29

Replication of the Hepatitis Virus Genome

Christoph Seeger and William S. Mason
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

Five strains of hepadnavirus have been identified. The prototype is human hepatitis B virus (HBV), first described as a primary cause of post-transfusion hepatitis (Blumberg et al. 1967). Very similar viruses have been isolated from woodchucks (Summers et al. 1978) and Beechey ground squirrels (Marion et al. 1980), and more distantly related viruses have been described in domestic ducks (Mason et al. 1980) and gray herons (Sprengel et al. 1988). With a genome size of 3 kbp, the hepadnaviruses are among the smallest of animal viruses. Despite this, these viruses are able to reproduce to high levels and to maintain a chronic, productive infection, often in the face of a vigorous immune response. Infection and replication take place primarily in the hepatocyte, the major parenchymal cell of the liver; are noncytopathic; and may persist for the lifetime of the host. All of these viruses are blood-borne and are poorly transmittable except by contact with the blood or blood-contaminated products from an infected individual. Nonetheless, infection can efficiently spread through a population, as revealed by the fact that more than 200 million people are currently infected by human hepatitis B virus.

Hepadnaviral DNA replication is of considerable interest because it occurs via reverse transcription of a viral RNA (Summers and Mason 1982), the pregenome, but in virtually all other details, differs from the reverse transcription pathway evolved by retroviruses. In fact, the hepadnavirus provirus is an episomal, covalently closed circular (CCC) DNA and not the integrated DNA of the retrovirus, and there is no evidence for a role of integration in hepadnavirus replication. Since most retroviruses are only able to complete provirus integration in dividing cells, utilization of an episomal template for transcription of hepadnaviral RNAs probably reflects the fact that the host cell is normally in G_0, dividing every 3–6 months to facilitate replacement of hepatocytes dying through random, cytopathic events (MacDonald 1960; Grisham 1962; Fourel et

DNA Replication in Eukaryotic Cells

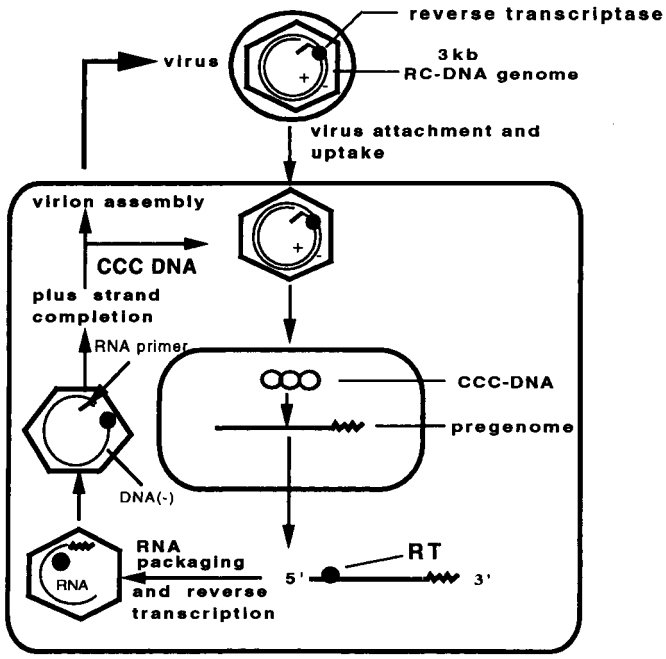

Figure 1 Reverse transcription in the replication of hepadnaviruses. The details of this replication cycle are described in the text. More detailed models of DNA replication intermediates are presented in Figs. 2–4.

al. 1994; Kajino et al. 1994). Schematically, hepadnavirus replication takes place through the series of steps shown in Figure 1. Virus, with an open circular, partially double-stranded DNA genome, infects the hepatocyte. The DNA is transported to the nucleus and converted to the covalently closed form, which serves as a template for the transcription of three (avian hepadnavirus) or four (mammalian hepadnavirus) classes of viral RNA, including the greater-than-genome-length molecule referred to as the pregenome. These RNAs are then transported to the cytoplasm, where viral assembly and DNA synthesis take place. The pregenome is packaged into subviral core particles along with a virus-encoded reverse transcriptase (RT), and viral DNA is then synthesized. Core particles with mature DNA then interact with viral envelope proteins, and virions are formed by budding into the endoplasmic reticulum, from where they are transported to the cell surface and released. Alternatively, the core particles may migrate to the nucleus to amplify the copy number of CCC DNA (Tuttleman et al. 1986), a process that is highly regulated, apparently to prevent virus replication from reaching levels that are cytocidal (Fig. 1) (Summers et al. 1990, 1991).

In this chapter, the process of DNA synthesis within virus nucleocapsids is discussed in detail. This is then related to factors that control CCC DNA amplification. Finally, the structure of the viral RT is reviewed, with respect both to known activities of this polypeptide and to functions that may potentially be carried out by this enzyme.

