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Hepatitis B Virus X Protein Produced in *Escherichia coli* Is Biologically Functional

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The hepatitis B virus X gene product *trans* activates transcription from a variety of viral and cellular regulatory elements. We expressed the complete, nonfused X protein in *Escherichia coli* and showed it to be active in *trans* activating a human immunodeficiency virus long terminal repeat-linked chloramphenicol acetyltransferase reporter gene.

Hepatitis B virus (HBV) contains an open reading frame called X, located between nucleotide positions 1376 and 1840 (in subtype adw), and encodes a protein of 154 amino acids or 16,560 daltons (9). The precise role of X protein during HBV infection is not clear. However, recent studies have established a *trans*-activating function for this protein (6–8, 13). With various cell lines, cotransfection studies using reporter gene systems have shown that the X gene product can activate transcription from the following regulatory elements: the HBV enhancer (1, 7), the simian virus 40 enhancer (8, 13), the human immunodeficiency virus (HIV) long terminal repeat (LTR) (6, 7, 10), the Rous sarcoma virus LTR (7, 13), the human T-lymphotropic virus type LTR (13), the herpes simplex virus thymidine kinase promoter (13), and the beta interferon gene promoter (11). This *trans*-activating property has also been established in human liver cells that stably express the X gene (7), as well as by integrated X gene sequences isolated from hepatocellular carcinoma tissue (12). Deletion mutagenesis studies on the HIV LTR (7, 10) have mapped the X-responsive elements within the enhancer region containing two direct repeats of the sequence GGGACTTTCC. These sequences are target sites for a transcription factor, NF-KB (5). Sequences bearing close homology to the NF-KB binding site are also present in at least some of the other X-responsive regulatory elements.

Little is known about the mechanisms of X-mediated regulation. Our interest is detailed structure-function analysis of the X protein to elucidate domains important for its activity. Two important prerequisites for this are the availability of large amounts of the active protein and an assay system to assess its functional activity. Therefore, we expressed the HBV X protein in *Escherichia coli* and purified it to homogeneity. Further, by using a simple cellular uptake assay, we demonstrated that the protein synthesized in *E. coli* displays a *trans*-activating function in cultured mammalian cells.

An expression system based on T7 RNA polymerase (F. W. Studier, A. H. Rosenberg, and J. J. Dunn, Methods Enzymol., in press) was used in this study. An *Nco*I-*Bgl*II fragment of HBV (nucleotides 1375 to 1987) was cloned between the *Nco*I and *Bam*HI sites of the vector pET-8C (Studier et al., in press) to yield plasmid pET-X (Fig. 1A). This placed the entire X open reading frame, including methionine codon 1, directly downstream of the T7 promoter and the ribosome-binding site in the vector. Expression was done in BL21 (DE3), which is a protease-deficient (Lon^- $ompT^-$) strain of *E. coli* containing a bacteriophage lambda lysogen that, on induction with isopropyl-β-D-thiogalactopyranoside (IPTG), expresses the T7 RNA polymerase gene. A 16- to 17-kilodalton protein was inducibly expressed in cells containing plasmid pET-X but not in cells with control plasmid pET-8C (Fig. 1B).

To establish that the 16- to 17-kilodalton protein is in fact the HBV X protein, a Western immunoblotting experiment was performed. Polyclonal antisera against two synthetic peptides from the HBV X protein were raised in rabbits. These peptides included amino acids 100 to 114 (HBX100) or 144 to 154 (HBX144) and were coupled to keyhole limpet hemocyanin or purified protein derivative, respectively, before injection. Proteins synthesized in transformed *E. coli* induced with IPTG were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. On probing with anti-HBX144, the 16- to 17-kilodalton band was seen only in pET-X transformed cells (Fig. 2A). Furthermore, a linear response to IPTG induction was also seen. Similar results were also obtained with anti-HBX100 (data not shown). To further establish the specificity of the anti-peptide sera, a cross-competition Western analysis was done. Nitrocellulose strips containing total *E. coli* lysates were incubated with either antiserum without or with the specific or the nonspecific peptide. The results (Fig. 2B) showed that a major protein band corresponding to the X protein monomer was eliminated by the specific peptide (lanes 3 and 7) but not by the nonspecific peptide (lanes 4 and 6). This showed that the expressed protein was indeed the HBV X protein. Two extra bands seen with anti-HBX100 may have been due to bacterial proteins with homology to the HBX100 peptide sequence.

