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A System for Studying the Selective Encapsidation of Hepadnavirus RNA

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All hepadnaviruses produce multiple genome-length RNA species, only one of which is encapsidated into subviral core particles prior to reverse transcription. To study the encapsidation mechanism, we developed a system in which the packaging of genetically marked target genomes of duck hepatitis B virus is mediated by factors supplied from a separate (helper) plasmid that encodes encapsidation functions. In the helper plasmid, the synthesis of the viral core and polymerase proteins was driven by a simian virus 40 promoter; the RNA produced by this construct was itself inefficiently packaged and was not active as a template for reverse transcription. Cotransfection of this construct with mutant genomes bearing frameshift lesions in either core or polymerase cistrons resulted in the successful packaging and reverse transcription of the mutant genomes. This system should allow definition of both the *cis*- and *trans*-acting elements of the encapsidation pathway.

Hepadnaviruses are small DNA viruses that replicate preferentially in liver cells and are involved in the pathogenesis of hepatocellular carcinoma (9). A key step in the life cycle of the hepadnaviruses is the generation of a small circular DNA genome by reverse transcription of an RNA intermediate (23; for review, see reference 9). As in retroviruses and caulimoviruses (other viruses that employ reverse transcription), this cytoplasmic reaction does not occur freely in solution but rather takes place within subviral core particles composed of the major nucleocapsid protein (core antigen) together with the viral polymerase. Presumably this arrangement serves not only to maximize the efficiency of template use but also to sequester the reaction from cellular mRNAs, reverse transcription of which could be deleterious to the host (8). As such, a critical event in the replicative cycle of the virus is the correct encapsidation of the appropriate viral RNA.

All hepatitis B viruses synthesize two size classes of virus-specific RNAs, either genomic or subgenomic in length (2, 6, 16). The subgenomic transcripts serve as mRNAs for the viral surface proteins and are not encapsidated into cores. The genomic transcripts are a family of RNAs that act both as mRNAs (for the viral core and polymerase proteins) and as the template for reverse transcription (2, 9). In the mammalian hepadnaviruses, three such transcripts exist. All are unspliced, terminally redundant, and 3′ coterminal; they differ by only 16 to 27 nucleotides (nt) at their 5′ termini, owing to the heterogeneity of their transcription start sites (6, 16). Detailed studies of the fine structure of viral DNA led to the inference that only the shortest of these RNA species can serve as a template for viral DNA synthesis (13, 21). In subsequent studies of the ground squirrel hepatitis virus (GSHV), we showed that this selection is imposed at the level of encapsidation; although all three genomic RNA species are found on polyribosomes, only the shortest RNA can be detected within cytoplasmic cores (7). The mechanisms by which this selection is made have remained mysterious. In retroviruses, a definable sequence element found only in encapsidated RNAs is responsible for selective packaging (1, 14, 15). However, it is clear that sequence elements alone cannot dictate packaging in hepadnaviruses, since the longer genomic RNAs contain all the sequences present in the shorter species and yet are not packaged (7).

To better understand the pathways of viral RNA packaging, we have developed an experimental system based on the duck hepatitis B virus (DHBV). The system uses an expression vector designed to produce all the viral gene products; this construct is used to provide encapsidation functions in *trans* to mutant RNA pregenomes. In this paper we report that this system allows the correct encapsidation of pregenomic viral RNA and makes possible the further dissection of elements required either in *cis* or in *trans* for this important assembly reaction.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England BioLabs (Beverly, Mass.) and used according to the manufacturer's directions. Radionuclides were purchased from Amersham Corp. Rabbit antibody to DHBV cores was the generous gift of J. Newbold (University of North Carolina); rabbit anti-DHBV surface antigen was kindly supplied by W. Mason (Fox Chase Cancer Center, Philadelphia).

Plasmids. pD2G is a tandem dimer of the European strain of DHBV (22) inserted at the *Eco*RI site of the vector pBS(−) (Stratagene, Inc., San Diego, Calif.). p442 is a tandem *Eco*RI dimer of DHBV carrying *pol* mutant 253 of Chang et al. (4); this mutant is a −1 frameshift in the region of *pol* immediately 3′ to the core open reading frame (ORF), generating a *pol* termination codon 10 nt downstream. The mutation also creates a unique *Pst*I site at the site of the frameshift. pDfs1 is a monomeric DHBV genome carrying a frameshift mutation in the unique region of ORF C. This mutation was generated by ablation of the unique *Nsi*I site at nt position 2845 (22) with T4 DNA polymerase, followed by self-ligation and recloning. The structure of both of these mutants was verified by DNA sequencing.

