Recombinant Human Hepatitis B Virus Reverse Transcriptase Is Active in the Absence of the Nucleocapsid or the Viral Replication Origin, DR1

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The double-stranded DNA genome of hepatitis B virus (HBV) is reverse transcribed from the viral pregenome RNA template by a virally encoded reverse transcriptase enzyme (RT) that possesses both priming and elongation activities. Prior efforts have failed to express an active form of HBV RT outside the nucleocapsid in animal cells or to release it from viral nucleocapsids, thus restricting the characterization of this important enzyme. Here, we have engineered epitope-tagged HBV RT proteins and expressed them in *Xenopus* oocytes via a synthetic RT mRNA which does not include the viral capsid protein or the known initiation site for viral DNA synthesis, DR1. We demonstrate the production of an immunoprecipitable 96-kDa HBV RT protein and show, using a simple in vitro RT assay, that oocyte lysates containing this protein possess an activity that (i) catalyzes an RNA-dependent deoxynucleotide triphosphate polymerization reaction by using an as-yet-unidentified RNA template and (ii) is sensitive to the RT inhibitors actinomycin D and phosphonoformate. Experiments with the chain terminator ddATP suggest that a significant amount of chain elongation occurs in our in vitro reaction. Electrophoretic analysis reveals a heterogeneous array of RT reaction products with sizes ranging from about 100 bases to far larger than that of the input RT mRNA. These products appear to contain covalently bound protein, consistent with the notion that the RT protein may have primed their synthesis. We conclude that HBV RT activity can be uncoupled from both the nucleocapsid and the replication origin, DR1. Our results raise the possibility that unless HBV employs novel mechanisms to regulate its constitutively active RT, cellular RNAs may be reverse transcribed during HBV infection, with potential implications for the development of HBV-related liver cancer. The use of the oocyte system should facilitate studies of HBV RT, including the development of HBV RT inhibitors for antiviral therapy.

The reverse transcriptase enzyme (RT) of human hepatitis B virus (HBV) has properties that set it apart from its retroviral counterparts. Although retroviral RTs replicate their genomes postinfection within particulate complexes that may (2) or may not (5) contain the viral capsid protein, the HBV RT is unusual in being able to convert its pregenomic RNA template into double-stranded DNA inside the newly assembled viral nucleocapsid (33) in a process that specifically depends on the HBV p21.5 capsid or core protein (11, 18, 24, 36). Although the HBV pregenome RNA exhibits a retrovirus-like arrangement of open reading frames (ORFs), in which the RT ORF overlaps the 3' end of the core ORF, genetic studies (3, 26) reveal that HBV RT is translated as a discrete species; there is no evidence for a ribosomal frameshifting event that would yield a core-RT precursor analogous to the retroviral gag-pol fusion proteins (14). The final unusual feature of the HBV RT protein is that it possesses an intrinsic priming activity (see below) that initiates first-strand DNA synthesis and leaves RT (or at least part of it) covalently linked to the viral DNA (9).

In addition to playing a pivotal role in the initiation and elongation phases of reverse transcription, RT is essential for the selective uptake of the pregenome RNA into the nucleocapsid (1, 13). RT, along with p21.5 and the pregenome RNA, is clearly a participant in the undefined hierarchy of interactions that direct the assembly of the replicative nucleocapsid. Unfortunately, attempts to study RT at a

biochemical level have been stymied by the lack of well-defined antibodies against RT and also by the failure to release active RT from nucleocapsids (21) or to express it in a nucleocapsid-free form in animal cells. In this study, we set out to remedy this situation by expressing, in *Xenopus* oocytes, a tagged RT molecule bearing an in-frame foreign epitope to render it immunoprecipitable. We describe the successful accomplishment of this goal and further show that the resultant RT protein unexpectedly displays RT activity.

