JOURNAL OF VIROLOGY, Apr. 1997, p. 2996–3004 Vol. 71, No. 4
0022-538X/97/$04.00+0

Transcomplementation of Nucleotide Priming and Reverse Transcription between Independently Expressed TP and RT Domains of the Hepatitis B Virus Reverse Transcriptase

ROBERT E. LANFORD,* LENA NOTVALL, HELEN LEE, AND BURTON BEAMES

Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas 78227

Received 1 October 1996/Accepted 7 January 1997

Hepadnavirus polymerases initiate reverse transcription in a protein-primed reaction that involves the covalent linkage of the first deoxyribonucleotide to the polymerase polypeptide. We recently expressed human hepatitis B virus (HBV) reverse transcriptase (pol) in insect cells by using the recombinant baculovirus system. The purified protein is active in nucleotide priming and reverse transcription reactions. In this report, we demonstrate that the tyrosine residue at amino acid number 63 within the TP (terminal protein) domain of the polymerase is the site of covalent linkage of the first nucleotide of minus-strand DNA. Analysis of pol polypeptides with mutations in the TP and RT (reverse transcriptase) domains indicated that both domains were required for in vitro nucleotide priming activity. Polymerase proteins with mutations in the TP and RT domains were not capable of complementing each other in the nucleotide priming reaction, suggesting that transcomplementation between full-length polypeptides was not possible. However, when the TP and RT domains were expressed as separate polypeptides, they formed a highly stable complex that was active in nucleotide priming and reverse transcription. The presence of an epsilon stem-loop dramatically increased the nucleotide priming activity in transcomplementation assays, even though full-length pol displayed similar activities in the absence and presence of epsilon. These data raise the possibility that in the transcomplementation assay, epsilon may play a role in the formation of a functional complex between TP and RT, rather than being required only as the template for nucleotide priming. The results indicate that using the baculovirus system, it is possible to dissect the protein-protein and protein-RNA interactions required for HBV genome replication.

Hepadnaviruses replicate their nucleic acid through a reverse transcription step (11, 26, 41, 44). The hepatitis B virus (HBV) reverse transcriptase, designated pol, is composed of four domains. From the amino terminus, the domains are: (i) the TP (terminal protein) domain, which becomes covalently linked to negative-strand DNA by virtue of the protein-primed initiation of reverse transcription; (ii) the spacer domain, which is tolerant of mutations; (iii) the RT (reverse transcriptase) domain, which contains the YMDD consensus motif for reverse transcriptases; and (iv) the RNase H domain (3, 34). The mechanism of genome replication for hepadnaviruses has been determined by a variety of methods. The initial step appears to be the recognition of the pregenomic RNA by the polymerase. This recognition occurs best in *cis*, wherein pol binds its own mRNA in an event that appears to be cotranslational (2, 12, 13, 15, 17, 32). The essential RNA sequence for this interaction, designated epsilon, is present at both ends of the terminally redundant pregenomic RNA (13, 15, 17, 32). Although epsilon is present on both ends of pregenomic RNA, only the 5′ copy appears to function in packaging (13, 15). The epsilon sequence in itself is sufficient to induce the packaging of foreign RNA sequences by HBV pol (13, 15), but an additional sequence is required for packaging in the duck HBV (DHBV) system (6, 13). The packaging of pol is dependent on an RNA molecule possessing a 5′ copy of epsilon (4); thus, neither pol nor pregenomic RNA can be packaged in the absence of the other.

The second critical event in genome replication involves a priming reaction in which a nucleotide becomes covalently linked to pol (3, 5, 29, 47, 50). The addition of the first four nucleotides is templated by a sequence in a bulge in the 5′ copy of epsilon (49, 51). The possibility that the priming reaction occurs prior to encapsidation has not been excluded. The primed pol complex is translocated to a complementary sequence in the 3′ copy of DR1, where the synthesis of minus-strand DNA resumes (8, 24, 30, 37, 39, 40, 49, 51, 54).

The synthesis of minus-strand DNA terminates at the 5′ end of pregenomic RNA (37, 54). The RNA template is degraded by the RNase H activity of pol with the exception of a short terminal oligoribonucleotide. This oligoribonucleotide is then translocated, in the second translocation step, to a homologous site, DR2, on minus-strand DNA, where it serves as the primer for plus-strand DNA (25, 27, 38, 43). Once plus-strand DNA synthesis has reached the 3′ end of minus-strand DNA, a third and final strand translocation to the 5′ end of minus-strand DNA occurs, resulting in a noncovalently closed, partially double stranded, circular DNA molecule. The synthesis of plus-strand DNA is only partially completed in mature virions, yielding the gapped DNA substrate that is filled in following infection of susceptible cells or during the endogenous polymerase assay (16, 19, 45).

Recently, several systems which permit the direct analysis of pol function in the absence of viral replication and other viral proteins have been described (23, 42, 47, 50). Two of the systems utilize the DHBV pol, and both have demonstrated reverse transcriptase activity that is template dependent and protein primed. One of the DHBV systems utilizes in vitro

* Corresponding author. Mailing address: Department of Virology and Immunology, Southwest Foundation for Biomedical Research, 7620 N.W. Loop 410, San Antonio, TX 78228. Phone: (210) 670-3245. Fax: (210) 670-3329. E-mail: rlanford@icarus.sfbr.org.

translation of DHBV pol to obtain lysates that contain a functional pol (50), while the other system packages an active fusion protein of DHBV pol in a virus-like particle from the yeast retrotransposon Ty1 (47). Both systems yield pol that possesses accurate protein-primed, reverse transcriptase activity that synthesizes minus-strand DNA originating at epsilon and DR1 (49, 51). The pol mRNA in both of these systems contains a 3′ copy of epsilon, but surprisingly no 5′ copy of this sequence is required in these systems. The realization that nucleotide priming occurs at epsilon prior to translocation to DR1 was first appreciated in studies with these two systems (49, 51). More recently, a third pol system that utilizes purified human HBV pol that is expressed via the baculovirus-insect cell expression system has been described (23). The purified HBV pol is active for in vitro protein priming and reverse transcriptase reactions. Surprisingly, the in vitro translation system for pol has not been successfully used for the human counterpart, HBV, nor have reports been published on the expression and purification of a functional form of DHBV pol by using a conventional expression system.

In this report, we describe the expression of the HBV pol TP and RT domains as independent polypeptides in insect cells by using the recombinant baculovirus system. When expressed in the same cells, TP and RT formed a stable complex that upon purification was competent for nucleotide priming and reverse transcription. The product of this reaction was a TP polypeptide with covalently attached minus-strand DNA. Transcomplementation between TP and RT was enhanced if the transcript encoding one of the polypeptides possessed an epsilon stem-loop.

