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X-Region-Specific Transcript in Mammalian Hepatitis B Virus-Infected Liver

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In vitro gene expression systems for hepatitis B virus have demonstrated that the virus genome is capable of producing an X-region-specific transcript of approximately 0.7 kilobases (kb). However, this transcript has not been detected in virus-infected cells. We now report the presence of a heterogeneous X-region-specific transcript of approximately 0.65 kb that is found primarily in the nucleus of liver cells infected with the woodchuck hepatitis virus. Interestingly, the majority of the transcripts are not polyadenylated. The transcript, which represents <1% of total virus-specific RNA, is found in animals with both acute and chronic woodchuck hepatitis virus infections. While it is probable that the 0.65-kb transcript is involved in the expression of the X gene protein, it may also direct the translation of a protein encoded by a newly identified open reading frame, ORF5, that is present in all hepadnavirus genomes analyzed.

The hepatitis B virus (HBV) genome, and the genomes of other hepadnaviruses of mammals, contains at least four long open reading frames (ORFs) that encode the virus core, surface, and polymerase proteins, as well as a protein, termed X, with an unknown function. It is now known that the X protein is produced during the course of virus infection and that the host can mount an antibody response against this virus protein (5, 6, 12, 18, 22, 27, 29, 34). Recent in vitro studies suggest that the X protein may be involved in the regulation of virus gene expression in a manner analogous to the *trans*-activating proteins of other viruses (36, 39). Although, in theory the X protein could be translated from any of the known virus mRNAs, in vitro gene expression experiments (2, 10, 11, 31, 33, 35, 38) suggest that an X-region-specific transcript can be produced in vivo. However, no such transcript has been identified in infected cells.

In this report, we demonstrate that an X-region transcript is produced during the course of active virus replication. The transcript may have gone undetected until now due to its low abundance and the fact that the majority of molecules lack poly(A) tails and remain in the nucleus of the infected cells.

MATERIALS AND METHODS

Experimental animals. The woodchucks (*Marmota monax*) used in this study were housed at the laboratory animal facility of the College of Veterinary Medicine, Cornell University, or at SEMA, Inc., Rockville, Md. Woodchuck no. 267 (WC267) and WC809, with no serological evidence of past or present infection with woodchuck hepatitis virus (WHV), served as the negative controls. WC220, trapped in the wild, was positive for woodchuck hepatitis surface antigen (WHsAg), and woodchuck hepatitis core antibody (WHcAb) and negative for woodchuck hepatitis surface antibody (WHsAb). WC1482, WC1636, WC1643, WC1651, and WC1664 were inoculated with WHV 3 to 7 days after birth and maintained in isolation. Blood samples were obtained at monthly intervals and serological assays performed as described previously (28). WC1651, WC1482, and WC1643, which were serially WHsAg and WHcAb positive, were sacrificed at 4, 7, and 11 months, respectively, after infection. WC1636 and WC1664, which were transiently positive for WHsAg and seroconverted to WHsAb, were sacrificed at 7 months and 11 months, respectively, after infection.

RNA preparation. Whole-cell RNA was isolated following homogenization of the liver tissue in 4 volumes of 4 M guanidine isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion (17). Poly(A)$^+$ RNA in loading buffer (20 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5 M NaCl) was selected by two rounds of oligo(dT)-cellulose chromatography (Type 7, Pharmacia). Poly(A)$^-$ RNA was obtained by extensive washing with loading buffer. Columns were then rinsed with washing buffer (20 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.1% SDS, 0.1 M NaCl), and poly(A)$^+$ RNA was eluted with 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–0.1% SDS (17).

Fractionation of liver cells was done as previously described (20). RNA was purified from the nuclear and cytoplasmic fractions as described above. Digestion of samples with pancreatic RNase (Boehringer Mannheim Biochemicals) or DNase I (Promega) was performed as described earlier (17).