The packaging plasmid pDP20 was generated from plasmid pSV45 (17), a construct carrying the hepatitis B virus (HBV) (subtype adw2) pre-*S*, *S*, and *X* genes (*Bst*EII-*Bgl*II, nt 2824 to 1987) driven by the simian virus 40 (SV40) early promoter (343-nt *Pvu*II-*Hin*dIII fragment). The HBV structural genes were deleted by cleavage with *Xba*I (adjacent to

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the SV40 promoter) and *Bam*HI (within the HBV *X* gene). This plasmid, pSV-XP, retains the SV40 promoter, the HBV polyadenylation signal, and an intervening *Sal*I site. A *Cla*I linker was then added to this *Sal*I site. To generate pDP20, a monomer DHBV genome permuted at its *Acc*I site within the precore was cloned into this *Cla*I site, after conversion of the DHBV *Acc*I site to *Cla*I by linker addition. Clones in which the DHBV genome was in the correct transcriptional orientation relative to the SV40 early promoter were then identified by restriction mapping, and one such clone, pDP20, was used for subsequent experiments.

Cell culture and transfections. Huh7 human hepatoma cells (25) were grown in DM 160 medium (24) with 10% fetal calf serum (GIBCO Laboratories) and passaged twice weekly at a 1:3 dilution. DNA transfections were performed by the calcium phosphate coprecipitation method exactly as described previously (11).

RNA preparation and hybridization analysis. Polyadenylated [poly(A)$^+$] total cellular RNA was extracted from Huh7 cells 48 h posttransfection as described previously (11). Briefly, cells were lysed in 1% sodium dodecyl sulfate (SDS) and proteinase K (250 μg/ml), and their DNA was sheared by multiple passages through a 26-gauge needle; poly(A)$^+$ RNA was then selected by annealing to oligo(dT)-cellulose (11).

RNA from approximately 3×10^6 cells was electrophoresed through a 1% agarose–2.2 M formaldehyde gel, transferred in 20× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) to a nylon membrane (Genescreen; New England Nuclear Corp.) and cross-linked to the filter with UV light. Filters were then annealed to radiolabeled DNA probes prepared by randomly primed DNA synthesis from pD2G templates with synthetic random oligonucleotide primers. The specific activity of the probes was typically 1×10^8 to 4×10^8 cpm/μg of DNA. Following hybridization, the filters were washed and prepared for autoradiography as described before (11).

Isolation of nucleic acid from cytoplasmic cores. At 120 h posttransfection, cells were washed twice with phosphate-buffered saline and then lysed in 1 ml of 0.5% Nonidet P-40–20 mM Tris (pH 7.5)–150 mM NaCl–10 mM EDTA. Nuclei were removed by centrifugation for 3 min in an Eppendorf microfuge at 4°C. Anti-DHBc (3 μl) was then added to the supernatant and incubated for 1 h on ice; following this, 30 μl of protein A-Sepharose 4B beads (Pharmacia) was added, and incubation was continued for 15 min. The beads were then collected by centrifugation for 2 min in a microfuge and washed three times with 1 ml of the above lysis buffer. After one further wash in TE(10 mM Tris [pH 7.5], 1 mM EDTA), the pellet was suspended in 0.5 ml of TE with 1% SDS and 200 μg of proteinase K per ml. After incubation for 1 h at 37°C, the beads were removed by centrifugation, and the supernatant was extracted with 1 volume of phenol and then with 1 volume of $CHCl_3$. The aqueous phase was then precipitated with 2 volumes of ethanol after addition of 20 μg of carrier yeast RNA. The ethanol precipitate was washed with 70% ethanol, dried under vacuum, and then suspended in 20 μl of TE for further analysis.

In some experiments (e.g., Fig. 4), cores were prepared by a modification of the method of Pugh et al. (18). Briefly, 1 ml of cytoplasmic extracts prepared in 10 mM Tris (pH 7.5)–1 mM EDTA–50 mM NaCl–8% sucrose–0.25% Nonidet P-40 were adjusted to 8 mM $CaCl_2$ and 6 mM $MgCl_2$ and digested with micrococcal nuclease (30 U/ml) (BM Biochemical) and RNase-free DNase (1 U/ml; Promega) for 15 min at 37°C. Cores were then precipitated by adding 330 μl of 26% polyethylene glycol–1.4 M NaCl–60 mM EDTA, incubating at 4°C for 1 h, and pelleting in an Eppendorf microfuge for 4 min (18). Resuspended cores in 100 μl of 10 mM Tris (pH 7.5)–8 mM $CaCl_2$–6 mM $MgCl_2$ were then redigested with micrococcal nuclease plus RNase-free DNase as above for an additional 10 min at 37°C, and then core nucleic acid was extracted as above.