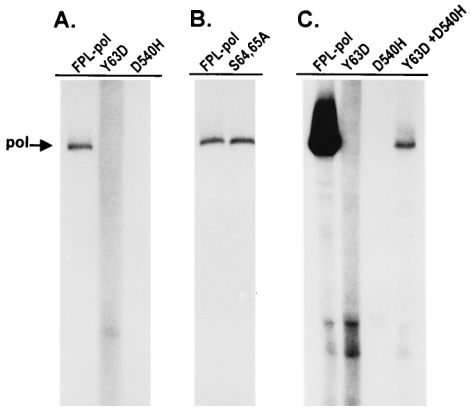

FIG. 1. Nucleotide priming occurs at tyrosine 63 of HBV pol; both the TP and RT domains of pol are required for nucleotide priming; and full-length mutant pol polypeptides cannot transcomplement each other. (A) Pol polypeptides were purified from insect cells infected with FPL-pol, Y63D, and D540H as described in Materials and Methods. The purified proteins were subjected to nucleotide priming reactions in a buffer containing 5 μCi of ^{32}P-labeled TTP and 100 μM unlabeled dATP, dCTP, and dGTP for 30 min at 30°C, and the products were analyzed by SDS-PAGE and autoradiography. (B) Purified pol proteins from cells infected with FPL-pol and S64,65A were used in nucleotide priming reactions as described above. (C) Pol polypeptides were immunoprecipitated from insect cells infected with FPL-pol, Y63D, and D540H and coinfected with Y63D plus D540H. The immune complexes were washed three times with TNG, with the second wash containing 1 M NaCl. Nucleotide priming reactions were conducted with the pol polypeptides still bound to the affinity beads, using the conditions described above. Following priming reactions, the beads were washed once more in TNG, and pol polypeptides were eluted in SDS-gel sample buffer. Samples were analyzed by SDS-PAGE and autoradiography.

MATERIALS AND METHODS

Cells and viruses. The *Spodoptera frugiperda* Sf9 cell line was cultivated in spinner culture as previously described (22). The cultivation medium was TNMFH (46) supplemented with 5% fetal bovine serum and 0.1% pluronic F68 prior to infection and was changed to Grace's medium supplemented with 2% fetal bovine serum and 0.1% pluronic F68 after infections. The methods for growth, isolation, and assay of recombinant baculoviruses were as previously described (46), with the exception that some of the viruses were generated by using the Bac to Bac system (Gibco BRL, Gaithersburg, Md.) in which transposition in bacteria creates the recombinant baculovirus genome rather than homologous recombination in insect cells (28).

Plasmid constructs. HBV sequences of the *ayw* subtype are numbered as designated by Galibert and coworkers (10). The FLAG-Pol-Stem Loop (FPL-pol) construct was previously described (23). The amino terminus of the pol open reading frame (ORF) was fused in frame with the FLAG epitope (International Biotechnologies Inc., New Haven, Conn.) such that the sequence Met Asp Tyr Lys Asp Asp Asp Asp Lys Leu preceded the polymerase Met at nucleotide (nt) 2309 (Fig. 1), and the HBV sequences in the construct continued through 3182/1 to nt 1988, which resulted in the presence of DR1 and the epsilon stem-loop structure found 3′ of the pol ORF on pregenomic RNA. FPL-pol was cloned into the baculovirus transfer vector pBacPAC9 (Clontech, Palo Alto, Calif.) for the isolation of recombinant baculoviruses. The FPΔ3′-pol construct was generated from FPL-pol by deletion of all HBV sequences downstream of the *Fsp*I site (nt 1804), thus removing DR1 and epsilon. The Y63D mutant was created by using PCR mutagenesis to change pol codon 63 from tyrosine to aspartic acid. The D540H mutant was created by the same process, changing codon 540 from aspartic acid to histidine. The mutations were then reconstructed into the FPL-pol transfer vector to produce recombinant baculoviruses with the respective mutations. The FTP334L-pol construct was generated by cleavage at the *Xho*I site (nt 129) and filling in the ends with Klenow enzyme to yield a frameshift mutation. This resulted in a virus encoding the first 334 amino acids of pol with nine additional non-pol amino acids and still containing all downstream HBV RNA sequences, including DR1 and the epsilon stem-loop. The FTP334 construct was generated by cleavage of FPL-pol with *Xho*I (nt 129) and at a *Pst*I site engineered at nt 1988, filling in the ends with Klenow enzyme, and ligating the blunt ends to generate a virus similar to FTP334L-pol but lacking all HBV sequences downstream of pol amino acid 334. The TP199 construct, described previously (1), contains the first 199 amino acids of pol but lacks a FLAG epitope and all HBV sequences downstream of pol amino acid 199. FTP199 was similar to TP199 but contained the FLAG epitope. FΔTPL was constructed from FPL-pol by cleavage with *Bsp*EI (nt 2331) and *Bgl*II (nt 2839) and religation to create an in-frame deletion removing pol amino acids 8 to 175, thus removing the TP domain. FΔTPΔ3′ (lacking both TP and epsilon) was generated from FΔTPL by deletion of all HBV sequences downstream of the *Fsp*I site (nt 1804). Pol 177-832 and Pol 199-758, described previously (1), contain pol amino acids 177 to 832 and 199 to 758 fused to polyhedrin leaders of four and three amino acids, respectively, and thus lack the FLAG epitope and the HBV sequences downstream of the pol ORF. The baculovirus expressing the pregenomic RNA was created by placing a cDNA copy of pregenomic RNA downstream of the polyhedrin promoter. Transcripts from this construct will contain a leader sequence of 120 nt from the polyhedrin transcript that precedes the pregenomic RNA start site.

SDS-PAGE and immunoblot analysis. Insect cell lysates and purified pol polypeptides were disrupted in electrophoresis sample buffer containing 2% sodium dodecyl sulfate (SDS) and 2% 2-mercaptoethanol and were heated to 100°C for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (18, 20). Gels from in vitro assays for pol function were stained with Coomassie blue, dried, and autoradiographed. For immunoblot analysis, proteins were electrophoretically transferred to a Flurotrans polyvinylidene difluoride blotting membrane (Pall Biosupport, Glen Cove, N.Y.), and membranes were processed as previously described (21), using a rabbit polyclonal antibody to full-length pol (23) followed by ^{125}I-protein A (NEN, Boston, Mass.).

Immunoaffinity purification of pol. Pol polypeptides were purified as previously described (23). Briefly, spinner cultures were harvested 48 h postinfection with recombinant baculoviruses. Cells were washed three times with phosphate-buffered saline. The cell pellet was extracted with PEB (phosphate-buffered saline containing 10% glycerol, 0.5% Nonidet P-40, protease inhibitors [100 μM leupeptin, 1 mM Pefabloc, 10 μM aprotinin, 10 μg of pepstatin per ml, and 1 mM EDTA), 50 U of RNasin [Promega] per ml, and 5 mM dithiothreitol). The clarified extract was passed over an affinity column containing the M2 monoclonal antibody (International Biotechnologies), and the column was washed sequentially with TNG (100 mM Tris HCl [pH 7.5], 30 mM NaCl, 10% glycerol), TNG with 1 M NaCl, and TNG. Bound polypeptides were eluted with 0.1 M glycine (pH 3.0)–10% glycerol, collected in 1-ml fractions, and neutralized with 67 μl of 0.8 M Tris HCl (pH 8.4)–3% Triton X-100–80 mM dithiothreitol. Purified pol polypeptides were frozen at −70°C until use.

