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The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element

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The X protein of hepatitis B virus (HBV) stimulates transcription of a large number of viral enhancers. This protein augments the activity of the HBV enhancer through a specific *cis* element, termed X responsive element (XRE). Multimers of XRE exhibit enhancer activity which is further stimulated by X. XRE binds multiple cellular transcription factors one of which is the C/EBP. We have constructed the DB gene containing the DNA-binding domain of the C/EBP. This gene efficiently represses the enhancer activity of the XRE by competitive displacement of the XRE-binding factors. Under these conditions, X was found to have only a partially stimulatory effect on transcription, suggesting that the XRE-binding proteins are required for the activity of X. In contrast, an X–DB hybrid protein that binds to the XRE is a strong transcription factor and acts without additional XRE-binding proteins. Furthermore, studies of X mutants revealed that the carboxy-terminus of the protein is required for this activation. These data show that X directly stimulates the cellular transcription machinery, possibly by protein–protein interaction with the XRE-binding factors.

Key words: C/EBP/hepatitis B virus X gene/hybrid genes/transcription factor activating domain/viral transactivator

Introduction

Most and perhaps all known viruses possess at least one open reading frame which codes for transactivator – a transcription regulatory protein. Despite the detailed characterization of the genes encoding viral transactivators and, in a few cases, their products, little is known about their mechanism of action. The emerging picture drawn from the study of several transactivators is that their activity is exerted through specific *cis* elements that are often found at the enhancers and promoters of the corresponding viral genomes. This distinctive property to act through specific *cis* elements is also shared by cellular transcription factors. However, unlike the cellular transcription factors, the viral transactivators, with a few exceptions, do not seem to contain a DNA-binding domain which is required for specific interaction with the DNA. The apparent paradox of transactivators lacking a DNA-binding domain yet acting through a specific DNA sequence can be easily resolved by assuming either that the viral products catalytically activate cellular factors associated with the corresponding *cis* elements, or that the viral transactivators associate with the *cis* elements by specific protein–protein interactions (Kovesdi *et al.*, 1986; Preston *et al.*, 1988; O'Hare *et al.*, 1988). In the latter case, the function of the viral transactivators is reminiscent of that of the activating domain of cellular transcription factors, a domain that directly interacts with and activates the transcription machinery (Ptashne, 1988).

The X protein of the hepatitis B virus (HBV) transactivates the enhancer of its own genome and that of several heterologous viruses (Spandau and Lee, 1988; Seto *et al.*, 1988; Twu and Robinson, 1989; Zahm *et al.*, 1988; Colgrove *et al.*, 1989). Recently, we have shown that the E element of the HBV enhancer (Shaul *et al.*, 1985; Shaul and Ben-Levy, 1987; Ben-Levy *et al.*, 1989) is sufficient to confer responsiveness to the X protein, and have hence designated it X responsive element (XRE; Faktor and Shaul, 1990). Interestingly, the XRE shows sequence similarity with the *cis* elements found in the LTRs of the human T-cell leukemia viruses (HTLV-I and II). These elements are responsive to the transactivators of the HTLVs, taxI and II (for review see Varmus, 1988) and were designated taxRE. The sequence similarity between XRE and taxRE is of functional significance, as was demonstrated by the ability of taxI to act also through XRE and *vice versa*, of X to act through taxRE (Faktor and Shaul, 1990). Despite the fact that both transactivators act through sequence specific *cis* elements, none possesses any DNA-binding activity (Nyborg *et al.*, 1988; Varmus, 1988; and our unpublished observation). Interestingly, however, the corresponding *cis* elements do bind several cellular factors (Tan *et al.*, 1989; Faktor *et al.*, 1990). In HBV, the intrinsic enhancer activity of XRE (the E element) is determined by these cellular factors (Ben-Levy *et al.*, 1989; Faktor *et al.*, 1990). One of the XRE-binding cellular proteins is the C/EBP. This liver-restricted transcription factor binds the XRE with a high affinity (Landschulz *et al.*, 1988; Dikstein,R., Faktor,O. and Shaul,Y., submitted). The gene of C/EBP was cloned and its DNA-binding domain was extensively characterized and localized to its carboxyl terminus (Landschulz *et al.*, 1989). We took advantage of this XRE-binding cellular protein to elucidate the mechanism of action of the X protein as a transcription transactivator. We report here that the XRE-binding cellular factors are required for X to function as a transactivator. Interestingly, however, a hybrid protein of X fused to the DNA-binding domain of C/EBP is a strong transactivator independent of these proteins. In this hybrid protein, X acts as an efficient activating domain, suggesting that X is capable of direct interaction with the transcription machinery. Furthermore, like the activating domains of a large number of transcription factors, a stretch of acidic residues within X is essential for its function. Our results suggest that the HBV X protein stimulates transcription by formation of protein–protein interactions with the XRE-binding factors.

