer activity in context of the TK promoter. In construct III the HBV enhancer was cloned in the opposite orientation. In all cases luciferase activity was determined 24 h later. The histogram represents the relative luciferase activity, where 100 is the basal activity without p53. Reporter plasmids (represented by roman numerals) and amounts of the wild-type and mutant p53 effector plasmids are indicated below. A schematic representation of each reporter plasmid is shown at the bottom. 'R-S' indicates the *EcoRV*-*StuI* fragment of the extended enhancer, containing the p53-binding site. 'En' indicates the *StuI*-*SphI* fragment containing the minimal HBV enhancer, lacking the p53-binding region. 'Xp', 'Md2p' and 'TKp' refer to the X (*MscI*-*NcoI* fragment) the mouse *mdm2* promoter and HSV TK gene promoters, respectively. In Hep3B cells deletion of the *EcoRV*-*StuI* fragment reduced the enhancer activity by 20%. The Xp reporter shows 10-fold lower activity than the enhancer-containing reporter.

Fig. 5. The HBV enhancer p53-binding element (R-S) responds to p53 in a context-dependent manner. Hep3B cells were transiently transfected by the combinations of indicated luciferase reporter and p53 expression plasmids. (A) Relative luciferase activity, where 100 is the basal activity without p53. The reporter plasmids (represented by roman numerals) and amounts of p53 effector plasmids are indicated below. (B) Schematic representation of the different reporters.

activity suggests that HBV gene expression and, hence, replication (since the pregenomic viral mRNA is the template for replication) should be repressed by overproduction of p53. HepG2 cells transfected with HBV DNA were treated with *cis*-platinum, a DNA-damaging agent that induces p53 overproduction (Fritsche *et al.*, 1993), and the viral transcripts were analyzed. HepG2 cells were used because they contain wild-type p53 and support HBV gene expression. A significant reduction in the level of the viral transcripts was obtained mainly in the treated cells (Figure 9). The effect of *cis*-platinum was even greater when the X open reading frame was knocked out (lanes 7–12). This observation is in agreement with a previous report that pX can block p53 activity (Lee *et al.*, 1995). Western analysis confirmed that p53 but not β -tubulin was overproduced by *cis*-platinum. These data suggest that physiological levels of p53 repress HBV gene expression.

Discussion

In this study we report that the p53 tumor suppressor protein binds the HBV enhancer and modulates its activity. The strategic position of the p53-binding region between the previously reported UE1 site (Shaul and Ben-Levy, 1987) and the minimal enhancer region, and its sequence conservation at the homologous position in other hepadnaviruses that infect mammals (data not shown), suggest a potential function in modulating the enhancer activity. The role of p53 was tested in two different human p53-null cell lines, the Hep3B hepatoma (Ponchel *et al.*, 1994) and the Saos2 osteosarcoma (Masuda *et al.*, 1987; Chen *et al.*, 1990). In both lines the p53 site repressed the

Fig. 6. The p53 responsive element of the *mdm2* gene (MPRE) confers p53-dependent repression in the context of the HBV enhancer. (A) Various combinations of luciferase reporters and p53 expression plasmids were transiently transfected into Hep3B cells. Basal activity in the absence of p53 was taken as 100. Reporter plasmids (represented by roman numerals) and amounts of p53 effector plasmids are indicated below. (B) Schematic representation of the different reporters.

Fig. 7. Transcriptional repression by wild-type p53 is dependent on an intact enhancer EP element. Details are as in Figure 5. The altered sequences of the EP^mEn and NF1b^mEn, bearing mutations at the EP and the NF1b sites, respectively, are boxed.