To purify the expressed X protein, cells from cultures induced for 3 h were suspended in 5 to 10% of the culture volume of 50 mM Tris (pH 8.5)–10 mM EDTA. After one freeze-thaw cycle, the cells were disrupted by sonication and the lysate was centrifuged at 14,000 × *g* for 15 min. The pellet was suspended in the above-described buffer containing 30% (wt/vol) sucrose and collected by centrifugation. This pellet was dissolved in the above described buffer containing 6 M urea and 5 mM 2-mercaptoethanol. At this stage, the mixture was dialyzed against phosphate-buffered saline (pH 7.2) containing 1 mM 2-mercaptoethanol and used

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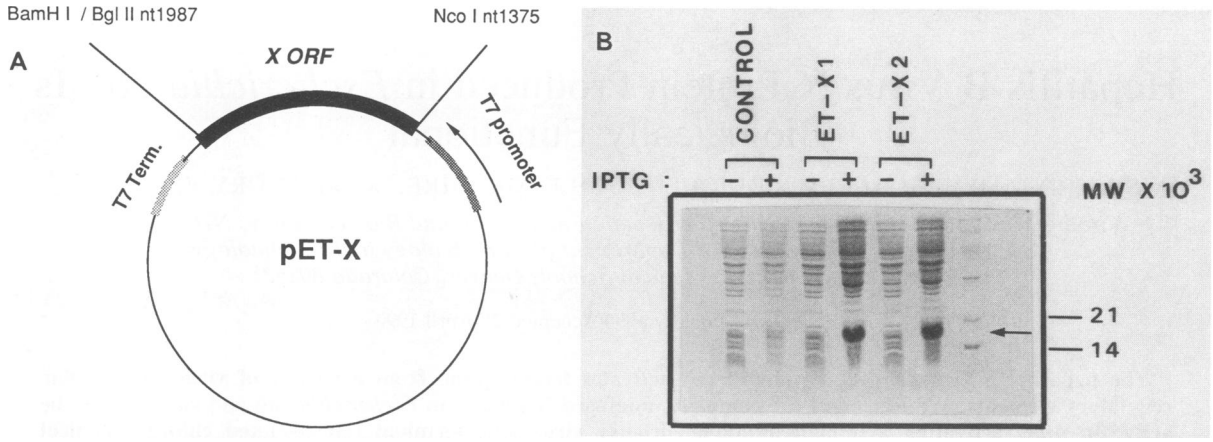
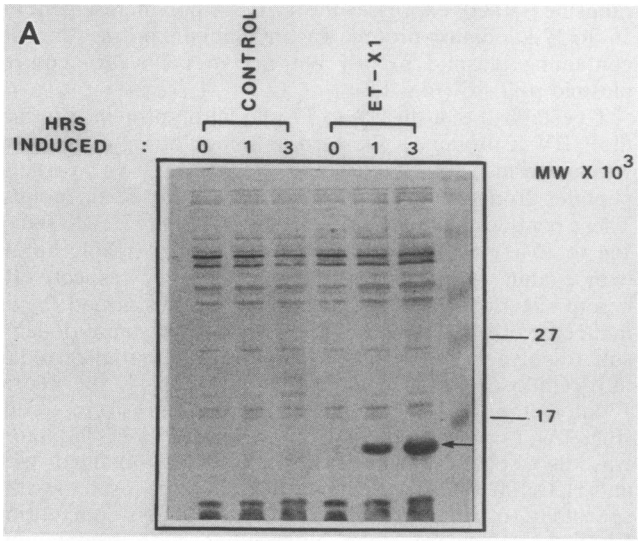
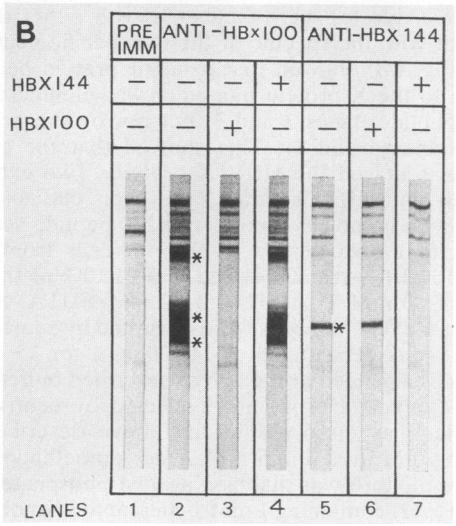