Polymerase assays. The polymerase assays were performed as previously described (23) with 10 μl of purified pol polypeptide in the elution/neutralization buffer supplemented to contain 10 mM $MgCl_2$, 100 μM unlabeled deoxyribonucleoside triphosphates (dATP, dGTP, and dCTP), and 5 μCi of [α-^{32}P]TTP (3,000 Ci/mmol; NEN). Alternatively, polymerase reactions were conducted with immunoprecipitated pol polypeptides still bound to the M2 affinity beads. The beads were suspended in TNM (100 mM Tris HCl [pH 7.5], 30 mM NaCl, 10 mM $MgCl_2$) containing the above-described nucleotide mixture. Assays were routinely performed at 30°C for 30 min unless stated otherwise.

RESULTS

Nucleotide priming requires the tyrosine at amino acid 63 in the TP domain and the YMDD motif in the RT domain. Studies with in vitro-translated DHBV pol have previously mapped the site of covalent linkage of the first nucleotide of minus-strand DNA to a tyrosine at amino acid 96 within a GxY motif in the TP domain of pol (53, 56). A similar motif is found in HBV pol at amino acid 63. To determine if tyrosine 63 in HBV pol was the site of covalent linkage of the first nucleotide of minus-strand DNA, we created a point mutation changing the tyrosine to an aspartic acid (Y63D). A recombinant baculovirus was generated, and pol polypeptide was purified from infected insect cells. The purified polypeptide was tested in the nucleotide priming-reverse transcriptase assay in which pol was incubated in a buffer containing [^{32}P]TTP and unlabeled dATP, dCTP, and dGTP. The reaction products were analyzed by SDS-PAGE, Coomassie blue staining, and autoradiography. This variant of FPL-pol was defective in nucleotide priming, as determined by the lack of a labeled band at the position of pol polypeptide in the gel (Fig. 1A). The possibility that disruption of pol function was due not to elimination of the nucleotide priming site but to local effects on pol structure was deemed unlikely, since a double mutant changing adjacent serine residues 64 and 65 to alanine residues (S64,65A) had no detrimental effect on pol function in the in vitro assays (Fig. 1B). In addition, analysis of cyanogen bromide digests of FPL-pol following the in vitro nucleotide priming reaction localized the covalently bound, labeled nucleotide to a fragment spanning amino acids 2 to 111 (data not shown). Since we have previously demonstrated that in vitro nucleotide priming labels a tyrosine residue (23), and the labeled cyanogen bromide fragment contains only two tyrosine residues, with the tyrosine at amino acid 63 being within the only GxY motif, the assumption that this is the site of nucleotide priming is warranted.

Studies with in vitro-translated DHBV have also demonstrated that the YMDD motif of the RT domain was required for nucleotide priming (56). Mutation of amino acid 540 of FPL-pol at the first aspartic acid of YMDD to histidine (D540H) also resulted in an HBV FPL-pol defective in the in vitro nucleotide priming assay (Fig. 1A). These results confirm that both the TP and RT domains of HBV pol are required for nucleotide priming.

Lack of transcomplementation between the TP and RT domains in full-length pol polypeptides. To determine whether the functional TP domain of the D540H pol mutant was able to interact with and complement the functional RT domain of the Y63D pol mutant in nucleotide priming reactions, the two purified mutant polypeptides were mixed in vitro and subjected to a nucleotide priming reaction. No labeling of the pol polypeptides occurred in this reaction despite intense labeling of wild-type (WT) FPL-pol in parallel reactions (data not shown). To test the possibility that the mutant polypeptides require interaction in vivo, perhaps in a cotranslational event, insect cells were coinfected with the two baculoviruses expressing the mutant polypeptides, and pol was immunoprecipitated from cell extracts by using the M2 monoclonal antibody reactive with the FLAG epitope. In these experiments, instead of using purified pol polypeptides, we conducted nucleotide priming reactions with immunoprecipitated pol polypeptides while the proteins were still bound to the M2 affinity beads. Under these conditions, FPL-pol yielded a positive nucleotide priming reaction indistinguishable from that observed with purified pol preparations (Fig. 1C). The single infections with Y63D and D540H failed to yield a positive nucleotide priming reaction (Fig. 1C). Coinfection with the two mutant viruses yielded a weak pol reaction. Although this could represent partial complementation between the two defective viruses, we believe that this reaction is due to a minor amount of WT virus generated by homologous recombination. Data from coinfections with viruses encoding truncated forms of pol support this conclusion (see below) but do not exclude the possibility that low-level complementation is occurring. Staining of the gels with Coomassie blue confirmed the presence of comparable amounts of pol polypeptides in the immunoprecipitates of FPL-pol and the coinfection of Y63D and D540H, indicating that sufficient pol was present for an in vitro nucleotide priming reaction. These results confirm observations previously reported for DHBV that TP and RT mutants are not capable of transcomplementation for nucleotide priming (56).

Transcomplementation of nucleotide priming with TP and RT domains expressed as independent polypeptides. Although full-length pol polypeptides were not capable of interacting and complementing each other in the nucleotide priming assay, we reasoned that the TP and RT domains may have strong interactions that are formed during or soon after translation. The fact that pol has a preference for a *cis* interaction with its own mRNA in genome packaging (2, 12, 13, 15, 17, 32) suggests that pol folding and RNA interaction may be cotranslational events. Such a folding scheme would highly favor intramolecular interactions of TP and RT over intermolecular interactions between different pol polypeptides. This would prevent transcomplementation in our experiments if the mutant TP and RT domains were still competent for their respective protein-protein interactions. To explore this possibility, we constructed baculoviruses that would independently express the TP and RT domains.

We produced several constructs which contained different TP and RT domains, because the exact functional boundaries of TP and RT have not been determined (Fig. 2). Initially, four constructs which expressed the TP domain were made. One construct (TP199) has been described previously (1) and contains the amino-terminal 199 amino acids of pol without a FLAG epitope. A new version of this virus which contained the amino-terminal FLAG epitope (FTP199) was produced for immunoprecipitation and affinity purification. In the event that the spacer domain was required for proper folding or interaction with the RT domain, we produced another TP virus (FTP334) which encoded a polypeptide with the amino-terminal 334 amino acids of pol, containing both the TP and spacer domains. In the event that DR1 and epsilon were required for activity, a virus similar to FTP334 was produced by introducing a frameshift mutation into FPL-pol at amino acid 334, so that the construct would still contain downstream HBV RNA sequences, including the ε sequence at the end of the viral transcript. This virus was designated FTP334L to indicate the presence of an epsilon stem-loop at the 3′ end of the transcript. A single virus containing the RT and RNase H domains was produced and was designated FΔTPL to indicate the deletion of the TP domain but the presence of a FLAG epitope and an epsilon stem-loop. This virus was created by an in-frame deletion of pol amino acids 8 to 175 of FPL-pol.