MATERIALS AND METHODS

Plasmids. All constructs were generated by standard recombinant DNA techniques. To create plasmid pSP64T-RT, the bulk of the RT gene and a small piece of 3' flanking sequence were mobilized as a restriction fragment and the 5' end of the gene was rebuilt via a synthetic oligonucleotide adaptor. The two fragments were coligated downstream of the SP6 promoter in plasmid pSP64T (16). Polymerase chain reaction oligonucleotide insertion was used to create epitope fusions bearing in-frame versions of either the FLAG (Immunex) epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) or the L epitope (Asp-His-Gln-Leu-Asp-Pro-Ala-Phe-Gly-Ala-Asn-Ser) corresponding to residues 27 to 38 of the viral LS protein (HBV subtype adw). These epitopes were introduced immediately after the initiating RT Met to create the FLAG-RT and L-RT constructs. In the RT-L and RT-FLAG constructs, the epitopes were inserted between RT amino acids 174 and 175 at the unique *Bst*EII site present in the RT

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gene (HBV subtype adw). Mutant fsL-RT is a frameshift mutant which is based on the L-RT epitope fusion construct (see Results) and contains a 1-base deletion located 11 residues downstream from the initiating Met codon (i.e., just beyond the L epitope), resulting in a termination codon 15 residues later. All constructs were verified by DNA sequencing.

RNA transcription and translation methodology. Capped SP6 mRNAs were prepared and concentrated 2-fold (for in vitro translations) or 15-fold (for oocyte studies) as described elsewhere (28). In vitro translations were performed with reticulocyte lysates supplemented with [³⁵S]methionine (NEN-Dupont) and the appropriate RNA as recommended by the manufacturer (Promega). Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on either 10 or 8% gels and detected by exposing the dried gels to X-ray film. Immunoprecipitation of proteins bearing the L epitope was performed (30) with 1 µg of anti-L monoclonal antibody MA 18/7 (12). *Xenopus* oocyte microinjection and labeling methodologies have been described earlier (30). Batches of 8 to 12 healthy oocytes were injected with ~40 nl of the appropriate mRNA per oocyte and labeled metabolically with 0.75 mCi of a mixture of [³⁵S]methionine and [³⁵S]cysteine (Expresslabel; NEN-Dupont) per ml for 12 to 16 h at 17°C. Lysates were prepared in 200 µl of homogenization buffer (30) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 µM leupeptin-hemisulfate, and 2 µg of aprotinin per ml, clarified by centrifugation, and immediately subjected to immunoprecipitation and SDS-PAGE essentially as described above.

In vitro reverse transcription assays. Care was taken to inject oocytes with equal amounts of the L-RT or fsL-RT mRNAs, which were generally prepared in parallel and checked for concentration by agarose gel electrophoresis and ethidium bromide staining. LS mRNA was generally used as a control in amounts that were at least equimolar with the appropriate RT mRNA. Microinjected oocytes were incubated for 12 to 14 h and then homogenized in a standard HBV polymerase buffer (50 mM Tris-HCl [pH 8.0]-40 mM MgCl₂-50 mM NH₄Cl-1% [vol/vol] Nonidet P-40-0.3% [vol/vol] 2-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, and 1 µg of aprotinin per ml. For RT assays, 10-µl samples of clarified (Eppendorf centrifuge; 15,000 × g, 10 min) lysates (≈0.5 oocyte) were diluted in 100 µl of polymerase buffer containing 0.2 mM each dCTP, dGTP, and dTTP (ultrapure; Pharmacia) and ~10 µCi (34 to 125 nM) of [α-³²P]dATP (NEN-Dupont). Assays using other labeled nucleotides were performed analogously. After 1 h at 37°C, the reactions were stopped by the addition of EDTA (to 25 mM) and the reaction mixtures were treated with trichloroacetic acid-sodium PP_i (10 and 1%, wt/vol, respectively) in the presence of 50 µg of carrier tRNA. The precipitates were collected by filtration through GF/C membranes (Whatman). The filters were washed extensively and dried, and radioactivity was measured by Cerenkov counting.

For the RT time course experiment, oocytes were injected with either L-RT mRNA or an LS mRNA control (see Results). At the appropriate time points, batches of unlabeled oocytes were homogenized and subjected to the RT assay.

Analysis of the nucleic acid products of reverse transcription. Large-scale RT assays were performed with L-RT- and fsL-RT-expressing lysates essentially as described above. Aliquots (one-quarter) of each reaction mixture were extracted twice with phenol with or without prior digestion for

45 min at 37°C with 1 mg of proteinase K per ml in the presence of 0.5% (wt/vol) SDS. Nucleic acids were then precipitated, washed, dissolved in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), electrophoresed through 1% agarose in 0.5× TBE (44.5 mM Tris, 44.5 mM borate, 0.5 mM EDTA), and visualized by autoradiography of the dried gel. The remaining halves of the RT reaction cocktails were deproteinized (as described above), adjusted to 10 mM sodium phosphate (pH 6.8), and loaded onto small columns of hydroxylapatite (Biogel HTP; Bio-Rad). After extensive washing with loading buffer, single- and double-stranded nucleic acids were eluted from the column with successive steps of 127 and 187 mM sodium phosphate (pH 6.8), respectively, according to the manufacturer's instructions. Samples of each eluate were quantitated by scintillation counting. The remaining nucleic acids were precipitated (8) with 5 mM EDTA and 0.1% (vol/vol) cetylpyridinium bromide and analyzed by agarose gel electrophoresis as described above.

