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Hybrid hepatitis B virus–host transcripts in a human hepatoma cell

(integrated viral sequences/transcription initiation and termination/multiple promoter sites/DNA transposition/oncogenesis)

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ABSTRACT **The human PLC/PRF/5 hepatoma cell line (the Alexander cell) contains at least seven copies of hepatitis B virus (HBV) DNA integrated in its genome; but it selectively expresses the HBV surface antigen (HBsAg) gene and perhaps low levels of the core gene. We have prepared a cDNA library from PLC/PRF/5 cell poly(A)$^+$ RNA and isolated clones containing HBV sequences. Hybridization experiments show that the great majority of HBV-specific RNAs in this cell line contain HBsAg coding sequences and are presumably derived from the HBsAg gene. Primer extension experiments show that these HBsAg mRNAs are, however, derived from multiple initiation sites in the HBsAg gene and involve two promoters: one at the 5′ end of the gene that can produce a protein of 45 kDa, and one located in the pre-S region that can produce two proteins of 31 kDa and the mature HBsAg, 25 kDa, respectively. The HBV RNAs are hybrid RNA species that contain HBV sequences at their 5′ ends and host DNA sequences at the 3′ ends. The great majority of these hybrid RNAs are transcribed from two closely related yet distinct HBV integrants. The viral–host sequences of these two related hybrid RNAs suggest that the related HBV sequences were generated from a parental fragment via duplication, translocation, and mutagenesis. These processes may play a role in HBV-related oncogenesis.**

Hepatitis B virus (HBV) causes acute and chronic hepatitis in human beings and has long been suspected to be the major cause of human hepatocellular carcinoma (1). The genomic DNA of HBV is partially double-stranded. It comprises two strands of unequal length (2, 3). The nucleotide sequences of the cloned HBV DNA as well as the related woodchuck, duck, and ground squirrel hepatitis virus DNA have been determined (4–9).

HBV DNA can integrate apparently randomly into a host cell genome (10–12). The Alexander cell line (PLC/PRF/5) is a human hepatoma cell line that produces HBV surface antigen (HBsAg) (13) and perhaps low levels of HBV core antigen (HBcAg) (14), but undetectable levels of other HBV gene products. Analysis of the integrated HBV DNA shows that there are no full-length integrated HBV viral genomes, instead, fragments of HBV DNA, frequently rearranged, are integrated in at least seven sites of the PLC/PRF/5 cell genome (11–13, 15, 16). In this paper, we report studies on the HBV-related RNAs reflected in clones in a cDNA library prepared from PLC/PRF/5 cells that hybridize with HBV probes. These studies allow us to predict the precise genetic origin of most of the transcripts.

MATERIALS AND METHODS

Enzymes and Cloning Vectors. Terminal deoxynucleotidyltransferase and G-tailed pBR322 were from New England Nuclear. Other enzymes used were from Bethesda Research Laboratories, New England Biolabs, and Boehringer Mannheim.

Preparation of Alexander Cell RNAs. Confluent Alexander cells were chilled on ice, washed twice with phosphate-buffered saline and once with Tris saline (0.15 M NaCl/10 mM Tris·HCl, pH 7.2). The cells were then lysed with Tris saline containing 0.5% Nonidet P-40 (Sigma). Nuclei were removed by slow-speed centrifugation, and sodium dodecyl sulfate was added to a final concentration of 0.5%, followed by phenol/chloroform extraction and ethanol precipitation. Total cellular RNA isolated this way was passed through an oligo(dT)-cellulose column (Collaborative Research, Waltham, MA) twice for the isolation of poly(A)$^+$ RNA (17).