Primer extension analysis. A synthetic 22-nt oligonucleotide (spanning positions 2660 to 2638 within the *C* gene) was used as the primer for primer extension analysis of pregenomic RNA. For 5′-end labeling, 50 ng of primer was incubated with 60 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 1.2 μl of T4 polynucleotide kinase (Pharmacia) in 66 mM Tris (pH 7.6)–10 mM $MgCl_2$–10 mM dithiothreitol–0.2 mg of bovine serum albumin per ml–1 mM spermidine hydrochloride (total reaction volume, 13 μl). After 60 min at 37°C, the reaction was terminated by heating to 65°C for 10 min. Then, 10 ng of labeled primer was coprecipitated with RNA in 2 volumes of ethanol. After the pellet was washed in 70% ethanol and dried under vacuum, 20 μl of hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.6], 400 mM NaCl, 1 mM EDTA, 80% formamide) was added, and the nucleic acid was denatured at 90°C for 2.5 min. The primer was then allowed to anneal to the RNA for 2 h at room temperature. The annealed mixture was then diluted with 10 volumes of water and reprecipitated with ethanol. The rinsed and dried pellet was suspended in 25 μl of reverse transcription buffer (100 mM Tris hydrochloride [pH 8.3], 100 mM KCl, 10 mM $MgCl_2$, 28 mM mercaptoethanol, 0.4 mM each dNTPs, 1 μl of RNasin) with 0.5 U of avian myeloblastosis virus reverse transcriptase (Seikagaku America, Inc.) per μl and incubated for 1 h at 42°C. The reaction was quenched by the addition of 75 μl of TE and extracted with phenol. The aqueous phase was precipitated with ethanol, and the rinsed and dried pellet was suspended in loading buffer and electrophoresed through a 6% polyacrylamide sequencing gel, which was then dried and autoradiographed.

Protein radiolabeling and immunoprecipitation. At 3 days posttransfection, cells were washed once in phosphate-buffered saline and incubated in methionine-free DME-16 medium. After 3 h of incubation at 37°C, 100 μCi of [^{35}S]methionine per ml was added, and incorporation was allowed to proceed for 4 h. Following this, cytoplasmic extracts were prepared, immunoprecipitated with the indicated antibody, and electrophoresed as described before (12, 17).

RESULTS

Figure 1A schematically depicts the coding organization of the genome of DHBV, the avian hepadnavirus we used for our studies. Three ORFs are present, encoding the surface (pre-S/S), core (C), and polymerase (P) proteins. As in other hepadnaviruses, the surface proteins are normally expressed from two subgenomic mRNAs, while the core and polymerase products are presumed to be translated from genomic mRNA templates (2).

Experimental strategy. Our approach to the study of hepadnaviral packaging has as its goal the separation of the components of the reaction required in *cis* from those required in *trans*. To this end, we constructed a system in which a transcriptionally competent genome defective for the production of one or more viral proteins can be packaged in *trans* by a cotransfected "packaging" plasmid which can supply the missing functions(s). In order to maximize the

