An internal segment (residues 58–119) of the hepatitis B virus X protein is sufficient to activate MAP kinase pathways in mouse liver

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Abstract The human hepatitis B virus X protein (HBx) is known as a dual-specificity transactivator stimulating the transcriptional machinery in the nucleus and signal transduction pathways in the cytoplasm. HBx-induced activation of mitogen-activated protein kinase (MAPK) signaling cascades is considered to play an important role in hepatitis B virus-mediated hepatocarcinogenesis. Herein, we have identified the regions of HBx that are crucial for activating such signaling cascades in vivo. A truncated mutant incorporating regions C–E (amino acids 58–140) was as effective as the full-length HBx in activating MAPKs and enhancing activator protein-1 binding activity. While deletion of region C (amino acids 58–84) or D (amino acids 85–119) led to a drastic loss of function, region E (amino acids 120–140) was dispensable for the activation of signaling cascades. Overall, these findings provide the first evidence for the requirement of domain 58–119 of HBx in transmitting mitogenic signals to the nucleus in vivo.

Key words: Activator protein-1; Extracellular signal-regulated kinase; Hepatitis B virus; Hepatitis B virus X protein; c-Jun N-terminal kinase; Mitogen-activated protein kinase

1. Introduction

Persistent infection by hepatitis B virus (HBV) often leads to hepatocellular carcinoma by a yet unknown mechanism [1]. Nonetheless, the 154 residue long polypeptide hepatitis B virus X protein (HBx), encoded by HBV, is an important etiological factor in viral carcinogenesis [2]. Comprehensive studies show HBx is a pleiotropic transcriptional transactivator [2]. Besides the cognate HBV promoter, HBx transactivates transcription from a diverse range of viral and cellular promoters, such as long terminal repeats of human immunodeficiency and Rous sarcoma virus [3,4], the promoters of proto-oncogenes [5–7] and host RNA polymerase II and III promoters [8,9]. As HBx does not bind to double-stranded DNA, such broad gene-regulatory functions are attributed to protein–protein interactions with other cellular factors. Consistently, HBx is shown to directly interact with a multitude of nuclear proteins such as specific components of the basal transcription machinery [10], the bZip family of transcription factors [11], tumor suppressor factor p53 [12], and UV-damaged DNA binding protein [13]. Alternatively, HBx manifests its transactivation function by modulation of signaling cascades involving mitogen-activated protein kinases (MAPKs) [14], Src-dependent kinases [15], protein kinase C [16], and Jak1-STAT pathways [17]. HBx-mediated transactivation functions are therefore so diverse that a unifying mechanism of its action remains elusive. Nevertheless, a dual mode of HBx action has been demonstrated in accordance with its both nuclear and cytoplasmic localization [18,19]. Thus HBx acts as both a nuclear and a cytoplasmic transactivator; while nuclear HBx affects transcription directly by interacting with specific transcriptional machinery at the promoter level, the cytoplasmic HBx influences indirectly by modulating various intracellular signaling [18]. Furthermore, the finding that a variant of HBx engineered to relocate exclusively to the nucleus via a nuclear localization signal (HBx-NLS) fails to activate cytoplasmic signaling cascades, but, in contrast, fully retains its nuclear activity in stimulating HBV enhancer I, implies that the nuclear and cytoplasmic activities of HBx are independent functions [18] and may involve different domains of HBx. The regions of HBx involved in its interaction with a number of nuclear proteins have been mapped [2,10–13]. We have identified a region on HBx encompassing residues 58–140 as the minimal nuclear transactivation domain [20]. However, in sharp contrast, the structure–function studies on its cytoplasmic activities lack such extensive investigation under in vitro and in vivo settings.

Exploiting the membrane-fusogenic ability of Sendai-viral F-glycoprotein and high affinity of terminal β-galactose containing ligands (present on F-glycoprotein) for asialoglycoprotein receptor on the hepatocyte surface, we have developed an efficient viral-based delivery system (F-virosomes[1]) to transfer foreign genes to hepatocytes both in vitro and in vivo [21–24].

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Abbreviations: AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase kinase; NLS, nuclear localization signal; RSV-LTR, Rous sarcoma virus long terminal repeat

[1] Process for Producing a Targeted Gene (1997) US Patent 5,683,866.