Northern (RNA) blot hybridization. RNA was denatured with 6% formaldehyde–50% formamide–1× MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) at 65°C for 7 min, fractionated by electrophoresis through a 1.5% agarose gel in 2% formaldehyde and 1× MOPS, and then transferred to Hybond-N nylon membranes (Amersham Corp.). Membranes were prehybridized in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2.5× Denhardt solution, 0.1% SDS, 1 mM EDTA, 5 mM NaH_2PO_4, and 200 μg of denatured calf thymus DNA per ml at 58°C for 4 h. Hybridization with radiolabeled probe was performed in the above buffer at 58°C for 24 h. After hybridization, the membrane was washed twice in 1× SSC–0.1% SDS for 5 min at room temperature and three times in 0.1× SSC–0.1% SDS at 70°C for 30 min per wash. The membrane was exposed to X-ray film (XAR [Eastman Kodak Co.] or Cronex [Du Pont Co.]) for autoradiography.

Construction and radiolabeling of probes. WHV DNA (14)

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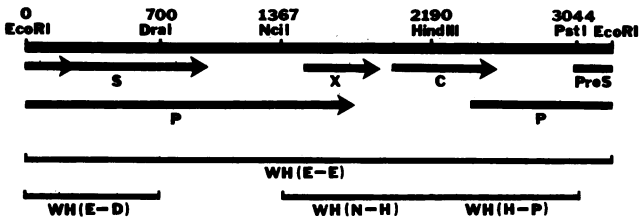

FIG. 1. Location of the sequences used as molecular hybridization probes. The WHV (14) genetic map is expressed in the linear configuration with the single *Eco*RI site as 0. Arrows indicate genes on the negative DNA strand: the surface (S), polymerase (P), X, core (C), and pre-surface (PreS) ORFs. The regions of the WHV genome used as molecular hybridization probes are as follows: WH(E-E), the complete WHV genome *Eco*RI-*Eco*RI; WH(E-D), the *Eco*RI (position 0)-*Dra*I (position 700) fragment; WH(N-H), the *Nci*I (position 1367)-*Hin*dIII (position 2190) fragment; WH(H-P), the *Hin*dIII (position 2190)-*Pst*I (position 3044) fragment.

was subcloned into the vectors pTZ (Pharmacia) or pGEM (Promega) by standard methods (17). The complete WHV genome [WH(E-E)] and the *Eco*RI-*Dra*I [WH(E-D)] and *Nci*I-*Hin*dIII [WH(N-H)] regions were cloned into the vector pGEM (Fig. 1). WHV region *Hin*dIII-*Pst*I [WH(H-P)] was cloned into the vector pTZ (Fig. 1). Radiolabeled RNA transcripts were prepared by using SP6 or T7 RNA polymerase according to the instructions of the supplier. The specific activities of probes were 1×10^9 to 2×10^9 cpm/μg.

RESULTS

Nuclear WHV RNA in infected liver. During woodchuck hepatitis virus (WHV) replication and gene expression two virus-specific RNA transcripts are produced (Fig. 2A). These transcripts possess poly(A) tails, are approximately 2.3 and 3.6 kilobases (kb) in size (Fig. 2B), and share the same 3′ terminus (23). The 2.3-kb mRNA serves as the template for the translation of the virus envelope, or surface, protein. The 3.6-kb transcript serves as the template both for the reverse transcription for genome replication and for the translation of the nucleocapsid (core) and polymerase proteins. Although in theory either of these two virus transcripts could be used to translate the X ORF, previous experiments using in vitro expression systems suggest that an X-region-specific transcript may be produced during the course of virus replication and gene expression. However, an X-region-specific transcript has not yet been identified in virus-infected cells. We attempted, therefore, to identify an X gene transcript by using specific virus probes and various cell and RNA fractionation techniques.

To identify an X-region-specific transcript in virus-infected cells, the whole-cell RNA of WC220 was examined by Northern blot hybridization with ^{32}P-labeled WH(N-H) RNA as a probe (Fig. 1). The two major WHV RNA species were observed in whole-cell RNA as expected (Fig. 3, lane 1). A longer exposure of the blot revealed the presence of a heterogeneous population of RNA transcripts smaller in size than the 2.3-kb WHsAg transcript. Isolation of liver cell nuclei and examination of RNA by Northern blot hybridization revealed a nucleic acid band with an average electrophoretic mobility of 0.65 kb (Fig. 3, lane 2). To verify that this band represented a genuine RNA transcript, and not contaminating DNA, the nuclear RNA preparation was digested with either DNase I or RNase A. While digestion with DNase I (Fig. 3, lane 3) resulted in no change, digestion with RNase A (Fig. 3, lane 4) completely eliminated the