Fig. 8. The EP element blocks activation of the mouse *mdm2* promoter by p53. Details as in Figure 5. The black bar in (B) represents the core region of the mouse *mdm2* p53-dependent promoter. EP and EP^m are as defined in Figure 7. 'Gal4' is UAS^G, conferring specific binding of the yeast Gal4 transactivator.

enhancer activity and this repression depended on wild-type p53. It is well established that p53, a strong activator of transcription, has a general repressive effect on promoters that lack a corresponding binding site, probably by sequestering basal transcription factors through protein-protein interactions. To our knowledge this is the first example of p53 acting as a sequence-specific transcriptional repressor. It is unlikely that this is due to non-specific sequestration of transcription factors, for several reasons. First, we used very low amounts of transfected p53 plasmid to reduce the probability of such sequestration. Indeed, under these conditions the X and TK promoters were only marginally affected. Second, we showed that the repression activity is fully dependent on the p53-binding site; upon its removal, no p53 effect was retained despite the fact that the basal enhancer activity remained the same. Third, we demonstrated that this behavior can be reproduced with the heterologous p53-binding DNA element of the *mdm2* gene. Finally, and perhaps most significantly, the positive transcriptional effect of DNA-bound p53 could be restored by mutating the EP element of the HBV enhancer.

In the context of the HBV enhancer, p53 represses transcription in an EP-element-dependent manner. However, in the context of the *mdm2* promoter, this element blocks the positive action of p53 but does not repress promoter activity. A likely explanation is that, in the former case, p53 and EP block each other's activity (Figure 9). Under these conditions the major positive role that the EP-binding proteins play in cooperation with the

Northern Blotting

Western Blotting

Fig. 9. HBV gene expression is reduced in cells treated with *cis*-platinum. HepG2 cells were transfected with a plasmid that contains two tandem copies of HBV full-length DNA, either wild-type or a mutant with a stop codon at position 27 of the X gene open reading frame (X-HBV, lanes 7–12). Cells were treated with 2 μ g/ml *cis*-platinum for the indicated time (in hours) before harvesting. RNA and proteins were extracted and analyzed. A ³²P-labeled HBV DNA probe was used to detect the known viral transcripts and GAPDH probe to quantify the RNA in each lane. For Western analysis, anti human p53 (1801+DO-1) and anti- β -tubulin (clone no. TUB2.1, Sigma) antibodies were used.

E and the GB element-binding proteins is eliminated as a result of p53 binding. Consequently, the contribution of the EP element to the rate of transcription is totally abolished, allowing p53 binding to result in transcriptional repression. On the other hand, in the context described in Figure 8, EP has no positive contribution to the activity of the *mdm2* promoter. Hence, its inactivation by p53 binding is not expected to repress transcriptional. Indeed, despite the positive role played by the EP element in the context of the enhancer (Ostapchuk *et al.*, 1989; Dikstein *et al.*, 1990a), this element has no enhancer activity in isolation. Clearly, the EP element is also responsible for inactivating the transcriptional activation function of DNA-bound p53. How can the EP element modify the mode of action of the DNA-bound p53? Although not formally proven, it is very likely that this involves the activity of EP-associated proteins. Two proteins are known to associate with this element. RFX1 was reported to bind the HBV enhancer at the EP site and activate it (Siegrist *et al.*, 1993). However, this protein cannot activate an artificial enhancer made of multimers of the EP element (Katan *et al.*, 1997). Accordingly, it has been reported that EP functions only in cooperation with other viral enhancer elements (Dikstein *et al.*, 1990a; Garcia *et al.*, 1993). Thus, RFX1 is not a conventional activator and

Fig. 10. A model to explain the role of the EP element in p53-mediated transcriptional repression by the HBV enhancer. The distinct enhancer elements are shown. R-S is the p53-binding region, and GB, EP and E are the HNF4/RXR, RFX1/c-Abl and basic luciferase zipper binding sites, respectively. (A) In the absence of p53 the EP element cooperates with the E and GB elements to mediate transcriptional activation (Dikstein *et al.*, 1990a; Garcia *et al.*, 1993). (B) In the presence of p53, an interfering interaction is generated between p53 and the EP-binding proteins. This interaction blocks the positive activity of p53, and simultaneously eliminates the cooperation between EP and other enhancer elements.