FIG. 1. (A) Schematic illustration of expression vector pET-X. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins present in *E. coli* transformed with plasmid pET-8C (control) or pET-X (two independent clones, ET-X1 and ET-X2). Cells were grown at 37°C to an A_{600} of 0.7 to 1.0 and induced with (+) or without (−) 0.4 mM IPTG for 3 h. Total bacterial proteins were analyzed on a 15% polyacrylamide gel after staining with Coomassie blue. The arrow indicates the presence of a new 16- to 17-kilodalton protein in pET-X-transformed cells on IPTG induction is indicated. ORF, Open reading frame; nt, nucleotide; Term., terminator; MW, molecular weight.





in functional assays. Alternatively, further purification was performed by preparative isoelectric focusing. The urea-solubilized protein mixture was diluted to 48 ml such that the final buffer strength was 10 mM Tris (pH 8.5)–2 mM EDTA–1 mM 2-mercaptoethanol. After adding 2 ml of pH 3 to 10 ampholines (Bio-Rad Laboratories), the mixture was electrofocused in a Bio-Rad Rotofor preparative isoelectric focusing cell for 5 h at 4°C. The X protein peak fractions focused in the pH range of 7.9 to 8.6 (fractions 12 to 14); the peak fraction (no. 13) had a pH of 8.3, which is close to the theoretical pI of 8.5. This gave electrophoretically pure X protein (Fig. 3).

The only known function of X protein is to *trans* activate certain *cis*-acting regulatory elements. Of these, the HIV LTR is the best characterized. We used a HeLa cell-derived line (LTR3CAT) stably transfected with a plasmid containing the bacterial gene for chloramphenicol acetyltransferase (CAT) under control of the HIV LTR sequences. The assay systems used were those described earlier—direct cellular uptake of proteins (4) or scrape loading of cells with proteins (2). Figure 4 shows the results of expression of the gene for CAT in the LTR3CAT cell line. Whereas very low background CAT activity was seen in the cells (lanes 1 and 2),

FIG. 2. Western analysis of X protein expression. (A) Control and pET-X-transformed *E. coli* cells were induced, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the legend to Fig. 1B. The filter with transferred protein was blocked with 1% nonfat milk in phosphate-buffered saline for 2 h followed by successive incubations with a 1:100 dilution of anti-HBX144 (in phosphate-buffered saline-milk) and a 1:500 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (in phosphate-buffered saline) for 2 to 3 h each. The blot was then developed by using 4-chloronaphthol as the substrate. MW, Molecular weight. (B) Nitrocellulose strips containing total proteins from pET-X-transformed *E. coli* were incubated with preimmune sera (1:100), anti-HBX100 (1:100), or anti-HBX144 (1:250). For competitions, peptide HBX100 or HBX144 was also included in the primary antibody incubation at a concentration of 0.5 mg/ml. The secondary antibody and color development were as described above. Bands eliminated by specific peptides (*) and the position of the X protein monomer (arrow) are indicated.

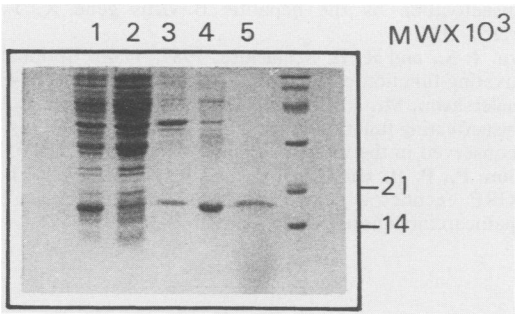


FIG. 3. Purification of HBV X protein. A representative progress of purification is shown. Lanes: 1, total cell lysate; 2, postsonication supernatant; 3, 30% sucrose wash of postsonication pellet; 4, pellet from sucrose wash in 6 M urea; 5, pooled fractions 12 to 14 from isoelectric focusing. MW, Molecular weight.