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Using this system, we have recently established a role of HBx in activating cytoplasmic MAPK signaling cascades in normal adult hepatocytes and discerned such activation to be functionally important for HBx-mediated hepatocellular carcinoma (submitted). In this study, we examined the effects of various HBx mutants on mitogenic signal transduction pathways and identified for the first time its domain crucial for such activities in liver cells in vivo.

2. Materials and methods

2.1. Expression vector of HBx and its mutants

Using the full-length HBV template (adw subtype) and the following oligonucleotide primers: forward, 5′-CGGAATTCATGGCTGCTAGGCTGT-3′ ($X0_f$) and reverse, 5′-CGGAATTCTTAGGCAGAGGTGAAAAAG-3′ ($X0_r$), the HBx gene was amplified as described earlier [20]. This cDNA (a 471-bp *Eco*RI fragment of the HBx gene, henceforth called X0) was cloned into plasmid vector pSG5 under the control of the SV40 promoter (Stratagene). Deletion mutants of X0 (X7, X9 and X10) were constructed by site-directed mutagenesis using single-stranded X0 template. The truncated form X15 was constructed by PCR using the following primer: forward, 5′-CGGAATTCCATATGCTCCCCGTCTGTGCCTTC-3′ ($X15_f$) and reverse, 5′-CGGAATTCGGATCCTTATTTGTGCCTACAGCCTCCTAA-3′ ($X15_r$) and cloned into pSG5. All plasmids were isolated using the Qiagen Megaprep kit.

2.2. Administration of DNA-loaded virosomes, insulin and PD98059 into mice

X0 or its mutant DNAs were loaded inside F-virosomes following our published methodologies [24]. Female BALB/c mice (12 weeks old) weighing ~18 g were injected i.v. into the tail vein with 2 μg each of DNA-loaded virosomes and kept for 48 h before being killed. 20 μg of insulin (Sigma) in phosphate-buffered saline (PBS) was injected i.v. into mice 1 h prior to death. Intravenous administration of PBS containing 75 μM PD98059 (New England Biolabs) in 0.37% dimethyl sulfoxide (DMSO) was injected 5 h before the X0/insulin-injected mice were killed. Injection of PBS alone (mock injection), pSG5 vector DNA-loaded F-virosomes and PBS with 0.37% DMSO served as appropriate negative controls. During all experiments, Delhi University guidelines were followed and animals were kept under standard pathogen-free conditions and were provided food and water ad libitum. Liver biopsies from animals were carried out as described below. A portion of each liver was kept frozen separately for isolating total RNA and preparing nuclear extracts. Each experiment was carried out at least three times independently.

2.3. Monitoring expression of HBx and its mutants

Separation of parenchymal cells (hepatocytes) was accomplished as described by us earlier [24]. Hepatocytes free from detectable red blood cells were collected and immediately stored at −70°C until use. Total RNA was isolated from hepatocytes using Trizol (Gibco-BRL). The primer sets designed for X0 and its mutants were as follows: for X0 and X15 specific transcripts $X15_f$ and $X15_r$; for X7 and X9 transcript, $X0_f$ and $X15_r$; for X10 transcript, $X15_f$ and $X0_r$. Gene-specific 'antisense' primers were employed for reverse transcription using DNase I (Gibco-BRL)-treated RNAs and Superscript™ RNase H^- reverse transcriptase (RT) (Gibco-BRL). PCR amplification (35 cycles) of the RT products was performed using high fidelity Platinum Taq DNA Polymerase (Gibco-BRL) with a cycling profile of 94°C for 45 s, 62°C for 45 s (or 55°C for 45 s in the case of β-actin primers, Stratagene), 72°C for 1 min and final extension at 72°C for 10 min. Specific amplified products of length 270 bp for X0 and X15, 351 bp for X7, 321 bp for X9 and 228 bp for X10 DNA were visualized by ethidium bromide staining on a 1.5% agarose gel. As an internal control, β-actin mRNA was also amplified by RT-PCR for each sample and a product of 650 bp was obtained. Specificity of the RT-PCR products was further ensured by Southern hybridization using a ^{32}P-labelled X0 DNA fragment as the probe.