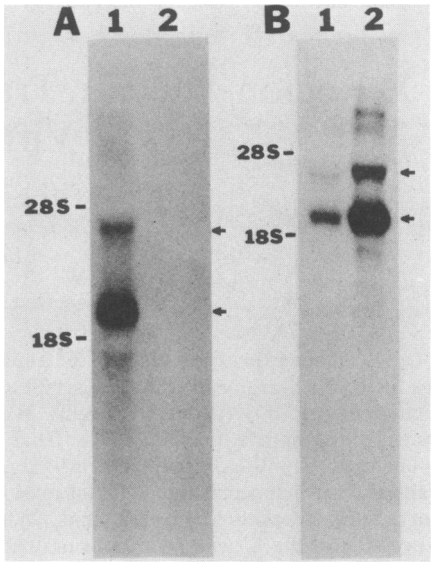

FIG. 2. WHV transcripts in infected liver. (A) Whole-cell RNA was isolated from an uninfected (WC809, lane 2) and a WHV-infected (WC1651, lane 1) woodchuck and analyzed by Northern blot hybridization with a ^{32}P-radiolabeled probe consisting of the entire WHV genome (see Materials and Methods). (B) Poly(A)-selected mRNA (lane 2) from WC1651 was compared to whole-cell RNA (lane 1). The positions of 28S and 18S rRNA are shown. Arrows point to the major 3.6- and 2.3-kb WHV transcripts (the pregenome and surface protein transcripts, respectively) in this and subsequent figures. We also note the presence of several transcripts of >3.6 kb which may represent the dimeric and trimeric forms of the pregenome transcript.

hybridization signal. This confirms that the 0.65-kb nucleic acid band is RNA.

Next, we attempted to determine whether the 0.65-kb RNA was specific for virus-infected cells and whether it was expressed in both acutely and chronically infected animals. Since it is known that a region of the X gene sequence can hybridize with human cellular DNA (42), we verified that the transcript was of viral, and not cellular, origin. To prove that the 0.65-kb transcript was present only in WHV-infected cells, we compared uninfected and WHV-infected liver tissue. Northern blot hybridization with probe WH(N-H) demonstrated that there was no such RNA species in either whole-cell or nuclear RNA of uninfected woodchucks (Fig. 4, lanes 1 and 2) or in two woodchucks with antibody to WHsAg (Fig. 4, lanes 7 to 10) because of a previous WHV infection. However, in addition to the expected 2.3- and 3.6-kb transcripts of whole-cell RNA from animals infected with WHV (Fig. 4, lanes 3 and 5), a 0.65-kb transcript was found in the nuclear RNA of both acutely (Fig. 4, lane 4) and chronically (Fig. 4, lane 6) infected woodchucks. The intensity of the hybridization signal of the 0.65-kb RNA of the acutely infected animal was stronger than that in the chronically infected animal. However, we estimate that the amount of the 0.65-kb transcript, relative to the 2.3- and 3.6-kb transcripts, was similar in the two animals and was <1% of the total virus-specific RNA in the infected cells. Therefore, the 0.65-kb transcript is a minor RNA transcript that is synthesized during active virus replication.

The 0.65-kb RNA is X region specific. Although the 0.65-kb nuclear transcript was the size expected if transcription began at an X gene promoter and terminated at the site used by the other virus transcripts, it was possible that the

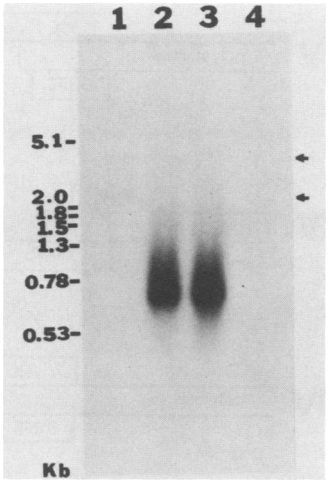

FIG. 3. Nuclear RNA transcripts. WHV RNA, purified from whole cells or nuclei of chronically infected liver tissue of WC220 was fractionated by electrophoresis on a horizontal 1.5% agarose gel and then transferred to a nylon membrane and hybridized with probe WH(N-H). Lane 1 contains 30 μg of whole-cell RNA, while lane 2 contains 30 μg of nuclear RNA. Nuclear RNA (30 μg) was treated with 2 U of DNase I for 20 min at 37°C in the presence of RNase inhibitor (lane 3) or treated with 50 μg of pancreatic RNase per ml (lane 4). The size of marker RNA molecules is given in kilobases. The small arrows give the positions of the 3.6- and 2.3-kb WHV transcripts. Isolation of DNA from nuclei revealed the presence of supercoiled WHV DNA (19) and served as a demonstration of the purity of the nuclei preparation.