Preparation of the cDNA Library. First-strand cDNA synthesis using the oligo(dT) primer and reverse transcriptase was as described (18). Reverse transcription was stopped by adding EDTA to a final concentration of 25 mM, followed by phenol/chloroform extraction. The unincorporated triphosphates were removed by using the ammonium acetate precipitation method of Okayama and Berg (19). The cDNA synthesized was self-primed and used directly for second-strand synthesis. Details of the reaction conditions using both the Klenow fragment of *Escherichia coli* DNA polymerase I and reverse transcriptase have been described (20). Double-stranded cDNA synthesized this way was then digested with nuclease S1 (100 units per μg of double-stranded cDNA) in 400 mM NaCl/1 mM $ZnSO_4$/35 mM Na acetate, pH 4.5, at room temperature for 60 min, and then size-fractionated on a Biogel A-150 m column (Bio-Rad). Double-stranded cDNA >600 base pairs (bp) long was used for tailing and cloning experiments. Tailing reactions were done in 1 mM $CaCl_2$/1 mM 2-mercaptoethanol/140 mM Na cacodylate/30 mM Tris·HCl, pH 7.6/100 μM dCTP. Eighteen units of terminal deoxynucleotidyltransferase was used for each microgram of double-stranded cDNA. The annealing of the C-tailed double-stranded cDNA to the G-tailed, *Pst* I-cut pBR322 were carried out as described (20). The annealing mixture was directly used for transformation into *E. coli* strain MC1061 (gift of C. M. Rice). About 90% of the transformants contain inserts with an average size of 800 bp. The transformants were stored in microtiter plates and were screened as described (21).

RNA Blot Analysis. RNA was electrophoresed in 1% agarose formaldehyde gels (20). RNA transfer was done as described by Alwine *et al.* (22); 1–3 × 10^7 cpm of [^{32}P]DNA probe was used for each analysis. The hybridizations were done in 50% formamide/3× Denhardt's solution/5× NaCl/Cit/200 μg of salmon sperm DNA per ml at 42°C. The hybridized filters were washed in 0.1× NaCl/Cit/0.1% NaDodSO$_4$ at 50°C (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinyl pyrrolidone; 1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate).

Primer Extension and Dideoxy Sequencing. The preparations of 5′ end-labeled primers from plasmids were carried out essentially as described (20). After electrophoretic separation in 5% preparative acrylamide/8 M urea gels, the prim-

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Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; HBcAg, HBV core antigen; bp, base pair(s); kb, kilobase(s).

ers were further purified on Elutip-d columns (Schleicher & Schuell) according to the manufacturer's recommendations. Approximately 1×10^7 cpm of primers were incubated with 10 μg of Alexander poly(A)$^+$ RNA in 0.4 M NaCl/1 mM EDTA/10 mM Pipes, pH 6.4, containing 80% formamide at 37°C overnight after heating at 65°C for 5 min. The annealed primer and RNA were then ethanol-precipitated twice and resuspended in 40 μl of H_2O; 8 μl of the primer/RNA mixture was used for each primer extension or dideoxy sequencing reaction. The reaction mixture of primer extension experiments contained 1 mM dNTP/2 mM dithiothreitol/4 mM Na pyrophosphate/50 mM KCl/8 mM $MgCl_2$/50 mM Tris·HCl, pH 8.3/20 units of reverse transcriptase. The reactions were carried out at 42°C for 1 hr and were stopped by phenol/chloroform extraction and ethanol precipitation. The samples were then resuspended in 5 μl of Maxam–Gilbert sample buffer and heated at 90°C for 2 min before running on a gel (23). The dideoxy sequencing experiments were done the same as primer extension experiments except that 100 μM dNTP, 10 μM respective dideoxy nucleotides, and 2 units of reverse transcriptase were used.

RESULTS

Most HBV RNAs in Alexander Cells Contain HBsAg Coding Sequences. Poly(A)$^+$ RNA prepared from PLC/PRF/5 cells was subjected to RNA gel analysis (22) using different regions of the cloned HBV DNA as probes. Most of HBV RNA in Alexander cells migrates between 28S and 18S rRNAs (Fig. 1). The pattern obtained with the entire HBV DNA as a probe (Fig. 1*A*, lane 1) is similar to that obtained when a 1.3-kilobase (kb) *Bam*HI fragment (Fig. 1*B*, fragment I) containing the coding HBsAg sequence was used (Fig. 1*A*, lane 2). When the 790-bp *Bgl* II/*Eco*RI fragment containing 20 bp of the 3′ end of the HBcAg coding sequence and extending through most of the HBsAg pre-S region (Fig. 1*B*, fragment III) was used as a probe (Fig. 1*A*, lane 3), several less intense bands were detected. These results indicate that either the intergenic region of HBcAg and HBsAg and/or the pre-S region of the HBsAg gene is transcribed in Alexander cells. When a fragment containing the HBcAg coding region (the 420-bp *Bgl* II fragment, fragment II, Fig. 1*B*) was used as a probe, only a faint band of ≈3 kb was observed (Fig. 1*A*, lane 4). The majority of HBV-related RNAs thus appear to be derived from the HBsAg region. The 3-kb band cross-reacting with the HBsAg probe does not hybridize with the core-surface intergenic probe (the *Bgl* II/*Eco*RI fragment, fragment III, Fig. 1*B*). Since the major transcription termination sites are ≈60 nucleotides upstream from the fragment II (24–26) and since there are no putative polyadenylylation sites (A-A-U-A-A-A) in fragment II or fragment III (4, 5, 7, 8), it seems likely that this is a fused RNA species containing host sequences at the 3′ end.

