suggest that their interactions may be evaluated with use of this structure. A detailed comparison of the structure of FKBP and the FKBP-FK506 complex is likely to provide additional insights into the mechanism of rotamase catalysis. The determination of the molecular interactions of the binary complex reported herein with protein targets implicated in studies of signaling mechanisms could provide profound insights into the biological properties of these molecules (*18*).

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HBV X Protein Alters the DNA Binding Specificity of CREB and ATF-2 by Protein-Protein Interactions

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The hepatitis B virus (HBV) X gene product trans-activates viral and cellular genes. The X protein (pX) does not bind independently to nucleic acids. The data presented here demonstrate that pX entered into a protein-protein complex with the cellular transcriptional factors CREB and ATF-2 and altered their DNA binding specificities. Although CREB and ATF-2 alone did not bind to the HBV enhancer element, a pX-CREB or pX–ATF-2 complex did bind to the HBV enhancer. Thus, the ability of pX to interact with cellular factors broadened the DNA binding specificity of these regulatory proteins and provides a mechanism for pX to participate in transcriptional regulation. This strategy of altered binding specificity may modify the repertoire of genes that can be regulated by transcriptional factors during viral infection.

CONTROL OF EUKARYOTIC GENE TRANscription is a tightly regulated process mediated by nuclear factors whose availabilities are determined by cell type, differentiation state, and cell cycle (*1*). During viral infection, this system of coordinate regulation is perturbed by the activity of one or more viral gene products. In many cases, these viral proteins activate cellular transcription factors that interact with cis-acting elements present in viral promoters and enhancers, resulting in the expression of viral genes (*1, 2*).

Human HBV infects hepatocytes and causes acute and chronic liver disease. Infection is associated with the development of hepatocellular carcinoma (*3*). The small genome of this virus encodes four genes whose transcriptional activities are controlled by at least four promoters and two enhancer elements (*3, 4*). The 16.5-kD product of the HBV X gene, pX, is a transcriptional trans-activator capable of elevating transcription governed by a variety of viral and cellular control elements (*5, 6*). Similar to the herpes virus VP16 or adenovirus *E1A* gene products (*7, 8*), pX does not bind independently to responsive DNA sequences. An alternative mechanism through which pX might activate gene expression is through protein-protein interactions with cellular transcription factors (*7*).

Several investigators have described transcriptional stimulation of reporter genes linked to the HBV enhancer element that is mediated by pX (*5, 6*). The NF-κB sequence motif within the human immunodeficiency virus long terminal repeat is the target of pX-mediated activation (*6*). A cryptic NF-κB element is located within the HBV enhancer at nucleotide 984. Although a reporter construct containing this HBV sequence is activated tenfold compared to control plasmids (Fig. 1), purified NF-κB does not bind to this sequence motif (*9*). A second pX-responsive element was also localized within the HBV enhancer (Fig. 1). This region contains overlapping binding sites for multiple transcription factors, including NF-1, C/EBP, AP-1, CREB, and ATF (*10*). In

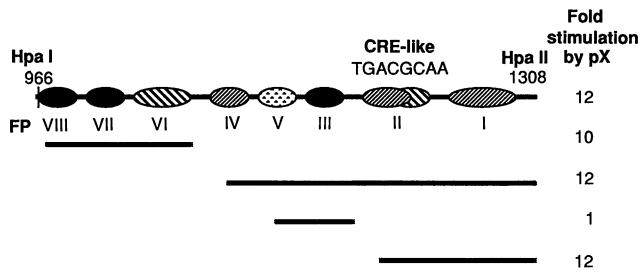

Fig. 1. Elements of the HBV enhancer that are pX-responsive. Luciferase gene expression (*18*) was under the control of the simian virus 40 (SV40) early promoter, which was linked to elements within the HBV enhancer. HepG2 or a stably transformed pX-expressing cell line (GET) was transfected with the HBV enhancer constructs indicated by the black lines, either alone or with a plasmid that encodes pX (*19*). Fold stimulation of transcription in the presence of pX is compared to basal levels achieved with the SV40 early promoter alone. Transfection efficiency was normalized with the pSV2β-galactosidase plasmid. FP, footprint sites protected in the DNase I assay, numbered in the order of their discovery.

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vitro binding of C/EBP, AP-1, and NF-1 to HBV enhancer sequences does not require pX (*9, 10*). Therefore, we focused on the interactions between pX and members of the CREB-ATF family.

