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Replicative Intermediates of Hepatitis B Virus in HepG2 Cells That Produce Infectious Virions

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Clonal cells derived from HepG2 cells transfected with a plasmid containing hepatitis B virus (HBV) DNA secrete hepatitis B surface antigen particles, nucleocapsids, and virions (M. A. Sells, M.-L. Chen, and G. Acs, Proc. Natl. Acad. Sci. USA 84:1005–1009, 1987) which elicit acute hepatitis in chimpanzees (G. Acs, M. A. Sells, R. H. Purcell, P. Price, R. Engle, M. Shapiro, and H. Popper, Proc. Natl. Acad. Sci. USA 84:4641–4644, 1987). We report here the initial characterization of the viral nucleic acids produced in this culture system. Kinetic analyses of nuclear, cytoplasmic, and extracellular HBV DNAs were performed by Southern blotting with radiolabeled HBV strand-specific probes. The results from these analyses indicate that at the stationary cellular growth phase, there is a dramatic increase in the rate at which HBV DNA accumulates. Incomplete double- and single-stranded forms of the HBV genome were detected in the nuclear and cytoplasmic fractions as well as in the extracellular medium. In addition, the nuclear DNA apparently includes multiple complete copies of the HBV genome chromosomally integrated and full-length covalently closed circular HBV DNA. Multiple HBV-specific polyadenylated RNAs with lengths of 3.5, 2.5, and 2.1 kilobases were identified by Northern (RNA) blot analysis. S1 nuclease mapping and primer extension identified a single 3′ end and multiple unique initiation sites corresponding to nucleotides just 5′ to the pre-S1 region, as well as upstream and within the pre-S2 and precore regions. The nucleic acid profile obtained from these analyses is essentially a facsimile of that obtained by studying liver tissue from HBV-infected individuals.

Hepatitis B virus (HBV) is one of a family of hepadnaviruses that share the ability to cause liver damage but that differ in their host range specificity. Like all hepadnaviruses, HBV replicates predominantly in hepatocytes in vivo (39), which results in either acute or chronic infection. The virion has a genome composed of partially double-stranded, relaxed circular (RC) DNA approximately 3.2 kilobases (kb) in length that is held in a circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands.

Examination of intrahepatic viral DNA revealed that a disproportionately high level of long- (minus-) strand DNA is present compared with short- (plus-) strand DNA (4, 23, 26, 27). Further studies identified these DNA forms as viral replicative intermediates which are localized to viral nucleocapsids in the cytoplasm of infected cells (40). Virion DNA synthesis is carried out in these particles, which are precursors of the virion core. The minus strand of the genome appears to be made from a template of viral pregenome RNA (40) with a protein primer (15), while the plus strand is synthesized from a DNA template (40) with an oligomer of viral RNA as the primer (21). Nucleocapsid cores containing incomplete plus strands are coated by the envelope proteins and are exported from the cell as mature virions. Although the predominant form of packaged extracellular HBV DNA is RC DNA and the majority of intracellular DNA is incomplete minus strands, RNA-DNA hybrids have been detected in serum-derived HBV particles (26).

Another major form of viral DNA, a covalently closed circular (ccc) DNA molecule which is not covalently bound to protein, was identified in the nucleus of infected cells (4, 23, 27, 46). During experimental in vivo infection of ducks with duck hepatitis B virus, ccc DNA precedes the appearance of other virus-specific single-stranded (SS) DNA or RNA (24, 42), supporting the notion that synthesis of the ccc DNA initiates the replication cycle with ccc DNA used as the template for synthesis of viral pregenome RNA.

During infection, the long (minus) DNA strand serves as the template for transcription of two major classes of virus-specific polyadenylated RNAs in approximately equivalent amounts (7, 8). The smaller of the mRNAs, with a length of approximately 2.1 kb, codes for the major protein of the viral envelope, hepatitis B surface antigen (7), and the middle protein, which is composed of the major protein plus 55 amino acids at the NH_2 terminus (37, 38). A third class of transcripts, the 2.5-kb polyadenylated RNAs, represents a minor portion of HBV-specific RNA in cells supporting viral replication in vitro (41, 48) and in the liver of infected chimpanzees (personal observation). It is possible that this RNA is initiated at the start site located just upstream from the first AUG of the pre-S1 region of the genome (31, 36) and is used to translate the large envelope protein, which is composed of the entire hepatitis B surface antigen moiety, with approximately 174 additional amino-terminal amino acids (29). The second major class of polyadenylated RNAs, the 3.5-kb transcripts, is of greater than genome length and has recently been shown to initiate in infected chimpanzees and human livers just downstream of the beginning of the precore region (47). This mRNA can serve not only as a template for translation of the hepatitis B core antigen and the viral polymerase but also as the pregenome template for reverse transcription of the minus strand of the HBV genome. All of these viral transcripts appear to use a common polyadenylation site at nucleotide (nt) 1935 (8).

Although much information has been obtained by analyzing infected tissue, animal model systems, and the expres-

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sion of one or more viral genes in tissue culture, a thorough exploration of the molecular biology of HBV by genetic and biochemical methodologies has long awaited the recently developed tissue culture viral replication systems (9, 35, 41, 43, 48). We have demonstrated that the cells of one of these lines (2.2.15), which was developed in our laboratory (35), assemble and secrete complete virions that cause hepatitis in chimpanzees (1). We describe here the viral nucleic acids identified in 2.2.15 cells and their growth medium. The profile of replicative intermediates identified in these cultured cells is fundamentally similar to that obtained by studying liver tissue from HBV-infected individuals.

MATERIALS AND METHODS

Cells and culture conditions. The establishment of the clonal cell line designated 2.2.15 by HBV DNA transfection of the human hepatoblastoma cell line HepG2 and the analysis and maintenance of these cells have been previously described (35).

Isolation and analysis of DNA. Chromosomal DNA was isolated from confluent cultures of 2.2.15 cells by the method of Jeffreys and Flavell, including treatment with 50 μg of RNase A per ml for 2 h at 37°C (18). Extrachromosomal DNA was prepared from cells which were maintained for approximately 12 days after forming a monolayer. This DNA was isolated after the cells were lysed in a solution of 20 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, 5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), and 1% sodium dodecyl sulfate by following the method described by Hirt (17). To enrich for ccc forms, DNA was isolated from cytoplasmic and nuclear fractions of 2.2.15 cells by two extractions with phenol, followed by two extractions with phenol and chloroform (1:1) without prior treatment with proteinase K.