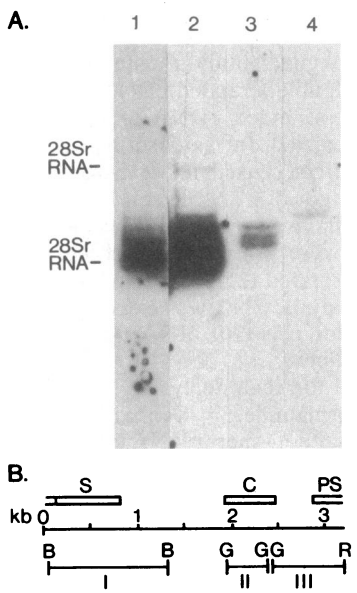

FIG. 1. RNA blot analysis of Alexander poly(A)$^+$ RNA with different regions of HBV DNA as probes. Lanes: 1, the entire cloned HBV DNA; 2, fragment I shown in *B*; 3, fragment III shown in *B*; 4, fragment II shown in *B*. All the probes used were subcloned into pBR322 vector or its derivatives. B, *Bam*HI; G, *Bgl* II; R, *Eco*RI; PS, pre-S region.

In two recent studies, HBV DNA transfected into heterologous cells was found to direct the transcription of B gene-specific RNA 1.1-kb long (25, 27). In one case, evidence was presented that the B RNA was generated through the splicing of the putative bicistronic mRNA of the HBsAg (25). In our RNA gels (Fig. 1), no HBV RNA of this size was detected. Our results thus give no support for the formation in significant quantities of this 1.1-kb RNA in Alexander cells.

Multiple Transcription Initiation Sites for HBsAg mRNAs. To determine the transcription initiation sites of HBsAg mRNAs, primer extension experiments were carried out on the poly(A)$^+$ RNA of PLC/PRF/5 cells, using as a primer the 120-bp *Xba* I/*Xho* I restriction fragment of one of the cDNA clones pAL2 (see below), containing the initiator methionine codon of the HBsAg protein and 27 bp of its 5′ flanking sequence (see Fig. 5 for map). Three major groups of extended cDNA fragments were detected migrating between the 234- and 278-bp size markers (Fig. 2*A*). In addition, a cDNA of ≈650 nucleotides was observed. To locate the transcription initiation sites more precisely, a set of nucleotide sequencing ladders derived from a dideoxy sequencing experiment using the same primer and templates was run in parallel on the gel with the extended cDNA fragments (Fig. 2*B*). A schematic representation of the transcription initiation sites is presented in Fig. 3. These are in almost exact agreement with the three groups of sites identified by Standring *et al.* (26).

The 3′ end of the large cDNA (Fig. 2*A*), corresponding to the 5′ end of the mRNA, was mapped more precisely by doing a primer extension experiment using the 96-bp *Bam*HI/*Alu* I fragment located ≈510 nucleotides upstream from the *Xba* I site (see Fig. 5). A single major product ≈120 nucleotides long was observed (Fig. 2*C*). This result indicates that the 5′ end of the RNA is ≈40 bp upstream from the initiation codon of the pre-S region (Fig. 3). This coincides with the putative promoter region containing a "TATA" box that was previously detected in *in vitro* transcription experiments using truncated HBsAg genes (28) and in *in vivo* experiments using a simian virus 40 expression vector (29). A densitometric analysis suggests that the large transcript accounts for only ≈2% of the transcripts of the gene (Fig. 2*A*); the dominant transcription activity is associated with the promoter within the pre-S region. A rough estimate of the proportion of activity of the three groups of sites within the pre-S region promoter is 59%, 24%, and 15% (Fig. 2*A*).