2.4. ERK assay

Standard published protocols [14] were followed for the extracellular signal-regulated kinase (ERK) assay with some modifications. To summarize, hepatocytes were lysed in a buffer composed of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1.0% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 50 mM β-glycerol phosphate, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1.5 mM $MgCl_2$ and 20 μg/ml each of aprotinin and leupeptin (Sigma) in conjunction with Complete™ protease inhibitor cocktail (Roche). Protein concentration in the lysates was estimated by the Bradford assay. ERK/MAPK activity was monitored by in vitro phosphorylation of exogenous substrate myelin basic protein (MBP) (Gibco-BRL). In brief, 5 μg each of protein lysate was included in a reaction containing 5 μg MBP in kinase buffer (20 mM Tris–HCl, pH 7.5, 40 mM $MgCl_2$, 10 μM ATP (Sigma)) and 2 μCi [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and incubated for 30 min at 30°C. The reaction was stopped by the addition of Laemmli sample buffer and resolved on 15% SDS–PAGE. The upper half of the gel was subjected to Western analysis with anti-ERK antibody (Santa Cruz Biotechnology) and was developed by the ECL detection system. The lower half of the gel containing MBP protein was dried and exposed to X-ray films. To check for equal amounts of MBP in each lane, dried blots were stained by Coomassie blue (CB). The stained bands corresponding to MBP protein were excised and radioactivity was measured by liquid scintillation counting to calculate fold activation.

2.5. Detection of phosphorylated ERK and JNK by Western blotting

The levels of active (phosphorylated) forms of ERK (P-ERK) and c-Jun N-terminal kinase (P-JNK) in the hepatocyte cell extracts (5 μg) were resolved in 15% SDS–PAGE and subjected to immunoblotting with anti-phospho-p44/42 MAPK antibody and Phospho Plus® SAPK/JNK Antibody (Cell Signaling Technology) respectively. The corresponding blots were stripped and reprobed with anti-ERK and anti-JNK antibodies.

2.6. Preparation of nuclear extracts and gel mobility shift assay

Nuclear extracts from each liver lobe and HepG2 cells were prepared as described by Lassar et al. [25]. Electrophoretic mobility shift assays (EMSA) were carried out essentially following a published protocol [14]. The synthetic oligonucleotides used as a probe or competitor DNA in this assay consist of a double-stranded TRE sequence (Promega). Binding reactions with 36 μg of liver nuclear extracts or 6 μg of HepG2 cell extracts were done using 10 000 cpm of ^{32}P-labeled probe for 60 min at 4°C followed by incubation for 5 min at 25°C. Competition binding experiments were performed with 50-fold molar excess of unlabeled oligonucleotide. The reaction mixtures were resolved on 4% non-denaturing polyacrylamide gel, dried and visualized by autoradiography. For antibody-supershift studies, 4 μg of antiserum (Santa Cruz Biotechnology) to either c-Jun/c-Fos or normal IgG was added after completion of the binding reaction and incubated at 28°C for 20 min. Some nuclear extracts (70 μg) were subjected to 15% SDS–PAGE followed by Western blotting to detect c-Jun and c-Fos protein levels using specific antibodies (Santa Cruz Biotechnologies) and visualized by ECL detection.

3. Results

3.1. Basis for selecting HBx mutants

Based on sequence homology with other mammalian hepadnaviruses, the HBx sequence (154 amino acids) has been divided into six regions (A–F) (Fig. 1). Earlier, we had constructed a panel of HBx mutants and demonstrated that deletions of regions A, B, and F could not abrogate the HBx-mediated transactivation of the Rous sarcoma virus long terminal repeat (RSV-LTR). On the other hand, deletion of regions C, D, and E resulted in significant loss of activity. Further, a truncated mutant, X15 harboring only regions C–E (amino acids 58–140), was able to stimulate RSV-LTR quite efficiently, suggesting an important role of residues 58–140 of HBx in RSV-LTR transactivation [20]. Considering that HBx-mediated transactivation occurs via a dual mechanism, we investigated whether the same domain of HBx is required for activating the MAPK pathways. Based on this rationale,

