Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2513–2517, April 1987
Microbiology

Expression of the hepatitis B virus X gene in mammalian cells

(*in vivo* transcription/chloramphenicol acetyltransferase assay/protein analysis/cell fractionation/immunofluorescence)

ALEEM SIDDIQUI, SHAHID JAMEEL, AND JOHN MAPOLES

Department of Microbiology and Immunology, University of Colorado School of Medicine, Denver, CO 80262

Communicated by Theodore T. Puck, December 23, 1986

ABSTRACT **The hepatitis B virus (HBV) DNA contains an open reading frame (ORF) designated X, which has the capacity to encode a protein of 16,560 Da (subtype adw). Such a protein has not been identified in either HBV particles or infected human livers, and therefore its role in the viral life cycle remains unknown. We report here the expression of the HBV X ORF in cultured cells using recombinant vectors. A protein of 16 kDa was identified by means of an antiserum prepared against a synthetic peptide and with human antisera from hepatitis B patients as well as those with hepatocellular carcinoma. Cell fractionation and immunofluorescence studies suggest a probable association with cytoskeletal components. Our studies further located a promoter sequence upstream of the X ORF, which directs the transcription of a 0.7- to 0.8-kilobase X-specific RNA in transfected human hepatoma cells.**

Hepatitis B virus (HBV) is the causative agent for viral hepatitis B and the infection has been linked to hepatocellular carcinoma in humans (1, 2). An understanding of the biology of this virus has been severely limited because of the inability to propagate it *in vitro*. Molecular cloning and nucleotide sequencing have identified at least four open reading frames (ORFs): S/pre-S, C, X, and Pol (2). The S and C regions have been studied by expression via recombinant vectors in both prokaryotic and eukaryotic cells (2–6); the expression and identification of X and Pol gene products has not yet been accomplished. In the viral DNA, the X ORF lies between nucleotide positions 1380 and 1842 (Fig. 1*A*) and overlaps the two direct repeats and the region of cohesive termini (1, 2). It can encode a polypeptide of 154 amino acids of 16,560 Da. Previous studies have described a 28-kDa protein in HBV-infected livers, which was recognized by an antiserum prepared against a synthetic peptide from the X ORF (9). Antibodies containing anti-X reactivity from hepatitis B carriers were also detected (7, 10). While these studies demonstrate the coding potential of the X ORF and its association with viral infection, expression of the authentic X protein has not been achieved. Furthermore, the elements in HBV DNA responsible for the expression of X ORF are not known. We have studied the expression of this region in monkey COS cells and in human liver HepG2 cells by using a transient expression system. We describe here the synthesis of a 16,000-Da protein, consistent with the coding capacity of the X ORF (2). This expression was achieved through the use of either an upstream native promoter sequence or with strong heterologous promoter/enhancer elements.

MATERIALS AND METHODS

DNA Constructions. The HBV genome used in our studies was derived from an adw subtype and its cloning has been described (11). Plasmid pQ3 was from R. Kaufman, (Genetics Institute, Boston) and pSV2CAT was from Bruce Howard (National Institutes of Health). Two types of recombinant vectors were constructed for X gene expression (Fig. 1*B*). Plasmid pMNX contains an *Acc* I/*Taq* I [1076–2020 nucleotides (nt)] fragment, which has ≈300 base pairs (bp) of 5′ flanking sequences, including the enhancer element (12, 13), the X ORF (1380–1842 nt), followed by a polyadenylylation signal sequence (1920 nt). This plasmid was designed to investigate the possible existence of a promoter upstream of the X region. In plasmid pAdX, the X ORF was placed under the control of the adenovirus major late promoter, simian virus 40 (SV40) enhancer, and virus-associated (VA) genes I and II (14). An *Eco*RI/*Sal* I fragment [3.3 kilobases (kb)] from plasmid pQ3 was subcloned into pML (15) to produce plasmid pMLAd. This does not include the dihydrofolate reductase gene or the SV40 polyadenylylation signal sequences present in pQ3. The *Nco* I/*Taq*I (1380–2020 nt) fragment of HBV, which contains the X ORF and the native polyadenylylation signal, was then introduced into pMLAd to generate the expression vector pAdX (Fig. 1*B*). COS cells (16) were used for expression of both the vectors pAdX and pMNX, whereas HepG2 human hepatoma cells (17) were used for the expression of pMNX.