Pol polypeptides were purified from insect cells infected with each of these viruses with the exception of TP199, which lacked a FLAG epitope for affinity purification. Comparison of the purified proteins by SDS-PAGE and Coomassie blue staining indicated that each of the pol polypeptides was expressed well and could be affinity purified (Fig. 3A). The FPL-pol polypeptide had an apparent molecular weight (MW) of 84,000; the FTP199 polypeptide had an MW of approximately 26,500; FTP334 and FTP334L polypeptides had apparent MWs of 40,700; and the FΔTPL polypeptide had an apparent MW of

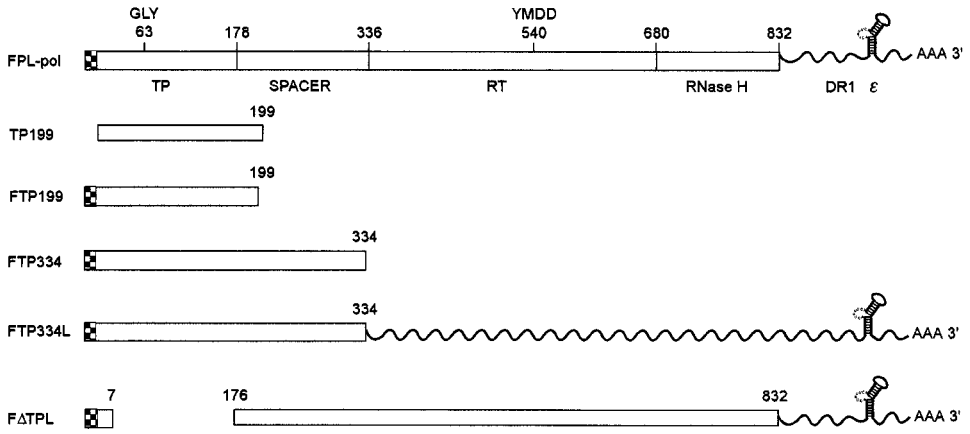

FIG. 2. Structures of pol constructs. The structure of the FPL-pol transcript from the baculovirus construct is depicted. The HBV polymerase ORF is represented as a rectangular box. At the amino terminus, the hatched section represents the 10-amino-acid FLAG epitope. The putative boundaries of TP, spacer, RT, and RNase H domains are indicated above the polymerase ORF, as are the positions of the GLY and YMDD motifs of the TP and RT domains, respectively. The 3′ end of the transcript indicates the presence of DR1 and epsilon (ε) regions. The structures of the pol transcripts and pol-encoding domains of some of the baculoviruses used in this study are depicted below FPL-pol. Constructs containing a FLAG epitope are designated by F, and those containing a 3′ epsilon sequence are designated by L.

64,300. Several cellular or baculoviral proteins were present in the purified pol preparations. The distinction of these proteins from pol polypeptides was accomplished by Western blotting with an antiserum produced against full-length pol (Fig. 3B) and by the fact that they were identical in size in each of the pol preparations, while the size of the pol polypeptides varied as expected. These proteins have been described previously with regard to FPL-pol preparations (23). The largest of the non-pol proteins has an apparent MW of 110,000 and fortuitously reacts with the M2 monoclonal antibody by Western blotting (23). The other three prominent proteins had MWs of 70,000, 56,000, and 46,000. Although these proteins appear to be specifically associated with pol in the purified preparations (data not shown), the nature of their association with pol is not clear, since they are present in pol preparations in which very little overlap exists in the pol amino acid sequences, for example, the seven-amino-acid overlap between FTP199 and FΔTPL.

To test the ability of the TP and RT polypeptides to interact with and complement each other in nucleotide priming reactions, insect cells were coinfected with one of the TP-expressing viruses and FΔTPL (RT function). Pol polypeptides were immunoprecipitated with M2 affinity beads, and priming reactions were conducted with the pol polypeptides still bound to the beads. Coinfection of cells with FΔTPL and either TP199 or FTP199 yielded positive priming reactions in which the TP polypeptide was intensely labeled by covalent attachment of labeled nucleotides (Fig. 4A). The TP polypeptides formed a labeled smear extending well up the gel, suggestive of elongation of the priming product by reverse transcription. These data demonstrate that two functional domains of pol required for nucleotide priming can be independently expressed and still form a complex capable of nucleotide priming. The fact that the TP199 polypeptide, in conjunction with the FΔTPL polypeptide, yielded a reaction similar in intensity and characteristics to the FTP199 polypeptide indicated that the complex formed between TP and RT was quite strong. This conclusion

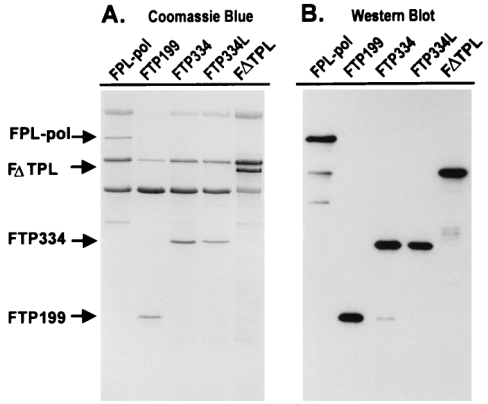

FIG. 3. Analysis of purified pol proteins. Sf9 cells were harvested 48 h postinfection with the recombinant baculoviruses FPL-pol, FTP199, FTP334, FTP334L, and FΔTPL. A cell extract was prepared with a detergent extraction buffer (PEB), and a clarified extract was purified on an M2 monoclonal antibody immunoaffinity resin as described in Materials and Methods. Samples from the purification were analyzed by SDS-PAGE and Coomassie blue staining (A) or by Western blotting with an antiserum produced against full-length pol followed by ^{125}I-protein A (B). The positions of the various pol polypeptides are indicated on the left.

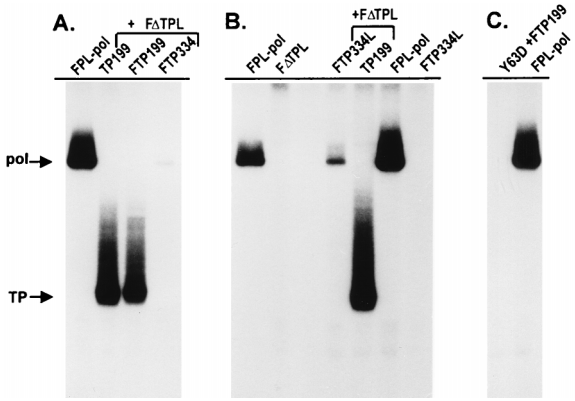

FIG. 4. The TP and RT domains of pol transcomplement each other for nucleotide priming when expressed as separate polypeptides. Pol polypeptides were immunoprecipitated from insect cells infected or coinfected with the various baculoviruses indicated at the top. Nucleotide priming reactions were conducted with pol polypeptides still bound to the affinity beads as described in the legend to Fig. 1. The positions of the FPL-pol (pol) and TP199 (TP) polypeptides are indicated on the left.

was drawn because the TP199 polypeptide lacks a FLAG epitope and was thus present in the immunoprecipitate due to interaction with FΔTPL, and the immunoprecipitates were washed three times with TNG buffer, with one of the washes containing 1 M NaCl. Additional studies revealed that the TP-RT interaction was not affected by a wash with 1 M urea and was only partially disrupted by a 3 M urea wash.

Although FTP199 was competent for transcomplementation with FΔTPL for nucleotide priming and reverse transcription, coinfections with FTP334 and FΔTPL did not yield a positive reaction. A weak reaction product was observed at the position of the gel of FPL-pol. This product of the nucleotide priming reaction was presumably due to homologous recombination between the FTP334 and FΔTPL viruses to generate WT FPL-pol. This product was observed in numerous reactions with various intensities (Fig. 4B, FTP334L + FΔTPL) whenever sufficient overlap between the TP- and RT-expressing viruses existed to permit homologous recombination. This product was not observed in the reactions from coinfections between FTP199 and FΔTPL, because insufficient overlap is present for efficient recombination. The inability of the FTP334 polypeptide to participate in the transcomplementation reaction was not due to low levels in the immunoprecipitate; the FTP334 polypeptide was expressed well and was present in the immunoprecipitate, as determined by Coomassie blue staining of the gel. One possible interpretation of these data is that a sequence between amino acids 199 and 334 is inhibitory to the TP function in these transcomplementation assays.