For those samples which were analyzed at 3-day intervals, DNA was isolated separately from the nuclear and cytoplasmic fractions as well as from the medium. Every 3 days after 3×10^5 cells were plated onto each of 11 25-cm^2 flasks, the medium was changed on all but one culture. The medium from the remaining culture was collected, incubated for 1 h at 4°C in the presence of 10% (wt/vol) polyethylene glycol (molecular weight, 8,000), and centrifuged at 10,000 × *g* for 10 min. The resulting pellet was suspended in 10 mM Tris hydrochloride (pH 7.5) and 10 mM EDTA. The cells of this culture were removed by trypsinization, counted, and disrupted by homogenization in a solution of 10 mM Tris (pH 8.0), 3 mM $MgCl_2$, 320 mM sucrose, and 0.3% Triton X-100. The nuclei were pelleted by centrifugation at 1,000 × *g* for 5 min. The total DNA from this pellet (nuclear fraction) and from the supernatant (cytoplasmic fraction), as well as from the resuspended pellet prepared from the growth medium (extracellular fraction), was purified by treatment with 1% sodium dodecyl sulfate and 400 μg of proteinase K per ml for 18 h at 37°C. The samples were then deproteinized by two extractions with equal volumes of phenol, followed by two extractions with phenol and chloroform (1:1).

Resuspended polyethylene glycol pellets, prepared as described above from medium exposed to 2.2.15 cells for 6 days after a monolayer was formed (approximately 15 days in culture), were centrifuged in 1.6 g of cesium chloride and were fractionated as previously described (35). DNA contained within fractions corresponding to densities between 1.18 and 1.44 g/ml was purified by direct deproteinization and extraction as described above.

To assay endogenous polymerase activity in the viral particles secreted into medium exposed to 2.2.15 cells from 15 to 18 days after plating (approximately 6 to 9 days after the cells had formed a monolayer), a sample of medium was incubated in the presence of 200 μM each dATP, dGTP, and TTP, 0.1% Triton X-100, 50 mM $MgCl_2$, and 100 μCi [α-^{32}P]dCTP (3,000 Ci/mmol) for 2 h at 37°C. The reaction products were then centrifuged on isopycnic cesium chloride gradients and treated as described above.

The DNA isolated from all samples was precipitated in the presence of ammonium acetate and ethanol and stored at −20°C overnight, at which time the samples were centrifuged. The resulting pellets were then washed, dried, and suspended in 10 mM Tris (pH 7.5). The purified DNA samples were electrophoresed in agarose gels either as isolated or after digestion with the indicated restriction endonucleases by using the conditions recommended by the supplier (New England BioLabs, Inc., Beverly, Mass.). The agarose gel containing the DNA products of the endogenous polymerase reaction were dried and autoradiographed for 12 h at −70°C with intensifying screens. For Southern blotting analysis, the DNA was transferred to Zeta-Probe nylon (Bio-Rad Laboratories, Richmond, Calif.) according to the method of Reed and Mann (32). Hybridization was performed at 45°C with HBV DNA radiolabeled by nick translation with [α-^{32}P]dCTP or at 50°C with ^{32}P-labeled strand-specific RNAs transcripted from subgenomic fragments of the HBV genome. The fragments were cloned into the pSP64/pSP65 in vitro transcription vector system. The radiolabeled RNAs were prepared as suggested by the supplier (Promega Biotec, Madison, Wis.).

Isolation and analysis of HBV-specific polyadenylated RNA. Total RNA was isolated from confluent cultures of 2.2.15 and HepG2 cells by the guanidinium thiocyanate procedure of Chirgwin and co-workers (10). Polyadenylated RNA was separated from the remaining RNA by chromatography on oligo(dT)-cellulose (2). This RNA was electrophoresed through 1% agarose gels containing 1.1 M formaldehyde by the method described by Lehrach and co-workers (20) and transferred to a nitrocellulose membrane (22). The blots were hybridized either at 50°C with radiolabeled HBV DNA or at 60°C with ^{32}P-labeled strand-specific RNA transcripts representing subgenomic fragments of the HBV genome.

S1 nuclease mapping was performed by the method of Berk and Sharp (3), and primer extension was performed by the method of Broom and Gilbert (5). The denatured reaction mixtures were electrophoresed through 8 and 4% polyacrylamide gels containing 7 M urea for the S1 nuclease protection and the primer extension analyses, respectively. DNA probes were prepared by phosphorylation of restriction fragments with [γ-^{32}P]ATP, followed by digestion with a second restriction enzyme, yielding fragments in which only one strand was labeled at the 5′ end (25).

RESULTS

Restriction analysis of chromosomally integrated HBV DNA sequences. To analyze the manner in which vector DNA and, in particular, HBV DNA sequences had integrated into the chromosomes of these cells, restriction analysis was performed on the total nuclear DNA. A comparison of the hybridization of 2.2.15 cell DNA on Southern blots (Fig. 1) and DNA from pDo1THBV-1, the transfection vector used to derive the 2.2.15 cell line (35) (data not shown), with radiolabeled HBV DNA indicated that the entire region of the vector which carries four copies of the HBV genome had integrated into the chromosomes fully intact and, again, apparently with a single minor rearrangement. The majority

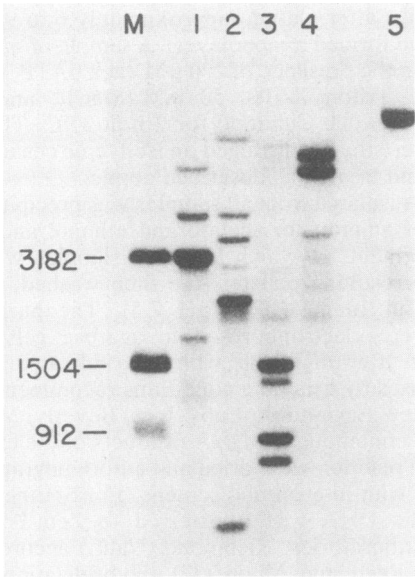

FIG. 1. Restriction analysis of chromosomally integrated HBV DNA sequences. DNA isolated from 2.2.15 cells was electrophoresed on a 1.0% agarose gel, transferred to nylon, and probed with radiolabeled full-length HBV DNA. Each lane was loaded with 10 μg of DNA either undigested (lane 5) or digested with *Eco*RI (lane 1), *Bgl*II (lane 2), *Bam*HI (lane 3), or *Hin*dIII (lane 4). The size markers (lane M) consisted of a mixture of 20 pg of *Eco*RI and 100 pg of *Eco*RI-*Bam*HI-digested pHBV-1 (16). Molecular masses are expressed in base pairs.