transcript was spliced and contained sequences from other regions of the virus genome. Therefore, to demonstrate that the transcript was X-region specific, Northern blot hybridization was performed on nuclear RNA isolated from WC220 liver tissue using radiolabeled probes from various regions of the virus genome (Fig. 1). In each hybridization, whole-cell RNA (i.e., RNA that contains the 2.3- and 3.6-kb virus transcripts) was used as a positive control. We could not detect the 0.65-kb transcript with the probe WH(E-D), which encompasses sequences within the S gene and ends 805 base pairs 5′ to the X gene initiation codon. Also, probe WH(H-P), which starts 150 base pairs 3′ of the polyadenylation signal (ATAAA) and ends 60 base pairs 5′ to the pre-surface gene initiation codon, did not hybridize with the 0.65-kb RNA species (data not shown). However, both probes did hybridize to the 2.3- and 3.6-kb mRNA transcripts and to heterogeneous low-molecular-weight nuclear RNA (i.e., molecules at various stages of transcription). The 0.65-kb RNA transcript did hybridize to all of the probes containing X gene sequences [e.g., WH(E-E) and WH(N-H)]. Our results are consistent with those of the in vitro studies that mapped the 5′ end of the transcript to a region of the genome that is 3′ to the surface gene (31, 34). Therefore, the 0.65-kb transcript that we found in WHV-infected livers is probably similar to the X-region transcript identified in recombinant gene expression systems.

Polyadenylation of the X-region transcript. If the 0.65-kb transcript serves as the mRNA for the translation of the X protein it is likely that at least some of the transcripts become polyadenylated. Analysis of poly(A)$^+$ and poly(A)$^-$ RNA from the nuclear and cytoplasmic cell fractions revealed that the majority of the 0.65-kb RNA species did not possess poly(A) tails of sufficient size to be selected by an oligo(dT)-cellulose column (Fig. 5, lane 4). Poly(A) selection did, however, identify a new species of nuclear WHV RNA of approximately 0.9 kb (Fig. 5, lane 4). The length of this poly(A)$^+$ RNA is consistent with that of a 0.65-kb transcript with a poly(A) tail of standard length. Thus, it is likely that the 0.9-kb transcript represents the polyadenylated form of the 0.65-kb X-region transcript that is in the process of being exported to the cytoplasm for translation. However, a significant proportion of the X-region transcripts are not polyadenylated and appear to accumulate in the nucleus of infected liver cells.

DISCUSSION

In this report, we describe an X-region-specific transcript in WHV-infected liver cells. Although an analogous transcript has been identified in gene expression systems, it has not been identified previously in vivo. This may be due to its low abundance (<1% of total virus transcripts) and to the fact that a significant fraction of the transcripts lack poly(A) tails and remain the cell nucleus. Apparently, some of the X region transcripts become polyadenylated, because we detected a 0.9-kb RNA species when we selected for poly(A)$^+$ nuclear RNA. These transcripts logically represent the 0.65-kb transcript with a poly(A) tail. Although we could not detect the 0.9-kb transcript in poly(A)-selected whole-cell RNA from WHV-infected livers, such a transcript has been identified in the in vitro expression systems. There are several explanations for the inability to detect the 0.9-kb transcript in whole-cell extracts. First, the transcript is very low in abundance and could easily be obscured by the high background level of heterogeneously sized RNA molecules present (Fig. 3). Also, it is possible that the transcript has a short half-life in infected liver. Therefore, the 0.9-kb transcript is only detectable by selection for poly(A)$^+$ nuclear