The cellular transcription factors CREB and ATF-2 (or CRE-BP1) participate in the inducible response of several promoters and enhancers to adenosine 3′,5′-monophosphate (cAMP) or through the activity of the adenovirus E1A protein (*1, 2, 8, 11*). These factors bind to a consensus cAMP response element (CRE) with the sequence TGACGTCA (*1, 2, 11*). A CRE-like sequence (TGACGCAA) is located within the HBV enhancer. With an electrophoretic mobility shift assay, independent binding of pX to HBV enhancer sequences was not detected (*9*). In addition, neither CREB nor ATF-2 bound to the CRE-related sequence in the HBV enhancer (Fig. 2A). However, inclusion of pX in the binding reactions that contained either CREB or ATF-2 resulted in a DNA-protein interaction (Fig. 2A). This interaction was abolished with an excess of unlabeled enhancer DNA (*9*). That pX is an integral part of the protein-DNA complex is supported by two additional lines of evidence. First, sequential dilution of pX in the reaction mixture resulted in an equivalent decrease in the number of complexes formed (Fig. 2B). Second, addition to the binding reaction of an antibody to pX (*12*) retarded the mobility of the complex (Fig. 2C). We obtained similar results using serum with antibodies to CREB (*9*). Addition of pre-immune serum to the binding reactions did not alter the mobility of the complex (*9*).

The HBV DNA sequence recognized by the protein complex was defined with deoxyribonuclease I (DNase I) protection analysis. Although no protection of the HBV enhancer sequence was observed when CREB, ATF-2, or pX were present alone in the binding reactions, a distinct pattern of protection was observed after addition of pX to reactions that contained CREB or ATF-2 (Fig. 2E). The protected regions overlapped the sequences that correspond to the CRE-like element in the HBV enhancer. Under similar conditions, both CREB (Fig. 2D) and ATF-2 (*13*), in the absence of pX, bound to a consensus CRE sequence from the human α-gonadotropin gene promoter, verifying that the recombinant proteins exhibited DNA binding specificity. We have obtained similar results using CREB derived from a Baculovirus expression system (*13*), suggesting that potential differences in post-translational modification did not influence interactions between CREB, pX, and HBV DNA. These observations support a model in which CREB and ATF-2 alone have weak affinity for the HBV CRE-like element; the presence of pX would stabilize their binding. This could result from a conformational change induced by protein-protein interactions or by a modification conferred by an enzymatic activity associated with pX.

We investigated the ability of purified ATF-2 to interact directly with pX by utilizing a sensitive assay for analyzing protein-protein interactions (*13*). In this assay, we used ^{125}I-labeled recombinant peptides in solution to detect direct protein-protein interactions with proteins immobilized on a nitrocellulose filter. We immobilized ATF-2, pX, or control BL21 bacterial proteins on a nitrocellulose filter under nondenaturing conditions. Incubation of the filters with ^{125}I-labeled ATF-2 peptide re-