To assay for promoter activity in the 5′ flanking sequences of the X ORF, we used the bacterial chloramphenicol acetyltransferase (CAT) expression system (18). The sequences upstream of the X ORF were cloned 5′ to the CAT gene between *Acc* I and *Hin*dIII restriction sites in the vector pSV0CAT (18). The HBV sequences in the resulting CAT plasmids are as follows: 1, pXPEnCAT, *Acc* I/*Nco* I (1076–1380 nt); 2, pXPCAT, *Sph* I/*Nco* I (1236–1380 nt); 3, pEnCAT, *Acc* I/*Sph* I (1076–1236 nt).

All enzymes were from either Boehringer Mannheim Biochemicals or Bethesda Research Laboratories.

RNA Analysis. The methods used for S1 nuclease mapping and RNA blot analysis have been described (8, 15).

CAT Assays. CAT assays were performed according to the method of Gorman *et al.* (18).

Protein Analysis. COS cells or Hep G2 cells were transfected with appropriate expression vectors using either the DEAE-dextran/chloroquine method (19) or the calcium phosphate coprecipitation method (20). Forty-two hours posttransfection, cells were labeled with 250 μCi (1 Ci = 37 GBq) of [^{35}S]methionine for 6 hr, collected by scraping, and fractionated as described in the figure legends. For fractionation, cells were lysed either by Dounce homogenization in a hypotonic buffer (0.05 M Tris·HCl, pH 7.5/0.025 M KCl/0.005 M $MgCl_2$) (TKM buffer) or with detergents to produce nuclear and cytoplasmic fractions. The lysates were immunoprecipitated in RIPA buffer (0.1 M NaCl/0.05 M Tris·HCl, pH 7.5/1% Nonidet P-40/1% deoxycholate/0.1% $NaDodSO_4$) with the indicated antisera, adsorbed with staphylococcal protein A, and analyzed on 18% $NaDodSO_4$/polyacrylamide gels. Gels were soaked in 0.5 M sodium

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; ORF, open reading frame; nt, nucleotide(s); CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; VA, virus associated.