functions in a context-dependent manner. It contains both activation and repression domains which may allow it to act as a dual-function regulator (Katan *et al.*, 1997). It is therefore possible that, in the presence of p53, RFX1 might acquire a negative role. Indeed, cotransfected RFX1 increases the p53-mediated repression of M-EP4-Luc reporter plasmid (data not shown). The second EP-associated protein is the proto-oncoprotein c-Abl (Dikstein *et al.*, 1992), which interacts directly with RFX1 (Agami and Shaul, 1998). This protein has tyrosine kinase activity, and EP-associated proteins have been reported to be tyrosine phosphorylated (Dikstein *et al.*, 1996). c-Abl can stimulate the transcription of p53 target genes and this does not require tyrosine kinase activity but does require p53 binding (Yuan *et al.*, 1996). The fact that the kinase domain of c-Abl is dispensable for this activation suggests that the ability of c-Abl to phosphorylate Pol II C-terminal domain (Duyster *et al.*, 1995) is not important in this process. Preliminary results suggest that cotransfected c-Abl can partially decrease the p53-mediated repression activity (data not shown); further experiments are required to evaluate this possibility.

Although we have not directly measured the affinity of p53 for HBV DNA, results from EMSA and competition experiments indicate that it is weaker than that for the *mdm2* site (data not shown). Hence, efficient binding to this element may require relatively high concentrations of p53, and repression of the enhancer by p53 should be relevant only under conditions that increase the cellular level of p53. This is often the situation in cells exposed to various types of stress, in particular those that lead to DNA damage (reviewed in Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996). We show here that under such stress conditions, HBV gene

expression is indeed sharply reduced. How inactivation of viral gene expression by p53 might serve the viral needs is unknown.

The observation that the transcriptional effect of DNA-bound p53 is context-dependent may also have implications for the function of p53. At present we do not know whether an EP-like element is present in any potential p53 target gene. Based on the molecular scenario described here, such elements may be anticipated in promoters of genes whose activity is specifically down-regulated by p53 (Murphy *et al.*, 1996). No such cellular genes, subject to sequence-specific transcriptional repression by p53, have yet been found.

Materials and methods

Plasmids

EXpLuc was constructed by inserting the HBV *StuI*-*NcoI* fragment (nt 1115–1373), containing the minimal enhancer X promoter, between the *SmaI* and *NcoI* sites of the plasmid pGL3-basic (Promega). (R-S)EXpLuc was prepared by inserting the HBV *EcoRV*-*NcoI* fragment (nt 1043–1373) into the same vector. XpLuc, which includes the HBV X promoter, was constructed by inserting the HBV *MscI*-*NcoI* fragment (nt 1217–1373) between the *SmaI* and *NcoI* sites of pGL3-basic. 4(R-S)XpLuc was prepared by inserting four repeats of the *EcoRV*-*StuI* fragment in a head-to-tail orientation upstream of the X promoter in the XpLuc reporter. Md2pLuc was prepared by inserting the *ApaI*-*NsiI* fragment from md2.9CAT0 (Juven *et al.*, 1993) into the *SmaI* site of pGL3. MPRE-XpLuc was constructed by subcloning the *ApaI*-*PvuII* fragment from Md2pLuc and inserting it upstream of the X promoter in the XpLuc reporter. This fragment, containing the p53 response element of the *mdm2* promoter, was inserted upstream of EXpLuc to construct MPRE-EXpLuc. To construct TK-based reporter plasmids, the HBV fragment was cloned at the *HindIII* site of the TK promoter. The enhancer mutant reporters (MPRE-NF1tm-EXpLuc and MPRE-EPtm-EXpLuc) were prepared by replacing the wild-type enhancer fragments with the mutated counterparts that had been previously generated (Dikstein *et al.*, 1990a). The reporters, M-EP4-luc, M-EP3tm-Luc and M-G5-Luc, contain respectively: four tandem repeats of EP element (5'-CCCCGTGCTGG-GCAACGGCC-3'), three tandem repeats of an EP-related sequence that does not bind the EP proteins and is referred to as a mutant (5'-CAAGTGTGCTGACGCAACCCCCAC-3') and five tandem repeats of the yeast Gal4-binding element (5'-GGAGGACAGTACTCCGC-3'). They were prepared by cloning these repeats into the *PvuII* site of the *mdm2* promoter, which resides between the p53 response elements and the basal promoter. The effector plasmids pCMVp53^{wt} and pCMVp53tm (Eliyahu *et al.*, 1989; Shaulian *et al.*, 1992), express the corresponding mouse polypeptides under the transcriptional control of the cytomegalovirus immediate early enhancer-promoter. The mutant p53 encoded by pCMVp53tm bears point mutations at positions 168 and 234 within the DNA-binding domain of the protein.