Molecular Cloning of HBV Transcripts in PLC/PRF/5 Cells. A cDNA library prepared from poly(A)$^+$ RNA derived from PLC/PRF/5 cells was screened with HBV DNA probes. Seven positive colonies were detected out of 17,000 screened. This frequency suggests that HBV-specific mRNAs represent only 0.05% of the total mRNA population. The HBV-specific cDNA clones termed pAL1 through pAL7 have a size range of 300–1900 bp. The maps of these clones are summarized in Fig. 5. Portions of each cDNA clone were sequenced; as expected, all contained HBV sequences, and in addition, five of the seven clones (pAL1, pAL2, pAL4, pAL6, and pAL7) contained non-HBV (presumably host) DNA sequences at their 3′ termini. Prelimi-

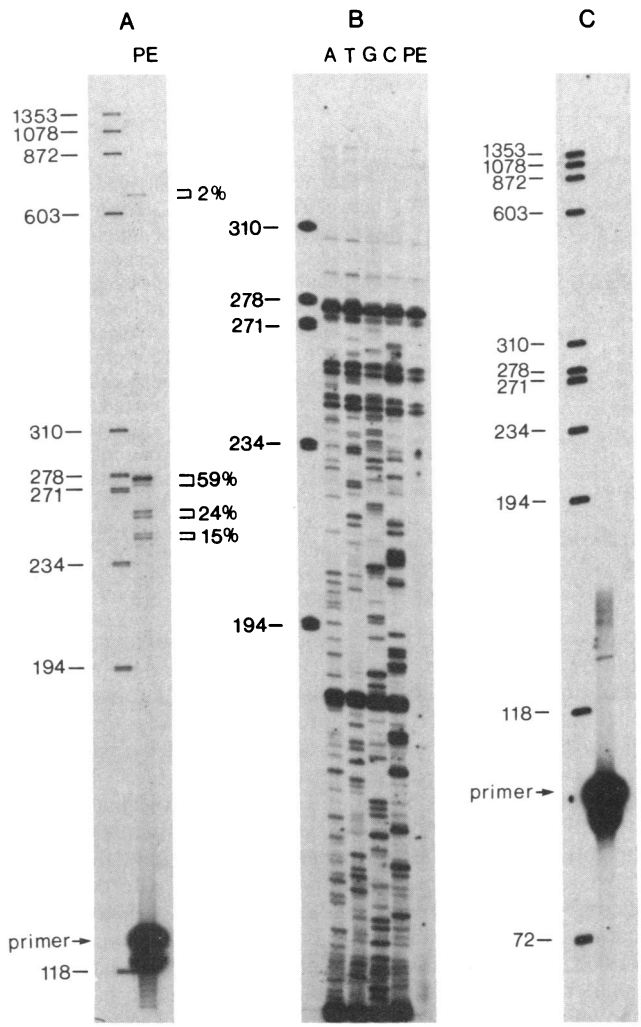

FIG. 2. Localizations of multiple transcription initiation sites of HBsAg mRNAs. (*A*) Primer extension experiment using *Xba* I/*Xho* I primer isolated from pAL2 (Fig. 5) and the Alexander RNA templates. Numbers are molecular size markers (in bp). Relative intensities of extended cDNA bands as determined by densitometer scanning are shown in percentages. (*B*) Dideoxy sequencing experiments. The same extended cDNA products from *A* are run in the PE lane. A, T, G, and C indicate the respective sequencing ladders. (*C*) Primer extension experiment using the *Bam*HI/*Alu* I fragment isolated from genomic clone 26 (see Fig. 5). The *Eco*RI/*Hin*dIII fragment of clone 26 was subcloned into pBR322. The 5′-end-labeled *Bam*HI/*Alu* I fragment was subsequently isolated from the subclone and used for the primer extension experiment.

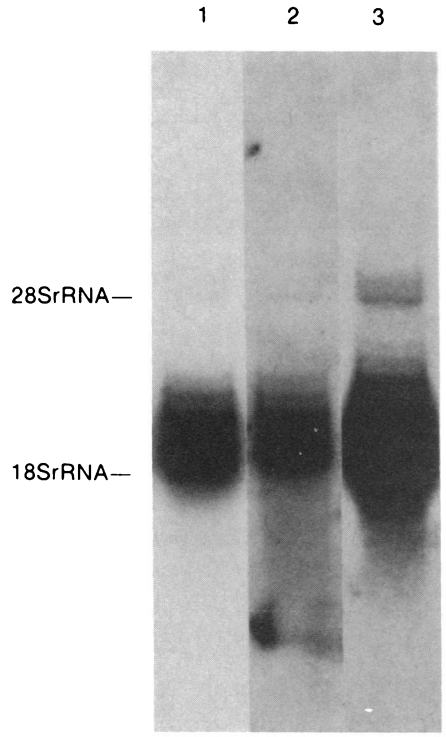

FIG. 4. RNA blot analysis of Alexander poly(A)$^+$ RNA with the subcloned host sequence of pAL2 as the probe. Lane 1, the entire cloned HBV DNA; lane 2, the subcloned host sequence of pAL2; lane 3, a longer exposure of lane 1.

