JOURNAL OF VIROLOGY, Oct. 1987, p. 3322–3325
0022-538X/87/103322-04$02.00/0

Vol. 61, No. 10

Expression of the Precore Region of an Avian Hepatitis B Virus Is Not Required for Viral Replication

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Received 21 May 1987/Accepted 26 June 1987

The core-antigen-coding region of all hepadnaviruses is preceded by a short, in-phase open reading frame termed precore whose expression can give rise to core-antigen-related polypeptides. To explore the functional significance of precore expression in vivo, we introduced a frameshift mutation into this region of the duck hepatitis B virus (DHBV) genome and examined the phenotype of this mutant DNA by intrahepatic inoculation into newborn ducklings. Animals receiving mutant DNA developed DHBV infection, as judged by the presence in hepatocytes of characteristic viral replicative intermediates; molecular cloning and DNA sequencing confirmed that the original mutation was present in the progeny genomes. Infection could be efficiently transmitted to susceptible ducklings by percutaneous inoculation with serum from mutant-infected animals, indicating that infectious progeny virus was generated. These findings indicate that expression of the precore region of DHBV is not essential for genomic replication, core particle morphogenesis, or intrahepatic viral spread.

The hepadnaviruses are a group of small, enveloped DNA viruses that produce acute and chronic infections of hepatocytes and that replicate their DNA by reverse transcription of RNA intermediates (5, 21). To date, members of this group have been recovered from hosts of several species, including humans, woodchucks, ground squirrels, and ducks (19). All of these viruses share a similar genomic organization, which includes at least three open reading frames (5, 19, 21). One of these encodes the multiple surface proteins of the envelope, another encodes the reverse transcriptase required for replication, and a third encodes the major structural protein of the viral nucleocapsid or core (core antigen). The mammalian viruses harbor an additional coding region (termed X) of unknown function.

In all hepadnaviral genomes, the coding region for core antigen is preceded by an in-phase, contiguous open reading frame of 29 to 43 codons known as precore (Fig. 1A). The expression of this region is predicted to generate core-related polypeptides with additional amino acids at their N termini. There is good evidence that precore expression does indeed occur in vivo. All hepadnavirus-infected liver cells produce at least two sets of transcripts containing core sequences. One RNA initiates within the precore region and therefore can encode only the core antigen. The other set of transcripts initiates upstream of the precore ATG codon and hence could encode the larger precore polypeptide (1, 3, 11, 24; R. Sprengel and H. Will, unpublished data).

Extensive work with human hepatitis B virus (HBV) indicates that precore polypeptides can be generated from such RNAs and that their properties differ from those of core antigen. Several studies (9, 12, 13) have used heterologous promoters to drive the expression of either precore or core gene products in cultured cells transfected with subgenomic fragments of cloned HBV DNA. When protein synthesis is initiated at the core ATG, the resulting protein is cytoplasmic in location, while initiation of translation at the precore ATG results in the targeting of the gene product to a membranous compartment (12); after further proteolytic processing (9; D. Standring, J. Ou, and W. Rutter, personal communication), the products are secreted into the medium. Similar products (collectively termed hepatitis B e antigen) are observed in the serum of HBV-infected patients, arguing strongly that this pathway is functional in vivo (5, 21). These results suggest that the HBV precore region encodes a signal sequence that can function to direct core antigen determinants to novel subcellular locales. Similarly, we have recently demonstrated that the serum of ducks infected with duck hepatitis B virus (DHBV) also contains secreted polypeptides derived from the expression of the DHBV precore region (R. Sprengel and H. Will, manuscript in preparation). However, the functional role of precore gene products in authentic viral replication in productively infected cells has not yet been examined.

Progress in dissecting the roles of the individual viral gene products in vivo has been hampered by the absence of convenient cell culture systems for viral growth. However, the observation that cloned viral DNA can initiate productive infection of susceptible hosts following intrahepatic inoculation (16, 17, 22) has allowed the development of a simple strategy for the mutational analysis of viral functions. Briefly, lesions in the region of interest are generated by site-directed mutagenesis in vitro, and the resulting mutant genomes are tested for viability in vivo by intrahepatic transfection. We have previously used this strategy to examine the role of short direct repeats (DRs) of viral DNA in genomic replication (15). In this study, we present a similar analysis of the hepadnaviral precore region. Although the limited host range of HBV (which includes only humans and higher primates) makes this approach difficult for the human virus, the animal hepadnavirus models are ideally suited to