Effects of RNase A and inhibitors of reverse transcription. For RNase A and RT inhibition experiments, we used lysates prepared from RT-L- or LS-microinjected oocytes. To test the RNA dependence of RT activity, RT-L and LS lysates from two oocytes were incubated with or without 20 µg of RNase A (DNase-free) per ml for 10 min at 37°C. Subsequently, double-scale, 200-µl RT assays were performed essentially as described above, except that a simplified RT buffer (50 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 60 mM NaCl, 10 mM dithiothreitol) replaced the HBV polymerase buffer and the reactions were conducted for 30 min rather than 1 h. RT inhibition studies were performed under the same conditions in assays supplemented with (i) actinomycin D (100 µg/ml; Sigma), (ii) phosphonoformate (PFA) (1 mM; foscarnet; Sigma), or (iii) ddATP (0.83 µM; Boehringer Mannheim) (see the legend to Fig. 5). The RT nucleic acid products were purified by deproteinization, extraction, and precipitation, resolved by agarose gel electrophoresis, and visualized by autoradiography of the dried gels, as described above. The amounts of ³²P-labeled products present in each lane or in selected regions of each lane were quantitated by scanning the dried gels with a phosphorimager (Molecular Devices).

RESULTS

Strategy for expression of an immunoprecipitable HBV RT. The structure of the HBV pregenome RNA, which serves as the reverse transcription template as well as the mRNA for both the 21.5-kDa capsid (C) protein and the RT protein, is shown in Fig. 1a. The HBV RT protein (with a predicted molecular mass of ~94.7 kDa) is encoded by a contiguous ~2.4-kb RT ORF that overlaps the 3' end of the C ORF. The S ORF, which specifies three related envelope (S) proteins, lies completely within the RT ORF but utilizes a different reading frame. During HBV infection, these S proteins are translated from a class of subgenomic mRNAs (32). Repeats found at each end of the pregenome RNA contain specific sequence elements crucial for HBV replication, such as the encapsidation signal, ϵ , which ensures the selective packaging of the pregenome RNA into the nucleocapsid (15), and the replication origin, DR1. In the proposed (25, 27) HBV replication scheme, the amino-terminal domain of the multifunctional (20) RT protein (Fig. 1b) initiates first-strand (minus-strand) DNA synthesis at the 3' copy of the short direct repeat DR1. RT remains covalently attached to the

FIG. 1. Structure of HBV pregenome RNA and synthetic RT mRNA. (a) Pregenome RNA (~3.5 kb) with capped 5' (filled circle) and polyadenylated 3' (AAA) ends. Redundant sequences (R) bear one copy each of the short direct repeat DR1 and an encapsidation signal (ϵ). DR2 is a further copy of DR1 located before the 3' R. The 3' DR1 element and DR2 provide the initiation sites for first-strand (minus-strand) and second-strand (plus-strand) HBV DNA synthesis, respectively (27). Also shown are ORFs for the p21.5 core protein (C-ORF) and the overlapping RT protein (RT-ORF); S-ORF (see below) is present in this RNA but is not shown. The region between the vertical dotted lines represents the sequence that was used to create plasmid pSP64T-RT (see below). (b) Structure of RT mRNA. The RT ORF and a short piece of 3' flank (line) lie between the *Xenopus* β -globin (β G.) 5' and 3' untranslated sequences. Patterned segments within the RT ORF represent four proposed (20) domains: the terminal protein (TP) domain, the tether region, the RT activity domain, and the RNase H domain. S-ORF, shown below RT-ORF, encodes the three HBV envelope proteins; the approximate location of the natural L epitope is indicated by an asterisk. Arrows indicate approximate locations at which the indicated epitopes or a termination codon were introduced.

elongating minus-strand DNA. Second-strand (plus-strand) DNA synthesis is primed by an RNA primer and initiates at DR2, which is identical in sequence to DR1 but is located near the 3' end of the RT ORF.