Cell extracts and protein preparation

Nuclear extracts and recombinant baculovirus-expressed p53 were prepared as previously described (Zauberger *et al.*, 1993). Nuclear extract was prepared from cells of Clone 6, overexpressing the temperature sensitive mouse p53 mutant p53Val135 (Michalovitz *et al.*, 1990). The cells were maintained at 32°C for 24 h before preparing the extracts, to induce the accumulation of wild-type-like p53 protein.

Protein-DNA interaction assays

For each electrophoretic mobility shift assay (EMSA), 50 ng of recombinant p53 was mixed (where appropriate) with 50 ng of the DNA competitors (see below) and 25 ng of the p53-specific monoclonal antibody pAb421 and binding buffer (10 mM HEPES pH 7.0, 4 mM MgCl₂, 100 mM KCl, 4% Ficoll 400, 5% glycerol, 1 mg bovine serum albumin, 0.05 mM dithiothreitol, 0.05 mM ZnCl₂ and 0.05 mM EDTA). This mixture was incubated for 10 min on ice, and then about 1 ng of end-labeled probe was added and the incubation was continued for another 20 min on ice. The reactions were loaded on a 5% non-denaturing polyacrylamide gel. Electrophoresis was carried out in 0.5× TBE buffer at 4°C. The competitors used were GLN-LTR (CAGG-

ACATGCCCGGGCAAGCCCAT; Zauberman *et al.*, 1993) and TGT3 (TGTGCCAAGTCTGTTTGCTGAC; Ori and Shaul, 1995).

For immune selection assays, a mixture of ³²P-labeled DNA fragments was incubated either with p53 from Clone 6 nuclear extracts or with recombinant p53, immunoprecipitated with PAb421; the precipitated DNA fragments were analyzed as previously described (Zauberman *et al.*, 1993).

DNase I footprinting

The p53-DNA binding conditions were as described for EMSA. Either nuclear extract or recombinant p53 was mixed with poly(dI-dC) and the PAb421 antibody. After 10 min of preincubation on ice, about 5 ng (10⁵ c.p.m.) of DNA probe, end-labeled by filling in, was added and incubation was continued for another 20 min in a total volume of 20 µl. The digestion step was performed by adding 1 ng of DNase I (DPRF, Worthington) in a total volume of 40 µl of 2.5 mM CaCl₂ and 5 mM MgCl₂, at room temperature for 1 min (Shaul *et al.*, 1986). The digestion was stopped by mixing with 410 µl stop solution (10 mM Tris-HCl pH 7.5, 45 µl sodium acetate pH 5.2, 10 µg tRNA), and 450 µl of phenol-chloroform-isoamylalcohol (25:24:1 ratio by volume). Following ethanol precipitation, the digested DNA was loaded on a 6% polyacrylamide denaturing sequencing gel.

Cell culture and DNA transfection

HepG2, Hep3B and Saos2 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (Gibco Laboratories) containing 100 U of penicillin and 100 µg of streptomycin per milliliter, supplemented with 8% fetal bovine serum. Transfection was carried out by the calcium phosphate method as previously described (Haviv *et al.*, 1995). About 6 h before transfection, 2×10⁵ cells were plated per 6 cm dish. Each transfection reaction contained a constant amount of 5 µg DNA consisting of 1 µg reporter, 1 µg SV40 β-galactosidase, wild type or mutant p53 as indicated, and carrier (pBluescript SK). Saos2 and Hep3B cells were harvested 24 and 48 h after transfection, respectively, and processed as described (Ori and Shaul, 1995). For analysis of HBV gene expression, HepG2 cells were transfected with 15 µg of 2×HBV plasmid per 10 cm dish. This plasmid contained a dimer of HBV DNA ligated head-to-tail at the unique *EcoRI* site. RNA and protein were extracted using TRI Reagent (Molecular Research Center).

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