Replication of a mutant hepatitis B virus with a fused X–C reading frame in hepatoma cells

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We have previously described a mutant hepatitis B virus (HBV) with a fused X–C open reading frame (ORF) resulting from a single nucleotide insertion in the X–C overlapping region. A stably transformed cell line producing HBV particles, HepG2-K8, was established by transfecting the human hepatoma cell line HepG2 with a plasmid carrying four tandem repeats of the mutant HBV genome. The virus particles secreted into the culture medium were characterized by density gradient centrifugation and electron microscopy. The particles, similar to Dane particles by morphology and density, contained the mature HBV genome and endogenous DNA polymerase activity. Six HBV-specific transcripts of 4-0, 3-5, 2-2, 2-1, 1-2 and 0-9 kb were detected in HepG2-K8 cells by Northern blot analysis. cDNA cloning and sequence analysis of X mRNA showed that an elongated X ORF encoding 193 amino acids was created by a frameshift mutation in the 3'-terminal region of the wild-type X ORF and that the formation of an in-frame termination codon (TAA) resulted from polyadenylation. This elongated X gene product exerted transcriptional trans-activation.

Hepatitis B virus (HBV), a member of the hepadnavirus group, is a small, enveloped virus containing a partially double-stranded DNA genome. Based on nucleotide sequence analysis, four open reading frames (ORFs) have been identified and designated the pre-C/C, P, pre-S/S and X genes (Tiollais et al., 1985; Ganem & Varmus, 1987). The smallest ORF, X, is conserved among all mammalian hepadnaviruses and has been shown to be expressed during virus infection (Kay et al., 1985; Moriarty et al., 1985; Meyers et al., 1986). It has been demonstrated that X protein is a transcriptional activator of various enhancer–promoter combinations, including the HBV enhancer and core gene promoter, the simian virus 40 (SV40) enhancer and early promoter, and the β-interferon gene (Twu & Schloemer, 1987; Spanaud & Lee, 1988; Zahm et al., 1988). A mutant HBV genome (subtype adr) has been cloned and its complete DNA sequence determined (Rho et al., 1989). DNA sequence analysis revealed a single nucleotide insertion in the X–C overlapping region, which resulted in a fused X–C ORF.

To test whether the mutant HBV with the fused X–C ORF is able to replicate in a hepatoma cell line and whether X gene-related transcripts are involved in the production of altered forms of X protein, we transfected plasmid pHBV4-neo into the human hepatoma cell line HepG2. The recombinant vector pHBV4-neo was constructed by combining four tandem repeats of the SV40 enhancer/promoter, the neo' gene, the SV40 poly(A) signal and the mutant HBV genome. G418-resistant colonies appeared with an efficiency of 5 × 10⁻⁵; and 20 G418-resistant clones were obtained. Of these, the HepG2-K8 clone, producing the greatest amounts of HBsAg and Hbc/HBeAg was further analysed.

To characterize the integrated HBV DNA in HepG2-K8 cells, chromosomal DNA was isolated (Jeffreys & Flavell, 1977) and hybridized with ³²P-labelled HBV DNA. Digestion with HindII, which does not cleave the mutant HBV DNA, generated fragments larger than 13 kb in length (Fig. 1 a, lane 2). Cleavage of HepG2-K8 cell DNA with BamHI (lane 3) or XhoI (lane 4) yielded a major band of 3-2 kb. Digestion with BglII (lane 5), which does not cleave in the vector portion of pHBV4-neo, generated the expected mutant HBV fragments of 2-78 and 0-41 kb. The restriction patterns obtained by digestion with both XhoI and BglII showed the presence of high M, DNA bands representing host–virus hybrid fragments. Thus, it was suggested that the junction of the head-to-tail tetramer was conserved in the integrated HBV DNA.