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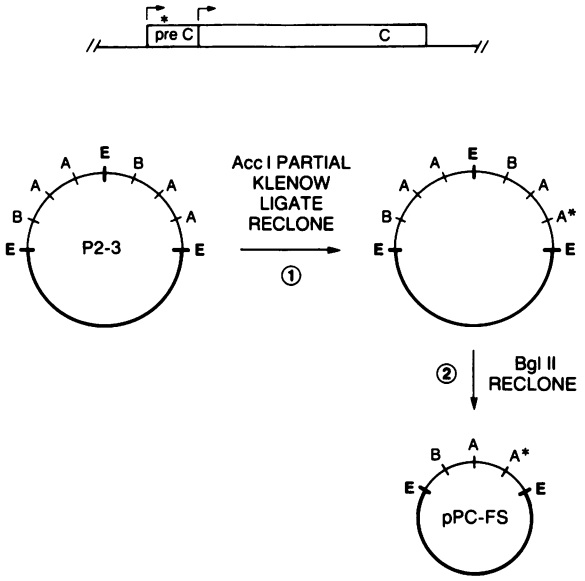

FIG. 1. Construction of precore frameshift mutation in DHBV DNA. (A) The organization of the precore and core-antigen-coding regions of DHBV. □, Coding regions; →, ATG codons; PreC, precore region; C, core-antigen-coding frame; *, site of precore frameshift mutation. (B) Construction of precore frameshift lesion. Plasmid p2-3 is a tandem dimer of DHBV DNA, with restriction sites as indicated. E, *Eco*RI; A, *Acc*I; B, *Bgl*II. The manipulations at each step of the construction are described in detail in the text and are indicated next to the arrow depicting each step. A*, Precore *Acc*I site inactivated by the 2-base-pair insertion.

this form of analysis. The avian system is particularly attractive, since susceptible ducklings are readily available and the incubation period of DHBV infection is only 2 to 3 weeks (versus 2 to 3 months for the mammalian viruses). Accordingly, we used DHBV as the experimental system for these studies.

To address the role of precore sequences in vivo, we constructed a frameshift mutation in the DHBV precore region and tested the mutant DNA for infectivity. The scheme for construction of the mutant is detailed in Fig. 1B. Briefly, plasmid p2-3, bearing a tandem dimer of the DHBV genome (generous gift of W. Mason), was linearized by partial digestion with *Acc*I; one of the two *Acc*I cleavage sites in DHBV DNA lies within the precore region (8). The termini of gel-purified linear molecules were then repaired with DNA polymerase I (Klenow fragment), religated, and cloned. Clones which had lost the appropriate *Acc*I site in the precore region were identified by restriction mapping. The wild-type copy of the DHBV genome was then excised with *Bgl*II to generate clones bearing only the mutant genome. In one of these (pPC-fs), the presence of the expected two-base insertion was verified by DNA sequencing (data not shown).

For infectivity testing, mutant DHBV genomes were liberated from the plasmid vector by *Eco*RI digestion, and the released molecules were self-annealed and ligated at low DNA concentrations (1 to 10 μg/ml) to promote recircularization. A 5-μg sample of this DNA (in 0.2 ml of 500 μg of DEAE-dextran per ml) was percutaneously injected into the livers of each of six virus-free ducklings (SPAFAS, Inc., Norwalk, Conn.) 1 day posthatching. Four control ducklings were similarly exposed to wild-type DNA. Three weeks later, all ducks were sacrificed and their liver DNA was examined for progeny DHBV sequences by Southern blotting with ^{32}P-labeled DHBV DNA as a probe. Five of the six mutant-infected samples (and one of the four controls) revealed the characteristic array of replicative intermediates typical of productively infected hepatocytes (20), i.e., asymmetric, protein-linked, heterogeneous forms and fully duplex monomeric circles lacking covalently attached protein (two representative examples are shown in Fig. 2C). To verify that the replicative forms seen in mutant-infected livers were of mutant origin, two experiments were carried out. First we examined the restriction pattern of the intrahepatic circular duplex viral DNA (Fig. 2A). Liver DNA from all five infected animals was prepared by phenol extraction in the absence of proteinase K; this procedure eliminates the abundant protein-linked heterogeneous viral DNA forms (6), which would otherwise obscure the cleavage pattern. *Acc*I cleavage of the remaining viral DNA in all cases revealed the presence of only one *Acc*I site; mapping of this site relative to the unique *Eco*RI and *Bgl*II sites of DHBV confirmed that the missing *Acc*I site was that in the precore region (representative results for one of the five mutants are shown in Fig. 2B).