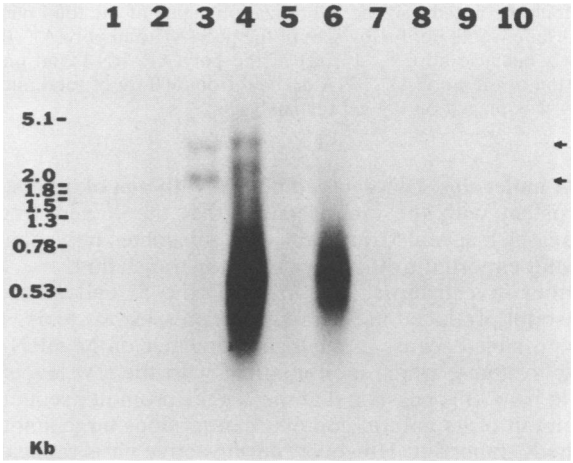

FIG. 4. Autoradiogram of the 0.65-kb RNA transcript in WHsAg- and WHsAb-positive livers. Northern blot hybridization of 30 μg of whole-cell (lanes 1, 3, 5, 7, and 9) and nuclear (lanes 2, 4, 6, 8, and 10) RNA was performed as described in Materials and Methods with recombinant WH(N-H) as a hybridization probe. The 0.65-kb transcript is not present in the liver RNA of woodchucks without past history of WHV infection (WC267, lanes 1 and 2) or in woodchucks that have recovered from a previous WHV infection (WC1636, lanes 7 and 8; WC1664, lanes 9 and 10). However, a 0.65-kb transcript was present in woodchucks with acute (WC1482, lanes 3 and 4) or chronic (WC1643, lanes 5 and 6) WHV infections. It should be noted that the electrophoretic mobility of the 3.6- and 2.3-kb bands is changed due to the presence of abundant rRNA in some preparations of RNA.

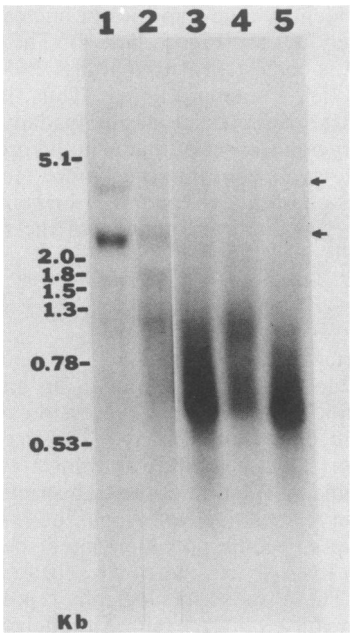

FIG. 5. Northern blot analysis of poly(A)$^+$ and poly(A)$^-$ RNA from the nuclei of WHV-infected cells. Total nuclear RNA (800 μg) was isolated from 40 g of liver tissue from the liver of WC220. The RNA was passed twice through an oligo(dT)-cellulose column as described in Materials and Methods. Northern blot analysis was performed with probe WH(N-H). Lane 1 contains 30 μg of whole-cell RNA; lane 2 contains 30 μg of cytoplasmic RNA; lane 3 contains 30 μg of total nuclear RNA; lane 4 contains 16 μg of poly(A)$^+$ nuclear RNA; and lane 5 contains 30 μg of poly(A)$^-$ nuclear RNA. The signal of 0.65-kb RNA in total nuclear RNA was approximately the same as that of poly(A)$^-$ nuclear RNA (lanes 3 and 5). Poly(A)$^+$ nuclear RNA contained less 0.65-kb RNA (lane 4). This suggests that 0.65-kb RNA is not extensively polyadenylated. It should be noted that the hybridization signal of the total nuclear RNA lane would not be the sum of the poly(A)$^+$ and poly(A)$^-$ RNA signals because all (i.e., 16 μg) of the poly(A)$^+$ RNA and only a fraction of the poly(A)$^-$ RNA derived from 400 μg of total nuclear RNA was placed on the gel for analysis.