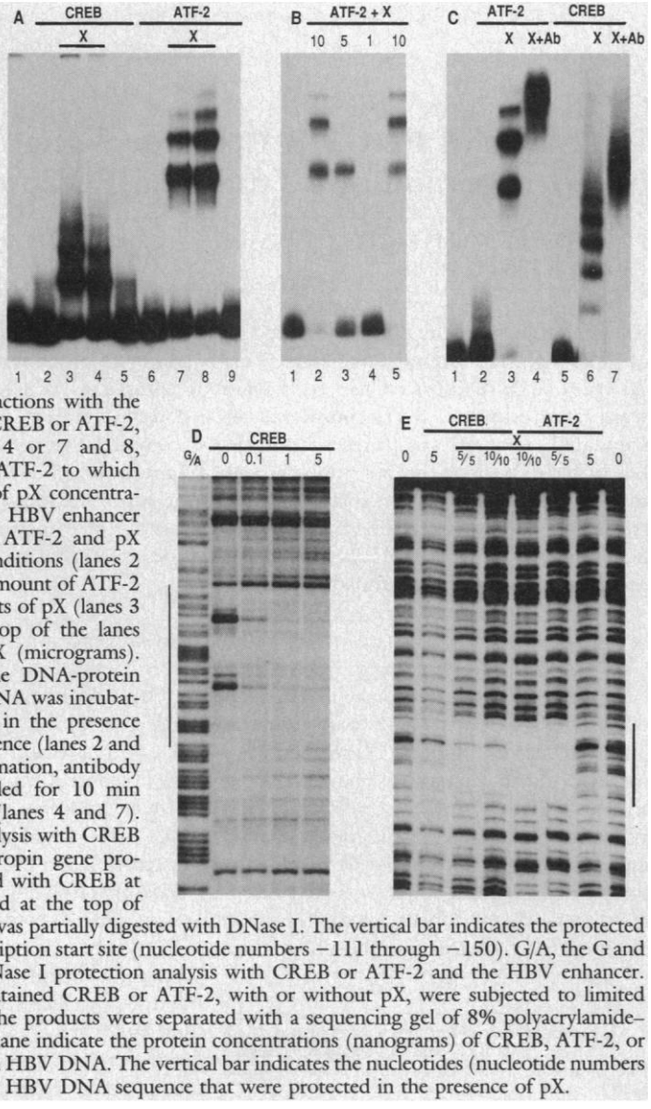

Fig. 2. Binding of CREB and ATF-2 to the HBV enhancer in the presence of pX. Partially purified proteins (*12, 13*) were mixed with an HBV enhancer fragment that was ^{32}P end-labeled (Acc I–Hpa II), and the complexes were separated by electrophoresis with a 5% polyacrylamide gel. In (A) to (C) lane 1 represents the probe in the absence of added protein (indicated by the arrow in the right margin). (**A**) Lanes 2 and 5, or 6 and 9, binding reactions with the HBV enhancer probe and CREB or ATF-2, respectively. Lanes 3 and 4 or 7 and 8, reactions with CREB and ATF-2 to which pX was added. (**B**) Effect of pX concentration on complex formation. HBV enhancer DNA was incubated with ATF-2 and pX under standard binding conditions (lanes 2 and 5) or with a constant amount of ATF-2 and with decreasing amounts of pX (lanes 3 and 4). Numbers at the top of the lanes indicate the amount of pX (micrograms). (**C**) Presence of pX in the DNA-protein complex. HBV enhancer DNA was incubated with ATF-2 or CREB in the presence (lanes 3, 4, 6, and 7) or absence (lanes 2 and 5) of pX. After complex formation, antibody (A6) to pX (*12*) was added for 10 min before gel electrophoresis (lanes 4 and 7). (**D**) DNase I protection analysis with CREB and the human α-gonadotropin gene promoter. DNA was incubated with CREB at the concentrations indicated at the top of each line (nanograms) and was partially digested with DNase I. The vertical bar indicates the protected region relative to the transcription start site (nucleotide numbers −111 through −150). G/A, the G and A sequencing lane. (**E**) DNase I protection analysis with CREB or ATF-2 and the HBV enhancer. Reaction mixtures that contained CREB or ATF-2, with or without pX, were subjected to limited treatment with DNase I. The products were separated with a sequencing gel of 8% polyacrylamide–urea. Numbers above each lane indicate the protein concentrations (nanograms) of CREB, ATF-2, or pX that were incubated with HBV DNA. The vertical bar indicates the nucleotides (nucleotide numbers 1199 through 1186) in the HBV DNA sequence that were protected in the presence of pX.

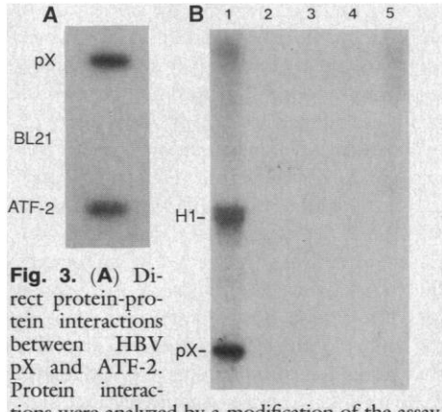

Fig. 3. (**A**) Direct protein-protein interactions between HBV pX and ATF-2. Protein interactions were analyzed by a modification of the assay described (*13*). Briefly, 500 ng of purified ATF-2 (350–505) recombinant peptide, 500 ng of purified pX, or 10 μg (20-fold excess) of BL21 bacterial lysate were immobilized on a nitrocellulose filter by the technique of slot blotting. The filter was incubated in nonfat dry milk for 1 hour to block nonspecific protein binding and then probed at room temperature for 8 hours with ATF-2 peptide that was ^{125}I-labeled, washed extensively, and subjected to autoradiography. (**B**) Protein kinase activity of pX. Immunoprecipitates immobilized on protein A–Sepharose beads (Pharmacia) were assayed for the presence of pX-specific protein kinase activity as described (*20*). Lanes 1, 3, and 4 represent the products of reactions with immobilized pX. Lanes 1, 2, and 5, calf thymus histone H1; lane 3, CREB; lane 4, ATF-2; lane 5, includes the products of a control reaction with extract prepared from the host bacterium BL21.

sulted in a specific interaction with immobilized ATF-2 and pX, but not with the bacterial lysate BL21 that lacked these proteins (Fig. 3A).