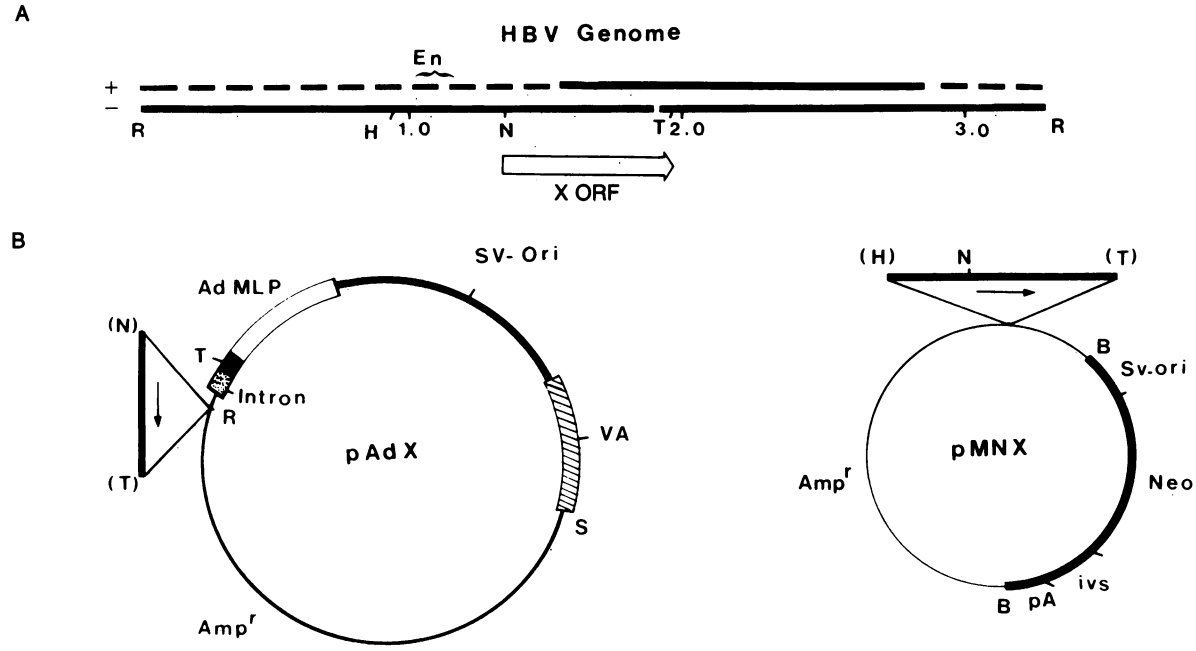

FIG. 1. Expression vectors. (*A*) Linear representation of the HBV genome. Dotted line refers to the single-stranded region in the HBV DNA molecule (1, 2). (*B*) Plasmid pAdX. HBV sequences in this vector include the X ORF and the polyadenylylation signal (*Nco* I/*Taq* I, 1384–2020 nt). The HBV fragment was cloned in plasmid pMLAd, which contains a *Sal* I/*Eco*RI fragment (3.3 kb) derived from plasmid pQ3 (7) and includes the adenovirus tripartite leader, a hybrid intron consisting of a 5′ splice site of the first exon of the tripartite leader and a 3′ splice site from a mouse immunoglobulin gene, two units of the SV40 origin including the SV40 enhancer and the adenovirus VA I and II gene regions. Plasmid pMNX contains an HBV *Acc* I/*Taq* I (1076–2020 nt) fragment cloned into the *Cla* I site of pMLneo (8). B, *Bam*HI; R, *Eco*RI; H, *Hpa* I; N, *Nco* I; T, *Taq* I; S, *Sal* I; Neo, neomycin resistance; Amp^r, ampicillin resistance; En, enhancer element; AdMLP, adenovirus major late promoter; VA, VA genes I and II; ivs, intervening sequences of SV40 small tumor antigen; pA, SV40 polyadenylylation signal; + and − indicate the polarity of the DNA strands.

salicylate for 1 hr, dried, and exposed to XAR-5 films at −70°C.

Immunofluorescence. COS cells transfected with pAdX were plated on tissue culture chamber slides 24 hr posttransfection, fixed in cold acetone, and incubated with anti-X peptide antiserum or preimmune serum. Second-stage antibody was fluorescein isothiocyanate-conjugated anti-rabbit IgG [affinity-purified F(ab′)$_2$ fragments; Cooper Biomedicals, Malvern, PA] at a 1:1000 dilution. Cells were visualized using a Zeiss Microscope 16, with IV FL vertical illuminator and Neofluor 40/0.75 objective.

RESULTS

Transcription of X RNA. We and others have previously noted a subgenomic transcript of ≈0.8 kb, which was speculated to correspond to the X ORF (15, 21–23). To demonstrate that the X ORF can encode a RNA and to investigate whether sequences upstream of the X ORF contained any transcriptional control elements, we constructed plasmid pMNX. The HBV sequences (*Acc* I/*Taq* I, 1076–2020 nt) in this vector included ≈300 bp located upstream of the X ORF, including the enhancer element. Transfection of HepG2 cells with pMNX resulted in the synthesis of a RNA of ≈800 nt (Fig. 2*A*). S1 nuclease mapping of this RNA using a *Hpa* I/*Ava* I probe radiolabeled at the 5′ end showed multiple initiations, some extending clearly into the enhancer region. The major initiation sites map approximately at nucleotide positions 1255, 1280, 1300, and 1310 (Fig. 2 *B* and *C*). To further confirm the presence of a promoter upstream of the X ORF, the CAT gene expression system was used (18). The activity of the CAT gene in transfected cells can be conveniently measured and is correlated with steady-state mRNA levels (24, 25). Fig. 2*D* shows the construction of the CAT plasmids used in this study. Plasmid DNAs were introduced into HepG2 cells, and the CAT activity was measured. Results of representative CAT assays are shown in Fig. 2*E*. The *Acc* I/*Nco* I fragment (1076–1380 nt, plasmid pXPEnCAT) directed efficient expression of the CAT gene in HepG2 cells. To further define the promoter element, this fragment was cleaved at the *Sph* I site and the resulting fragments were cloned 5′ to the CAT gene to produce plasmids pEnCAT (*Acc* I/*Sph* I, 1076–1236 nt) and pXPCAT (*Sph* I/*Nco* I, 1236–1380 nt). It should be noted that the HBV fragment in plasmid pEnCAT contains only the enhancer element, the boundaries of which have been described (12, 13). As shown in Fig. 2*E*, both the fragments directed efficient expression of the CAT gene. From these results and from the S1 nuclease analysis of X gene transcripts, it is clear that the promoter activity extends into the enhancer region. Both these fragments were inactive when placed in an antisense orientation with respect to the CAT gene (data not shown). When assayed in HeLa cells, the X promoter was <10% as efficient as in HepG2 cells, indicating a tissue-specific character (S.J., unpublished results).