We expressed the RT protein using a synthetic RT mRNA (Fig. 1b). Briefly, this SP6 mRNA contains a minimal RT ORF and a short piece of 3' flanking sequence (corresponding to the region of the pregenome RNA included between the dotted lines in Fig. 1a) embedded between the translationally enhancing *Xenopus* β -globin flanking sequences (16). According to the HBV replication model (described above), RT mRNA should not be a template for reverse transcription, since it lacks both ϵ and DR1. To monitor RT production, RT was tagged with either the FLAG (19) epitope or an epitope (L) (29) from the HBV large surface (LS) protein. Epitopes were inserted in frame with RT either into the amino terminus (FLAG-RT and L-RT fusions) or between RT residues 174 and 175, which lie at the border of a nonfunctional tether region (20) of the RT (RT-FLAG and RT-L fusions). We also created a mutant L-RT (fsL-RT) bearing a frameshift near the 5' end of the L-RT ORF. In this mutant the L-RT protein translation terminates shortly after the frameshift. The epitope and frameshift positions are shown in Fig. 1b, which also summarizes the approximate boundaries of the known RT subdomains (20).

Expression of HBV RT in reticulocyte lysates and in frog oocytes. Upon translation in reticulocyte lysates (Fig. 2a), the capped L-RT and FLAG-RT mRNAs, but not fsL-RT mRNA, gave the predicted ~96-kDa 35 S-labeled proteins, as did RT-FLAG and RT-L mRNAs (data not shown). The RT RNAs were translated less efficiently than mRNAs encoding the HBV LS and C proteins. Anti-L monoclonal antibody MA 18/7 (12) specifically immunoprecipitated both native and denatured forms of L-RT (Fig. 2b) and RT-L but not wild-type RT (data not shown). No 96-kDa band was seen when MA 18/7 was omitted or replaced with a control monoclonal antibody of the same isotype (data not shown). Curiously, we were unable to specifically immunoprecipitate FLAG-RT or RT-FLAG fusions with any of four different anti-FLAG antibodies under a wide variety of immunoprecipitation conditions (data not shown).

FIG. 2. Expression of RT in vitro and in *Xenopus* oocytes. (a) Translation of mRNAs encoding the L-RT, fsL-RT, FLAG-RT, LS, and C HBV proteins in reticulocyte lysates supplemented with [35 S]methionine. Proteins were visualized by autoradiography after SDS-PAGE. (b) Monoclonal anti-LS antibody MA 18/7 (mAb) immunoprecipitates both native and denatured L-RT protein. The 35 S-labeled L-RT products were immunoprecipitated from 10- μ l portions of in vitro translation reactions either in native form (lanes 1 and 2) or after denaturation (lanes 3 and 4) (by boiling for 2 min with 1% [wt/vol] SDS). MA 18/7 was omitted in lanes 2 and 4. Immunoprecipitation of FLAG-tagged RTs was attempted unsuccessfully (data not shown) under the same conditions with commercially available M1 and M2 monoclonal anti-FLAG antibodies (IBI) and with an M5 monoclonal antibody and a rabbit anti-FLAG serum kindly provided by Immunex (Seattle, Wash.). (c) Only oocytes microinjected with L-RT mRNA synthesize an immunoprecipitable 96-kDa RT protein. Translations of the fsL-RT and LS mRNAs are shown. The positions of prestained (a and b) or 14 C-labeled (panel c) marker proteins (sizes in kilodaltons) (Bethesda Research Laboratories) are indicated.

FIG. 3. *Xenopus* oocytes express an enzymatically active HBV RT. Crude oocyte lysates stimulate incorporation of radiolabeled deoxynucleotide triphosphates into acid-insoluble products (A and B). (A) Incorporation of [α - 32 P]dCTP or [α - 32 P]dATP directed by lysates of oocytes injected with 15 $\times$ L-RT, 1 $\times$ RT, LS, or no mRNA (none). (B) Enhanced incorporation activity depends on the L-RT protein. Shown is a comparison of the activities present in lysates from oocytes injected with 15 $\times$ L-RT and 15 $\times$ fsL-RT mRNAs. Lysates of LS-expressing oocytes provide a control. (C) Immunoprecipitated L-RT protein retains activity. Proteins were immunoprecipitated from fresh L-RT and fsL-RT lysates with anti-L monoclonal antibody (MA 18/7) and immediately assayed for RT activity as described in Materials and Methods. (D) RT activity time course. Unlabeled L-RT or LS proteins were synthesized in oocytes for the indicated times. At each time point, batches of five oocytes were homogenized and subjected to RT in vitro activity assays. Further details are supplied in Materials and Methods.