This result rules out contamination of the input DNA with wild-type sequences or reversion of the mutant to the wild type during replication but does not exclude the occurrence of a second-site (compensatory) frameshift mutation in precore, which could restore the reading frame to the wild type. To examine this possibility, we digested the liver DNA of one mutant-infected animal with *Eco*RI. We recovered fragments of 3.0 to 3.5 kilobases (kb) from agarose gels and cloned these into lambda gtWES. Progeny plaques annealing to ^{32}P-labeled DHBV DNA sequences were picked, and a 1.3-kb *Xba*I fragment spanning the precore region was subcloned from one of these into pSP65 (10). In this plasmid vector, the sequence of the entire precore region was determined by the chain termination method. The original two-base frameshift mutation was still present in the recovered mutant DNA (Fig. 3); further sequencing (data not shown) revealed no additional changes between the precore ATG and the first out-of-phase terminator (at position 2631) following the frameshift lesion.

The presence of replicative forms in intrahepatic DNA strongly implies that infectious virus generated in the initially transfected cells successfully spread to uninfected hepatocytes (16, 17). To confirm this inference, we prepared a cell-free homogenate from one mutant-infected liver and used this material to inoculate four uninfected 1-day-old ducklings by subcutaneous inoculation in the thigh. Examination of liver DNA from these animals 21 days later revealed the presence of typical DHBV replicative forms in all four samples; restriction analysis of two of these samples performed as outlined in Fig. 2 again confirmed the presence of the mutation (data not shown). We then tested for the presence of infectious virus in the serum of these recipients. A pool of serum from three of these mutant-infected animals was inoculated subcutaneously into three ducklings (0.1 ml per recipient). All three developed viremia, as judged by dot hybridization of recipient serum.

These experiments demonstrate that the expression of the precore region of DHBV is not essential for the uptake, replication, assembly, or intrahepatic spread of the virus in experimental infection in vivo. However, it is possible that precore expression might affect the efficiency of titer of virus infection or contribute to more specialized biological functions, such as tropism for extrahepatic viscera (7) or competence for vertical transmission; these possibilities are