Results

Cellular DNA-binding transcription factors are required for the transactivating effect of X

The E element of the HBV enhancer is sufficient to confer responsiveness to transactivation by X and was designated the XRE (Faktor and Shaul, 1990). We constructed a reporter plasmid, TKCAT3EG, in which regulation of two distinct promoters is under the control of XRE. This plasmid contains two reporter genes, globin and CAT. The expression of the former is controlled by the globin and the latter by the HSV TK promoters. A synthetic enhancer containing three 26 bp repeats of the XRE sequence (nucleotides 1186–1288 on the HBV sequence) is positioned at the 3′ end of both reporter genes. This reporter plasmid allows the determination of the effect of the transactivator simultaneously on two distinct promoters (β-globin and TK). The effect of X on the globin promoter is determined by RNA analysis and on the TK promoter by measuring CAT activity.

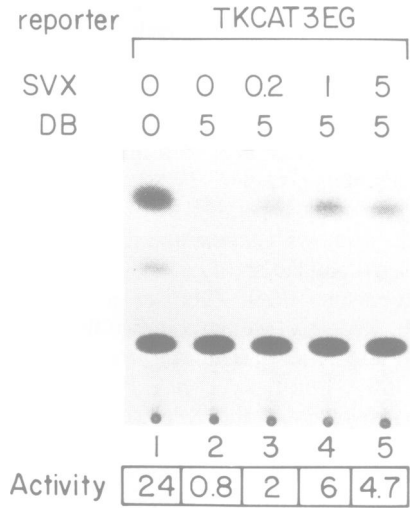

Fig. 1. Effect of the HBV X gene in the presence of a truncated C/EBP gene. Alexander cells were transfected with 5 μg of DB plasmid alone or in combination with different amounts of SVX plasmid, as indicated at the top of the lanes and the CAT activity was measured. TKCAT3EG (3 μg per plate) was used as a reporter plasmid (see text) and SV_2-DHFR control plasmid was added to reach an equal amount of DNA per plate. Cell extracts were prepared after 2 days and assayed for CAT activity by TLC. The activity indicates the percentage conversion of [^{14}C]chloramphenicol into the acetylated form.

We have previously reported that the XRE (the E element of the HBV enhancer) has a strong intrinsic enhancer activity which is determined by the XRE-binding cellular proteins (Ben-Levy *et al.*, 1989; Faktor *et al.*, 1990). Some of these proteins were identified as C/EBP (Johnson *et al.*, 1987), jun proteins and possibly CREB (Faktor *et al.*, 1990). Here we address the question of whether binding of these cellular factors is required for transactivation by X. A simple way to test this possibility is to determine the effect of X on the XRE in an environment which interferes with the binding of these transcription factors. Such an environment can be provided by over-expressing a truncated C/EBP. This truncated protein, which interacts with the XRE, is expected to displace competitively the XRE-binding proteins. Cells were transfected with the reporter plasmid and with or without DB, a plasmid that drives the synthesis of truncated C/EBP (DB gene, Figure 2). A dramatic reduction (>25-fold) in CAT activity was obtained in the presence of the DB plasmid (Figure 1, compare lanes 1 and 2). No reduction in the activity of a second reporter plasmid, in which six copies of the AP-1 binding site were used as a synthetic enhancer, was obtained (data not shown), suggesting that the truncated C/EBP protein specifically displaced the XRE-binding proteins. Next, we cotransfected an effector plasmid (SVX) that directs the synthesis of the

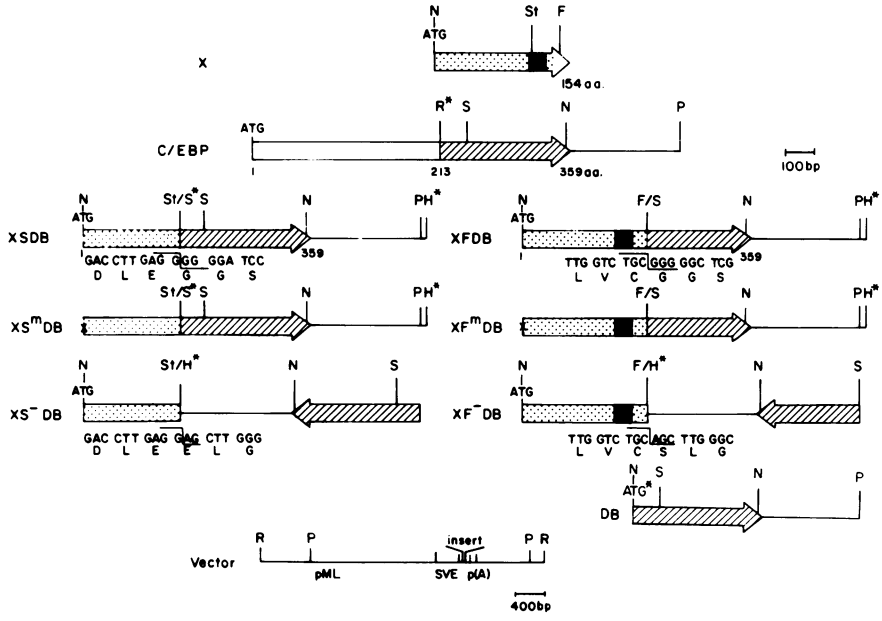

Fig. 2. Schematic representation of the structure of the chimeric X constructs used. The X gene is denoted by dots and the C/EBP gene sequences by diagonal lines. The black labeled region within the X gene encodes an acidic sequence. Each X fusion gene is shown as a bar and the predicted sequence in the vicinity of the junction with the X coding sequence is shown below it. The asterisks indicate the restriction cleavage sites originating from cloning manipulations. The vector used for these constructs is indicated at the bottom. N, *Nco*I; St, *Stu*I; F, *Fsp*I; R, *Eco*RI; S, *Sma*I; P, *Pst*I and H, *Hin*dIII.