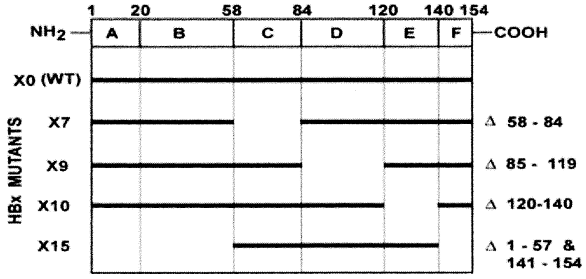

Fig. 1. Scheme of HBx mutants. Based on the homology with other mammalian hepadnaviruses, the 154 amino acids long wild-type (WT) HBx sequence (X0) has been divided into six regions (A–F). Deletion (Δ) mutants (X7, X9, X10) and a truncated mutant (X15) of HBx are shown schematically. A solid bar indicates the portion of HBx present in the mutant, with the deleted region shown as a gap. The light vertical lines depict the boundaries of the six regions of homology (A–F).

we selected a truncated mutant containing regions C–E (X15) as well as deletion mutants which were lacking either region C (X7), D (X9) or E (X10) and analyzed their transactivation potential in signaling cascades.

3.2. *In vivo ERK activity of HBx mutants in mouse hepatocytes*

We recently demonstrated that HBx expressed in vivo in mouse hepatocytes is able to activate ERKs constitutively resembling an oncogenic stimulus in function (submitted). Considering the central importance of ERK activation in tumors [26], it is pertinent to identify the region(s) of HBx involved in such activities. Thus expression vectors X0, X7, X9, X10, and X15 were delivered to mouse liver through F-virosomes (Fig. 2). Insulin, a well-known stimulator of ERK via Ras-Raf-MAPK cascades [27], was injected into mice to serve as a positive control (Fig. 2a,b, lanes 9 and 10). The cytostatic drug PD98059 [28], known to be a specific and efficient inhibitor of MAP kinase kinase kinase (MEK), the kinase that activates ERKs, was introduced into mouse liver to determine the requirement of MEK in HBx-mediated ERK activation (Fig. 2a,b, lanes 4 and 10). Hepatocyte lysates were analyzed for the phosphorylation of ERK1/2 as well as for in vitro kinase assay using MBP as the specific substrate (Fig. 2a,b). Consistent with our previous results [20], X15-expressing hepatocytes showed an elevated level of phosphorylated ERK along with an increased ERK functional activity over that of X0 (see lane 8 against lane 3), which can be accounted for by the deletion of the N-terminal trans-repression domain of HBx in X15 [29]. In contrast to X0, both X7 and X9 mutants showed drastic inhibition of phosphorylation in ERK1/2 proteins (Fig. 2a, upper panel, compare lanes 5 and 6 with lane 3), and also were poor activators of MBP phosphorylation (only 10–20% activity of X0) (Fig. 2b, upper panel, lanes 5 and 6). X10 mutant displayed 80% activity of full-length HBx protein (X0) in both assays (Fig. 2a,b, upper panels, lane 7). Interestingly, all the mutants exhibited total ERK levels comparable to vector and X0, eliminating the possibility of ERK levels contributing to variations in their activities (Fig. 2a,b, lower panels). In addition, equal protein loading in all lanes hereafter was verified by Ponceau S red staining (data not shown). Moreover, the presence of specific transcripts of all the HBx mutants, as checked by RT-PCR (Fig. 2d, upper panel) followed by Southern hybridization (data not shown), excluded the possibility of different activities exhibited by HBx mutants to be related to gene expression. A drastic loss of activity in both X7 and X9 thus suggests the essential requirements of regions C and D (amino acids 58–84 and 85–119) in HBx-mediated ERK activation. In conclusion, reten-