In subsequent experiments, we examined whether the addition of DR1 and epsilon to the FTP334 construct would correct the defect in nucleotide priming. Coinfection of cells with FTP334L and FΔTPL also failed to yield a complex positive for nucleotide priming at the position of the FTP334L polypeptide (Fig. 4B), although a positive reaction at the position of FPL-pol, due to recombination, was again observed. Control infections with FPL-pol and TP199 plus FΔTPL were again intensely positive, while control single infections with the FTP334L and FΔTPL viruses were negative, demonstrating that the reaction product from the coinfection that comigrated with FPL-pol was not a product of either virus alone.

The ability of FTP199 to complement the TP mutant Y63D was examined as well. Coinfection of insect cells with these viruses and immunoprecipitation of the pol polypeptides did not yield a positive nucleotide priming reaction (Fig. 4C). This finding confirms that the Y63D polypeptide is not available for interaction with an independently expressed TP domain and supports the conclusion that the weak priming reaction present from coinfection with Y63D and D540H was due to the creation of WT virus by recombination.

Dependence of transcomplementation on the presence of an epsilon stem-loop. HBV genomic replication is initiated by the protein priming event in which the covalent linkage of the first nucleotide to pol is templated by the 5′ epsilon sequence. In vitro nucleotide priming in the DHBV system is dependent on a 3′ copy of epsilon which cannot support packaging and/or genomic replication in vivo (35, 49, 51). The baculovirus system for HBV pol nucleotide priming also provides a 3′ copy of epsilon for nucleotide priming, but the dependence of priming on this sequence has not been apparent (see below). To determine whether the transcomplementation reaction between TP and RT was dependent on the 3′ copy of epsilon, this sequence was deleted from the FΔTPL construct to create FΔTPΔ3′. Coinfection of insect cells with FTP199 and either FΔTPL or FΔTPΔ3′ and immunoprecipitation of the pol polypeptides resulted in complexes competent for nucleotide priming with both FΔTPL and FΔTPΔ3′; however, the intensity of the reaction from complexes containing FΔTPΔ3′ was much less than that for complexes containing FΔTPL (Fig. 5A). The reduction in nucleotide priming activity was not due to lower levels of the FΔTPΔ3′ polypeptide, since Coomassie blue staining of the gel indicated that the two RT polypeptides were present in the immunoprecipitates at similar levels (data not shown). These data suggest that the transcomplementation reaction was facilitated by the presence of an epsilon stem-loop. The absolute dependence of this reaction on an RNA template coprecipitating with the pol polypeptides was demonstrated by the lack of nucleotide priming activity when the complex was pretreated with nuclease prior to the reaction (data not shown and reference 23).

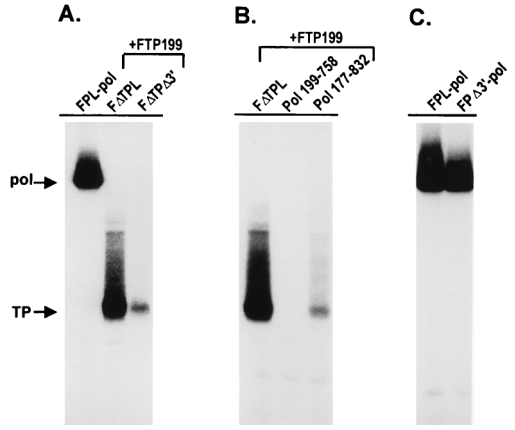

FIG. 5. The epsilon stem loop is needed for transcomplementation between the TP and RT domains but not for in vitro nucleotide priming per se. Insect cells were infected or coinfected with the various baculoviruses indicated at the top, and the pol polypeptides were immunoprecipitated with M2 affinity beads. Nucleotide priming-reverse transcriptase reactions were conducted with pol polypeptides bound to the affinity beads as described in the legend to Fig. 1. The positions of the FPL-pol (pol) and FTP199 (TP) polypeptides are indicated on the left. Although transcomplementation reactions were highly dependent of the presence of epsilon, nucleotide priming activities of full-length pol were similar in the presence and absence of epsilon.

Transcomplementation was also tested using two other RT constructs lacking a 3′ epsilon sequence. Baculoviruses expressing pol amino acids 199 to 758 and 177 to 832 have been described previously (1). Both constructs lack a FLAG epitope and HBV sequences downstream of the pol ORF. Coinfections between FTP199 and Pol 199-758 did not yield a complex competent for nucleotide priming, while coinfections between FTP199 and Pol 177-832 yielded complexes with greatly diminished nucleotide priming activity in comparison to coinfections with FTP199 and FΔTPL (Fig. 5B). The level of nucleotide priming activity for complexes containing Pol 177-832 was similar to that of FΔTPΔ3′. These viruses express nearly identical proteins except that Pol 177-832 lacks the FLAG epitope, which indicates that the presence of the FLAG epitope on either the TP or RT domain is sufficient to permit the immunoprecipitation of both polypeptides in a functional complex. The failure of Pol 199-758 to complement FTP199 was not due to low protein expression, since this polypeptide is expressed at higher levels than Pol 177-832. Since Pol 199-758 is truncated at both the amino and carboxyl termini in comparison to Pol 177-832, these data imply that either one or both of these regions, amino acid sequences between 177 and 199 and between 758 and 832, are required as part of the RT domain in the transcomplementation reaction.

The exact role that the epsilon stem-loop plays in the transcomplementation reaction is not clear. Comparison of

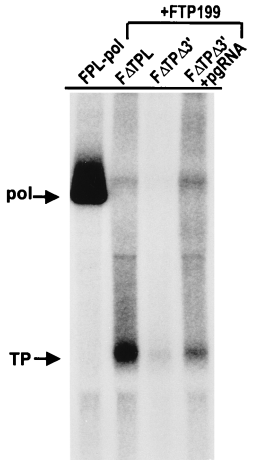

FIG. 6. The epsilon stem-loop can be supplied in *trans* for transcomplementation reactions between the TP and RT domains. Insect cells were infected or coinfected with the various baculoviruses indicated at the top. To supply epsilon in *trans*, a triple infection was conducted with FTP199, FΔTPΔ3′, and pgRNA, a baculovirus expressing pregenomic RNA with 5′ and 3′ copies of epsilon. The pol polypeptides were immunoprecipitated with M2 affinity beads. Nucleotide priming-reverse transcriptase reactions were conducted with pol polypeptides bound to the affinity beads as described in the legend to Fig. 1. The positions of the FPL-pol (pol) and FTP199 (TP) polypeptides are indicated on the left.

FPL-pol with a full-length pol construct lacking the 3′ epsilon, FPΔ3′-pol, revealed that for the full-length pol polypeptide, no decrease in the nucleotide priming reaction was apparent in the absence of epsilon (Fig. 5C). These data suggest that within this system, epsilon may play a role in the formation of a functional complex between individually expressed TP and RT domains.