HBV X protein together with the DB plasmid. Increasing amounts of the SVX plasmid were found to be only partially effective and the obtained activity was much lower than that observed in the absence of DB (Figure 1, lanes 3–5). These data suggest that the XRE-binding proteins are required for the activity of X. The requirement of these proteins raises the possibility that X acts through direct interaction with the XRE-binding factors. The 7-fold induction in CAT activity obtained by X in the presence of the DB protein might be due either to the fact that not all of the XRE sites are occupied by the DB or to an alternative mechanism for the action of X protein.

A chimeric X–DB is a potent transactivator

We next addressed the question of whether the negative effect of DB can be reversed by X targeted to the XRE. For this purpose, we engineered a set of X–DB hybrid genes. These chimeric genes contained most of the X ORF (143 out of 154 amino acids) joined in-frame with the DNA-binding domain of C/EBP (115 carboxy-terminal amino acids). The 11 amino acids missing at the carboxyl end are not essential for the transactivating function of X (Wollersheim *et al.*, 1988). The chimeric genes are expressed under the SV40 regulatory elements, were designated XFDB and XF^{m}DB; the latter bears a mutation at the first ATG of the hybrid gene (Figure 2).

Three plasmids were cotransfected into the cultured cells, the reporter plasmid TKCAT3EG, the various effector plasmids and the πSVHPα2 reference plasmid (Treisman *et al.*, 1983). The latter plasmid contains the α-globin gene under the control of SV40 regulatory sequences and serves as an internal control for transfection efficiency and integrity of expression. The extent of transactivation of the β-globin promoter was monitored by the RNase A/T1 protection technique and that of the TK promoter was monitored by CAT activity. For RNA analysis, labeled α- and β-globin RNA probes were synthesized *in vitro* with SP6 RNA polymerase from pSP6α132 and pSP6β350, respectively, to yield 222 and 520 ribonucleotide α- and β-probes. Correctly initiated transcripts should protect a 132 nucleotide fragment of the α-globin probe and a 353 nucleotide fragment of the β-globin probe (Figure 3, bottom). The activities of the β-globin and TK promoters under the control of the XRE were induced 6- and 3-fold, respectively, by SVX plasmid driving the synthesis of the native X (Figure 3, lanes 2 and 3). This induction depends on the presence of the XRE, since a reporter plasmid lacking the XRE is not responsive (data not shown). This finding is in agreement with our previous observation that the XRE is sufficient for responsiveness to X (Faktor and Shaul, 1990). Remarkably, the XFDB plasmid containing the DNA-binding domain of C/EBP fused to X acted as a strong transactivator of both the globin and the TK promoters (20- and 25-fold induction, respectively; Figure 3). This induction is 5- to 8-fold higher than that obtained with native X in the absence of DB. The control plasmid XF^{m}DB carrying a mutation at the first ATG was found to be non-functional (lane 7), confirming the requirement for a functional ORF for this transactivation activity.

To demonstrate that XFDB stimulated transcription by its interaction with the XRE, we analyzed the effect of this gene in the presence of the DB protein. We reasoned that the binding of XFDB to the XRE should result in suppression of the DB negative phenotype. Alexander cells were transfected with three plasmids, the reporter TKCAT3EG, DB and either XFDB or SVX. As expected, X is incapable of suppressing the negative effect of DB (Figure 4, lanes 2, 4 and 5). However, XFDB when cotransfected with DB