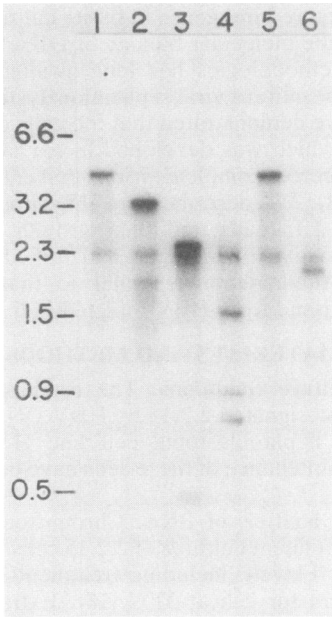

FIG. 2. Restriction analysis of extrachromosomal HBV DNA. Low-molecular-weight DNA isolated by the Hirt procedure from 2.2.15 cells was electrophoresed on a 1.0% agarose gel, transferred to nylon, and probed with radiolabeled full-length HBV DNA. Each lane was loaded with 10 μg of DNA either undigested (lane 1) or digested with *Eco*RI (lane 2), *Bgl*II (lane 3), *Bam*HI (lane 4), *Hin*dIII (lane 5), or *Hpa*II (lane 6). The size markers (in kilobase pairs) were those used in the analysis shown in Fig. 1 and a mixture of *Eco*RI- and *Bgl*II-digested pTHBV-1 (11).

of the HBV sequences were integrated as full-length genomes, as demonstrated by the 3,182-base-pair fragments obtained by *Eco*RI digestion (Fig. 1, lane 1). The integrity of the structure of the HBV DNAs was confirmed by the internal fragments of the tandems, which were obtained by both *Bgl*II (2,329-base-pair fragment) (Fig. 1, lane 2) and *Bam*HI (766-base-pair fragment) (Fig. 1, lane 3) digestion. *Hin*dIII digestion (Fig. 1, lane 4) yields fragments with lengths of 14, 10.2, and 3.8 kb, with the larger fragments hybridizing to the HBV probe more intensely. There are no *Hin*dIII sites in our HBV DNA; however, restriction of the transfection vector with this endonuclease produces a fragment with a unit length equal to that of the largest of these DNAs which contain the double tandems of the viral genome. The 10.2- and 3.8-kb fragments could have been generated by insertion and rearrangement of the vector DNA. Furthermore, both the undigested (Fig. 1, lane 5) and the endonuclease-digested (Fig. 1, lanes 1 to 4) DNA samples contained a low level of a heterogeneous population of HBV DNA, suggesting that episomal, replicating forms of HBV DNA are present in the nuclei of 2.2.15 cells.

Restriction analysis of extrachromosomal HBV DNA. To examine the nature of extrachromosomal HBV DNA, low-molecular-weight DNA was isolated from the cells, electrophoresed on a 1.0% agarose gel, and Southern blotted. The samples analyzed were either undigested (Fig. 2, lane 1) or digested with *Eco*RI (Fig. 2, lane 2), *Bgl*II (Fig. 2, lane 3), *Bam*HI (Fig. 2, lane 4), *Hin*dIII (Fig. 2, lane 5), or *Hpa*II (Fig. 2, lane 6). All fragments which could be expected from the digestion of ccc and RC HBV DNA were obtained. However, the majority of the HBV-specific DNA with an apparent mobility of 2.3 kb was refractory to digestion by all of the restriction enzymes used, suggesting that this DNA was SS in nature. When the same blot was reprobed with radiolabeled non-HBV DNA sequences from the transfection vector, no hybridization was observed (data not shown).

Detection and localization of ccc HBV DNA. To detect ccc DNA in nuclear (Fig. 3, lanes 1 and 2) and cytoplasmic (Fig. 3, lanes 3 and 4) fractions, DNA was isolated and analyzed without deproteinization. The samples were electrophoresed on a 1.4% agarose gel and Southern blotted; they were undigested (Fig. 3, lanes 1 and 3) or digested with *Eco*RI (Fig. 3, lanes 2 and 4). While the sample isolated from the nuclear preparation contained DNAs with apparent mobilities of 3.8 (the approximate mobility of RC HBV DNA on a 1.4% gel) and 2.3 kb which hybridized to the HBV DNA probe (Fig. 3, lane 1), that from the cytoplasmic fraction contained HBV-specific DNA with the mobilities of 3.8-kb fragments exclusively (Fig. 3, lane 3). However, upon *Eco*RI digestion, only a single 3.2-kb DNA fragment was detected in either sample (Fig. 3, lanes 2 and 4). The presence of a fragment with an apparent mobility of 3.8 kb, which is replaced by a single 3.2-kb DNA fragment after digestion with *Eco*RI, suggests that both samples of DNA contain a form of RC HBV DNA. The presence of DNA that migrates with 2.3-kb, double-stranded fragments (a mobility shared by supercoiled 3.2-kb ccc DNA), which can then be cut by *Eco*RI at a single site to yield a 3.2-kb fragment, argues strongly for the presence of supercoiled ccc HBV DNA in the nuclei of 2.2.15 cells.