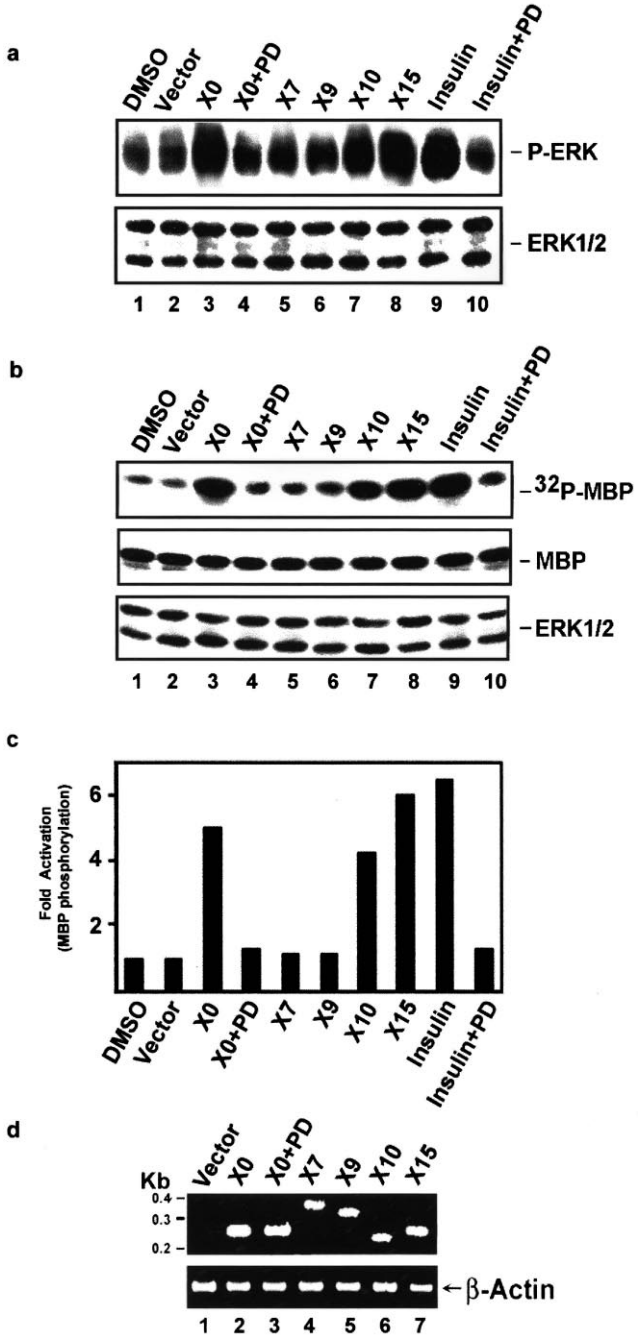

Fig. 2. Effect of HBx mutant genes on MAPK activity in vivo. a: Hepatocyte lysates (5 μg) were prepared 2 days after injection of X0, X7, X9, X10 and X15 DNA-loaded virosomes into mice as described in the text. Immunoblotting with anti-phospho-ERK antibody is shown in the upper panel and with anti-ERK antibody is shown in the lower panel. b: The profile of MBP phosphorylation (upper panel), CB-stained MBP (middle panel) and total ERK level (lower panel) was analyzed as described in the text. c: Fold activation in terms of MBP phosphorylation was calculated as described in the text. d: The expression of HBx and its mutants (upper panel) and the β-actin mRNA (lower panel) were examined as narrated in the text. Primers used: for X0 and X15, $X15_f$ and $X15_r$; for X7 and X9, $X0_f$ and $X15_r$; for X10, $X15_f$ and $X0_r$.

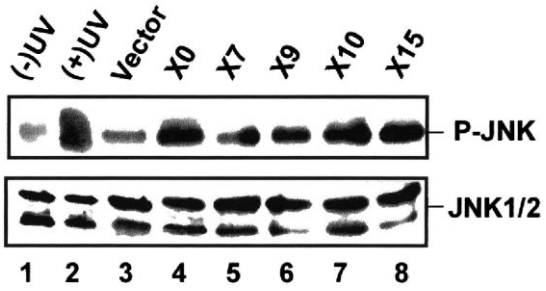

Fig. 3. Influence of HBx mutants on JNK activation. The same hepatocyte lysates (5 μg) as described in Fig. 2 were tested for activated JNK levels (P-JNK, upper panel) and total JNKs (JNK1/2, lower panel). (−) UV and (+) UV represent total extracts from unirradiated and irradiated 293 cells as negative and positive controls respectively.

tion of complete activity in X15 comprising regions C, D, and E defines residues 58–140 of HBx as the minimal functional domain involved in ERK activation.