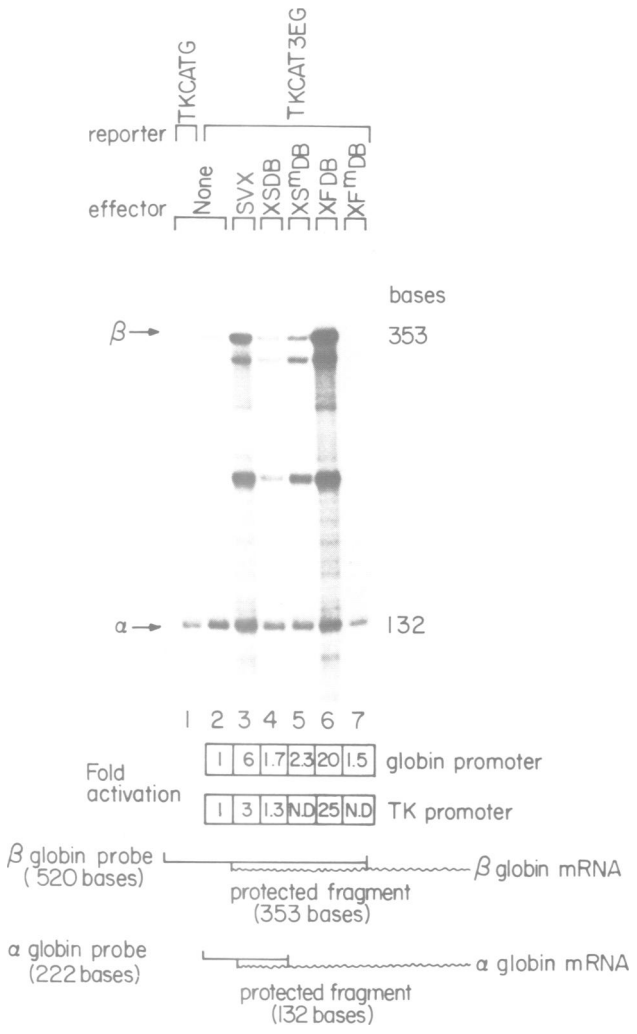

Fig. 3. Transactivation of XRE-globin promoter by chimeric X gene as analyzed by RNase A/T1 protection and CAT assay. **Top**: Alexander cells were cotransfected with the different effector plasmids (5 μg each) and TKCAT3EG reporter plasmid (3 μg), as indicated in the figure. As a control, TKCATG reporter plasmid was used; this is an enhancerless plasmid containing two reporter genes and promoters. The CAT gene is under the regulation of the TK promoter of HSV (−105 to +51) and the polyadenylation signal of SV40. The globin gene is under the control of its authentic promoter and polyadenylation sequences. The β-globin promoter is not effectively activated by X gene (Faktor and Shaul, 1990). β and α denote the protected β and α RNA bands. The activity was determined by scanning the autoradiogram with a densitometer and each value of β-globin mRNA synthesis was corrected according to the corresponding value of α-globin mRNA synthesis. The factor of increase in activation marked below each line was determined by measuring the ratio between the activity of the β-globin promoter obtained in the presence or the absence of the corresponding effector plasmids. The fold of activation of the TK promoter was measured by determination of CAT activity and the average results from 3–5 experiments are shown. **Bottom**: schematic representation of α and β RNA probes (straight lines), generated by SP6 RNA polymerase from pSP6α132 and pSP6β350 respectively (a gift of T.Maniatis). pSP6α132 contains an insert of 222 bp yielding a protected fragment of 132 bp of exon 1. pSP6β350 contains an insert of 520 bp yielding a protected fragment of 353 bp comprising exon 1 and part of exon 2. The probes were purified on 5% acrylamide gel.

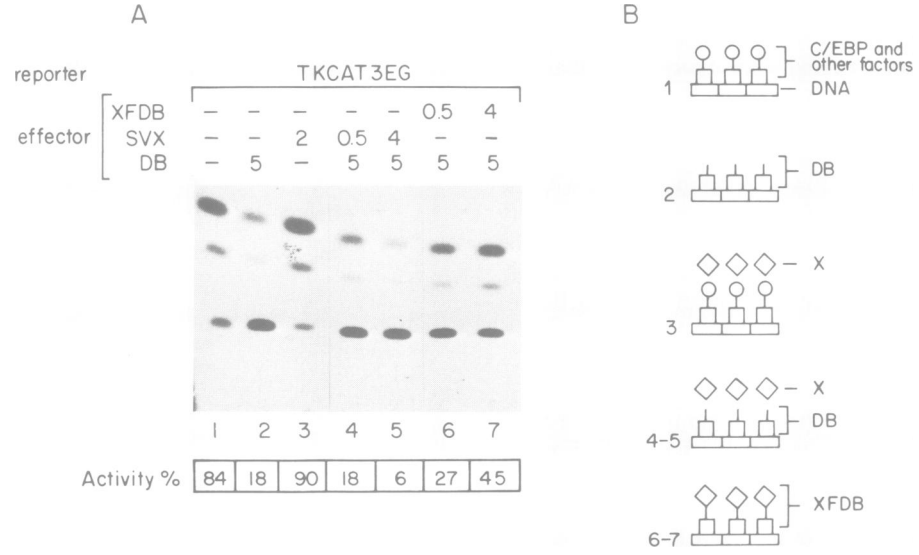

Fig. 4. Transactivation of the XRE-TK promoter by the chimeric X gene in the presence of DB, as analyzed by CAT activity. **A.** Alexander cells were cotransfected with different amounts of different effector plasmids, as indicated above each lane, together with 3 μg of TKCAT3EG reporter plasmid. The percentage conversion of [^{14}C]chloramphenicol to the acetylated form is shown as % activity. **B.** The complex interaction of the different effector proteins with the three copies of XRE (E), as obtained in the experiments done in panel B, are schematically shown with the corresponding lane numbers.

plasmid in a molar ratio of about one, suppressed 50% of the negative phenotype of DB (lanes 2, 6 and 7). We infer that the chimeric X−DB (XFDB) protein acts by direct interaction with XRE. Targeted X is, therefore, a strong transcription factor in the absence of additional XRE-binding proteins, further confirming that X has an intrinsic transcriptional activation activity.