To test the possibility that HepG2-K8 cells can produce mature HBV in vitro, HepG2-K8 cell culture medium was collected, concentrated and fractionated by caesium chloride density gradient centrifugation. When the fractions were analysed by radioimmunoassay (RIA) for HBsAg and Hbc/HBeAg, a peak of Hbc/HBeAg was
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Fig. 1. Characterization of HBV DNA from HepG2 cells transfected with a mutant HBV. (a) Southern blot analysis of chromosomal DNA from HepG2-K8 cells. HBV DNA labelled with \([\alpha-32P]dCTP\) by nick translation was used as a probe. Lane 1, undigested chromosomal DNA from HepG2-K8 cells; lanes 2 to 5, the same DNA digested with HindIII, BamHI, XhoI or BgIII, respectively. The arrowheads to the right indicate molecular size (kb). (b) HBV DNA in virus particles secreted from HepG2-K8 cells was analysed by gel electrophoresis and Southern blot hybridization. D1, D2 and S are likely to be a partially double-stranded open circular DNA, the linear form of the same DNA and ssDNA, respectively. (c) Virus particles from the culture medium of HepG2-K8 cells were purified and radiolabelled with \([\alpha-32P]dCTP\) by an endogenous polymerase reaction. The labelled HBV DNA was extracted and examined by agarose gel electrophoresis and autoradiography.

Fig. 2. CsCl density gradient centrifugation analysis of the HBV particles secreted from HepG2-K8 cells. RIA values determined for HBsAg (□) and HBc/HBeAg (○) particles of each fraction are given. The density of each fraction (●) is indicated. An electron micrograph of fraction 8 is presented in the inset. The bar marker represents 100 nm.

DNA isolated from the fraction containing virion-like particles was subjected to agarose gel electrophoresis and analysed by Southern blotting to investigate the nature of the HBV DNA contained within the virus particles. Replicative intermediates (D1, D2 and S) of HBV DNA were detected (Fig. 1b). D1, D2 and S are likely to be partially double-stranded open circular DNA, the linear form of the same DNA and ssDNA, respectively, as suggested by Tsurimoto et al. (1987). To investigate whether the HBV particles in the culture medium of HepG2-K8 cells contained endogenous DNA polymerase activity, the particles were purified from the medium and subjected to the endogenous polymerase reaction in the presence of \([\alpha-32P]dCTP\) (Kaplan et al., 1973). The labelled HBV DNA was isolated and analysed by agarose gel electrophoresis and autoradiography. The result indicated that HBV particles isolated from the medium were able to generate dsDNA of around 3-2 kb by gap filling of partially dsDNA (Fig. 1c) as previously reported (Sureau et al., 1986). These results suggested that the particles contain HBV DNA and DNA polymerase activity.

To identify HBV-specific transcripts we prepared poly(A)+ RNA from HepG2-K8 cells, and analysed HBV RNA by the Northern blotting. When the 0-64 kb BamHI–BglII fragment (nucleotides 1397 to 1981) was used as an X gene-specific probe, six transcripts were detected. 3-5 kb and 2-1 kb RNAs were the major transcripts, and were considered to be the pregenomic RNA and the mRNA for HBsAg, respectively. Additional minor transcripts of 4-0 kb, 1-2 kb and 0-9 kb were also detected (Fig. 3, lane 1). The 4-0 kb RNA probably consisted of the pregenomic RNA plus an extra copy of the X gene, as suggested by Guo et al. (1991). The 1-2 kb and 0-9 kb RNAs might correspond to the 1-3 kb RNA containing the C-terminal portion of the polymerase ORF and the X gene transcript, respectively (Suzuki et al., 1989). Using a C gene-specific probe (the BglII fragment, nucleotides 1982 to 2396), 4-0 kb, 3-5 kb and
2.2 kb RNAs were detected, of which the 2.2 kb transcript was shown to have a size distinct from that of the 2.1 kb transcript (Fig. 3, lane 2). Therefore, the 2.2 kb RNA, which reacts with both probes, may be a spliced HBV RNA (Suet al., 1989; Suzuki et al., 1989). Although the tetrameric HBV DNA used for transfection is linked to the SV40 enhancer/promoter, the transcription of viral RNAs would be predominantly directed by HBV regulatory elements, and the interaction between the X gene product and the SV40 enhancer would have no effect on viral expression and replication (Sureau et al., 1986; Sells et al., 1987; Tsurimoto et al., 1987).