Kinetic analysis of intracellular and extracellular HBV DNA production. To determine the rate and site of HBV DNA replicative-intermediate synthesis, the total nuclear, cytoplasmic, and extracellular DNA which was isolated from one 25-cm^2 flask at 3-day intervals was electrophoresed on a 1.4% agarose gel and Southern blotted. The DNA was

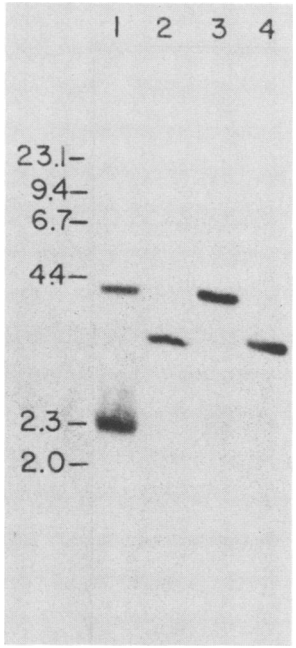

FIG. 3. Identification of ccc HBV DNA. Nondeproteinated DNA isolated from 2.2.15 cells was electrophoresed on a 1.4% agarose gel, transferred to nylon, and probed with radiolabeled full-length HBV DNA. Each lane was loaded with 20 μg of DNA isolated from either the nuclear (lanes 1 and 2) or the cytoplasmic (lanes 3 and 4) fraction. The electrophoresed samples were undigested (lanes 1 and 3) or digested with *Eco*RI (lanes 2 and 4). The size markers were *Hin*dIII-digested bacteriophage lambda DNA, indicated in kilobase pairs.

probed with minus-strand- (Fig. 4A, C, and E) and plus-strand-specific riboprobes, as well as with nick-translated, full-length HBV DNA (data not shown). The number of viable cells per flask from which the DNA samples were prepared every 3 days was determined by quantitating cells which excluded trypan blue (Table 1). From day 12 to day 30 of the experiment, the number of viable cells remained relatively constant. As evidenced by Southern blotting the nuclear DNA (Fig. 4A and B), it appears that the chromosomally integrated HBV DNA remains stable throughout this stationary phase of cellular growth. In contrast, there was a conspicuous increase in all low-molecular-weight HBV DNA in all DNA fractions during this time. A portion of extracellular and intracellular DNA apparently consisted of RC, linear partial SS, and SS HBV DNA (Fig. 4). The extracellular DNA (Fig. 4E and F) contains all of the replicative intermediates detected in the cytoplasmic fraction (Fig. 4C and D), although the latter has a lower proportion of mature forms of the viral genome (apparent mobilities of 5.0, 3.9, and 3.2 kb [linear double-stranded DNA]). DNA with a mobility of 2.3 kb (double-stranded linear DNA and supercoiled ccc DNA) anneals to both minus- (Fig. 4A) and plus- (Fig. 4B) strand-specific riboprobes, demonstrating that the sequences from both strands are present in this DNA. No such fragment from either the cytoplasmic or extracellular DNA hybridized to both of these riboprobes (Fig. 4C through F). However, both of these samples contained DNA with an apparent mobility of 2.1 kb that hybridized only to the minus-strand-specific riboprobes (Fig. 4C through F), indicating that it represents minus-strand HBV DNA in a single-stranded form.

Characterization of HBV DNA in extracellular particles. To

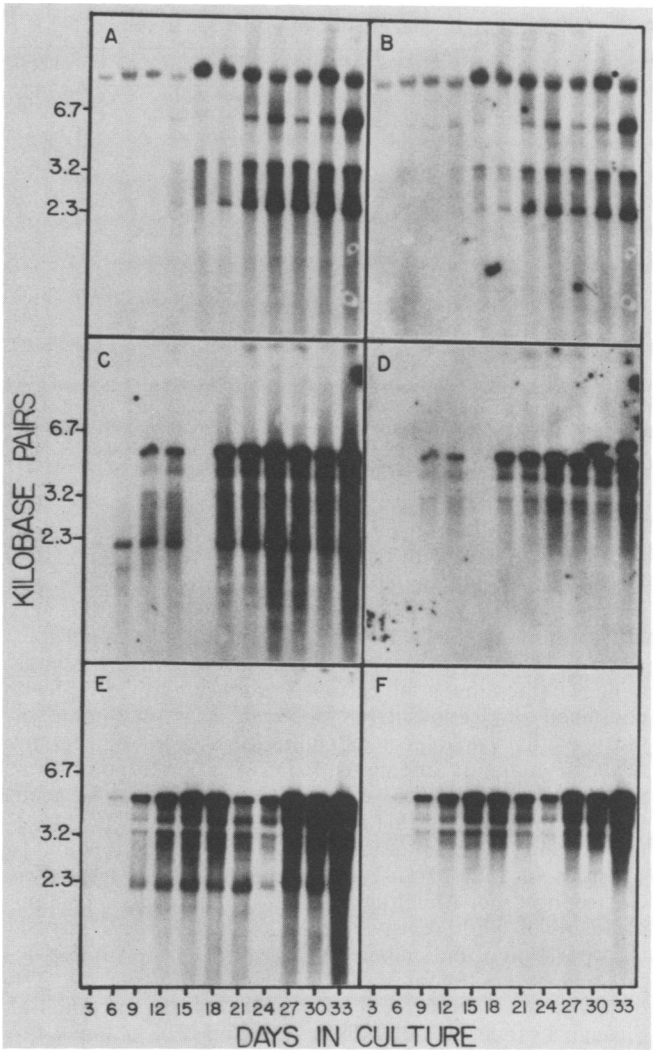

FIG. 4. Kinetic analysis of intracellular and extracellular HBV DNA production. Nuclear (A and B), cytoplasmic (C and D), and extracellular (E and F) DNA was isolated from one 25-cm^2 flask of 2.2.15 cells at 3-day intervals up to 33 days. At day 0, each flask was seeded with 3×10^5 cells. The total DNA sample isolated from each fraction was electrophoresed on a 1.4% agarose gel, transferred to nylon, and probed with either a minus- (A, C, and E) or a plus- (B, D, and F) strand-specific riboprobe. The DNA sample isolated from the cytoplasmic fraction at 15 days was lost during purification (C and D). The molecular size markers (indicated in kilobase pairs) consisted of a mixture of *Eco*RI- and *Eco*RI-*Bam*HI-digested pHBV-1 (16).

determine the precise nature of the HBV DNA that was contained within assembled viral particles, DNA was isolated from fractions of cesium chloride density gradients and was analyzed by Southern blotting (Fig. 5). The HBV DNA detected in fractions 10 to 13 (between 1.22 and 1.27 g/ml) represents predominantly mature viral DNA (Fig. 5B through D), while the principal forms of HBV DNA identified in fractions 14 to 19 (between 1.32 and 1.38 g/ml) represent either partially double-stranded molecules or SS DNA (Fig. 5B through D). The DNAs with high electrophoretic mobilities in fractions 14 to 19 most probably represent minus-strand DNA, since it hybridized with equal efficiency with nick-translated full-length HBV DNA (data not shown) and minus-strand-specific probes (Fig. 5B through D) but