The carboxyl end of the X within the hybrid X−DB is required for its activity

Recent work has demonstrated that transcription factors contain two parts; one directs DNA binding and the other, the activating domain, presumably interacts with components of the transcriptional machinery (for review see Ptashne, 1988). Acidic sequences are a characteristic feature of activating domains of several transcription factors (Ptashne, 1988; Mitchell and Tjian, 1989). Inspection of the carboxyl end of the X gene shows the presence of a stretch of acidic amino acids (Figure 6B). Therefore, we tested whether this region was required for the activity of chimeric X. For this purpose, we constructed a second set of chimeric genes which lacked the acidic region. The X gene was truncated at the *Stu*I site (removing the 45 carboxyl amino acids to which the DNA-binding domain of C/EBP (141 carboxy amino acids) was fused, in-frame, to give XSDB. In parallel, the first ATG of the X hybrid gene was mutated to generate the control plasmid XS^{m}DB (Figure 2). Remarkably, the XSDB plasmid lacking the acidic region showed a low transactivating activity as compared to that obtained with XFDB (1.7- versus 20-fold induction, Figure 3). Similar results were obtained by analyzing the effect of these chimeric genes on the TK promoter as monitored by CAT activity. XSDB had a low transactivation activity (~1.3-fold induction, Figure 3). Although the activity of XSDB is low, it is still significant, since this construct abolished the repression effect of the linked C/EBP DNA-binding domain. Thus, in accordance with the sequence composition of the activating domain

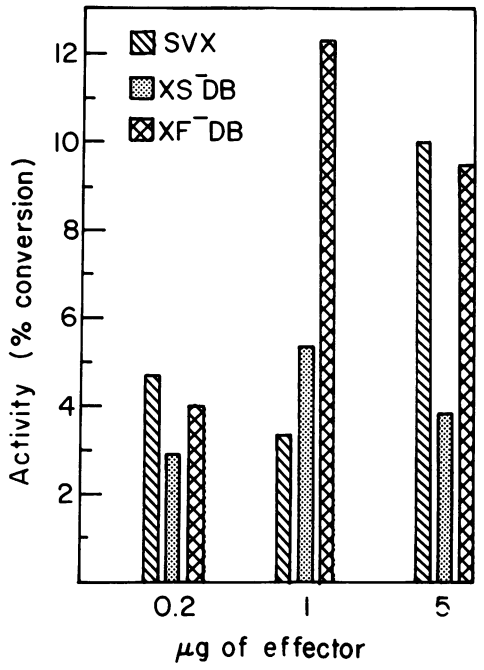

Fig. 5. A diagrammatic representation of the activities of native X and its mutant. HepG2 hepatoma cells were cotransfected with different amounts (0.2, 1 and 5 μg) of each effector plasmid, as indicated, together with pEX$_p$$^+$CAT reporter plasmid (Treinin and Laub, 1988) containing the HBV enhancer−X promoter complex, and CAT activity was determined.

of several transcription factors, the X stimulates transcription through a region rich in acidic amino acids.

The carboxyl end of the native X is required for its transactivation function

The data presented so far support the hypothesis that X acts by direct interaction with the transcription machinery through the XRE-binding proteins. In this case, one would expect

that the acidic region within X is also required for the activity of native X that lacks DNA-binding activity. The effect of the acidic region on the activity of X was analyzed by employing the same truncated X genes but without the functional DNA-binding domain of the C/EBP gene. These plasmids, XS$^-$DB and XF$^-$DB, contain the sequence of C/EBP but in an opposite orientation to the X gene (Figure 2). In this set of experiments, the EX$_p^+$CAT was employed as a reporter plasmid. In this plasmid, the expression of the CAT gene is driven by the HBV enhancer and the X gene promotor. In full agreement with the data obtained with the X−DB chimeric gene, a mutant of the native X gene lacking the carboxy-terminal 45 amino acids was not functional (Figure 5). Similar data were obtained by using the TKCAT3EG reporter plasmid and the Alexander cell line (data not shown). The requirement for the carboxy-terminus of the X protein for its transactivation function suggests that this region is a functional domain of X. Most probably, this domain acts to stimulate transcription by direct interaction with the transcription machinery. The importance of this region of X is also supported by its evolutionary conservation among the other members of the hepadna family (Figure 6C).

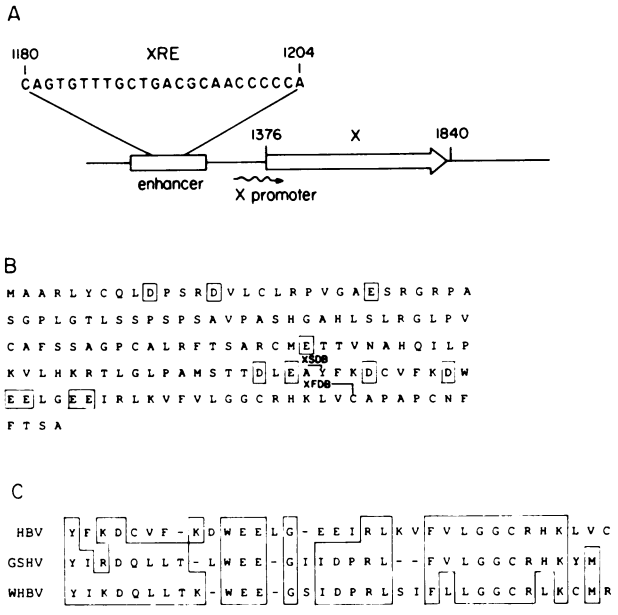

Fig. 6. The structure of the X gene and the predicted amino acid sequence of the whole or part of the X gene of different members of the hepadna family. **A.** The structure of the X gene, its position within the HBV genome (the nucleotide numbers are from the unique *Eco*RI site) and the sequence of XRE are shown. **B.** The predicted amino acid sequence of X gene subtype adw designated by the one letter code is shown. The acidic amino acids are boxed. **C.** Sequence comparison of the *StuI*−*FspI* region of X (bases 1707−1805 on HBV map) between the human hepatitis B virus (HBV, Valenzuela *et al.*, 1981), groundsquirrel hepatitis virus (GSHV, Seeger *et al.*, 1984) and woodchuck hepatitis virus (WHBV, Kodama *et al.*, 1985). Identical amino acids are boxed.