CREB and ATF-related proteins mediate gene expression after activation by protein kinases (*14*). The identification of a protein kinase activity associated with pX (*15*) suggests that CREB or ATF-2 may be a substrate for the pX kinase. However, under conditions in which phosphorylation of calf thymus histone H1 by pX was demonstrable, protein kinase activity was not evident when CREB or ATF-2 was used as substrate (Fig. 3B). Consistent with these observations, we have detected the pX-mediated binding of truncated forms of both CREB and ATF-2 in which potential phosphorylation sites have been deleted (*9, 16*). Taken together, these data demonstrate that the direct protein-protein interaction resulting in DNA-binding specificity is independent of the kinase activity of pX.

The data presented demonstrate that pX engages a CRE-like sequence in the HBV enhancer by direct protein-protein interactions with CREB or ATF-2, resulting in an altered DNA binding specificity. Like other viral transactivators, such as VP16 and E1A (*7, 8*), pX does not bind DNA directly. However, pX differs from E1A and VP16 in that neither of these proteins alters the specificity of the DNA-protein interaction. The inability of CREB or ATF to bind to the HBV CRE-like element in the absence of pX may be due to the lack of complete homology to the CRE consensus sequence, the contribution from the flanking sequences, or both. Because a number of purified proteins bind to the HBV enhancer, pX may participate in the selection of specific protein combinations whose interactions result in the alteration of transcriptional activity. Whether pX possesses an independent activating domain as suggested (*17*) or augments functional domains present in the protein complex remains to be determined. Within the context of the HBV genome, it is possible that trans-activation occurs by alternative mechanisms that are not mutually exclusive, but that are dependent on the availability of certain transcription factors during various stages of the cell cycle or of hepatocyte differentiation. The establishment of complexes between pX and cellular proteins allows these proteins to bind to sequences for which the affinity of these cellular factors may be weak. A consequence of this type of interaction could be an expanded repertoire of cellular genes that become activated during viral infection.

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Inhibition of PDGF β Receptor Signal Transduction by Coexpression of a Truncated Receptor

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A mutated form of the platelet-derived growth factor (PDGF) β receptor lacking most of its cytoplasmic domain was tested for its ability to block wild-type PDGF receptor function. PDGF induced the formation of complexes consisting of wild-type and truncated receptors. Such complexes were defective in autophosphorylation. When truncated receptors were expressed in excess compared to wild-type receptors, stimulation by PDGF of receptor autophosphorylation, association of phosphatidylinositol-3 kinase with the receptor, and calcium mobilization were blocked. Thus, a truncated receptor can inactivate wild-type receptor function by forming ligand-dependent receptor complexes (probably heterodimers) that are incapable of mediating the early steps of signal transduction.

WHEN PDGF BINDS TO ITS SPECIFIC receptor on the cell surface, the receptor protein becomes phosphorylated on tyrosine residues (autophosphorylation), and the conformation of the cytoplasmic domain of the receptor is altered so that the receptor can interact with and phosphorylate cytoplasmic signaling molecules (*1–4*). In response to PDGF, the receptor forms noncovalently linked receptor dimers (*5, 6*). Whether dimerization is required for receptor autophosphorylation and for generating cytoplasmic signals is not known.

The wild-type β receptor for PDGF expressed in Chinese hamster ovary (CHO-K1) cells by transfection of cDNA (*7*) underwent a ligand-induced increase in density detected by analysis on sucrose gradients (Fig. 1A). This shift was consistent with the formation of receptor dimers (*5*). Although we cannot exclude the possibility that the shift in sedimentation of the receptor was due to association of the receptor with other nonreceptor molecules, this seems unlikely since we observed a similar shift (*8*) in a receptor mutant that lacked tyrosine kinase activity and was unable to associate with cytoplasmic molecules (*4, 9*). Only the receptor that sedimented at 11.5*S* reacted with

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