nary hybridization experiments (Southern gel analysis) indicate that the 3′ sequences present in these hybrid clones are present in host DNA. Thus, the mRNAs represent hybrids between HBV and host DNA. The junctions of the HBV and host DNA sequences are at identical positions in the five hybrid clones. This suggests that they are derived from the same or closely related genes. The other two clones (pAL3 and pAL5) contained only HBsAg coding sequences. Because the overlapping sequences of these two cDNA clones and the overlapping sequences of pAL1 and pAL3 are identical, pAL3 and pAL5 are probably also derived from the same or similar integrated HBV DNA.

The HBV RNA in Alexander cells was subjected to RNA gel analysis using both the total HBV sequences and the host sequences of pAL2 (Fig. 4) as a probe. Both probes give similar hybridization signals; thus, most of the HBV specific RNA in Alexander cells contains the host sequences of pAL2 and thus must be transcribed from the same or similar HBV DNAs.

HBV Transcripts Contain Heterogeneous Termination Sites. cDNA clones pAL1, pAL2, and pAL6 contain a stretch of adenosine residues at their 3′ termini. Thus, they presumably comprise the 3′ termini of their respective mRNAs. The polyadenylylation sites of each cDNA clone are different (Fig. 5*B*). The consensus cleavage/polyadenylylation signal, A-A-T-A-A-A, is not present in any sequence; but there are many closely related sequences with only a single base substitution. This is probably responsible for the heterogeneous 3′ ends of the RNA (30, 31). It has recently been shown that replacing nucleotides within A-A-T-A-A-A results in the cleavage and polyadenylylation of RNA at incorrect sites (32, 33).

The heterogeneity of the PLC/PRF/5 HBV transcripts (Figs. 1 and 4) is presumably the result of complex transcription initiation and termination events. For example, an RNA

FIG. 3. Multiple transcription initiation sites of HBsAg gene. Arrows denote the transcription initiation sites located. Sequence shown is the prototype sequence of Valenzuela *et al.* (8). Brackets show possible transcription initiation sites. Initiation codons of P45 and P31 are shown in boldface type. The TATA box preceding transcription initiation site of P45 RNA is also shown in boldface type.