3.3. Regions of HBx involved in JNK activation

The JNKs are another important member of the MAPK family of proteins that are activated by stress and growth factors [30]. As our recent studies showed the role of HBx in activating JNKs in mouse liver, we further attempted to identify the domain(s) of HBx involved in their activation. We assessed the levels of phosphorylated JNKs in the hepatocytes expressing HBx and its mutants as above. Untreated and UV-treated 293 cells were used as negative and positive controls respectively (lanes 1 and 2). As shown in Fig. 3, upper panel, HBx mutants influenced JNK activation in a pattern consistent with ERK activation. X15, which includes regions C–E (amino acids 58–140), displayed increased levels of P-JNK, even higher than that induced by X0 (compare lanes 3, 4 and 8). While X7 and X9 mutants showed P-JNK levels comparable to that of vector (compare lane 3 with lanes 5 and 6), X10 exhibited activity close to that of X0 (compare lane 7 with lanes 3 and 4). The total JNK level remained the same for all the lysates, reflecting that the variations observed in JNK activities are not the consequences of changes in JNK expression levels (Fig. 3, lower panel, lanes 3–8). Taken together, these results show that the minimal functional domain of HBx required for JNK activation also converges to residues 58–140.

3.4. Minimal functional region(s) of HBx involved in increased DNA binding potential of AP-1 in vivo

The activator protein-1 (AP-1) family of transcription factors are activated by the integrated effects of activated ERKs and JNKs [30]. In accordance with this, we next questioned whether HBx-mediated enhanced AP-1 activity involves MAPK activation. Nuclear extracts prepared from the liver lobes from injected animals as used previously (Figs. 2 and 3) were allowed to bind to radiolabelled AP-1 DNA (Fig. 4a, panel i). Serum-starved and serum-induced HepG2 nuclear extracts contributed as controls for the induction of AP-1 binding activity (lanes 8 and 9). As apparent, both X7 and X9 mutants of HBx showed a marked mitigation of AP-1 activity almost comparable to the baseline (compare lanes 1, 4 and 5). By contrast, X10 displayed enhanced activity, albeit less than X0 (lanes 3 and 6), while the truncated mutant X15 with region C–E of HBx was as effective as the full-length HBx (compare lanes 3 and 7). To assess the specificity of the AP-1 binding complex, a supershift assay was also performed (Fig. 4a, panel ii). Addition of antibody to c-Jun and c-Fos to the nuclear extracts resulted in 'supershifted' complexes. The results on AP-1 binding activity were supported further by respective levels of c-Jun and c-Fos (Fig. 4b,c, panel i). Specifically, we found that both X7 and X9 mutants showed c-Jun and c-Fos protein levels close to basal (compare lane 1 with lanes 3 and 4). While X10 induced c-Jun and c-Fos levels comparable to X0 (lanes 2 and 5), X15 stimulated both c-Jun and c-Fos over X0 (lanes 2 and 6). The levels of c-Jun and c-Fos observed in various mutants thus validated the observed AP-1 binding activity. Overall, these results are in parallel to those obtained from the mutants' influence on ERK and JNK activation. Altogether, they suggest that the minimal functional region of HBx (amino acids 58–140) required for enhanced AP-1 binding activity converges to the region needed for both ERK and JNK activation.

4. Discussion

HBx is unique among viral transactivators as it can modulate gene expression by a dual mechanism, viz. by directly influencing the transcription apparatus and also by acting upon various signal transduction pathways. Although the molecular mechanism of its mode of interference into the transcription machinery has been well investigated, the mechanism by which it stimulates various signaling cascades is not known. Recently, we have established, in vivo, that HBx is a potent activator of MAPK pathways in mouse liver (submitted). In order to have a better understanding of the HBx transactivation mechanisms, we aimed our present study to determine the regions of HBx required for activating signaling cascades. We identified residues 58–140 of HBx as the minimal functional domain needed for HBx activation of MAPKs and the subsequent downstream events. Our study thus helped to delineate the promiscuous transactivation mechanism of HBx. The additional significance of our data lies in the fact that the observations have been made in the natural, physiological environment of hepatocytes, in the whole animal. Consistent with our previous study on HBx transactivation of RSV-LTR [20], the present work demonstrates that truncated mutant X15 spanning domains C–E (amino acids 58–140) of HBx is as effective as the wild-type HBx (X0) in activating cytoplasmic signaling cascades. Since the functional domain (amino acids 58–140) of HBx required to activate cytoplasmic cascades is also essential for activating the transcriptional machinery [10,11,29,31], it may be inferred that the nuclear and cytoplasmic transcriptional activation functions of HBx grossly occur via a common domain. We find that while deletions involving either region C (X7) or D (X9) resulted in almost complete loss of function, deleting region E (X10) only moderately obliterated HBx ability to activate MAPK cascades. This is apparently in contrast with our previous studies, where a significant abrogation of RSV-LTR transactivation occurred by deleting region E [20]. This contradiction can be consolidated by the fact that the region encompassing amino acid residues 118–133 of HBx is acidic in nature and is likely to be essential for HBx nuclear transcriptional activities, such as its role as coactivator of transcription factors [32]. This would suggest no involvement of this region in transcrip-