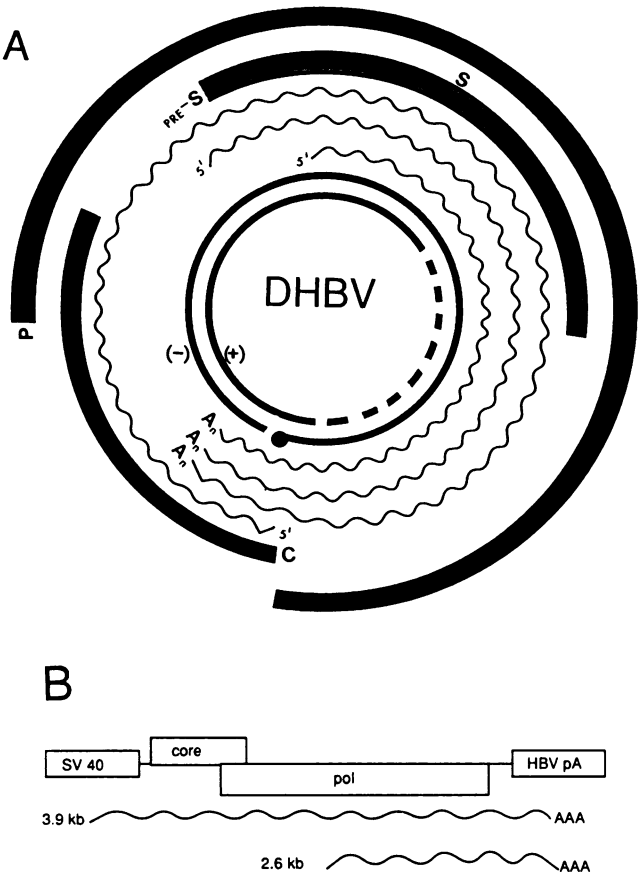

FIG. 1. Genetic organization of DHBV (A) and a candidate DHBV packaging vector (B). (A) Physical and genetic map of DHBV. The central portion depicts the DHBV genome, as found in viral cores and virions. The dashed line indicates a region of partial single-strandedness; in many DHBV particles, the plus strand is nearly complete. Wavy lines indicate the major transcripts seen in infected liver (2). These include both genomic (3.3 kb) and subgenomic (2.0 and 1.8 kb) species. Heavy bars indicate positions of the major viral coding regions: C, core antigen; P, polymerase; pre-S/S, surface proteins. (B) Structure of packaging plasmid pDP20. An SV40 fragment containing the early promoter is followed by a unit-length DHBV genome permuted at the *Acc*I site within precore. 3′ to this are sequences from HBV containing the viral polyadenylation signal (HBV pA). Wavy lines beneath the diagram depict the approximate extent of the two classes of transcript, genomic and subgenomic; although shown as a single 2.6-kb RNA, the subgenomic class actually consists of two species, as in wild-type DHBV (cf. panel A).

efficiency of this complementation, the donor plasmid's RNA was designed to be inefficiently packaged, so as not to compete with the packaging of the target RNA.

As a target for packaging, we constructed a plasmid carrying a unit-length DHBV genome bearing a frameshift lesion in ORF C. Upon transfection into permissive cells, such genomes (after liberation from their plasmid backbone) direct the production of pregenomic RNAs which cannot package themselves owing to their inability to synthesize core antigen (4), but which can be packaged by functions supplied in *trans* (see below).

In the absence of decisive information on which viral proteins are required in *trans* for encapsidation, we designed a packaging vector that would produce both classes of transcript and therefore presumably direct the synthesis of all essential viral gene products. The structure of the resulting construct, pDP20, is shown in Fig. 1B. In this plasmid, a unit-length DHBV genome permuted at the *Acc*I site within the precore region was cloned downstream of an SV40 early promoter; 3′ to the DHBV genome is a heterologous polyadenylation signal derived from HBV. The DHBV promoters for the surface transcripts are intact and thus should direct the production of functional subgenomic mRNAs. An authentic pregenomic RNA cannot be produced, since the core promoter has been interrupted by plasmid sequences. However, an analog of the genomic transcripts can be generated from the SV40 promoter. This RNA would contain the intact coding regions for core and polymerase, but differs from authentic pregenome at its 5′ end, having lost 47 nt of DHBV sequences from the precore region; it is therefore unable to direct the synthesis of the precore protein. The RNA has also acquired 89 to 93 nt of SV40 and polylinker sequences at its 5′ end; the heterogeneity of added SV40 sequence is due to the multiple start sites of SV40 early mRNA (initiating at SV40 nt 5150 and 5154 [5, 10, 26]).

The absence of the precore gene product should not affect packaging in *trans*, since we (3) and others (19) have previously shown that this product is dispensable for viral replication. However, the changes in genomic RNA structure at its 5′ end may diminish the packaging of this RNA in *cis*. The DHBV sequences missing from the 5′ end of the RNA include both the normal cap site and a copy of the DR1 element. (These sequences are still present in the transcript, but owing to the structure of the clone are present only at its 3′ end.) We anticipated that these changes would adversely affect the packaging of the transcript, since more modest changes at the 5′ end of ground squirrel hepatitis virus RNAs have previously been noted to strongly impair encapsidation (7). In addition, owing to the presence of HBV sequences at their 3′ ends, both classes of RNAs produced by pDP20 are expected to be ca. 600 nt longer than their wild-type counterparts. This, too, might further decrease packaging of the donor genomic RNA.