We next examined the translation of concentrated L-RT mRNAs injected into *Xenopus* oocytes (Fig. 2c). L-RT mRNA, but not the fsL-RT and LS mRNAs, directed production of a $\sim$ 96-kDa 35 S-labeled L-RT protein that was immunoprecipitated by MA 18/7 and appeared to be identical to the in vitro product by SDS-PAGE. Interestingly, all three of these RNAs generated immunoprecipitable 39- and 42-kDa LS proteins (31) which were not detected for control oocytes (data not shown). For the L-RT and fsL-RT mRNAs, these LS species presumably arise by internal initiation of translation, which seemingly increases upon abrogation of the RT ORF.

Production of an enzymatically active RT in oocytes. We next tested whether the oocyte-produced RT proteins had RT activity. Crude oocyte lysates were incubated in a polymerase buffer (20) in the presence of one α - 32 P-labeled and three unlabeled deoxynucleotide triphosphates. No template or primer was added. After incubating the mixture for 1 h at 37°C, we measured the conversion of 32 P into a trichloroacetic acid-precipitable form. Remarkably, this simple RT assay revealed (Fig. 3A) that lysates from oocytes

injected with concentrated L-RT mRNA gave up to 20-fold-stronger signals than lysates from oocytes that were either uninjected or injected with concentrated LS mRNA. Similar results were obtained whether the α - 32 P-labeled deoxynucleotide was dATP, dCTP (Fig. 3A), or dGTP (data not shown). Another representative experiment (Fig. 3B) revealed an $\sim$ 10- to 19-fold-stronger signal for oocytes injected with L-RT mRNA than for those injected with the LS and fsL-RT mRNAs. These results have been confirmed in $\sim$ 20 independent experiments (data not shown). Positive signals were seen only for oocytes injected with concentrated mRNAs encoding any of the RTs and typically varied from 4- to 10-fold above the background level, although we have seen signals as faint as 2-fold and as strong as 20-fold. A wide variety of concentrated mRNAs encoding other proteins were tested, but all gave negative results (Fig. 3 and data not shown).

The likelihood of a link between the L-RT protein and the putative RT activity was strengthened by the results of an immunoprecipitation experiment (Fig. 3C) using antibody MA 18/7 and lysates from L-RT- or fsL-RT-expressing oocytes. Both the washed immunoprecipitates and samples of the original lysates were then assayed for RT activity. The untreated L-RT lysate displayed 13-fold more activity in the RT assay than did the fsL-RT control (data not shown). After immunoprecipitation, this activity difference increased to $\sim$ 18-fold, despite a low overall recovery of RT activity by this method.

We also tested (Fig. 3D) how the level of RT activity present in oocytes depends on the time allowed for protein synthesis postinjection. Batches of oocytes were injected with either L-RT mRNA or LS mRNA, incubated for 3, 6, 10, or 25 h prior to lysis, and then immediately subjected to the RT assay. In this experiment, RT activity exceeding the LS control background level was first detected after $\sim$ 10 h of incubation and was still seen after 25 h, though at reduced levels; further experiments have verified that 12 to 14 h postinjection is the optimal time for assaying HBV RT activity in oocytes (data not shown). A parallel analysis of L-RT protein synthesis in oocytes (by steady-state labeling of the oocyte proteins with [35 S]Met and [35 S]Cys followed by immunoprecipitation and SDS-PAGE) suggested that the level of L-RT protein is maximal between 6 and 10 h and diminishes slightly by 25 h (data not shown). Thus, there is a general correlation between the levels of RT activity and quantities of the 96-kDa L-RT protein, but the large amount of L-RT protein with no detectable RT activity seen at the 6-h time point suggests that the emergence of RT activity may lag behind protein production.