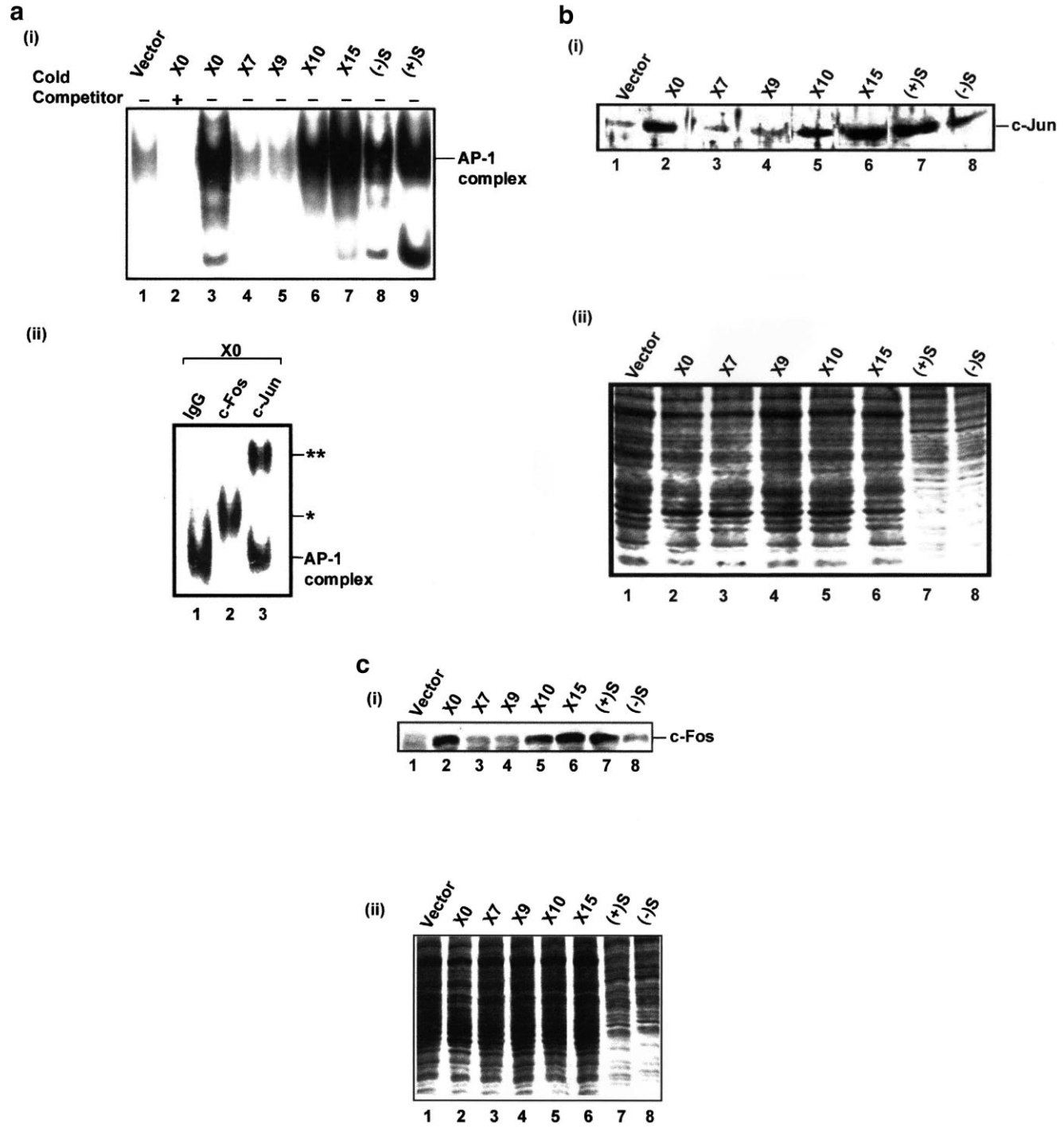

Fig. 4. AP-1 DNA binding activity of HBx mutants. a i: EMSA was performed with nuclear extracts (36 μg for liver extracts and 6 μg for HepG2 extracts) prepared as described in the text. (−) and (+) indicate the absence and presence of cold competitor respectively during the DNA-Protein interaction. ii: Specificity of the AP-1 binding was confirmed by a 'supershift' assay as mentioned in the text. (*) and (**) represent the supershifted AP-1 complex in the presence of c-Fos (lane 2) and c-Jun antibodies (lane 3) respectively. 'IgG' indicates the normal rabbit serum taken as negative control. b and c: Nuclear extracts (70 μg for liver extracts and 30 μg for HepG2 extracts) were subjected to immunoblotting for detection of c-Jun protein and c-Fos protein levels using specific antibody respectively (panel i). Panel ii reflects equal protein loading by Ponceau S red staining of the corresponding blots. (−) S and (+) S stand for HepG2 extracts following serum starvation or induction respectively.

tional activation via cellular signaling cascades. However, region E of HBx has been shown to bind to the 19S proteasome complex, which is closely related to the COP9 signalsome, a complex containing a weak canonical MAPK kinase site [33,34]. HBx probably binds this complex via region E and contributes moderately to the activation of MAPK cascades, thus explaining the partial reduction of activity in X10. These results therefore demonstrate that while the minimal essential region of HBx involved in activating MAPK cascades maps to region 58–119, region E (amino acids 120–140) integrates ad-

ditional events to restore the wild-type activity of HBx. Altogether, it may be mentioned that regions C, D, and E (amino acids 58–140) of HBx are involved in modulating transcription through cytoplasmic signaling cascades. This conclusion is supported by the observations that similar regions of HBx are involved in activation of the Jak1-STAT signaling pathway [17] and region 90–122 has been shown to interact with protein kinase C binding protein (XAP3), presumably facilitating HBx to activate the protein kinase C pathway in the cytoplasm [35].