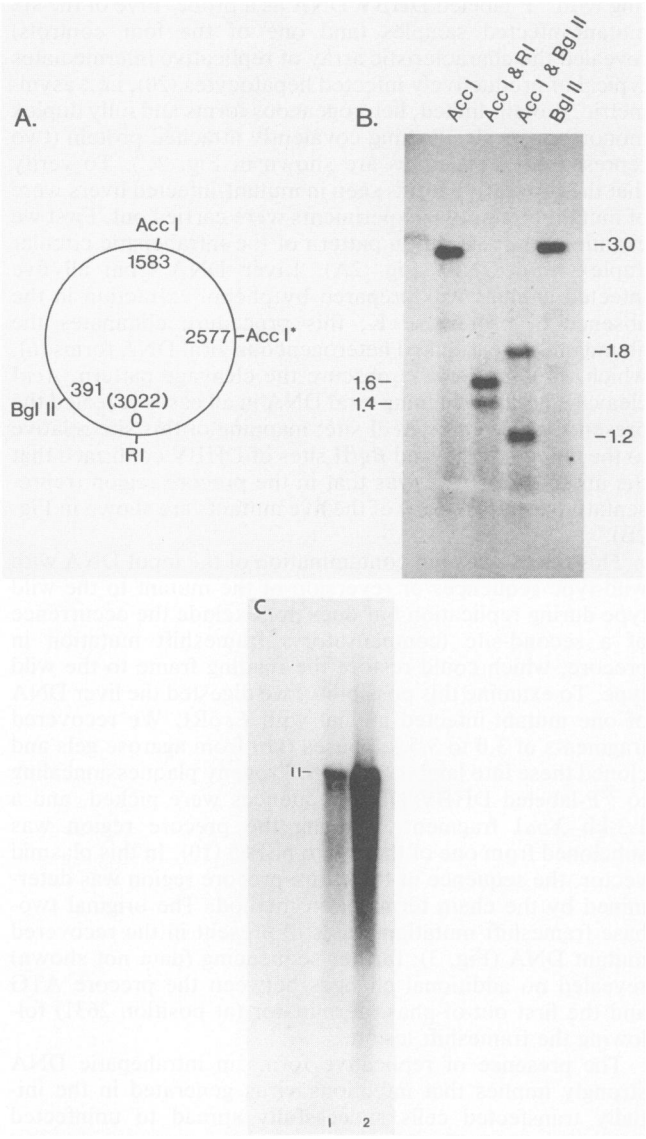

FIG. 2. Viral DNA forms in the livers of ducks transfected with mutant genomes. (A) Restriction map of relevant sites within DHBV DNA. Nucleotide positions are numbered with respect to the unique *Eco*RI site (8). *, *Acc*I site eliminated by the precore frameshift lesion. (B) Cleavage analysis of duplex DHBV DNA in the liver of animal 5 at 3 weeks posttransfection with pPc-fs DNA. Liver homogenates in 1% sodium dodecyl sulfate were extracted with phenol chloroform (1:1) without prior proteinase K treatment, as previously described (16). A 10-μg portion of the resulting relaxed circular DNA was digested with the indicated enzymes; the sample in the left lane was undigested. Product DNA was electrophoresed through 1% agarose, transferred to nitrocellulose, hybridized to ^{32}P-labeled DHBV DNA, and autoradiographed as previously described (16). The sizes of the fragments (in kilobases) are indicated. (C) Liver DNA prepared from animals 5 (lane 1) and 6 (lane 2) 3 weeks posttransfection with pPC-fs DNA. Liver homogenates were treated with 1% sodium dodecyl sulfate and 500 μg of proteinase K per ml for 3 h prior to phenol extraction; 10 μg of the resulting DNA was digested with *Pvu*II, which does not cleave within DHBV. Product DNA was electrophoresed through 1% agarose, transferred to nitrocellulose, and hybridized to ^{32}P-labeled DHBV DNA as in panel B. ||, Position of fully duplex open circles of DHBV DNA.

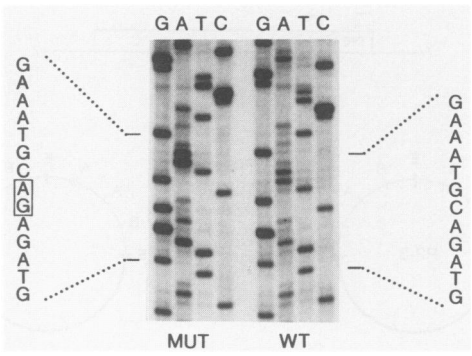

FIG. 3. Nucleotide sequence of the precore region of cloned DHBV DNA recovered from liver of a mutant-infected duck. A 1.3-kb *Xba*I fragment of DHBV DNA was excised from a lambda gt WES clone of the mutant viral DNA and subcloned into the *Xba*I site of pSP65 (10). A clone in which the precore region was adjacent to the SP6 promoter region was identified. The sequence of the entire precore region was determined by the dideoxy method by using a primer complementary to the SP6 promoter sequence. Left panel, a portion of the sequence showing the region spanning the lesion in recovered mutant (MUT) DNA; right panel, corresponding sequence of wild-type (WT) DHBV DNA. The sequences depicted are of minus-strand polarity.

currently being explored. The availability of viable precore mutant viruses will also make possible an assessment of the impact of precore proteins on viral persistence and on the pathogenic potential of DHBV.

We do not know at present whether these findings can be extrapolated to the mammalian viruses, including HBV. As noted previously, DHBV was chosen for these studies because of experimental tractability. However, DHBV is the most diverged of the hepadnaviruses at the sequence level (8, 18). Its 35-kilodalton core antigen is distinctly larger than those of the mammalian viruses (20 to 22 kilodaltons) and within the DHBV precore/core open reading frame, there is little amino acid homology to the cognate region of HBV (18). On the other hand, the DHBV precore region does contain a consensus signal peptidase recognition sequence (R. Colgrove, personal communication), and the virus does generate secretory precore products analogous to the e antigen of HBV. Additional experiments will be required to directly test whether the mammalian viruses require functional precore polypeptides for replication; we are currently constructing analogous mutations in the ground squirrel hepatitis virus precore region to examine this issue further.

Our findings with the DHBV precore gene recall an interesting parallel in murine retrovirology. The *gag* gene of Moloney murine leukemia virus encodes the nucleocapsid or core proteins of the virus, and its ATG initiator is preceded by an upstream initiator codon that is also expressed in vivo. Translation from the upstream initiator gives rise to *gag*-related proteins which enter the secretory pathway, are glycosylated, and are expressed on the cell surface and in the medium (2). Interestingly, as for DHBV, mutational ablation of these upstream sequences does not impair viral replication or infectivity (4, 14).

The experiments reported here represent the first demonstration of a nonessential region within the genome of a hepadnavirus. The existence of such a region is surprising given the small size (3.0 kb) and extremely compact coding organization of the genome; in DHBV (as in HBV), every nucleotide in the genome is in at least one coding region and 50% of the sequence is read in more than one frame. The fact

that the precore region is nonessential also suggests that insertions of exogenous DNA sequences into this region may be tolerated and raises the intriguing possibility that hepadnaviruses could be developed as genetic vectors for the delivery of foreign DNA to the liver.

These experiments were supported by Public Health Service grants (AI18782 and AM26743) from the National Institutes of Health to the authors and to the University of California at San Francisco Liver Center. H.E.V. is American Cancer Society Professor of Molecular Virology.

We thank Effie Meredith for outstanding assistance with the preparation of the manuscript.

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