TABLE 1. Quantitation of viable 2.2.15 cells[a]

Flask no.	Days in culture	No. of cells
1	3	8.4×10^5
2	6	2.4×10^6
3	9	4.0×10^6
4	12	8.6×10^6
5	15	7.8×10^6
6	18	7.9×10^6
7	21	7.3×10^6
8	24	1.1×10^7
9	27	8.9×10^6
10	30	9.1×10^6
11	33	5.2×10^6

[a] At day zero, each flask was seeded with 3×10^5 cells. At 3-day intervals, the total number of viable cells was quantitated for each flask of cells from which the intracellular and extracellular DNA was analyzed, as described in Materials and Methods. The total number of viable cells in each of these flasks was quantitated by counting cells which exclude trypan blue.

weakly, if at all, with plus-strand-specific riboprobes (Fig. 5C). In agreement with the results shown in Fig. 4, no HBV DNA was identified which hybridized to all probes and migrated with 2.3-kb DNA; therefore, it is likely that very little, if any, ccc HBV DNA is present in these samples.

Assay of endogenous DNA polymerase activity. To determine whether the viral particles produced by 2.2.15 cells contained endogenous DNA polymerase activity comparable with that capable of extending SS regions of genomic DNA in authentic infectious virions (19), incorporation of radiolabeled dCTP into extracellular DNA was assayed. DNA radiolabeled in this manner was isolated from isopycnic cesium chloride gradient fractions corresponding to densities of 1.12 to 1.47 g/ml (data not shown) and was electrophoresed. An autoradiogram of the dried gel containing samples with densities of 1.19 to 1.45 g/ml showed incorporation of radiolabeled dCTP into DNA, with a structure characteristic for the genome of HBV (Fig. 6). The majority of incorporation was detected in fractions 11 through 17 (densities of 1.24 to 1.39 g/ml; Fig. 6, lanes 4 to 10).

Detection of HBV-specific polyadenylated RNA. To identify the major classes of virus-specific transcripts produced by 2.2.15 cells, the polyadenylated RNAs from these cells and their corresponding control HepG2 cells were isolated, Northern (RNA) blotted, and hybridized to nick-translated, full-length HBV DNA and non-HBV DNA fragments from the transfection vector (data not shown), as well as to radiolabeled riboprobes (Fig. 7). The riboprobes used correspond to either nt 2508 to 3182 and 1 to 249 on the physical map of the HBV genome (which represents the pre-S1 and pre-S2 regions and a portion of the S regions) (Fig. 7, lanes 1 and 2) or nt 2169 to 2425 (which represents a major portion of the core region) (Fig. 7, lanes 3 and 4). Although multiple RNAs isolated from the 2.2.15 cells hybridized to the riboprobe representing the S regions (Fig. 7, lane 2), only RNA with lengths of 3.5, 2.5, and 2.1 kb were specific for HBV probes and did not hybridize when probed with non-HBV fragments from the transfection vector (data not shown). An identical sample of RNA from 2.2.15 cells contained only a singular class of RNAs (3.5 kb) that hybridized to the riboprobe representing the core region (Fig. 7, lane 4). None of the polyadenylated RNAs purified from the control cells hybridized to HBV DNA (data not shown) or to either of the riboprobes (Fig. 7, lanes 1 and 3). Polyadenylated RNA isolated from 2.2.15 cells that was probed with full-length HBV DNA (data not shown) displayed a hybridization pattern similar to that obtained when the riboprobe representing the S regions was used (Fig. 7, lane 2).

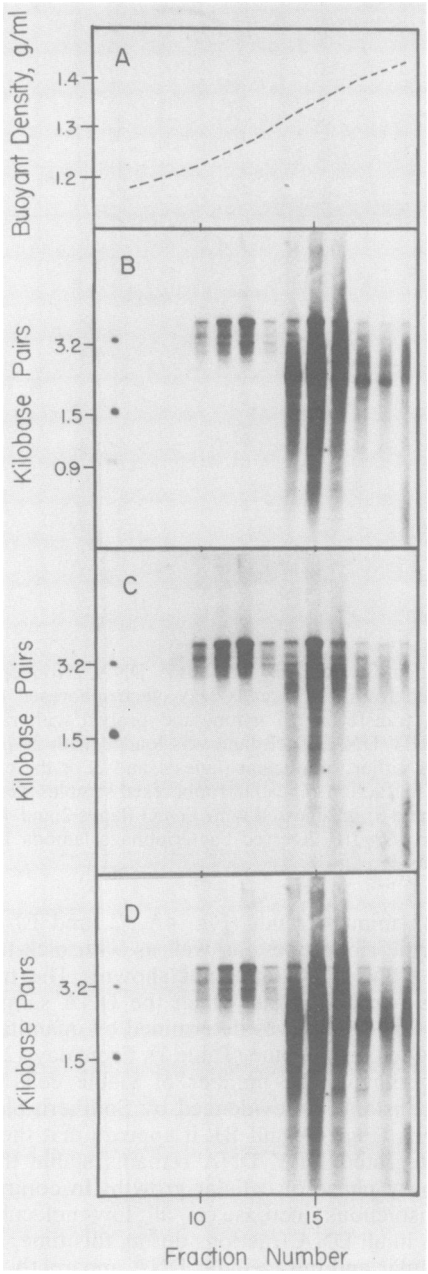

FIG. 5. Characterization of HBV DNA in extracellular particles. Fifty milliliters of 2.2.15 cell growth medium was pelleted by polyethylene glycol, centrifuged on cesium chloride gradients, and then fractionated as described in Materials and Methods. The density of each fraction was determined by refractive index (A). DNAs isolated from fractions with densities of 1.18 to 1.43 g/ml (fractions 7 to 19) were electrophoresed on a 1.0% agarose gel and transferred to nylon. The blots were probed with radiolabeled full-length HBV DNA (B) as well as plus- (C) and minus- (D) strand-specific riboprobes. The size markers (in kilobase pairs) consisted of those used in the analysis shown in Fig. 1.