Importantly, our studies also demonstrate that deletion of HBx region C (amino acids 58–84) or D (amino acids 85–119) not only abolishes activation of MAPKs in cytoplasm but also AP-1 binding activity in the nucleus, thus directly linking HBx-mediated enhanced AP-1 activity with activation of MAPKs. These observations suggest that HBx-induced nuclear and cytoplasmic effects may not be mutually exclusive and a cross-talk exists between the two transactivation functions. Studies by Johnson et al. which demonstrate that HBx-mediated enhancement of both RNA polymerase II- and III-dependent promoter activities occur through the involvement of Ras-mediated upregulation of TATA binding protein lend further support to this view [9]. It may be thus reasoned that concomitant interactions of HBx with cytoplasmic signal transduction components and nuclear transcriptional machinery account for HBx transcriptional activation.

Intriguingly, our data demonstrate for the first time that the mutational loss or gain of JNK stimulating activity is coupled with that of ERK activity. This congruent pattern of ERK and JNK activation makes it reasonable that HBx mediates activation of both kinases via a common upstream effector. Based on a recent study of MAPK activation to occur through JAK tyrosine kinases, via stimulation of Src kinases and adapter signaling molecules [36], we speculate that HBx-induced JAK activation serves as a common effector for increased ERK and JNK activities via Src and Ras. Integration of ERK and JNK activities manifested through elevated c-Fos and c-Jun levels results in increased binding activity of AP-1 that might lead to increased expression of genes responsible for cell proliferation and tumorigenesis. Therefore, the present study, defining the domain of HBx involved in signaling pathways, not only helps to dissect the complex mechanism of HBx transactivation, but also attempts to understand the significance of mitogenic cascades in HBV-associated liver carcinogenesis in the native hepatocyte environment in vivo.

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