there was an approximately eightfold increase on scrape loading of the X protein into these cells (lanes 3 and 4). The increase was even more pronounced, up to 30-fold, when the cells were simply overlaid with protein (lanes 5 and 6). This level of stimulation by X is consistent with the results obtained by others following DNA transfection (7). This *trans* activation appeared to be specific, as there was no increase in the levels of actin RNA when X protein-containing extract was loaded into LTR3CAT cells compared with control extracts (data not shown). Thus, the X protein produced in *E. coli* was biologically active. The HIV LTR also seems to be stimulated by the HBV X protein in the environment of lymphocytic and hepatic cell types (H. Maguire, unpublished data). Although cell-specific differences are expected, it appears that X can function as a standard *trans* activator in a variety of cell types.

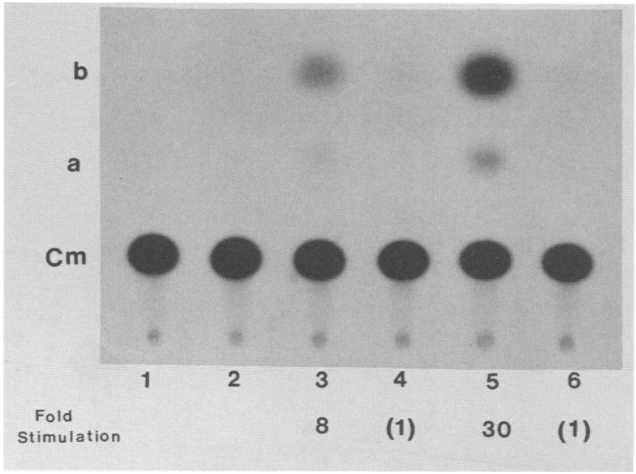


FIG. 4. Functional activity of X protein. Partially purified protein extracts were prepared from *E. coli* transformed with plasmid pET-X (X extract) or pET-8C (control extract); the protein content of the former was equivalent to that shown in Fig. 1 and 2. This preparation, at a total protein concentration of 100 μg/ml, was added to 2 ml of Dulbecco modified Eagle medium containing 10% fetal bovine serum and overlaid on a monolayer of LTR3CAT cells. For scrape loading, the cells were gently scraped off the plate in the above-described medium and then allowed to settle down. After 6 h at 37°C, 8 ml of fresh Dulbecco modified Eagle medium with 10% fetal bovine serum was added and the cells were harvested for CAT assay (3) after another 24 h. Lanes 1, 3, and 4 contained cells scrape loaded with Dulbecco modified Eagle medium, X, and control extracts, respectively. Lanes 2, 5, and 6 contained cells overlaid with Dulbecco modified Eagle medium, X, and control extracts, respectively. Fold stimulation of CAT activity over the background is indicated. Cm, [^{14}C]chloramphenicol; a, 1-acetate; b, 3-acetate.

Several possible mechanisms can be invoked to explain the *trans*-activating function of the HBV X protein. The protein may directly bind to target DNA sequences within a given response element, for example NF-KB-like sequences. This, however, is unlikely because of the rather ubiquitous nature of X-mediated *trans* activation. Not all of its target genes contain NF-KB-like sequences or other defined sequence homologies in their regulatory regions. We tried direct DNA-binding experiments with *E. coli*-expressed or in vitro-translated X protein and probes containing the HIV LTR, the HBV enhancer, or other subgenomic regions of HBV. However, no specific binding of X to a DNA fragment was observed (data not shown).

trans-Activator proteins that do not bind DNA directly may function by entering into complexes with other transcription factors. Protein-protein interactions are fundamental to regulation of transcription in eucaryotic cells. Without a DNA-binding function, it is likely that X protein activates transcription by interacting with or modifying other transcription factors. For example, for the HIV LTR, the NF-KB factor may be one such transcription factor influenced by X. It is known that under noninducing conditions or in nonlymphocytic cells, NF-KB is found in the cytoplasm, bound to an inhibitor (IKB) (5). HBV X protein may act, for example, by shifting the equilibrium in favor of free NF-KB. Availability of pure preparations of X protein will facilitate inquiry into its ability to interact with other proteins. More detailed analysis remains to be performed in terms of the domains of X protein essential for *trans* activation. Establishment of the good expression and assay systems reported here forms the basis for future studies.

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