To establish X gene-related cDNA clones, cDNA cloning of X mRNA from the HepG2-K8 cells was conducted using the Agt11 host vector system from a kit supplied by Boehringer Mannheim and a cloning kit purchased from Amersham, except for the primer, which was oligo(dT)15 connected to HindIII/EcoRI/SalI linker sequences (Gubler & Hoffman, 1983). We screened the clones with an X gene-specific probe (nucleotides 1370 to 1397) and a C gene-specific probe (nucleotides 1910 to 1932), and three positive clones were selected. DNA sequence analysis of the cDNA clones showed that two clones (cX-14) terminated 14 nucleotides downstream of the poly(A) signal (TATAAA) at position 1933, and one (cX-31) terminated 31 nucleotides downstream at position 1950. The 5' ends of the cX-14 clones and the cX-31 clone were at nucleotides 1310 and 1334, respectively (data not shown). In the sequence of the cX-31 cDNA, an in-frame termination codon TAA was created by polyadenylation (Fig. 4a). The resulting ORF could encode an elongated X protein of 193 amino acids, consisting of 151 amino acids encoded by the wild-type X ORF (wX) and an additional 42 amino acids encoded by the pre-C/C ORF region resulting from a frameshift mutation in the C-terminal region of the wild-type X ORF (Fig. 4a). To confirm that the elongated X protein was of Mr 21K, we expressed the elongated X ORF in Escherichia coli using the T7 expression system (Studier & Moffatt, 1986) and identified a protein of 21K by Western blotting with a rabbit anti-X protein antiserum (J. W. Shim & H. M. Rho, unpublished results).

Based on previous results showing that the C-terminal amino acids of X protein are dispensable for its function in trans-activation of the SV40 enhancer/promoter by the elongated X gene product, (a) The nucleotide sequence and deduced amino acid sequences of the C-terminal region of the elongated X ORF (eX) and wild-type X ORF (wX). The single-letter amino acid code is used. (b) Schematic maps of effector plasmids pHX and pHEx. The construction of the plasmids was confirmed by DNA sequence analysis. Numbers in parentheses indicate map positions on the HBV genome (Rho et al., 1989). Filled box, HBV enhancer/X promoter; open box, each ORF; open circle, HBV poly(A) signal; solid circle, HSV TK poly(A) signal. (c) CAT assay for the trans-activation function of the elongated X protein. HepG2 cells were cotransfected with 5 ~tg of pSV2CAT and 5 ~tg of one of plasmids pUC19, pHX and pHEx. Fold activation represents the ratio of the percentage of chloramphenicol acetylation in cotransfection with the X gene expression vector to that with control plasmid pUC19. All values are given as the mean ± standard deviation obtained from experiments carried out in sextuplicate. CM, [14C]Chloramphenicol; 1-AC, 1-acetylated; 3-AC, 3-acetylated.
as a transcriptional trans-activator (Levrero et al., 1990; Ritter et al., 1991), we examined the trans-acting activity of the elongated X protein. The reporter plasmid pSV2CAT, which contains the SV40 enhancer and early promoter driving the chloramphenicol acetyltransferase (CAT) gene, was cotransfected with either pHX or pHeX into HepG2 cells (Chen & Okayama, 1987) and a CAT assay was performed (Gorman et al., 1982). Plasmid pHX contained the wild-type X gene (Won et al., 1989) under the regulation of the HBV enhancer/X promoter and the poly(A) signal of the herpes simplex virus thymidine kinase gene (HSV TK); pHeX was similar but contained the elongated X gene (Fig. 4b). As shown in Fig. 4(c), the CAT activities derived from cells cotransfected with pHX (lane 2) or pHeX (lane 3) were significantly greater than those from cells cotransfected with the control vector pUC19 (lane 1). Therefore, it was evident that the elongated X protein had the trans-activating function. Plasmid pHeX may produce a transcript terminating 14 nucleotides downstream of the HBV poly(A) signal within the elongated X ORF. The transcript which lacks the translation termination codon could theoretically produce a protein with a poly(A)-encoded C-terminal polylysine tail, which is thought to be non-functional.

Our results demonstrate that the mutant HBV with a deletion of the stop codon of the X ORF resulting in a fused X–C ORF is able to replicate in HepG2 cells. Since X gene frameshift mutants of ground squirrel hepatitis virus failed to grow in animal hosts (Ganem & Varmus, 1987), the X protein may play an important role in the virus life cycle. The trans-acting activity of the elongated X protein suggests that it also plays an important role in replication and gene regulation. Experiments on infectivity and viral DNA synthesis are in progress.

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References


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