Analysis of nucleic acid products of the RT reaction. Next, the products from the in vitro RT assay were deproteinized with proteinase K and phenol and then analyzed by agarose gel electrophoresis. In a representative experiment (Fig. 4a), L-RT-expressing lysates clearly generated more 32 P-labeled products (lane 1) than did the fsL-RT control (lane 3). Although small products (including discrete species with apparent sizes of $\sim$ 150 and 600 bases) predominated, the array of RT products had lengths which extended to at least 23,000 bases. When proteinase K treatment was omitted, phenol extraction eliminated the specific L-RT products (Fig. 4a, lane 2) but not most of the fsL-RT background (lane 4). Since HBV DNA genomes bearing covalently bound RT protein are extracted into phenol (9), this result is consistent with the notion that the RT protein had primed DNA synthesis in oocytes and remained covalently bound to the products. The deproteinized L-RT and fsL-RT products

FIG. 4. Analysis of nucleic acid products of reverse transcription reactions. (a) Most of the in vitro labeled ^{32}P -RT products are covalently linked to protein. Lanes 1 and 2, L-RT reaction products; lanes 3 and 4, fsL-RT reaction products. Addition (+) (lanes 1 and 3) and omission (-) (lanes 2 and 4) of proteinase K (P.K.) are indicated above the panel. Positions of DNA size markers are shown on the left. (b) Separation of single-stranded (ss) and double-stranded (ds) RT products by hydroxylapatite chromatography. Lane 1, ss L-RT products; lane 2, ss fsL-RT products; lane 3, ds L-RT products; lane 4, ds fsL-RT products. (c) Quantitation of ss and ds products by Cerenkov counting.

were further characterized by hydroxylapatite chromatography, which permits resolution of single- and double-stranded nucleic acid species. The two species were then analyzed by agarose gel electrophoresis (Fig. 4b) and quantitated by scintillation counting (Fig. 4c). The data revealed that ~20% of the RT product eluted at 127 mM sodium phosphate, as expected for single-stranded material, most of which appeared to be short (Fig. 4b, lane 1). The remaining ~80% of the product eluted at 187 mM sodium phosphate (Fig. 4b, lane 3), consistent with its being double-stranded, and included both short and long species. Preliminary analyses

with restriction enzymes and RNase H suggest that the latter consists of both DNA-DNA and RNA-DNA duplexes. The bulk of the fsL-RT background species were in the form of double-stranded material (Fig. 4b, lane 4).

Further characterization of the RT reaction. To reassure ourselves that our assays are measuring bona fide RT activity, we turned to tests that have been used historically to define RT reactions (Fig. 5). For these tests, we used an optimized 30-min RT assay performed in a standard RT buffer rather than the more complex HBV polymerase buffer (see Materials and Methods), and RT-L mRNA rather than

FIG. 5. Effects of RNase A and inhibitors of reverse transcription. In vitro RT production appears to be mostly RNA dependent (a) and is repressed by known inhibitors of HBV RT (b through d). Lysates of RT-L- and LS-injected oocytes (indicated above the lanes) were used for this experiment. ^{32}P -labeled products were generated by in vitro RT reactions after treatment (+) with 20 μg of RNase A per ml (panel a, lanes 2 and 4), 100 μg of actinomycin D (Act.D) per ml (panel b, lanes 2 and 4), 1 mM PFA (panel c, lanes 2 and 4), or 0.83 μM ddATP (panel d, lanes 2 and 4). In each panel, lanes 1 and 3 show the corresponding products from untreated (-) control reactions. Reaction products were analyzed by agarose gel electrophoresis and visualized by autoradiography. Lanes 5 and 6 in panel d show lighter exposures of lanes 1 and 2. The end-labeled *Hind*III fragments of λ phage DNA were used as size markers (M). Fragment sizes are given in kilobase pairs.

L-RT mRNA provided the source of RT protein, with LS mRNA-injected oocytes as the controls. The ^{32}P -labeled nucleic acid products of the *in vitro* RT reactions were treated with proteinase K and phenol-chloroform to remove protein, electrophoresed through agarose gels, and then visualized by autoradiography.

The data in Fig. 5 show that the untreated RT-L assay samples (lanes 1) always yielded more ^{32}P -labeled products than did the corresponding untreated LS controls (lanes 3). Phosphorimaging of the gels revealed that the ^{32}P activity in the RT-L lanes ranged from 6.8-fold (Fig. 5a) to 20.8-fold (Fig. 5d) higher than that in the corresponding LS lanes. The RT-L reaction products were more heterogeneous than the LS control products and included larger species. The control lane products (a discrete ~1.8-kb band and a smear of shorter species) are not dependent on LS mRNA, since essentially the same pattern was seen whether LS mRNA was omitted or replaced by another control RNA (data not shown). Thus, these species presumably represent background products generated in the lysates by an endogenous oocyte enzyme acting on oocyte nucleic acid species.