In the transcomplementation assays described above, epsilon was provided at the 3′ terminus of the transcript for the RT domain. To determine whether epsilon could be provided in *trans* from a third baculovirus construct, a baculovirus expressing a transcript similar to pregenomic RNA with 5′ and 3′ copies of epsilon was constructed. As before, infection of cells with WT FPL-pol or coinfection of cells with FTP199 plus FΔTPL resulted in intensely labeled polypeptides in the priming reactions, while the labeling of the TP polypeptide was dramatically reduced in a coinfection with FTP199 plus FΔTPΔ3′ (Fig. 6). A triple infection with FTP199, FΔTPΔ3′, and the virus expressing a pregenomic RNA resulted in partial restoration of the priming activity. The lower priming activity of the complexes from the triple infection in comparison to complexes from cells coinfected with FTP199 plus FΔTPL may be due to the difficulty in obtaining efficient triple infections. A single infection with the virus expressing pregenomic RNA did not yield a positive priming reaction (data not shown). Although this virus contains the ORFs of both core and pol, only core polypeptide was expressed at detectable levels in cells infected with this virus. These data demonstrate that epsilon can be provided in *trans* to TP and RT for the formation of a functional complex in transcomplementation assays.

DISCUSSION

Many viruses have proteins covalently bound the 5′ end of the RNA or DNA genomes. In most instances, these terminal proteins are involved in the protein priming of genome replication (reviewed in reference 36). The terminal protein provides a 3′ OH from serine, threonine, or tyrosine to replace the requirement for a 3′ OH from an RNA primer to initiate nucleic acid synthesis. The best-known examples are adenovirus and phage ϕ29. For the TP protein of adenovirus, the linkage to DNA is via serine with dCMP. The only other viral factors required for initiation are the DNA polymerase and template; however, two host transcription factors, NF1 (CTF) and NF3 (OTF-1), stimulate initiation. ϕ29 is the representative member of a group of phages using protein-primed initiation of DNA synthesis. The ϕ29 TP protein initiates via serine with dAMP, and again, the only other viral factors required are the DNA polymerase and template. Other groups of phages with TP proteins involved in initiation of DNA synthesis include PRD1, which initiates with dGMP linked to tyrosine; Cp1, which initiates with dAMP via a threonine; and HB-3. Linear double-stranded DNA plasmids also have TP proteins involved in initiation of DNA synthesis. Many RNA viruses have VPg proteins that are terminal proteins. The most notable examples are the picornaviruses and caliciviruses, but five groups of plant RNA viruses have VPg terminal proteins as well.

The TP domain of hepadnaviruses is unique in that it is part of the same polypeptide as the polymerase, which in this case is a reverse transcriptase. Despite differences in replication schemes, picornavirus VPg proteins share the GxY consensus motif with the hepadnavirus TP domains. The DHBV GxY motif at amino acid 96 is the site of nucleotide priming (53, 56), and we have now shown that nucleotide priming for HBV pol maps to the GxY motif at amino acid 63 (Fig. 1). Predictions based on other TP systems suggest that both the TP and RT domains are required for nucleotide priming, and this has been confirmed by mutagenesis of the TP and RT motifs (50, 56) (Fig. 1).

As with the DHBV pol system (56), full-length HBV pol proteins with mutations in the TP and RT domains were unable to complement each other in nucleotide priming reactions. These data are also consistent with earlier observations by Radziwill and coworkers (34), who did not observe complementation between HBV pol mutants during HBV replication. These data might have been predicted, since intramolecular interaction of TP and RT would be favored over intermolecular interaction between two different pol polypeptides. Thus, if the TP and RT domains have specific interactions, complementation may be possible only between incomplete pol ORFs. Indirect genetic data from DHBV suggested that the TP and RT domains may be able to complement each other in *trans*. A DHBV frameshift mutant that terminates the pol ORF to yield a TP protein of 307 amino acids and a pre-S–pol fusion protein replicates at 10% of WT levels (55).

A strong interaction between TP and RT was conclusively demonstrated by the formation of a stable TP-RT complex functional for in vitro nucleotide priming activity (Fig. 4). Since independently expressed TP and RT polypeptides interact to complement each other for nucleotide priming, a high preference for intramolecular interaction of the TP and RT domains must prevent intermolecular interactions between full-length pol polypeptides. This hypothesis implies that intramolecular folding is rapid and that mutations in the GLY and YMDD motifs do not interfere with interaction between the TP and RT domains. Several lines of evidence support a cotranslational folding of pol and interaction with its template. Hepadnavirus polymerases have a high degree of preference for binding in *cis* the pregenomic RNA that served as mRNA for pol (2, 12, 13, 15, 17, 32). DHBV pol requires interaction with the host chaperone Hsp90 in order to bind the epsilon stem-loop (14), presumably a cotranslational event. In addition, the binding of epsilon by in vitro-translated DHBV pol results in a conformational change that appears to be needed for enzy-

matic activity and can be monitored by protease resistance (48). In the HBV pol-baculovirus system, transcomplementation between TP and RT polypeptides requires coinfection of insect cells with baculoviruses expressing these proteins. In vitro mixing of the purified proteins, with or without additional template, does not result in a functional interaction between TP and RT, nor does the mixing of lysates from individually infected insect cells prior to immunoprecipitation (data not shown). Even with full-length FPL-pol, it has not been possible to form a functional pol-template complex in vitro. Removal of the endogenous template from purified HBV pol by nuclease treatment results in a loss of nucleotide priming activity (23), and this activity cannot be restored by supplementation with synthetic epsilon or pregenomic RNA (data not shown).

Pol is highly sensitive to mutations with respect to the viral replication cycle with the notable exception of the spacer region. The sensitivity to mutations may be a reflection of the requirement for a complex folding scheme that involves RNA binding as well as interaction of distant domains and perhaps interaction with the capsid protein. The packaging of pregenomic RNA and pol into a capsid requires the entire pol ORF, and yet none of the enzymatic functions appear to be essential (2, 7, 12). Despite the strict requirements for the pol ORF in replication, in vitro pol reactions do not require the full pol ORF. Mutagenesis of DHBV pol has been performed by both Wang and coworkers (52) and Pollack and Ganem (33). Pol can lose at least 74 amino acid from the amino terminus and still maintain in vitro activity, and the COOH terminus can be deleted up to amino acid 568. Although the amino acid sequences of HBV and DHBV pol are not highly conserved, the sequences can be partially aligned by making a number of deletions and insertions (34). The alignment suggests that HBV pol should tolerate an NH_2-terminal deletion of 41 amino acids and a COOH-terminal deletion to amino acid 607, but the difference in these viruses dictates that such assumptions be confirmed by mutagenesis studies. The observation that Pol 177-832, but not Pol 199-758, was competent for complementation of TP in nucleotide priming (Fig. 5) suggests that differences with DHBV may exist. The lack of activity of Pol 199-758 is due either to the absence of the pol amino acid sequence from 177 to 199, which is within the spacer domain, or to the absence of the sequence from 758 to 832. This represents a significant divergence from the predictions based on DHBV if the loss of activity is due to the truncated COOH terminus. Although the truncation at the amino terminus is within the spacer domain, the differences between TP199 and TP334 indicate that the spacer domain is not completely neutral with regard to TP function in transcomplementation assays. The additional sequence from 199 to 334 may induce improper folding of the TP domain, but this too will require experimental validation.