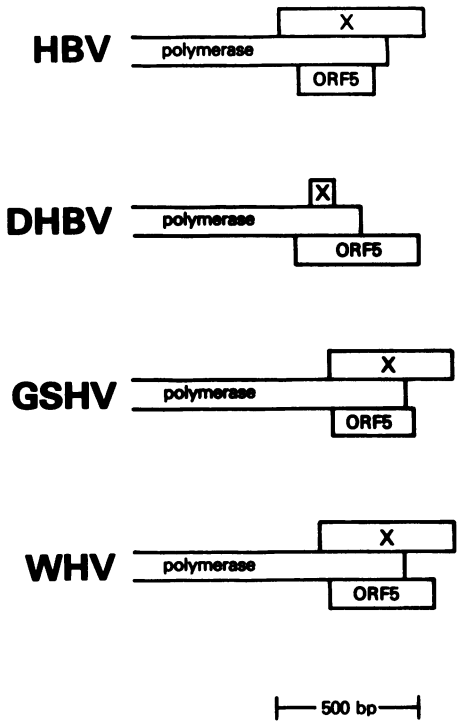

FIG. 6. Position of ORF5 on the hepadnavirus genome. A new ORF, ORF5, has been identified in an alternate translation frame within the X gene in HBV (3, 7, 8, 13, 25, 26, 29, 30, 40), DHBV (16, 37), ground squirrel hepatitis virus (GSHV) (32), and WHV (4, 9, 14) genome sequences. Although an initiation codon is not present in phase with this ORF, it could be translated into protein by a frameshift from the polymerase or X gene ORFs during translation. This may explain why the DHBV genome, which lacks an X gene sequence at the 3′ end of the linear virus genome (37), maintains the 5′ terminus of the X gene ORF. The sequence CCATGG, the consensus translation initiation sequence of eucaryotic genes (15), is present in the same position of the polymerase gene ORF in all 18 hepadnavirus genome sequences. In the mammalian hepadnaviruses this likely represents the initiation sequence of the X protein, but would not be expected to be maintained in the DHBV genome that lacks an X gene ORF at this position. A second means for translation of the ORF5 protein is that an initiation codon other than AUG could be used to begin translation. Indeed, the triplet ACG is capable of transcription initiation (1), and all ORF5 sequences possess an invariant ACG codon at the 5′ end of the putative gene sequence.

RNA molecules. Our data, combined with that of others, are consistent with the interpretation that the X gene region possesses a specific transcript which becomes polyadenylated and exported to the cytoplasm for translation.

Other investigators have mapped the 5′ end of the X transcript produced in the in vitro expression systems. This was possible because of the high abundance of the mRNA in these systems, which is in contrast with the low levels we see in vivo. It is possible that the X gene promoter sequence, taken out of its natural context, can produce large amounts of the X transcript. However, during active virus replication the promoter may be under stringent control. The general location of the promoter element has been determined (34, 38), but the exact sequences involved have not yet been precisely mapped. We found by computer-assisted nucleotide sequence analysis (19, 21) that there is a highly conserved domain immediately adjacent to the 5′ start site of the X transcript that may contain sequences essential for the function of the promoter element. Within a 60-nucleotide domain, 33 residues are invariant among 16 mammalian hepadnavirus isolates. Considering purine (R)-to-purine (i.e., A and G) and pyrimidine (Y)-to-pyrimidine (i.e., C and T) changes to be conservative substitutions for *cis*-acting elements, the 15-residue sequence GCYTGYYTTGCYCGC may represent the core element of the promoter. This is consistent with the observation that the triplets TGC, CTG, and GCT occur within 50 nucleotides of the start site of mammalian RNA polymerase II transcripts and are thought to represent sequences involved in polymerase binding (24). Interestingly, the nucleotides within this region of many hepadnavirus genomes are theoretically capable of forming a relatively stable hairpin structure ($G = -18$ kcal/mol). Therefore, this domain may play a role in the transcription of X-region sequences by RNA polymerase II.

Although the X-region transcript may be the template for the translation of the X gene protein, it may have other functions. One such function may be the expression of another virus protein encoded from a previously undetected ORF. ORF5 has been identified by our computer analysis of the HBV genome in 18 of 18 hepadnavirus sequences examined (Fig. 6). The ORF is 70 to 100 codons long and is found

in HBV and WHV genome sequences, as well as in those of the ground squirrel hepatitis virus and duck hepatitis B virus (DHBV). Experiments to determine whether this ORF represents a genuine gene sequence are ongoing in our laboratory.

Finally, it is possible that the X transcript also functions at the RNA level in virus replication and gene expression. Two complementary sequences that could hybridize to the X transcript are supercoiled virus DNA, the template for transcription, and an anti-sense virus transcript that may encode a sixth virus protein from the plus-DNA strand (21). A function at the RNA level would help to explain why the transcript remains in the nucleus and does not become polyadenylated at the same rate as the other virus transcripts. It is possible that the X-region transcript plays several important roles in virus replication and gene expression.

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