Protein Analysis and Subcellular Localization of X. To facilitate the identification and subsequent analysis of the X protein by NaDodSO$_4$/PAGE, we constructed the expression vector pAdX, in which the X ORF was placed under the transcriptional control of the adenovirus major late promoter, SV40 enhancer, and VA genes I and II (14). COS cells were used for the transient expression of this plasmid. Cells were transfected with pAdX, the cell lysates immunoprecipitated with appropriate antisera, and analyzed by NaDodSO$_4$/PAGE (Fig. 3*A*). A 16-kDa protein was specifically immunoprecipitated with an anti-X serum made against peptide 99 (9) (lane 6) and human sera from a patient with hepatocellular carcinoma (lane 4) or from a hepatitis B carrier (lane 2). These human sera were previously shown to contain

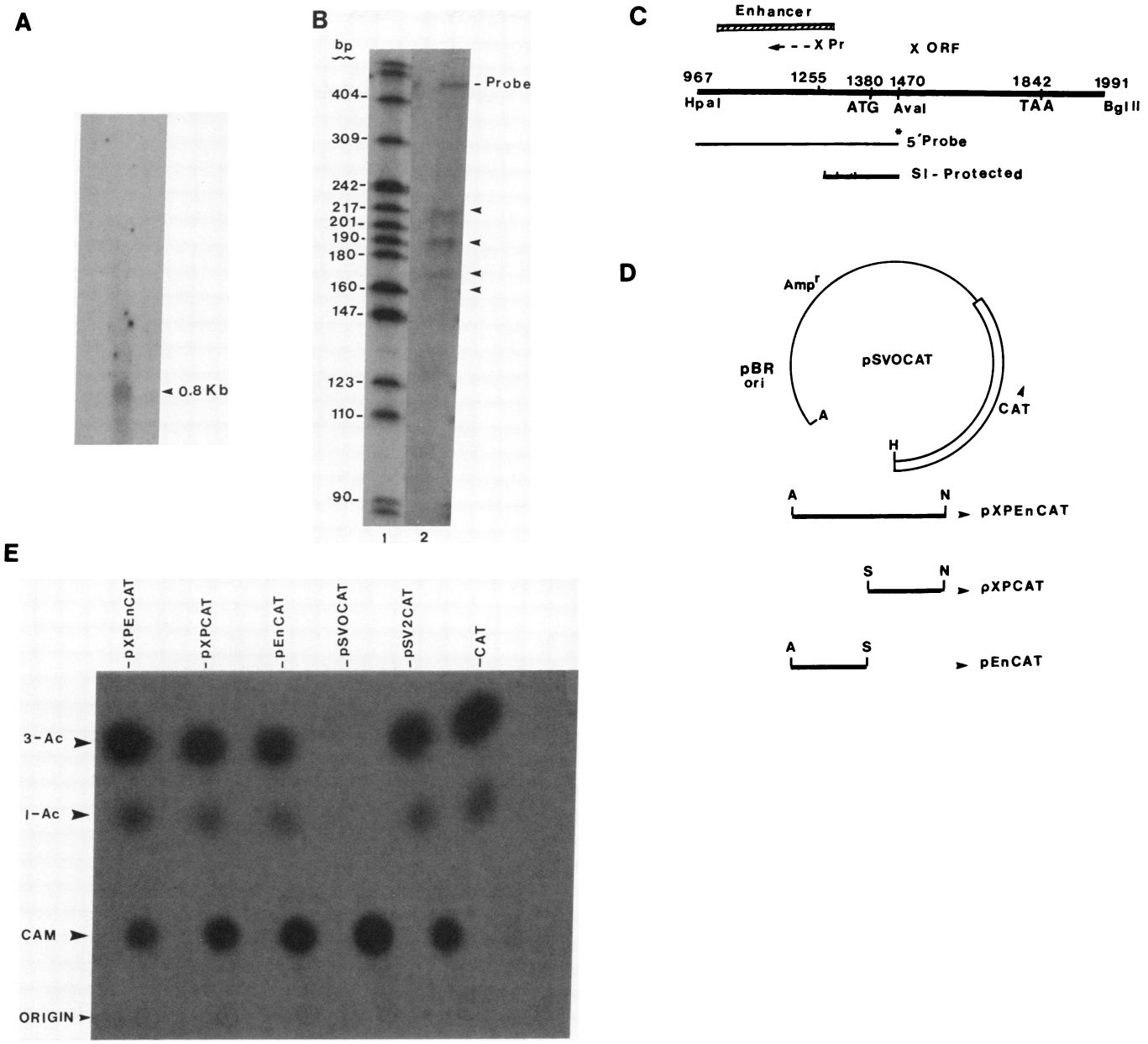

FIG. 2. Characterization of X RNA and promoter element. (*A*) Blot-hybridization analysis of 5 μg of poly(A)$^+$ RNA (8) from HepG2 cells transfected with plasmid pMNX. A ^{32}P-labeled probe containing the X ORF sequences was used. (*B*) S1 nuclease mapping analysis (19). Total cytoplasmic RNA (20 μg) from HepG2 cells transfected with plasmid pMNX was hybridized with a 5′-end-labeled *Hpa* I/*Ava* I (967–1470 nt) fragment and the S1 nuclease-protected fragments were analyzed on a 8% urea/polyacrylamide gel. Lane 1, *Hpa* II-cleaved 5′-end-labeled pBR322 DNA fragments as molecular weight standards. Lane 2, S1 nuclease protected fragments. Arrows indicate the major fragments. (*C*) Schematic illustration of the S1 nuclease probe and the fragments protected from S1 nuclease digestion by pMNX-directed RNA. The four major 5′ ends are indicated on the S1 nuclease-protected illustration. The approximate boundaries of enhancer region