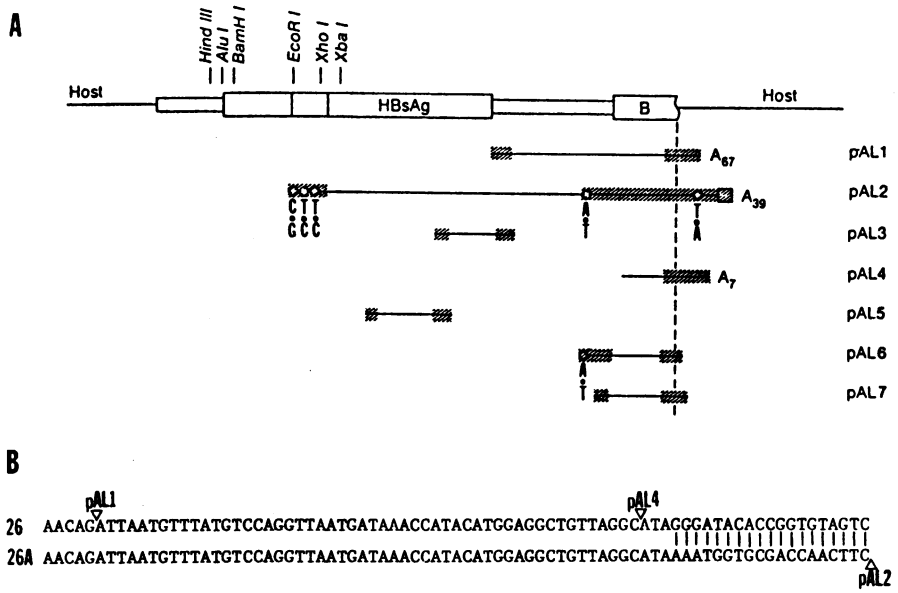

FIG. 5. (*A*) Structural relationship between cDNA clones isolated and genomic clone 26. Shaded regions indicate sequences determined by chemical methods. Circles represent point mutations. Letters below circles indicate sequence changes from clone 26 (top) to 26A (bottom). The 19 nucleotides adjacent to the poly(A) tail of pAL2 are boxed. Relevant restriction enzyme sites are indicated. (*B*) Comparison of sequences of clones 26 and 26A immediately adjacent to polyadenylylation site of pAL2. Arrows indicate polyadenylylation sites of different cDNA clones. (The clone 26 sequence was kindly provided by Pablo Garcia.)

>5 kb long hybridized to the pAL2 probe (Fig. 4, lane 2); thus, either a host promoter >2 kb upstream from HBV sequence is used, or, perhaps more likely, the polyadenylylation/termination site is >2 kb downstream from the HBV–host junction, or some combination of the two.

PLC/PRF/5 Cells Contain Transcripts Derived from Clone 26 and a Closely Related Analogue. The integrated HBV fragments present in PLC/PRF/5 cells have been isolated by molecular cloning (15), characterized by restriction enzyme mapping, and partially sequenced. Preliminary restriction mapping of clone pAL2 showed that it resembled one of the cloned HBV fragments (clone 26). All of the cloned sequences (pAL1–7) closely resemble the sequence of clone 26 and are significantly different from all others. The sequences of two of the clones, pAL1 and pAL4, are identical to clone 26. The sequence of two others, pAL2 and pAL6, are closely related to that of clone 26, but have several significant differences: at its 5′ end, clone pAL2 had three point mutations in 150 bp of sequence; in addition, there were two point mutations adjacent to the poly(A) tract of pAL2, one located in the host flanking sequence, the other located ≈600 bp upstream in the intergenic region between the surface antigen and B genes (Fig. 5). The cDNA clone pAL6 is short (≈500 bp). We infer that it does not contain the complete host flanking sequence of its mRNA template, because it does not contain a poly(A) tract. It contains, however, the identical point mutation 600 bp upstream from the poly(A) in pAL2. The cDNA clone pAL7 is short and the sequences are located between the two point mutations at the 3′ end of pAL2. It is improbable that pAL2 is derived from clone 26 by some process of error-prone transcription, because pAL2 and pAL6 share the same point mutation. Therefore, we propose that pAL1 and pAL4 are derived from clone 26, and pAL2 and pAL6 are derived from a related clone, which we term clone 26A.

This proposal is further supported by the presence of partially divergent host sequences at the 3′ end. pAL2 contains a divergent 19-bp sequence at the 3′ terminus (Fig. 5*B*). This is unlikely to be the result of a splicing event, because the consensus splicing signal near the 5′ end of the intervening sequences G-T is not found at the region in clone 26 where the sequence of pAL2 starts to diverge (34).

DISCUSSION

We have characterized the HBV-related RNA in the PLC/PRF/5 human hepatoma cell. The HBV RNAs comprise only a small proportion (0.05%) of the total cellular mRNAs; the great majority of this HBV RNA is derived from the surface antigen gene. This is consistent with the observations that the dominant HBV gene product of this hepatoma cell is HBsAg. The primer extension experiments demonstrate that the HBsAg gene-related transcripts in PLC/PRF/5 cells have heterogeneous 5′ ends. The majority originate in the central portion of the pre-S region. In agreement with Cattaneo *et al.* (24) and Standring *et al.* (26), we have found that most of the transcription initiation sites are clustered around a conserved methionine residue. Those located 5′ to this initiation methionine can transcribe RNA encoding a protein of 31,000 Da (P31). P31 and its glycosylated form have been isolated from mature HBV (Dane particles) and have been shown to bind polymerized serum albumin (35); this may play a crucial role for uptake of the particles into liver. The initiating sites located 3′ to the initiation codon of P31 can produce only the mature HBsAg protein of 25,000 Da. Unlike Cattaneo *et al.* and Standring *et al.*, we also find a minor transcription initiation site (only 2% of total mRNA) located ≈45 bp upstream from the first initiation codon of the pre-S region. This site is 20–30 bp downstream from T-A-T-A-T-A-A, a variant of the TATA sequence typically found in most eukaryotic promoters (36). This site also corresponds to the site detected in *in vitro* transcription experiments by Rall *et al.* (28) and in cellular expression experiments carried out by Laub *et al.* (29) in which the HBsAg gene was incorporated into a simian virus 40 vector. Translation of this mRNA would yield a 45,000-Da protein. This would explain conservation of the entire pre-S open reading frame among all known hepadna viruses (3). However, the function of this 45,000-Da protein remains a mystery. We postulate that this molecule, like P31, is located in the HBsAg particle.