Characterization of the packaging plasmid. To validate these predictions of the packaging vector design, we transfected Huh7 cells (known to be permissive for DHBV replication [11, 18]) with either wild-type DHBV DNA or pDP20 and examined the virus-specific RNAs and proteins produced. Poly(A)$^+$ RNA from each set of transfected cells was fractionated on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled DHBV DNA. Figure 2 shows the pattern of viral transcripts present in cytoplasmic extracts 2 days following transfection with either wild-type DHBV DNA (lane 1) or pDP20 DNA (lane 2). In both cases, two major classes of virus-related transcripts were seen, corresponding to genomic and subgenomic RNAs (under these conditions of electrophoresis, the wild-type pre-S and S subgenomic mRNAs are barely resolved from each other). As predicted, the transcripts generated by pDP20 were larger than their wild-type counterparts, by an amount corresponding to the size of the added HBV sequences at their 3′ termini.

We next examined the ability of these transcripts to serve as mRNAs for the synthesis of DHBV core and surface antigens. Huh7 cells transfected as above were radiolabeled with [^{35}S]methionine, and cytoplasmic extracts were precipitated with polyclonal antisera directed against either surface antigen particles (Fig. 3A) or cytoplasmic cores (Fig. 3B). The former antiserum was reactive primarily against the pre-S polypeptide, while the latter recognized only the DHBV core antigen. Precipitated proteins were analyzed by

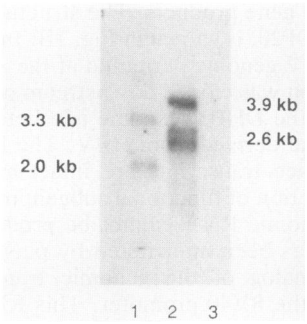

FIG. 2. Northern blot of DHBV-specific poly(A)$^+$ RNA from transfected Huh7 cells. RNA prepared 48 h posttransfection was electrophoresed through 1% agarose–2.2 M formaldehyde gels, transferred to nylon membranes, and annealed to [^{32}P]DHBV DNA. Lane 1, RNA from cells transfected with cloned dimeric wild-type DHBV DNA. Lane 2, RNA from cells transfected with pDP20. Lane 3, RNA from mock-transfected cells.

SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (Fig. 3). The levels of pre-S protein in pDP20-transfected cells were identical to those seen in a wild-type transfection. Core polypeptides were produced severalfold more abundantly from pDP20 than from the wild-type, perhaps owing to the strength of the SV40 promoter used to drive *C* mRNA production in this vector (cf. Fig. 4).

To determine whether the genomic RNAs generated by pDP20 were packaging-defective, we carried out more detailed analyses of their structure and distribution. Extracts of cells transfected with either wild-type DHBV DNA or pDP20 were divided into two equal portions. From one, total poly(A)$^+$ RNA was harvested. From the other, cytoplasmic cores were purified by polyethylene glycol precipitation and nuclease digestion (to remove exogenous DNA and RNA), following which their nucleic acid was then extracted (see Materials and Methods). From each sample, genomic RNA was then identified and quantitated by primer extension analysis, using as a primer a synthetic oligonucleotide complementary to the 5′ portion of the *C* ORF (Fig. 4). Examination of the RNA in the total cytoplasmic extract from wild-type DHBV-transfected cells (lane 1) showed a band of 131 nt in length, corresponding to the 5′ ends of wild-type pregenomic RNA, in accord with the previous mapping of Buscher et al. (2). (The band immediately above this in lane 1 is a strong stop to reverse transcription originating from priming at a second copy of the priming site located in the downstream terminal redundancy of the RNA.) As expected, the start sites of the cytoplasmic pDP20 genomic transcripts (lane 3) mapped to two distinct sites within SV40 DNA (5, 10) and were easily distinguished from the wild-type pregenome in this assay. When RNA extracted from purified cores was similarly examined (lanes 2 and 4), further differences became apparent. In the wild-type infection, it was clear that a large proportion of the RNA present in the extract was encapsidated within subviral cores (compare lanes 1 and 2); however, in pDP20-transfected cells, the fraction of the RNA that was encapsidated was approximately 10-fold less than for the wild type (cf. lanes 3 and 4), although a low level of packaged RNA (lane 4) was still detectable. Similar results were obtained when cores were purified by immunoprecipitation (R.C.H., unpublished data). These studies indicate that the genomic RNAs of pDP20 are indeed encapsidation-deficient, but the defect is incomplete.