Discussion

The capacity of the X protein of HBV to function as a transactivator has been demonstrated recently by several groups (Spandau and Lee, 1988; Seto *et al.*, 1988; Twu and Robinson, *et al.*, 1989; Zahm *et al.*, 1988; Colgrove *et al.*, 1989). We have shown that the X protein of HBV transactivates the enhancer of its own genome and have defined a specific element within this enhancer which is sufficient to confer responsiveness to X (Faktor and Shaul, 1990; Figure 6A). This element, termed XRE, has a strong intrinsic enhancer activity and responds to multiple stimuli (Ben-Levy *et al.*, 1989; Faktor *et al.*, 1990). XRE binds multiple cellular transcription factors, including C/EBP, which interacts with unusual avidity with the XRE (Johnson *et al.*, 1987), the jun proteins and perhaps CREB (Ben-Levy *et al.*, 1989; Faktor *et al.*, 1990). Mutational studies revealed that the binding of these cellular factors is crucial for the enhancer activity of this element (Dikstein,R., Faktor,O. and Shaul,Y., submitted).

In general, transcription factors can be divided into two distinct domains. One directs DNA binding (DNA-binding domain) and the other presumably acts by direct interaction with some component of the transcription apparatus, probably the TATA-binding factor TFIID (Sawadogo and Roeder, 1985). The latter is known as the activating domain. Each domain acts independently so that a truncated transcription factor, containing only the DNA-binding region, is capable of recognizing and interacting with the cognate DNA *cis* element with the same sequence specificity. In our system, such a truncated gene functions as a repressor, probably due to competitive exclusion of the endogenous factors. The strong repression of the enhancer activity of XRE obtained under these conditions suggests that the truncated C/EBP (the DB gene) is efficiently expressed in transfected cells and that the protein product is translocated to the nuclei and binds to XRE. The possibility that this protein binds to additional sites within the reporter plasmid was not excluded in our experiments, but this should not affect our conclusions. Clearly, X is incapable of fully relieving the repression effect of the DB gene when present as a native protein but can do so as a hybrid protein with an XRE-binding activity. The simplest way to explain these observations is to assume that X has a transcription-activating domain, but lacks a DNA-binding domain that can efficiently bind XRE. This conclusion was also confirmed by consistent failure to demonstrate direct interaction *in vitro* of X with XRE (unpublished). Thus, we believe that X activates the XRE-binding factors by generating a complex through protein−protein interaction. However, the possibility that X also exerts its effect by catalytic modification of the XRE−binding proteins, from inactive to active forms, is not completely ruled out.

We report here that a hybrid X−DB protein behaves as a strong transcription factor. In these hybrid proteins, X plays the role of the activating domain, and possibly acts to stimulate transcription by direct interaction with cellular components of the transcription apparatus, such as the TATA-binding factor TFIID (Lin *et al.*, 1988). Furthermore, the functional domain of X was localized to a charged portion (acid region). Acidic sequences are a characteristic feature of a large number of well characterized activating domains of either viral (Triezenberg *et al.*, 1988b; Sadowski *et al.*, 1988) or cellular factors (Mitchell and Tjian, 1989). The acidic region was also found to play an important role in the transactivation of the HBV enhancer by native X, suggesting that similarly to the hybrid X−DB, the native X also acts by direct interaction with cellular transcription machinery through the enhancer-binding proteins.

The mode of action of X as suggested here is reminiscent of that of VP16, a sequence-specific transactivator of HSV (Post *et al.*, 1981; Triezenberg *et al.*, 1988a). It was shown that VP16 activates transcription by protein−protein interaction with octamer transcription factors (Gerster and Roeder, 1988). Similarly to the X protein of HBV, a hybrid VP16−Gal4 gene which binds DNA was found to be a potent transcription factor. In this hybrid gene, the DNA-binding domain of the heterologous gene of the yeast Gal4 factor was used to study the role of VP16 (Sadowski *et al.*, 1988), whereas in our experiments the role of X was investigated in the context of its homologous XRE *cis* element and one of its cognate binding proteins. The biggest advantage of the homologous system used by us is that synergistic interaction of X with the other XRE-binding proteins can also be addressed. These studies are now in progress.