To test whether the polymerizing activity observed for the RT-L lysates is dependent on an RNA template, oocyte extracts were treated for 10 min at 37°C with 20 µg of RNase A per ml prior to the RT assay (Fig. 5a). As judged by phosphorimaging, this pretreatment eliminated 83% of the RT-L reaction products (Fig. 5a, lane 2) versus only 8% of the control reaction products (lane 4).

Actinomycin D (Fig. 5b) and PFA (Fig. 5c) were tested by adding them to RT assays to final concentrations of 100 µg/ml and 1 mM, respectively. The synthesis of ^{32}P -labeled products directed by the RT-L-expressing lysate was exquisitely sensitive to inhibition by actinomycin D and PFA (also known as foscarnet), which reduced label incorporation by 85 and 96%, respectively (Fig. 5b and c, cf. lanes 1 and 2). The control lane products were less sensitive to these drugs, showing reductions of 52% with actinomycin D (Fig. 5b, cf. lanes 3 and 4) and 56% with PFA (Fig. 5c, cf. lanes 3 and 4). These values are consistent with the known properties of these drugs (4, 17, 33) which preferentially inhibit viral polymerases and RTs but also affect cellular polymerases at the concentrations used in this study.

The final inhibitor tested, the chain terminator ddATP (Fig. 5d), reduced the overall production of RT-L-derived products by 35% (Fig. 5d, cf. lanes 1 and 2) and caused a 25% reduction in the background lane products (cf. lanes 3 and 4). These modest inhibition figures reflect the fact that the ddATP concentration used in this experiment (0.83 µM) was only about fourfold higher than the ^{32}P dATP concentration. Higher ddATP/ ^{32}P dATP ratios elicited a more severe curtailment of ^{32}P dATP incorporation (data not shown). An intriguing feature of the data in Fig. 5d, lanes 1 and 2, which is more easily seen in a shorter autoradiographic exposure (lanes 5 and 6), is that the extent of inhibition due to ddATP varies with the length of the RT-L-derived product, with the longer products being inhibited more severely than the shorter ones. Using the phosphorimager, we divided each of lanes 1 and 2 of the ddATP gels into three equal regions, corresponding to three product size classes (<0.1 to 0.5 kb, 0.5 to 2.2 kb, and 2.2 to >23 kb), and quantitated the ^{32}P activity present in each region. The levels of inhibition due to ddATP seen in these size classes were 20, 35, and 48%, respectively, whereas the LS controls gave a uniform ~25% inhibition for all three size classes. The fact that the longer products of the RT assay are more sensitive to chain termination with ddATP suggests that

there is significant chain elongation occurring in this *in vitro* reaction (22).

In summary, these experiments show that RT-L-containing oocyte extracts exhibit an RNA-dependent deoxynucleotide-polymerizing activity that (i) is distinct from an endogenous polymerase detected in control oocyte lysates and (ii) behaves as expected in response to drugs that have classically been used to define RT reactions. Taken together with the findings presented in the preceding sections, the results shown in Fig. 5 make a compelling case for the fact that we are dealing with the true HBV RT. The only puzzling feature of the data in Fig. 5 is an apparent discrepancy between the RNase A and actinomycin D results. The sensitivity of the RT reaction to RNase A seemingly implies that the HBV RT is copying an RNA template, and, hence generating a first-strand cDNA product. In contrast, the actinomycin D data imply that the main RT reaction product is double-stranded DNA, since this drug preferentially reduces second- rather than first-strand DNA synthesis (33). However, these results can perhaps be reconciled if the *in vitro* RT reaction starts with an initial phase of single-strand synthesis followed by a second phase of double-strand synthesis. Further experiments will be required to address this issue.

DISCUSSION

We have expressed in frog oocytes an immunoprecipitable form of human HBV RT in the absence of the viral nucleocapsid protein. Unexpectedly, oocyte lysates containing this soluble RT exhibited a deoxynucleotide polymerizing activity that (i) has the properties expected for a true RT and (ii) appears to remain covalently linked to the reverse transcription products, a characteristic and unusual feature of hepadnaviral polymerases. This result immediately argues against the capsid protein being a prerequisite for RT activity, even though genetic evidence indicates that the carboxyl-terminal subdomain of the viral capsid protein is required for DNA genome maturation in both the duck (24, 36) and human (18) viruses. We speculate that the involvement of the capsid protein in the replication process may be a helper function enabling HBV RT to overcome the formidable problems of topology and nucleic acid condensation that must arise during the conversion of single-stranded RNA into double-stranded DNA within the strict confines of the capsid. We have previously proposed that this help may come in the form of binding energy supplied by specific DNA-binding motifs present in the capsid protein carboxyl terminus (11).