and the X promoter are shown above. (*D*) Structure of HBV-CAT plasmids. The HBV fragments (*Acc* I/*Nco* I, *Sph* I/*Nco* I, and *Acc* I/*Sph* I) were inserted into pSVOCAT in the orientations shown. A, *Acc* I; N, *Nco* I; S, *Sph* I; H, *Hpa* I; Amp^r, ampicillin resistance. (*E*) CAT assays of lysates prepared from HepG2 cells transfected with the indicated plasmids. Conversion of [^{14}C]chloramphenicol (CAM) into its acetylated forms (1-Ac and 3-Ac) is shown for a 60-min reaction using 250 μg of protein from each transfected cell lysate.

anti-X reactivity (9). This protein band is absent in uninfected COS cells (lanes 8 and 9) and is not precipitated by normal human sera or preimmune rabbit serum (lanes 1, 3, and 5). Furthermore, labeling of transfected cells with [^{32}P]orthophosphate showed that X is not a phosphoprotein (data not shown).

To study the subcellular location of the X protein, pAdX-transfected COS cells were fractionated (Fig. 3). Cell fractionation by Dounce homogenization revealed the X protein to be associated with the nuclear pellet (Fig. 3*B*, lane 5). It was not found as a cytosolic protein or associated with cytoplasmic membranes (lanes 3 and 7). The X protein synthesized under the control of its native promoter (plasmid pMNX) shows a similar association with the nuclear pellet (Fig. 3*D*, lane 2). When pAdX-transfected COS cells were lysed by 0.5% Nonidet P-40 treatment and fractionated into detergent-soluble and insoluble portions, some of the X protein fractionated into the Nonidet P-40 soluble fraction (Fig. 3*C*, lanes 1 and 2), but a sizeable portion still remained associated with the nuclear pellet (lanes 8 and 9). In another experiment, when the nuclear pellet from Dounce-homogenized cells was extracted with 1% Nonidet P-40 and 0.5%