In the absence of a DNA-binding domain within X, we assume that its sequence-specific effect is mediated by protein−protein interaction. This implies that X must have an additional functional domain that acts to recognize and interact with one or more of the XRE-binding cellular factors. Elucidation of this domain is of great importance for understanding the mechanism of action of X. What possible advantage is there of bearing a domain that generates protein−protein interaction over bearing a DNA-binding domain? We imagine that by protein−protein interaction, the viral transactivator can activate a larger repertoire of genes. Perhaps this is the reason for the capacity of the X protein to be a general transactivator and to stimulate the activity of a large number of viruses (Twu *et al.*, 1989). Furthermore, by protein−protein interaction, the virus might control some additional cellular machinery. This possibility is underscored by the recent observations that viral transactivators interact with some essential proteins that control cell growth. The best studied example is the interaction of E1A and SV40 Tag with the product of the retinoblastoma locus (Whyte *et al.*, 1988; DeCaprio *et al.*, 1988). This protein presumably acts as a tumor suppressor, an activity which is abolished by interaction with the E1A or SV40 Tag (Green, 1989). This interaction seems, therefore, to be crucial for transformation of cells by these viral transactivators.

Finally, the observation that X is a strong transcription factor once targeted to DNA may have some relevance to the proposed oncogenic potential of this gene (Zahm *et al.*, 1988). Most integrated HBV genomes contain a truncated X which lacks the translation stop codon. This event may lead to generation of a viral−host hybrid gene in which a cellular ORF is joined in-frame with the X ORF as described by Zahm *et al.* (1988). It is very likely, therefore, that in this way X may acquire a DNA-binding domain and thus function as a strong transcription factor with a new sequence specificity. Some of the genes induced by this hybrid protein might be regulators of cell growth the unscheduled expression of which produces the neoplastic phenotype.

Materials and methods

Plasmid constructions

The structure of SVX plasmid has been described elsewhere (Faktor and Shaul, 1990). This plasmid was constructed by cloning the X gene of HBV (*BalI*−*BglII* fragment 1217−1987 on the HBV map) into the pSV_2-DHFR vector (Lee *et al.*, 1981) from which the *StuI*−*Bam*HI containing the DHFR gene had been deleted. To fuse the DNA-binding domain of C/EBP to the X gene, a *PstI* fragment of 0.8 kb coding for the 141 carboxyl amino acids of the C/EBP and 385 bp of 3′ untranslated sequences of this gene was excised from L20 phage (Landschulz *et al.*, 1988) and cloned at the *PstI* site of GEM-2 (Promega Biotech), to generate the GEM2-DB plasmid. This plasmid was partially cleaved with *SmaI* to cut within GEM-2 at the 5′ end of the inserted fragment, then cleaved with *Hin*dIII followed by a fill-in reaction using Klenow (Boehringer). This fragment was inserted into SVX from which the *StuI* (base 1702 in the HBV genome)−*HpaI* (base 2666 in the SV40 genome) region containing the small t-intron sequences of SV40 from the SVX plasmid had been removed. In this XSDB plasmid the X gene is truncated at the *StuI* site (base 1702 in the HBV genome) and fused to the truncated C/EBP gene at the *SmaI* site. The DNA-binding domain was similarly introduced into the same *StuI*−*HpaI* fragment in the opposite orientation to the X gene to give SX^-DB. Within this construct, a translation termination signal is found 18 bp away from the *StuI*−*Hin*dIII ligated site. The same *SmaI*−*Hin*dIII fragment was also cloned in the SVX^m plasmid, to give XS^mDB. This plasmid contains a deletion of the first ATG of the X gene which was generated by *NcoI* cleavage followed by mung bean nuclease treatment to remove the four protruding base pairs.

The second set of hybrid X−DB plasmids was constructed as follows: a complete digest of a *SmaI* filled-in *Hin*dIII fragment containing 115 carboxyl amino acids and 385 bp of 3′ untranslated region of the C/EBP gene was excised from the GEM2-DB plasmid. This fragment was inserted into SVX from which the *FspI* (base 1803 in the HBV genome)−*HpaI* (2666 in SV40 genome) region had been removed, to form the XFDB plasmid. The same fragment of the C/EBP gene was also cloned, in the opposite orientation to the 3′ end of the X gene, at the *FspI* site to form XF^-DB plasmid. Within this construct, a translation termination signal is found 78 bp away from the *FspI*−*Hin*dIII ligation site. The XF^mDB was constructed in a similar way, except that the SVX^m plasmid was used. For the construction of the DB gene, the partial *SmaI*−*Hin*dIII fragment of GEM2-DB was first cloned into a Bluescript plasmid containing a functional ATG within a *NcoI* site. The ATG was introduced into the Bluescript plasmid by cloning a synthetic oligonucleotide of 15 nt (gccaccatggctgca) at *Eco*RV−*Hin*dIII sites. In the resultant plasmid, the ATG is in-frame with the coding sequence of the C/EBP gene. These C/EBP sequences linked to the ATG were excised from the Bluescript plasmid by partial *SmaI*−*Hin*dIII cleavage and cloned into *StuI*−*Hin*dIII digested SV_2-DHFR vector.

Cell growth and transfection

Cells were cultured in Dulbecco-modified Eagle's minimal essential medium (GIBCO) containing 100 U/ml penicillin and 100 μg/ml streptomycin with 8% fetal calf serum. The cells were plated onto 5 cm plates one day prior to transfection, reaching 50−70% confluence. DNA was transfected using the $CaPO_4$ method, followed after 12 h by a glycerol shock (Honigwacks *et al.*, 1989). Cell extracts were prepared 24 h after the glycerol shock and CAT activity was determined (Gorman *et al.*, 1982). For RNA analysis, 10 cm plates were used and cytoplasmic RNA was prepared 48 h after transfection and analyzed by an RNase protection assay as described elsewhere (Ben-Levy *et al.*, 1989; Honigwacks *et al.*, 1989).