Our results are consistent with a recent report (35) that duck HBV RT protein produced by *in vitro* translation is enzymatically active in the absence of the viral nucleocapsid. However, our study provides the first demonstration (i) that this result holds true for the medically more important human enzyme, (ii) that such activity can be detected in an animal cell system, and (iii) that the activity can be unexpectedly uncoupled from regulatory elements such as the encapsidation signal, ϵ , and, most notably, the replication origin, DR1 (25, 27). There are some differences between the results seen with duck and human HBV RTs. Curiously, the duck RT activity was limited largely to the covalent addition of just four deoxynucleotides to the RT protein, while only trace amounts of longer products were seen. Although our results also suggest, albeit indirectly, that the HBV RT protein remains attached to the reverse transcription products and thus has presumably primed DNA synthesis, the oocyte-derived enzyme differs in its polymerizing capabilities in that it yields large amounts of products that are longer

than 100 nucleotides (some may be as long as 23 kb). We have not shown that these products arise exclusively in our *in vitro* assay (some may well have been started inside the intact oocyte), but the effect of ddATP on the reaction (Fig. 5d) argues strongly that our *in vitro* reaction can support chain elongation. Whether these disparate results reflect differences between the two systems or between the two enzymes and/or their templates or whether they are caused by unknown factors remains to be determined.

Among the many crucial questions that remain to be answered are those about the nature of the presumptive RNA reverse transcription template(s) copied in our system and the identity of the priming site(s) used in the absence of DR1. We do not yet know whether the reaction template is the input HBV RT mRNA or an unidentified endogenous oocyte RNA. The fact that some of the polymerized RT products are apparently longer than the injected RT mRNA could be taken to imply the use of a long oocyte mRNA template (or templates), but we certainly do not rule out the possibility that RT mRNA acts as its own substrate. To explain overlength products arising by reverse transcription of RT mRNA, we can invoke scenarios such as the reiterated copying of the RNA (via snapback priming) or the jumping of the reverse transcription machinery to additional templates. These issues are currently under investigation.

As we noted earlier, there is convincing evidence (3, 26) that HBV RT is translated as a discrete entity and not as a core-RT precursor. The constitutive activity of this discrete RT forces us to reconsider the proposed HBV replication scheme. It now becomes conceivable that reverse transcription of the HBV pregenome RNA initiates prior to capsid assembly and that a partially replicated genome could be the true substrate for selective inclusion into the capsid. Moreover, the notion that HBV replication may initiate outside the capsid challenges the widely held perception that the production of active viral RTs, and hence, the reverse transcription process, is restricted, or segregated, to the interior of viral nucleocapsids (6). Retroviruses and distantly related viruses such as yeast L-A double-stranded RNA virus (7) accomplish such segregation via a common mechanism. Briefly, these viruses elaborate inactive gag-pol RT precursors (14) which are activated (by proteolytic cleavage) only once they have bound their pregenome RNAs and have incorporated into the viral nucleocapsid. We speculate that unless HBV uses a novel kind of segregation strategy, the active HBV RT may come into contact with and reverse transcribe other viral and even cellular mRNAs during HBV infection. That HBV RT is not completely faithful to its pregenome template is suggested by the recent demonstration that subgenomic viral RNAs are reverse transcribed during viral infection (10, 34). The reverse transcription of cytoplasmic RNAs could have implications for the development of HBV-linked hepatocellular carcinoma (23).

The demonstration of a free human RT that is both highly active and immunoprecipitable opens up new avenues in the study of HBV biology and pathology. Our system should prove useful for characterizing the RT molecule and determining the identity of its active form, for examining its primase and RNase H subactivities, and for elucidating how it interacts with RNA. In addition, it should be feasible to extend our prior oocyte studies on HBV capsid assembly (28, 37, 38) to the key issue of how the RT protein, the capsid protein, and the pregenome RNA communicate to promote the assembly of a replication-competent nucleocapsid (25). Finally, the robust activity of the human HBV RT seen in

these studies makes the oocyte system attractive for testing novel RT inhibitors for antiviral therapy.

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