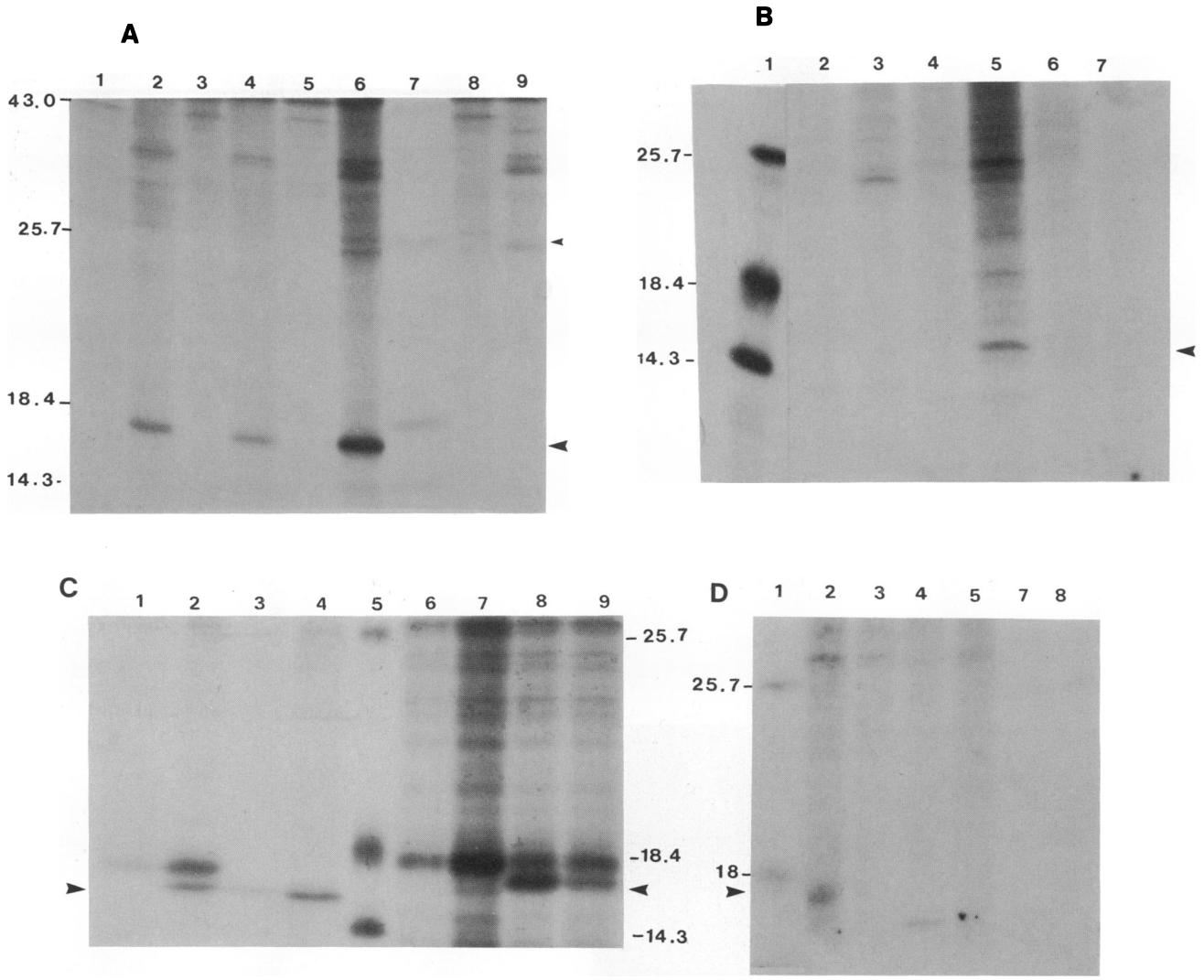

FIG. 3. NaDodSO$_4$/PAGE analysis of X protein. (*A*) Analysis of total COS cell lysates transfected with pAdX. Cells were sonicated in STE buffer (0.1 M NaCl/0.05 M Tris·HCl, pH 7.5/0.001 M EDTA), clarified in a microfuge at 13,000 rpm for 10 min, immunoprecipitated, and analyzed by NaDodSO$_4$/PAGE. Lanes: 1 and 3, normal human sera; 5, preimmune serum control; 2, human serum from hepatitis B carrier; 4, serum from a patient with HBV infection and hepatocellular carcinoma; 6, anti-X peptide antiserum (5); 7, molecular weight standard. Lanes 8 and 9 are untransfected COS cell lysates precipitated with anti-X peptide antiserum (lane 8) or with hepatitis B carrier serum (lane 9). (*B*) COS cells transfected with pAdX were lysed in a hypotonic TKM buffer and the supernatant was centrifuged in a Beckman airfuge at 100,000 × *g* to pellet the membranes. All fractions were either immunoprecipitated with anti-X peptide rabbit antiserum (lanes 3, 5, and 7) or with preimmune rabbit serum control (lanes 2, 4, and 6). Lanes: 2 and 3, supernatants after microfuge centrifugation; 4 and 5, nuclear pellet; 6 and 7, membrane preparation. (*C*) COS cells transfected with pAdX (lanes 1, 2, 8, and 9) or pMLAd (lanes 3, 4, 6, and 7) were lysed with 0.5% Nonidet P-40 and the nuclei were isolated by centrifugation at 5000 rpm for 5 min. Lanes: 1–4, Nonidet P-40 extracts; 6–9, Nonidet P-40-insoluble fractions; 5, ^{14}C-labeled molecular weight protein standards. Antisera used were as follows: lanes 1, 3, 6, and 9, anti-X antiserum prepared against β-gal-X fusion product (from J. Sninsky); lanes 2, 4, 7, and 8, anti-X peptide antiserum. (*D*) COS cells transfected with pMNX. Cell lysates were prepared as described in *B*. All fractions were immunoprecipitated with either anti-X peptide antiserum (lanes 2, 4, and 7) or preimmune serum control (lanes 3, 5, and 8). Lane 1, molecular weight standard. Fractions: lanes 2 and 3, nuclear pellet; lanes 4 and 5, cytoplasmic extract; lanes 7 and 8, membrane pellets. Arrowheads indicate the position of the X protein.