Determination of the 5′ and 3′ ends of HBV-specific polyadenylated RNA. The initiation and termination sites of the HBV-specific RNAs were defined by S1 nuclease protection analysis. Polyadenylated RNAs from 2.2.15 cells that were

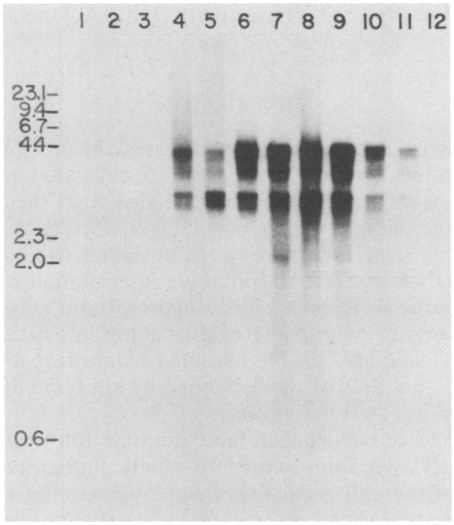

FIG. 6. Assay of endogenous DNA polymerase activity. The products of the endogenous polymerase reaction contained in 4.6 ml of culture medium were centrifuged in an isopycnic cesium chloride gradient. The gradient was fractionated, and the density of each fraction was measured by the refractive index. The DNA was isolated from fractions with densities of 1.19 to 1.46 g/ml and electrophoresed on a 1.4% agarose gel. The gel was dried and autoradiographed for 12 h at −70°C with intensifier screens. The size markers (in kilobase pairs) consisted of *Hin*dIII-digested bacteriophage lambda DNA.

annealed to a 5′-end-labeled *Rsa*I-*Xba*I fragment (nt 2508 to 3182 and 1 to 249, labeled at nt 249; Fig. 8A, lane 3) protected four discrete sizes of DNA from S1 nuclease digestion (630, 278, 250, and 230 nt in length), along with the initial 923-nt fragment (Fig. 8A, lane 1). The sizes of these fragments correspond to RNAs that either initiate at or, alternatively, are spliced at map positions immediately 5′ to

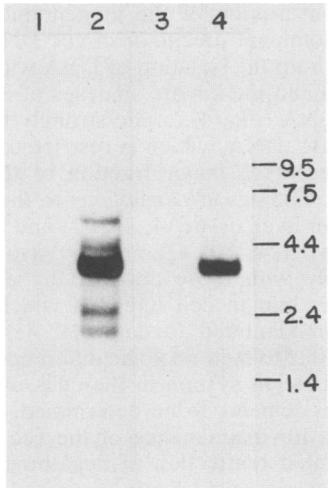

FIG. 7. Northern blot analysis of polyadenylated RNA extracted from HepG2 (lanes 1 and 3) and 2.2.15 (lanes 2 and 4) cells. Each lane of the gel contains 2.5 μg of polyadenylated RNA which was annealed with either a riboprobe corresponding to the pre-S1 and pre-S2 regions and a portion of the S regions (lanes 1 and 2) or a riboprobe corresponding to the core region (lanes 3 and 4). The size markers (in kilobases) consisted of an RNA ladder obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

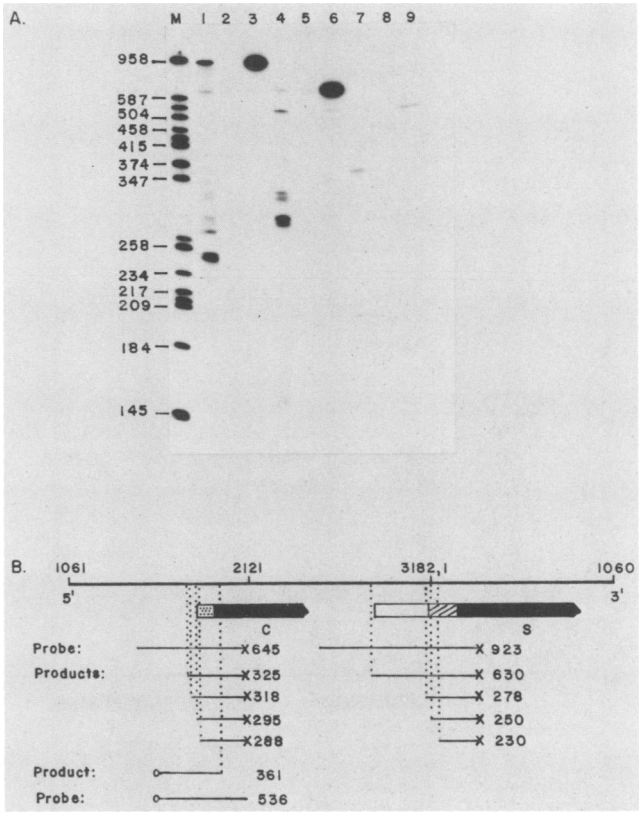

FIG. 8. S1 nuclease mapping of the 5′ and 3′ ends of the polyadenylated RNA in 2.2.15 cells. (A) Polyadenylated RNAs from 2.2.15 (lanes 1, 4, and 7) and HepG2 (lanes 2, 5, and 8) cells were annealed for 3 h at 52°C with labeled probes corresponding to nt 2508 to 3182 and 1 to 249, labeled at nt 249 (lanes 1 and 2), to nt 1465 to 2110, labeled at nt 2110 (lanes 4 and 5), and to nt 1574 to 2110, labeled at nt 1574 (lanes 7 and 8). The fragments which were protected from S1 nuclease digestion as well as the probes alone (lanes 3, 6, and 9) were electrophoresed on a denaturing 8% polyacrylamide gel. Fragment size markers (lane M; indicated in nucleotides) consisted of kinase-treated *Hae*III fragments of pTHBV-1 (11). (B) Schematic localization of the initiation and termination sites for the major viral transcripts, as mapped by S1 nuclease protection.

the pre-S region, just 5′ to or within the pre-S2 region, at nt 2801 ± 20, 3153 ± 5, 3181 ± 5, and 19 ± 5, respectively (Fig. 8B). The primary start sites in the transcripts correspond to nt 3153 and 3181. When a 5′-end-labeled *Ava*I-*Bst*NI fragment (nt 1465 to 2110, labeled at nt 2110; Fig. 8A, lane 6) was annealed to the polyadenylated RNAs from 2.2.15 cells, four discrete sizes of DNA were protected from S1 nuclease digestion (325, 318, 295, and 288 nt), along with the initial 645-nt fragment (Fig. 8A, lane 4). The two predominant fragments correspond to start sites at map positions immediately 5′ to and just within the precore region at nt 1815 ± 5, and 1822 ± 5, while the remaining fragments represent start sites upstream at nt 1785 ± 5 and 1795 ± 5 (Fig. 8B). Polyadenylated RNA from 2.2.15 cells that was annealed to a 3′-end-labeled *Bst*NI-*Ava*II fragment (nt 1574 to 2110, labeled at nt 1574; Fig. 8A, lane 9), a single-361-nt fragment was protected from digestion. The size of this fragment corresponds to RNAs that terminate at nt 1935. The polyadenylated RNA from HepG2 cells did not protect any portion of the probes described above (Fig. 8A, lanes 2, 5, and 8).