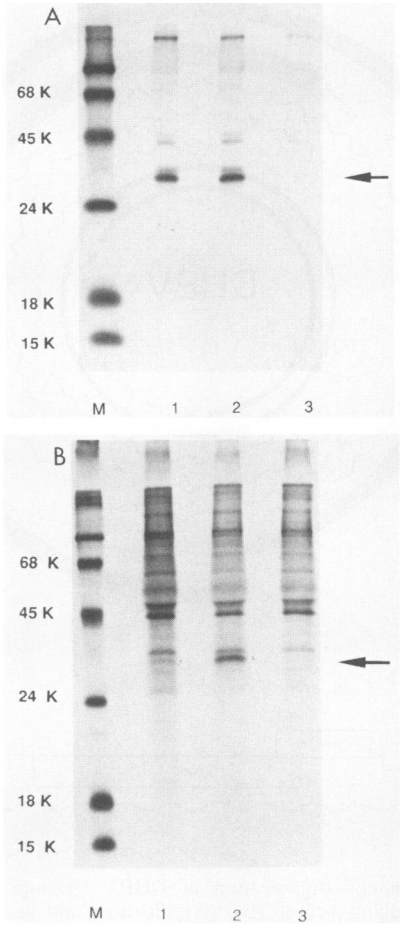

FIG. 3. Synthesis of viral proteins in transfected cells. Cells were transfected with wild-type DHBV DNA (lane 1), pDP20 DNA (lane 2), or no DNA (lane 3). Cells were radiolabeled with [^{35}S]methionine as described in Materials and Methods, and cytoplasmic extracts were immunoprecipitated with either anti-surface antigen (A) or anti-core antigen (B) antibody. Precipitates were analyzed by SDS-PAGE and autoradiography. Arrows depict the positions of the pre-S and C proteins in panels A and B, respectively. Sizes of standards are shown in kilodaltons (lanes M).

The ability of packaged pDP20 genomic RNA to serve as a template for reverse transcription was then assessed. Cytoplasmic core particles were immunoprecipitated from Huh7 cells transfected with DHBV or pDP20, and their nucleic acids were extracted as described in Materials and Methods. Equal portions of these preparations were electrophoresed through 1% agarose gels, and viral DNA sequences were identified by Southern blot hybridization. As shown in Fig. 5, under conditions in which wild-type DHBV genomes generated abundant replicative forms (lane 3), no progeny viral DNA was observed in cells that received pDP20 (lane 1). Even long exposures of such filters did not reveal viral DNA, indicating that even allowing for the defect in encapsidation, the genomic transcripts were not efficiently copied into DNA. Assuming that pDP20 produces functional polymerase (see below), this suggests that the genomic RNA generated by this construct is defective in *cis* for reverse transcription.

Packaging in *trans*. The above studies indicated that the pDP20 vector produces the transcripts and gene products expected from current knowledge of hepadnavirus gene

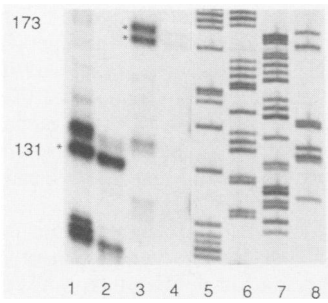

FIG. 4. Primer extension analysis of total and encapsidated pregenomic RNAs in transfected cells. Cells were transfected with either wild-type DHBV DNA (lanes 1 and 2) or pDP20 DNA (lanes 3 and 4), and each transfection was divided into two portions. From one, total cytoplasmic poly(A)$^+$ RNA was prepared; from the other, nucleic acid was extracted from purified cores. To each RNA sample a synthetic primer derived from the 5′ region of ORF C was annealed, and extension was carried out with reverse transcriptase; extension products were analyzed on 6% polyacrylamide–8 M urea gels. Lanes 1 and 3, Total RNA. Lanes 2 and 4, Core RNA. Lanes 5 to 8, Sequencing ladder generated by same primer on wild-type DHBV genome. Bands corresponding to start sites of genomic RNAs of wild type (lane 1) and pDP20 (lane 3) are indicated with asterisks; the nucleotide length of these species is indicated at left. Other bands in lane 1 are due to strong stops to primer extension resulting from priming at the 3′ copy of the priming site.

expression (9). However, they do not demonstrate whether the vector is capable of encapsidating other pregenomic RNAs in *trans*. To do this, we generated a plasmid (pDfs1) in which a frameshift mutation in the unique portion of the core gene was introduced into an otherwise intact DHBV genome. The mutation eliminates the unique *Nsi*I site within ORF C and hence has a characteristic restriction profile. After liberation of this genome from its plasmid vector and transfection into Huh7 cells, this construct produced normal levels of the correct pregenomic and subgenomic RNAs (data not shown), but could not synthesize viral DNA owing to the inability to make core particles (4). It therefore provides a convenient target RNA for packaging by pDP20 functions. Accordingly, we cotransfected Huh7 cells with