deoxycholate, a pattern similar to that shown in Fig. 3*C*, was obtained—i.e., the X protein was not entirely extractable with detergents (data not shown). We further analyzed the subcellular distribution of this protein by indirect immunofluorescence (Fig. 4). It is clear that X is not located in the nucleus but seems to be associated with the nuclear periphery and displays a diffuse cytoplasmic and occasionally a punctate distribution as shown in Fig. 4. The behavior of the X protein from cell-fractionation data suggests its association with the cytoskeleton (26, 27). Because of the limited resolution of fluorescent microscopy, the location of the X protein to substructures of the cytoskeleton cannot be determined. A definitive localization will require immunoelectron microscopy.

DISCUSSION

In this study, we describe the expression of the X gene of HBV by means of transient expression using recombinant vectors. The X gene was expressed either through its native or heterologous transcriptional regulatory elements. The HBV fragment in plasmid pMNX, which includes the X ORF with 300 bp of flanking 5′ sequences directed the synthesis of a RNA of ≈0.8 kb in Hep G2 human hepatoma cells. This is compatible with the size of a X-specific transcript. A subgenomic transcript of 0.8 kb has been noted in *in vivo* expression systems (15, 21–23). However, such a transcript has never been documented in infected livers. It is not clear whether this is due to the limited sensitivity of the mapping

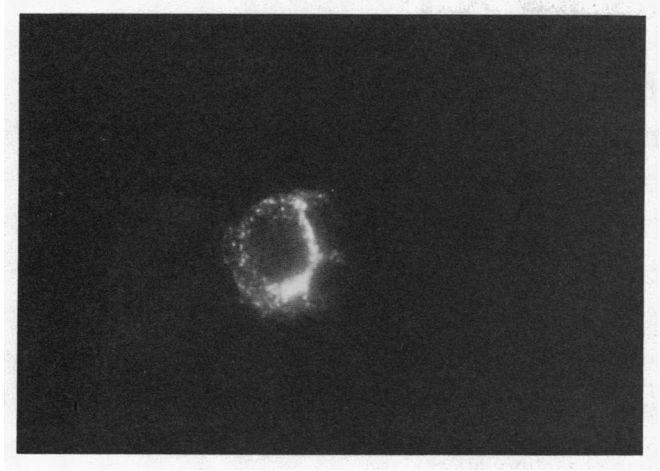

FIG. 4. Indirect immunofluorescence of the X protein in COS cells transfected with pAdX.

techniques used or is the result of a subtle regulation associated with viral infection of hepatic tissue.

Our results with expression of the bacterial CAT gene further strengthen the argument for a promoter element present upstream of the X ORF. This is in contrast to the suggestion that X mRNA may be a spliced transcript initiating in the S region (21). The X promoter appears to exhibit tissue specificity and its functional domains overlap with those of the enhancer, reminiscent of retroviral long terminal repeats. There appers to be no canonical "TATA" sequence in the vicinity of the initiation sites of the X transcripts. It should be noted that another HBV promoter, the internal hepatitis B surface antigen promoter, also lacks a TATA box and the transcripts initiating from this promoter also contain heterogeneous 5′ ends.

We have demonstrated here that the X ORF of HBV can encode a protein of ≈16 kDa and have obtained preliminary data on its subcellular location. The subcellular distribution of the X protein in this study suggests its association with the cytoskeleton and nuclear envelope. If this is its actual location in HBV-infected liver cells, we suggest that it may interact with the pregenomic RNA and may be involved in viral replication and assembly. It is possible that behavior of the X protein is different in the course of viral infection from that seen in our studies. The 28-kDa protein with anti-X reactivity reported by Moriarity *et al.* (9) may be an X-cellular fusion protein, since its size is much larger than the coding capacity of the X ORF. More HBV-infected liver tissues need to be analyzed to confirm the pattern of X gene expression. It is clear, however, that this gene is expressed at some stage of viral infection, as circulating antibodies to X have been demonstrated in some, but not all, hepatitis B carriers (7, 10). In a cotransfection experiment with HBV core antigen and X-expressing vectors, the X protein was not detected in core particles, as has been reported (28). Perhaps its inclusion requires the presence of intact viral nucleic acid.