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References

Ben-Levy,R., Faktor,O., Berger,I. and Shaul,Y. (1989) *Mol. Cell. Biol.*, **9**, 1804−1809.

Colgrove,R., Simon,G. and Ganem,D. (1989) *J. Virol.*, **63**, 4019−4026.

DeCaprio,J.A., Ludlow,J.W., Figge,J., Shew,J.-Y., Hung,C.-M., Lee, W.-H., Marsilio,E., Paucha,E. and Livingston,D.M. (1988) *Cell*, **54**, 275−283.

Faktor,O., Budlovsky,S., Ben-Levy,R. and Shaul,Y. (1990) *J. Virol.*, in press.

Faktor,O. and Shaul,Y. (1990) *Oncogene*, in press.

Gerster,T. and Roeder,R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6347−6351.

Gorman,C.M., Moffat,L.F., Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044−1051.

Green,M.R. (1989) *Cell*, **56**, 1–3.
Honigwacks,J., Faktor,O., Dikstein,R., Shaul,Y. and Laub,O. (1989) *J. Virol.*, **63**, 919–924.
Johnson,P.F., Landschulz,W.H., Graves,B.J. and McKnight,S.L. (1987) *Genes Dev.*, **1**, 133–146.
Kodama,K., Ogasawara,N., Yoshikawa,H. and Murakami,S. (1985) *J. Virol.*, **56**, 978–986.
Kovesdi,I., Reichel,R. and Nevins,J.R. (1986) *Cell*, **45**, 219–228.
Landschulz,W.H., Johnson,P.F., Adashi,E.Y., Graves,B.J. and McKnight,S.L. (1988) *Genes Dev.*, **2**, 786–800.
Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1989) *Science*, **243**, 1681–1688.
Lee,F., Mulligan,R., Berg,P. and Ringold,G. (1981) *Nature*, **294**, 228–232.
Lin,Y.-S., Carey,M.F., Ptashne,M. and Green,M.R. (1988) *Cell*, **54**, 659–664.
Mitchell,P.J. and Tjian,R. (1989) *Science*, **245**, 371–378.
Nyborg,J.K., Dynan,W.S., Chen,I.S.Y. and Wachsman,W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1457–1461.
O'Hare,P. and Goding,C.R. (1988) *Cell*, **52**, 435–445.
Post,L.E., Mackem,S. and Roizman,B. (1981) *Cell*, **24**, 555–565.
Preston,C.M., Frame,M.C. and Campbell,M.E.M. (1988) *Cell*, **52**, 425–434.
Ptashne,M. (1988) *Nature*, **335**, 683–689.
Sadowski,I., Ma,J., Triezenberg,S. and Ptashne,M. (1988) *Nature*, **351**, 563–564.
Sawadogo,M. and Roeder,R.G. (1985) *Cell*, **43**, 165–175.
Seeger,C., Ganem,D. and Varmus,H.E. (1984) *J. Virol.*, **51**, 367–375.
Seto,E., Yen,B.T.S., Peterlin,B.M. and Ou,J.-H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8286–8290.
Shaul,Y. and Ben-Levy,R. (1987) *EMBO J.*, **6**, 1913–1920.
Shaul,Y., Rutter,W.J. and Laub,O. (1985) *EMBO J.*, **4**, 427–430.
Spandau,D.F. and Lee,C.-H. (1988) *J. Virol.*, **62**, 427–434.
Tan,T.-H., Horikoshi,M. and Roeder,R.G .(1989) *Mol. Cell. Biol.*, **9**, 1733–1745.
Treinin,M. and Laub,O. (1987) *Mol. Cell. Biol.*, **7**, 545–548.
Treisman,R., Green,M.R. and Maniatis,T. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7428–7432.
Triezenberg,S.J., Kingsburg,R.C. and McKnight,S.L. (1988a) *Genes Dev.*, **2**, 718–729.
Triezenberg,S.J., LaMarco,K.L. and McKnight,S.L. (1988b) *Genes Dev.*, **2**, 730–742.
Twu,J.-S. and Robinson,W.S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2046–2050.
Twu,J.-S., Rosen,C.A., Haseltine,W.A. and Robinson,W.S. (1989) *J. Virol.*, **63**, 2857–2860.
Valenzuela,P., Quiroga,J., Zaldivar,J., Gray,P. and Rutter,W.J. (1981) In Fields,B., Jaenisch,R. and Fox,C.F. (eds), *Animal Virus Genetics*. Academic Press, Inc., New York, pp. 57–70.
Varmus,H. (1988) *Genes Dev.*, **2**, 1055–1062.
Whyte,P., Williamson,N.M. and Harlow,E. (1988) *Cell*, **56**, 67–75.
Wollersheim,M., Debelka,U. and Hofschneider,P.H. (1988) *Oncogene*, **3**, 545–552.
Zahm,P., Hofschneider,P.H. and Koshy,R. (1988) *Oncogene*, **3**, 169–177.

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