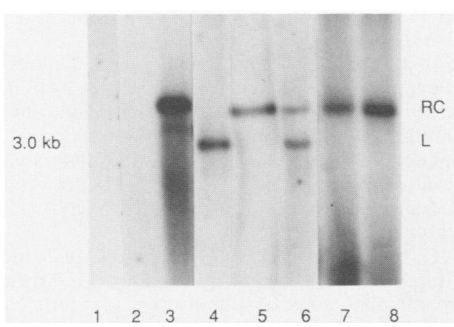

FIG. 5. Complementation of core-defective genomes by pDP20. Huh7 cells were transfected with 10 μg each of pDP20 (lane 1), pDfs1 (lane 2), wild-type DHBV DNA (lanes 3 and 4), wild-type DHBV plus pDfs1 (lanes 5 and 6), or pDP20 plus pDfs1 (lanes 7 and 8). Five days later, DNA was extracted from purified cores and examined by Southern hybridization with [^{32}P]DHBV DNA. DNA samples in lanes 4, 6, and 8 were cleaved with *Nsi*I prior to electrophoresis. (All samples were also exposed to *Dpn*I prior to electrophoresis to degrade residual input DNA from the transfection.) RC indicates position of relaxed circular DHBV monomer; L indicates position of unit-length linear DHBV molecule.

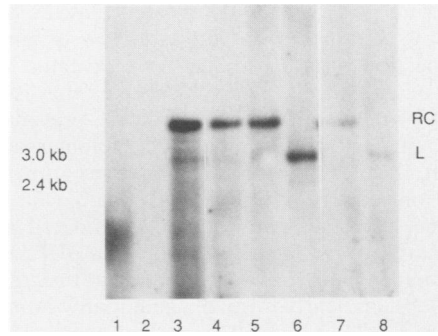

FIG. 6. Complementation of polymerase-negative genomes. Huh7 cells were transfected with 10 μg each of pDP20 (lane 1), p442 (lane 2), wild-type DHBV (lane 3), wild-type DHBV plus p442 (lanes 4 to 6), or pDP20 plus p442 (lanes 7 and 8). Encapsidated DNA was purified and treated with *Dpn*I to degrade residual input (transfected) DNA, and the samples in lanes 5 and 8 were digested with *Pst*I. The sample in lane 6 was digested with *Pst*I plus *Nsi*I; this will generate a 2.4-kb fragment only from encapsidated *pol*-defective genomes. DNA was then fractionated on 1.4% agarose gels and analyzed by Southern hybridization with ^{32}P-labeled DHBV DNA. RC denotes relaxed unit-length DHBV circle; L indicates unit-length DHBV linear molecule.

pDfs1 and either pDP20 or wild-type DHBV DNA; 5 days later, viral cores were precipitated and examined for viral DNA by Southern blotting. As shown in Fig. 5, neither pDfs1 alone nor pDP20 alone produced progeny viral DNA (lanes 1 and 2), but when the two were present together, abundant quantities of DHBV DNA were produced (lane 7). *Nsi*I digestion of this DNA revealed that all of the encapsidated genomes lacked the *Nsi*I site (lane 8), indicating that the encapsidated and reverse-transcribed genome was of mutant origin. Thus, pDP20 can efficiently supply core function in *trans*. The level of encapsidation of the coreless genome by pDP20 was comparable to that achieved by wild-type helper DNA (lane 5); in the latter case, *Nsi*I digestion revealed that, as expected, similar quantities of both wild-type and mutant DNA were encapsidated (lane 6).

Finally, we asked whether pDP20 could supply polymerase in *trans*. We and others have previously shown that polymerase is synthesized by de novo initiation at the first AUG codon in the P ORF, presumably from genomic RNA as a messenger (4, 20). Because the DHBV *pol* product is present at quantities too low to be directly visualized by immunoprecipitation with currently available sera, at present *pol* function can only be demonstrated by genetic complementation of *pol* mutants. Accordingly, we cotransfected Huh7 cells with a plasmid (p442) bearing a frameshift mutation in ORF P, together with either pDP20 or wild-type DHBV DNA as helper (Fig. 6). Mutant p442 generated a unique *Pst*I site in the portion of ORF P just 3′ to the core-*pol* overlap; since wild-type DHBV is not cut by *Pst*I, the mutant DNA again had a distinctive restriction pattern. When p442 DNA was present alone, no progeny viral DNA was produced (lane 2). However, when cotransfected with pDP20, small quantities of encapsidated DNA were found in cytoplasmic cores (lane 7). *Pst*I digestion confirmed that this DNA was of mutant origin (lane 8). The levels of encapsidated p442 DNA were reproducibly ca. 10-fold lower than those of the coreless mutant pDfs1 (compare Fig. 6, lane 8, with Fig. 5, lane 8). However, the same was true when the wild-type DHBV genome was used as the helper (Fig. 6, lanes 4 to 6). Restriction analysis of the cotransfection of the