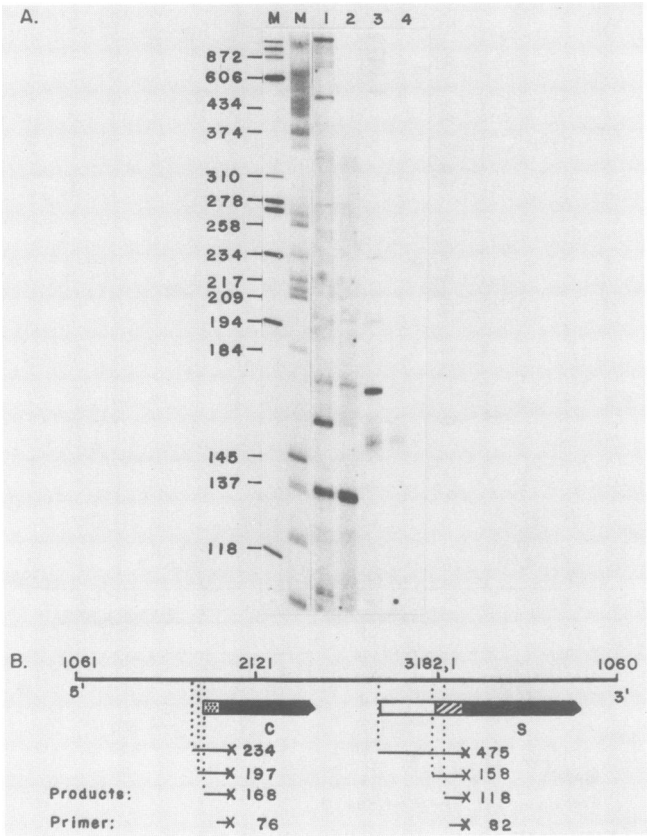

FIG. 9. Primer extension analysis of the 5′ ends of the polyadenylated RNA in 2.2.15 cells. (A) Polyadenylated RNAs from 2.2.15 (lanes 1 and 3) and HepG2 (lanes 2 and 4) cells were annealed for 3 h at 52°C with labeled probes and extended with avian myeloblastosis virus reverse transcriptase. The reaction products which were protected from nuclease digestion were electrophoresed on a denaturing 4% polyacrylamide gel. The RNA was annealed to probes corresponding to nt 47 to 129, labeled at nt 129 (lanes 1 and 2), and nt 1910 to 1986, labeled at nt 1986 (lanes 3 and 4). Fragment size markers (lanes M; indicated in nucleotides) consisted of kinase-treated *Hae*III fragments of phiX174 and pTHBV-1. (B) Schematic localization of the start sites for the major viral transcripts, as mapped by primer extension.

To confirm that the 5′ ends which were identified by the S1 nuclease protection analysis were authentic initiation sites, primer extension analysis was performed on samples of RNA from 2.2.15 and HepG2 cells (Fig. 9). A 5′-end-radiolabeled *Stu*I-*Xho*I fragment (nt 47 to 129, labeled at nt 129) was extended by using 2.2.15 cell RNA as the template. Three reaction products with lengths of 475, 158, and 118 nt were obtained which were unique to this sample (Fig. 9, lane 1). These products identified initiation sites at nt 2836 ± 5, 3153 ± 3, and 11 ± 2, the primary initiation site being at nt 3153, immediately 5′ to the pre-S2 region (Fig. 9B). The remaining fragments are also present in the RNA from HepG2 cells that was annealed to the same fragment (Fig. 9A, lane 2). A second extension reaction using a 5′-end-labeled *Tag*I-*Bal*II fragment (nt 1910 to 1986, labeled at nt 1986) as a primer yielded three products unique to the 2.2.15 cell RNA samples (Fig. 9A, lane 3) compared with that obtained with HepG2 cell RNA (Fig. 9A, lane 4). Of these products, the 168-nt fragment which corresponds to a start site at nt 1818 ± 2 was in greater abundance than were the 197-nt and 234-nt products which identify start sites at nt 1789 ± 3 and 1752 ± 3, respectively (Fig. 9A, lane 3, and Fig. 9B).

DISCUSSION

Over the past year, we have witnessed the development of several culture systems consisting of cells that produce a wide variety of replicative intermediates from the life cycle of HBV, including viruslike particles (9, 35, 41, 43, 48). The experiments described here were designed to characterize the viral DNA and RNA replicative intermediates in one of these systems which we established (35) and subsequently demonstrated to be capable of producing infectious virions (1). The nucleic acid profile obtained from these analyses is essentially the same as that obtained by studying liver tissue from HBV-infected individuals.

The 2.2.15 cells appear to have multiple full-length copies of the HBV genome chromosomally integrated stably throughout the cell growth cycle. However, the DNA isolated from nuclear and cytoplasmic fractions, as well as from the growth medium, contains a sizable increase in replicating forms of HBV DNA at the stationary phase of growth. The increased production of low-molecular-weight HBV DNA, identified during the first 15 days of our kinetic analysis, cannot be accounted for solely by cellular replication, since the HBV-specific DNA isolated over this time increased many fold over the increase in cell number. Furthermore, when the amount of total DNA per sample was held constant and analyzed similarly, the same overall increase is observed by us (34) and, in another HBV-producing cell line, by Sureau and colleagues (41). These findings support the suggestion, made previously (41), that the synthesis of viral replication intermediates is cell cycle dependent in tissue culture.