Thus, the HBsAg region of HBV contains two promoters controlling the transcription of three overlapping coding sequences. The three resulting mRNAs can produce three proteins with common carboxyl termini, but different NH_2-terminal extensions.

Seven of the integrated HBV sequences in PLC/PRF/5 cells have been isolated by molecular cloning and characterized in our laboratory (λ clones 8, 13, 14, 15, 25, and 27) (15). Three other clones have been isolated by others [at least two of which appear to be identical to clones isolated by us (10, 12)]. Restriction enzyme mapping and sequence studies of all five cDNAs containing HBV and host sequences show that they are closely related to only one of the clones (our clone 26).

Yosef Shaul and Orgad Laub, in collaboration with us, have recently discovered an enhancer-like element in the HBV genome that is located between the coding sequences of HBsAg and the putative B genes (unpublished observations) and affects the level of expression from the core gene promoter. This enhancer may also affect transcription from the promoter of the surface antigen gene as well; thus, the level of expression of the various integrated HBV sequences should be dependent on the presence of the enhancer as well as the promoter. Several of the integrated clones miss one or the other of the regulatory elements of the surface antigen gene (clones 8, 13, 14, 15, and 23) (15). Only two integrated fragments (clones 26 and 27) contain both the promoter and enhancer regions. The HBV sequences in clone 27 contain a set of inverted repeats in which two HBsAg coding sequences are joined at their 3′ flanking regions. Thus, transcription of this clone would generate a double-stranded RNA that is at least 4 kb long and would probably be inactive. The RNA analysis (denaturing gels) shows that transcripts of this size represent no more than 1% of the total HBV mRNA. The structure of this HBV DNA fragment in the genome or, alternatively, the lack of an appropriate polyadenylylation site in the flanking host sequences could explain a lowered level of transcription of this HBV DNA. In contrast, clone 26 contains HBV DNA linearly integrated without complicated rearrangements (Fig. 5). The normal cleavage polyadenylylation signal of HBsAg is located in the coding region of HBcAg gene (24–26), but this signal is deleted in all of the HBsAg fragments obtained from the PLC/PRF/5 cells. In clone 26, apparently weak termination signals in the host sequences are used. The cDNA sequence suggests that stops are made at different positions in the host sequence.

The sequence of several clones derived from the PLC/PRF/5 cells containing both HBV and host sequences revealed the presence of two distinct templates that could be distinguished by five discovered point mutations (pAL2) and a 19-bp unique sequence at the 3′ terminus. This finding is reminiscent of recent findings that two other cloned HBV DNAs (clones 8 and 23) share the same 5′ flanking host sequences for 84 bases and then diverge thereafter (39).

These abrupt transitions seen in clones 8 and 23 and 26 and 26A can be explained by duplication and translocation of the integrated sequence. Alternatively, the possibility that this sequence transition is caused by duplication followed by DNA insertion cannot be ruled out. The duplication process might occur at the DNA level or, alternatively, via an RNA intermediate; the latter pathway is particularly intriguing, because the HBV DNA polymerase is probably a reverse transcriptase (37). The sequence divergence of clone 26 and clone 26A (five specific single base changes in 700 bp compared) represents a high mutation rate; furthermore, it is not restricted to the HBV-specific sequences; a mutation is also found within the host sequence (Fig. 5). It is unlikely that these point mutations are the result of replication errors [estimated to be $\approx 1 \times 10^{-11}$ per replication; in reverse transcription, 10^{-3} to 10^{-4} per transcription (38)]. It appears that mutagenesis frequently accompanies or follows duplication and translocation.

It is possible that insertional mutagenesis may be causally related to HBV-associated hepatic carcinogenesis; the finding of relatively frequent transposition and the mutation in the region of transposition lends credibility to this possibility. However, promoter or enhancer insertion could also initiate oncogenesis by regulating the cellular expression of a fortuitously juxtaposed cellular oncogene.

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