wild type with p442 with *Pst*I revealed that the majority of the progeny genomes were of wild-type origin (lane 5); double digestion with *Pst*I plus *Nsi*I revealed only trace quantities of the 2.4-kilobase (kb) species that is the signature of the p442 genome (lane 6). We are presently investigating the reasons for the inefficient complementation of this *pol* mutant; irrespective of this, however, it is clear that pDP20 performed at least as efficiently as the wild-type in *pol* generation. (In fact, the levels of encapsidated *pol* mutant DNA appeared to be slightly greater with pDP20 than with wild-type helper, perhaps owing to the fact that pDP20 RNA competes less efficiently for packaging than wild-type helper pregenomes.)

DISCUSSION

We have described an experimental system in which the encapsidation of hepadnaviral pregenomic RNA can be observed and manipulated. The system functions efficiently; encapsidation of a coreless test genome by our packaging plasmid proceeded to the same extent as when wild-type DHBV served as the helper. Although packaging of a polymerase-deficient mutant by pDP20 was inefficient, the same was also true when wild-type helper was used. Similarly inefficient complementation of *pol* mutant genomes by polymerase expression vectors is also apparent in other studies of *pol* expression (see Fig. 5 in reference 20). We have recently discovered that products of the *P* gene are absolutely required for viral RNA packaging (R. Hirsch, J. Lavine, L. Chang, H. Varmus, and D. Ganem, manuscript in preparation). The inefficient complementation of mutant 442 by either wild-type helper or pDP20 may be due to inefficient association of polymerase proteins with core subunits or RNA in *trans*. Alternatively, the N-terminal amber fragment of the p442 polymerase may be able to interfere with normal *pol* function, giving the mutant a dominant negative phenotype. Strongly against the latter is the fact that p442 did not appear to reduce the yield of wild-type DHBV DNA when the two genomes were cotransfected (Fig. 6). Clearly, further studies are required to define the nature of the role played by *P* gene products in pregenome encapsidation.

The genomic RNA of pDP20, as expected, was defective for packaging, although the defect was only about 10-fold. However, those genomes that were encapsidated were not active as templates for reverse transcription. Several potential explanations for this result can be envisioned. First, these transcripts are not terminally redundant and hence carry only one copy of DR1 and its adjacent sequences, at the 3′ end of the molecule. These sequences are implicated in the priming of reverse transcription (9, 13, 21); since they are normally found at both ends of the transcript, priming can in principle proceed from either position. However, if priming normally occurs only from the 5′ copy of this site, then the absence of this site in the pDP20 transcript could account for the phenotype. Alternatively, the extent of the flanking DHBV sequence (30 nt) retained 3′ to DR1 in this RNA may be insufficient for priming, or the added 3′ sequences derived from HBV may impair priming by altering the secondary structure of the template or its interactions with core and polymerase proteins.

The ability to separate the participants in this assembly reaction into factors acting in *trans* and elements required in *cis* should allow further dissection of the nature of these participants. We are currently constructing deletion and point mutations affecting the structure of pregenomic RNA in pDfs1 to define the *cis*-acting elements required for encapsidation. As noted previously, it is improbable that primary sequence elements alone can specify selective encapsidation, and the nature of the signals involved is likely to be complex. Given the highly compact nature of the hepadnavirus genome, the definition of these elements should be greatly facilitated by divorcing *cis*- from *trans*-acting components. This system should also allow ready definition of the encapsidation limits of DHBV.

Formally, our approach to hepadnavirus packaging is analogous to that used in the generation of retrovirus vectors, where encapsidation-deficient genomes capable of generating all essential viral gene products are used to package defective viral genomes in *trans* (14). As in retrovirology, the development of a packaging system for hepadnaviruses also serves as a necessary first step in efforts to develop these viruses as genetic vectors. The marked hepatotropism of these viruses, together with their ability to produce persistent noncytocidal hepatic infections, makes them attractive candidates as vehicles for the delivery of foreign DNA to the liver. On the other hand, their small size and extraordinary genetic compactness pose formidable problems that may severely limit their practical utility. Elucidation of their packaging parameters and mechanism by the system described here should allow realistic assessment of the potential of hepadnaviruses as vectors in the future.

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