On the basis of several lines of evidence (24, 27, 30, 33, 44), it is presumed that the replication of several hepadnaviruses involves transcription of the terminally redundant pregenomic RNA from ccc DNA. By experimentally infecting duck liver cells in vitro, Tuttleman and colleagues (44) have shown that the RC DNA synthesized in the cytoplasm by reverse transcription of the pregenomic RNA is most likely the predominant precursor of ccc DNA. Our results, obtained from both the isolation of DNA without deproteinization (Fig. 3) and the kinetic analyses of intracellular and extracellular DNA (Fig. 4), argue strongly that 2.2.15 cells contain ccc HBV DNA, which is restricted to the nucleus, where it represents a major fraction of the HBV DNA. Although these findings are analogous to those obtained by studying human liver tissue (4, 26, 27) and duck liver cells infected in vivo (24, 30, 42) and in vitro (44, 45), they contrast sharply with those obtained by examining other HBV-producing human cell lines, in which no ccc HBV DNA has been identified to date (41, 43). Whether this discrepancy is due to methodologic differences or represents an altered replication system in the cells of some of these culture systems remains to be determined. It appears that the mechanism for maintenance of the ccc DNA pool depends on repeated reinfection of hepatocytes. In our cultures, this process is most likely restricted to recycling of HBV DNA or amplification of the ccc HBV DNA, since the parent cell line (HepG2) of 2.2.15 cells is seemingly refractory to infection by virions produced in vivo or in vitro (personal observations).

During an experimental in vivo infection of ducks with duck HBV, the ccc DNA was the first novel, virus-specific nucleic acid to appear in the nucleus prior to the accumula-

tion of either virus-specific RNA or SS DNA (24, 42). However, by analyzing the DNA produced in 2.2.15 cells over the course of 33 days, we obtained evidence that the accumulation of SS and RC HBV DNA in the cytoplasm precedes that of ccc DNA in the nuclei of the cultured cells. One rationale for this discrepancy might be that in our system, HBV DNA is integrated into the genome of 2.2.15 cells and can therefore act directly as a precursor to virion DNA, thus circumventing the necessity for the amplification of ccc DNA to produce substantial amounts of SS and RC HBV DNA. This same argument can be used to explain the lack of ccc in other culture systems, since it is conceivable that the integrated HBV DNA can negate the necessity for synthesis of ccc DNA entirely.

In naturally occurring infections in vivo, the overwhelming majority of extracellular HBV DNA is RC DNA, while the predominating intracellular intrahepatic DNAs are less mature forms consisting of SS (minus) and partially double-stranded viral DNA. However, in both cells infected in vitro with duck HBV (44, 45) and in HBV DNA-transfected human cell lines producing virions (35, 41, 43), a large fraction of the extracellular particles identified contain SS and partially double-stranded viral DNA. The well-defined intracellular localization of ccc DNA in 2.2.15 cells argues against a premature shedding of immature (corelike) particles from cell death in these systems. Why the seemingly coordinated maturation of the viral genome and export of particles in vivo have been altered in vitro is yet to be determined. Nevertheless, it will be of interest to establish the biological significance, if any, of this alteration in particle export.

As evidenced by the incorporation of radiolabeled dCTP into HBV DNA, it appears that the viral particles produced by 2.2.15 cells are rich in endogenous polymerase activity. The results obtained in the present study indicate that Dane particles and possibly core particles carry the enzyme activity. These findings contrast with those obtained previously, in which incorporation of radiolabel was obtained in complete Dane particles only (34). The most likely explanation for this discrepancy is methodologic. In the present study, endogenous polymerase activity was assayed prior to centrifugation of the virus particles in the presence of cesium chloride, while in the former experiments, the enzyme activity was assayed after centrifugation, a procedure that can damage the core particles, resulting in inactivation of the polymerase.

Analysis of the RNA in 2.2.15 cells revealed the presence of two major classes of transcripts (with lengths of 3.5 and 2.1 kb) with microheterogeneity at their 5′ ends. These two species have been identified in the livers from infected chimpanzees and humans (7, 8, 47). The third species of transcripts found in 2.2.5 cells (a 2.5-kb RNA) represents only a minor fraction of the viral RNAs in the liver of chimpanzees (personal observation). Will and colleagues did not detect this transcript in the livers of infected chimpanzees and humans (47). However, as previously discussed by these authors (47), the inability to demonstrate the presence of all the HBV RNAs and to perform a complete mapping of all the viral transcripts is most likely due to the limited availability of well-preserved HBV-infected liver tissue, an obstacle which is nullified by using HBV-producing cultured cells. The major 5′ ends of the 3.5- and 2.1-kb RNAs identified in the present study correspond well with those observed for ground squirrel hepatitis virus (12) and in another HBV-producing culture system (48). Whether there is a preferential packaging of one of the 3.5-kb RNAs in our system, analogous to the RNA packaging in ground squirrel hepatitis virus (13), remains to be determined. It appears certain, however, that the HBV transcripts in 2.2.15 cells use a singular polyadenylation site at nt 1935, the only 3′ end identified in our system as well as in in vivo studies of other hepadnaviruses (8, 12).

Thus, we have established that 2.2.15 cells produce not only infectious virions (35) but also all of the RNA and DNA intermediates so far identified in cells infected with other hepadnaviruses studied in vivo and in vitro (6, 12, 14, 23, 24, 28, 33, 40, 44, 46), in livers of HBV-infected chimpanzees and humans (4, 7, 8, 26, 27, 47), and in cultures of other HBV-producing human liver cell lines (9, 41, 43, 48). This cell line can now be used to further dissect the molecular events in viral replication by sequentially tracing and defining the interdependency of the synthesis and assembly of the viral proteins and particles as well as their secretion. Although several steps of the virus life cycle can be investigated in detail by using this system, the study of attachment, penetration, and uncoating of HBV awaits the development of an in vitro culture system consisting of cells that are receptive to infection by the intact virus, similar to that described for duck HBV (45). Nevertheless, the development and characterization of in vitro replication systems such as 2.2.15 cells has made possible the long-awaited scrutiny of HBV by genetic and biochemical analyses. Furthermore, 2.2.15 cells can be used in experiments designed to expand our understanding of the pathogenesis of hepatitis B and to define possible antiviral drugs for treatment of HBV-related disorders.

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