Hepadnavirus replication utilizes the 5′ copy of epsilon for both packaging and nucleotide priming (13, 15, 31, 49, 51). In the DHBV system, in vitro nucleotide priming utilizes a 3′ copy of epsilon or epsilon supplied in *trans*. The reaction is highly dependent on the presence of epsilon, and Hsp90 is required to facilitate the interaction between epsilon and pol (14). The lack of a requirement for a 5′ copy of epsilon suggests that the requirements for in vitro priming reactions are more relaxed than those imposed during genome replication. Surprisingly, the current system for in vitro nucleotide priming for full-length FPL-pol is not dependent on the presence of epsilon. In our initial characterization of FPL-pol, it was assumed that the addition of the 3′ copy of epsilon was at least partially responsible for our success in purifying active pol (23); however, in those studies a construct lacking epsilon was not evaluated. The current data suggest that the addition of a FLAG epitope and rapid affinity purification were the essential changes from our previous unsuccessful attempts to purify active pol (1). Although epsilon was not essential for nucleotide priming activity with full-length pol, our previous studies suggested that when present, epsilon may be used as the template for priming. Indeed, at least a portion of the DNA synthesized by FPL-pol in insect cells maps to DR1 by primer extension (23), which suggests that priming may have occurred at epsilon with strand translocation to DR1. HBV pol activity has been demonstrated in *Xenopus* oocytes in the absence of epsilon as well; however, the actual priming reaction has not been characterized in that system (42). These data again suggest that the requirements for in vitro priming are less stringent than in vivo, since an absolute requirement for the 5′ copy of epsilon in HBV genomic replication has been demonstrated in mutagenesis studies (9, 31, 35), and sequencing experiments have confirmed that the 5′ copy of epsilon is the template for nucleotide priming with HBV (31).

There are multiple potential reasons why full-length HBV pol in our system does not require epsilon for nucleotide priming, some of which we have explored. We have previously determined that the specific activity of purified pol is low in the in vitro nucleotide priming assay, with less than 1% of the molecules being active (23). The lack of a requirement for epsilon and the low specific activity of FPL-pol may be interrelated. Pol may not properly associate with epsilon, and this may cause the low specific activity. One possible explanation is that pol expressed in insect cells is lacking a critical mammalian or liver specific host factor. Yet, FPL-pol expressed in Huh7 cells by using a vaccinia virus vector has the same specific activity as FPL-pol expressed in insect cells (unpublished data). Alternatively, human HBV pol may have a requirement for a 5′ copy of epsilon for nucleotide priming. We have also expressed HBV pol with both 5′ and 3′ copies of epsilon in the vaccinia virus system and did not observe a significant increase in specific activity (unpublished data). The difference between in vitro reactions with DHBV and HBV pol may be due to inherent differences in the enzymes. Some differences between the two enzymes are obvious, since in vitro-translated HBV pol is not active in nucleotide priming, while DHBV pol is very active in this system. Major differences exist in the RNA sequences required for pregenomic RNA encapsidation as well (6, 13, 15). However, we have found that DHBV pol expressed in insect cells has a specific activity similar to that of HBV FPL-pol (unpublished data). The low specific activity may simply be a result of overexpression of the protein, such that inadequate levels of epsilon are available for the amount of pol translated; alternatively, inadequate levels of an essential host factor may be available, or overexpression may lead to improper folding for other reasons.

Finally, the discrepancies in requirements for in vitro priming between DHBV and HBV pol may be due to inherent differences in the assay systems. In the baculovirus system, total cellular RNA is available for pol to seek an alternative stem-loop structure for folding and priming. In the in vitro translation system, no RNA is present except for the single pol transcript, and in the yeast Ty-pol fusion system, pol is presumably encapsidated such that cellular RNA is not available. We have found that in vitro-translated DHBV pol retains approximately 10% of the priming activity in the absence of epsilon, indicating that even in this system, some epsilon independent priming is possible (data not shown). It is possible that even during HBV replication, pol occasionally primes by using an alternative stem-loop structure. The product of these reactions may be too rare to detect by the current methods of

analysis. Reverse transcription of cellular RNAs could even be involved in the development of hepatocellular carcinoma.

The current system of transcomplementation offers the opportunity for further definition of the protein-protein and protein-RNA interactions required for nucleotide priming. A role for the epsilon stem-loop that was not observed with full-length pol is apparent in this system. One possibility is that epsilon stabilizes the TP-RT complex and that a highly stable interaction is not required when both domains are present on the same polypeptide. Although the current system makes possible a dissection of HBV pol in a manner not previously possible, the inability to reconstitute a functional pol in vitro places restrictions on the types of analysis performed as well as the ease in which such studies can be performed. Hopefully, future studies will provide a method of in vitro reconstitution of this reaction.

ACKNOWLEDGMENT

This work was supported by grant CA53246 from the National Institutes of Health.