At present there is no evidence for X to be directly oncogenic in action, but a regulatory role in the multistage process of oncogenesis or transformation needs to be determined. From these studies, it is premature to speculate on a definite function of the X protein. There are, however, some interesting observations presented by Miller and Robinson (29) based on computer-assisted analysis of the nucleotide sequence. The X gene codon usage preference is similar to that of the genes of eukaryotic cells, whereas the codon usage of other HBV genes is similar to viral genes. It appears, therefore, that on an evolutionary scale the X gene was recently acquired from the host-cell genome. It is tempting to assign a trans-activation function to the X gene product, analogous to the pX gene product of human T-cell lymphotropic viruses. Both genes are located at the 3′ end on a linear genomic map and share similar codon preferences (29). Although our immunofluorescence studies do not localize the X protein inside the nucleus, where a trans-activator protein would be found, it is possible that the sensitivity of the technique precludes detection of very small amounts, which would be functionally sufficient for trans-activation. Finally, the studies presented here should stimulate interest in understanding the role of the X protein in the HBV life cycle and processes that lead to active infection and extrahepatic syndromes associated with HBV.

Technical assistance of Steve Loukin is acknowledged. We thank Drs. A. Moriarity and J. Sninsky for the generous gift of anti-X antisera, without which this work would not be possible; R. Kaufman for the gift of plasmid pQ3; and F. LaRosa for help with immunofluorescence. This investigation was supported by grants from the Milheim Foundation for Cancer Research and the National Institutes of Health.

1. Robinson, W. S. (1979) *Compr. Virol.* **14,** 471–526.
2. Tiollais, P., Pourcel, C. & Dejean, A. (1985) *Nature (London)* **317,** 489–495.
3. Standring, D., Rutter, W. J., Varmus, H. E. & Ganem, D. (1984) *Virology* **50,** 563–571.
4. Siddiqui, A. (1983) *Mol. Cell. Biol.* **3,** 143–146.
5. Ou, J.-h., Laub, O. & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1578–1582.
6. Roossinck, M., Jameel, S., Loukin, S. & Siddiqui, A. (1986) *Mol. Cell. Biol.* **6,** 1393–1400.
7. Kay, A., Mandart, E., Trepo, C. & Galibert, F. (1985) *EMBO J.* **4,** 1287–1292.
8. Weaver, R. & Weissman, C. (1979) *Nucleic Acids Res.* **7,** 1175–1182.
9. Moriarty, A., Alexander, H., Lerner, R. & Thornton, G. (1985) *Science* **227,** 429–433.
10. Meyers, M., Trepo, L., Nath, N. & Sninsky, J. (1985) *J. Virol.* **57,** 101–109.
11. Sninsky, J., Siddiqui, A., Robinson, W. & Cohen, S. (1979) *Nature (London)* **379,** 346–348.
12. Shaul, Y., Rutter, W. J. & Laub, O. (1985) *EMBO J.* **4,** 427–430.
13. Jameel, S. & Siddiqui, A. (1986) *Mol. Cell. Biol.* **6,** 710–715.
14. Kaufman, R. J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 689–693.
15. Siddiqui, A., Jameel, S. & Mapoles, J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 566–570.
16. Gluzman, Y. (1981) *Cell* **23,** 175–182.
17. Knowles, B., Howe, C. C. & Aden, D. F. (1980) *Science* **209,** 497–499.
18. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
19. Luthman, H. & Magnusson, G. (1983) *Nucleic Acids Res.* **11,** 1295–1308.
20. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52,** 456–467.
21. Simonsen, C. C. & Levinson, A. D. (1983) *Mol. Cell. Biol.* **3,** 2250–2258.
22. Gough, N. M. (1983) *J. Mol. Biol.* **165,** 683–699.
23. Saito, I., Yoshiaki, O. & Shimojo, H. (1986) *J. Virol.* **58,** 554–560.
24. Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6777–6781.
25. Walker, M. D., Edlund, T., Boulet, A. M. & Rutter, W. J. (1983) *Nature (London)* **306,** 557–561.
26. Brown, S., Levinson, W. & Spudich, J. A. (1976) *J. Supramol. Struct.* **5,** 119–130.
27. Davis, L. & Blobel, G. (1986) *Cell* **45,** 699–709.
28. Feitelson, M. (1986) *Hepatology* **6,** 191–198.
29. Miller, R. H. & Robinson, W. S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2531–2535.