REFERENCES

1. **Ayola, B., P. Kanda, and R. E. Lanford.** 1993. High level expression and phosphorylation of hepatitis B virus polymerase in insect cells with recombinant baculoviruses. Virology **194:**370–373.
2. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol. **64:**5324–5332.
3. **Bartenschlager, R., and H. Schaller.** 1988. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J. **7:**4185–4192.
4. **Bartenschlager, R., and H. Schaller.** 1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J. **11:**3413–3420.
5. **Bosch, V., R. Bartenschlager, G. Radziwill, and H. Schaller.** 1988. The duck hepatitis B virus P-gene codes for protein strongly associated with the 5′-end of the viral DNA minus strand. Virology **166:**475–485.
6. **Calvert, J., and J. Summers.** 1994. Two regions of an avian hepadnavirus RNA pregenome are required in *cis* for encapsidation. J. Virol. **68:**2084–2090.
7. **Chang, L.-J., R. C. Hirsch, D. Ganem, and H. E. Varmus.** 1990. Effects of insertional and point mutations on the functions of the duck hepatitis B virus polymerase. J. Virol. **64:**5553–5558.
8. **Condreay, L. D., T.-T. Wu, C. E. Aldrich, M. A. Delaney, J. Summers, C. Seeger, and W. S. Mason.** 1992. Replication of DHBV genomes with mutations at the sites of initiation of minus- and plus-strand DNA synthesis. Virology **188:**208–216.
9. **Fallows, D. A., and S. P. Goff.** 1995. Mutations in the Î sequences of human hepatitis B virus affect both RNA encapsidation and reverse transcription. J. Virol. **69:**3067–3073.
10. **Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay.** 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. Nature **281:**646–650.
11. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56:**651–693.
12. **Hirsch, R. C., J. E. Lavine, L.-J. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature **344:**552–555.
13. **Hirsch, R. C., D. D. Loeb, J. R. Pollack, and D. Ganem.** 1991. *cis*-acting sequences required for encapsidation of duck hepatitis B virus pregenomic RNA. J. Virol. **65:**3309–3316.
14. **Hu, J. M., and C. Seeger.** 1996. Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **93:**1060–1064.
15. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9:**3389–3396.
16. **Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson.** 1973. DNA polymerase associated with human hepatitis B antigen. J. Virol. **12:**995–1005.
17. **Knaus, T., and M. Nassal.** 1993. The encapsidation signal on the hepatitis B virus RNA pregenome forms a stem-loop structure that is critical for its function. Nucleic Acids Res. **21:**3967–3975.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
19. **Landers, T. A., H. B. Greenberg, and W. S. Robinson.** 1977. Structure of hepatitis B Dane particle DNA and nature of the endogenous DNA polymerase reaction. J. Virol. **23:**368–376.
20. **Lanford, R. E., and J. S. Butel.** 1979. Antigenic relationship of SV40 early proteins to purified large T polypeptide. Virology **97:**295–306.
21. **Lanford, R. E., K. D. Carey, L. E. Estlack, G. C. Smith, and R. V. Hay.** 1989. Analysis of plasma protein and lipoprotein synthesis in long-term primary cultures of baboon hepatocytes maintained in serum-free medium. In Vitro Cell. Dev. Biol. **25:**174–182.
22. **Lanford, R. E., and L. Notvall.** 1990. Expression of hepatitis B virus core and precore antigens in insect cells and characterization of a core-associated kinase activity. Virology **176:**222–233.
23. **Lanford, R. E., L. Notvall, and B. Beames.** 1995. Nucleotide priming and reverse transcriptase activity of hepatitis B virus polymerase expressed in insect cells. J. Virol. **69:**4431–4439.
24. **Lien, J., D. J. Petcu, C. E. Aldrich, and W. S. Mason.** 1987. Initiation and termination of duck hepatitis B virus DNA synthesis during virus maturation. J. Virol. **61:**3832–3840.
25. **Lien, J.-M., C. E. Aldrich, and W. S. Mason.** 1986. Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus-strand DNA synthesis. J. Virol. **57:**229–236.
26. **Loeb, D. D., and D. Ganem.** 1993. Reverse transcription pathway of the hepatitis B viruses, p. 329–355. *In* A. M. Skalka and S. P. Goff (ed.), Reverse transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Loeb, D. D., R. C. Hirsch, and D. Ganem.** 1991. Sequence-independent RNA cleavages generate the primers for plus strand DNA synthesis in hepatitis B viruses: implications for other reverse transcribing elements. EMBO J. **10:**3533–3540.
28. **Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins.** 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. **67:**4566–4579.
29. **Molnar-Kimber, K. L., J. Summers, J. M. Taylor, and W. S. Mason.** 1983. Protein covalently bound to minus-strand DNA intermediates of duck hepatitis B virus. J. Virol. **45:**165–172.
30. **Molnar-Kimber, K. L., J. W. Summers, and W. S. Mason.** 1984. Mapping of the cohesive overlap of duck hepatitis B virus DNA and of the site of initiation of reverse transcription. J. Virol. **51:**181–191.
31. **Nassal, M., and A. Rieger.** 1996. Bulged region of the hepatitis B virus RNA encapsidation signal contains the replication origin for discontinuous first-strand DNA synthesis. J. Virol. **70:**2764–2773.
32. **Pollack, J. R., and D. Ganem.** 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. J. Virol. **67:**3254–3263.
33. **Pollack, J. R., and D. Ganem.** 1994. Site-specific RNA binding by a hepatitis B virus reverse transcriptase initiates two distinct reactions: RNA packaging and DNA synthesis. J. Virol. **68:**5579–5587.
34. **Radziwill, G., W. Tucker, and H. Schaller.** 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. J. Virol. **64:**613–620.
35. **Rieger, A., and M. Nassal.** 1996. Specific hepatitis B virus minus-strand DNA synthesis requires only the 5′ encapsidation signal and the 3′-proximal direct repeat DR1*. J. Virol. **70:**585–589.
36. **Salas, M.** 1991. Protein-priming of DNA replication. Annu. Rev. Biochem. **60:**39–71.
37. **Seeger, C., D. Ganem, and H. E. Varmus.** 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science **232:**477–484.
38. **Seeger, C., and J. Maragos.** 1989. Molecular analysis of the function of direct repeats and a polypurine tract for plus-strand DNA priming in woodchuck hepatitis virus. J. Virol. **63:**1907–1915.
39. **Seeger, C., and J. Maragos.** 1990. Identification and characterization of the woodchuck hepatitis virus origin of DNA replication. J. Virol. **64:**16–23.
40. **Seeger, C., and J. Maragos.** 1991. Identification of a signal necessary for initiation of reverse transcription of the hepadnavirus genome. J. Virol. **65:**5190–5195.
41. **Seeger, C., J. Summers, and W. S. Mason.** 1991. Viral DNA synthesis. Curr. Top. Microbiol. Immunol. **168:**41–60.
42. **Seifer, M., and D. N. Standring.** 1993. Recombinant human hepatitis B virus reverse transcriptase is active in the absence of the nucleocapsid or the viral replication origin, DR1. J. Virol. **67:**4513–4520.
43. **Staprans, S., D. D. Loeb, and D. Ganem.** 1991. Mutations affecting hepadnavirus plus-strand DNA synthesis dissociate primer cleavage from translocation and reveal the origin of linear viral DNA. J. Virol. **65:**1255–1262.
44. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29:**403–415.
45. **Summers, J., A. O'Connell, and I. Millman.** 1975. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from dane particles. Proc. Natl. Acad. Sci. USA **72:**4597–4601.
46. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Tex. Agric. Exp. Stn. Bull. **1555:**1–48, plus appendix.
47. **Tavis, J. E., and D. Ganem.** 1993. Expression of functional hepatitis B virus

polymerase in yeast reveals it to be the sole viral protein required for correct initiation of reverse transcription. Proc. Natl. Acad. Sci. USA **90:**4107–4111.
48. **Tavis, J. E., and D. Ganem.** 1996. Evidence for activation of the hepatitis B virus polymerase by binding of its RNA template. J. Virol. **70:**5741–5750.
49. **Tavis, J. E., S. Perri, and D. Ganem.** 1994. Hepadnavirus reverse transcription initiates within the stem-loop of the RNA packaging signal and employs a novel strand transfer. J. Virol. **68:**3536–3543.
50. **Wang, G.-H., and C. Seeger.** 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell **71:**663–670.
51. **Wang, G.-H., and C. Seeger.** 1993. Novel mechanism for reverse transcription in hepatitis B viruses. J. Virol. **67:**6507–6512.
52. **Wang, G.-H., F. Zoulim, E. H. Leber, J. Kitson, and C. Seeger.** 1994. Role of RNA in enzymatic activity of the reverse transcriptase of hepatitis B viruses. J. Virol. **68:**8437–8442.
53. **Weber, M., V. Bronsema, H. Bartos, A. Bosserhoff, R. Bartenschlager, and H. Schaller.** 1994. Hepadnavirus P protein utilizes a tyrosine residue in the TP domain to prime reverse transcription. J. Virol. **68:**2994–2999.
54. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Büscher, R. Sprengel, R. Cattaneo, and H. Schaller.** 1987. Replication strategy of human hepatitis B virus. J. Virol. **61:**904–911.
55. **Wu, T.-T., L. D. Condreay, L. Coates, C. Aldrich, and W. Mason.** 1991. Evidence that less-than-full-length *cis* gene products are functional in hepadnavirus DNA synthesis. J. Virol. **65:**2155–2163.
56. **Zoulim, F., and C. Seeger.** 1994. Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. J. Virol. **68:**6–13.