REVERSE TRANSCRIPTION OF THE VIRAL GENOME

Packaging and Protein Priming of Minus-strand DNA Synthesis

Replication of the viral genome occurs in subviral core particles that are present in the cytoplasm of infected cells. It depends on the expression of core proteins that assemble into icosahedral capsids with T=3 and T=4 icosahedral symmetry (Onodera et al. 1982; Nassal and Schaller 1993; Crowther et al. 1994), RT polypeptide, and the presence of *cis*-acting sequences on pregenomic RNA. Assembly of subviral core particles is triggered by the formation of a complex between pregenomic RNA, the template for reverse transcription, and the viral polymerase (Bartenschlager et al. 1990; Hirsch et al. 1990). To initiate the packaging reaction, the polymerase first binds to the packaging signal, termed ε, which has been proposed to fold into an RNA hairpin with a loop and a bulge (Fig. 2) (Junker-Niepmann et al. 1990). Although pregenomic RNA bears copies of ε at either end, a consequence of a terminal redundancy, it is the copy at the 5′ end of the RNA template that acts as a signal for RNA packaging. Fusion of pregenomic RNA sequences of HBV that comprise ε with an unrelated RNA segment leads to the encapsidation of the hybrid RNA into core particles, provided that the polymerase is expressed in the same cell (Junker-Niepmann et al. 1990); in addition, specific mutations of the 5′, but not the 3′, copy of ε have been shown to prevent packaging (Knaus and Nassal 1993; Pollack and Ganem 1993). In the avian viruses, packaging depends on additional sequences on pregenomic RNA that are located approximately 1 kb downstream from ε (Hirsch et al. 1991; Calvert and Summers 1994). The role of these sequences in virus assembly remains to be determined.

The requirement for a DNA polymerase in RNA packaging is unique among retroviruses. How the polymerase facilitates the packaging reaction is not yet understood, although genetic studies have shown that the polymerase activity per se is not involved (Bartenschlager et al. 1990; Chang et al. 1990; Hirsch et al. 1990; Roychoudhury et al. 1991; Chen et al. 1992). Genetic evidence suggests that expression of the polymerase polypeptide and RNA packaging occur in *cis* (Bartenschlager et al. 1990;

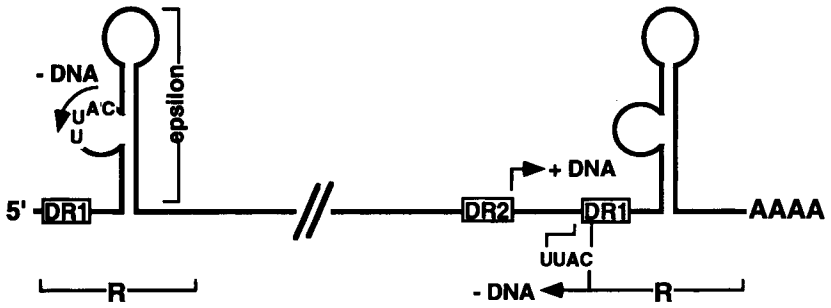

Figure 2 *cis*-Acting signals on pregenomic RNA. The RNA bears terminal repetitions (R) that contain copies of the packaging signal (ε), but only the 5′ copy has functional activity in vivo. The predicted stem-loop structure of ε is depicted as described by Junker-Niepmann et al. (1990). The positions of the 5′ ends of minus- and plus-strand DNAs and the UUAC motifs important for minus-strand DNA synthesis within ε and at DR1 are indicated.

Hirsch et al. 1990). This is probably a consequence of the dual role of pregenomic RNA as the mRNA for the translation of the RT and the template for reverse transcription (Huang and Summers 1991). Therefore, pregenomic RNAs encoding a structurally intact polymerase are preferred substrates for RNA packaging over RNAs with defective *pol* genes. Thus, the binding of polymerase with ε RNA may occur during or shortly after the translation of the *pol* gene product and induce the assembly of core particles. The core subunit, which is also translated from pregenomic RNA, and has RNA- and DNA-binding domains (Hatton et al. 1992), appears to function in packaging equally well in *cis* and in *trans*.

Following assembly of subviral core particles, minus-strand DNA is reverse-transcribed (Summers and Mason 1982). This step leads to the formation of a covalent linkage between the 5′ end of minus-strand DNA and protein (Gerlich and Robinson 1980). By analogy with the protein-priming mechanism first described for adenovirus DNA replication, it has long been speculated that this terminal protein was the primer for reverse transcription of hepadnavirus DNA (Rekosh et al. 1977; Gerlich and Robinson 1980; Molnar-Kimber et al. 1983; Bartenschlager and Schaller 1988). However, an understanding of the mechanism that controls the priming of viral DNA synthesis has only recently been obtained, owing to the discovery that enzymatically active RT of duck hepatitis B virus (DHBV) can be produced in a cell-free system (Wang and Seeger 1992). The major finding has been that ε RNA is not solely a signal for

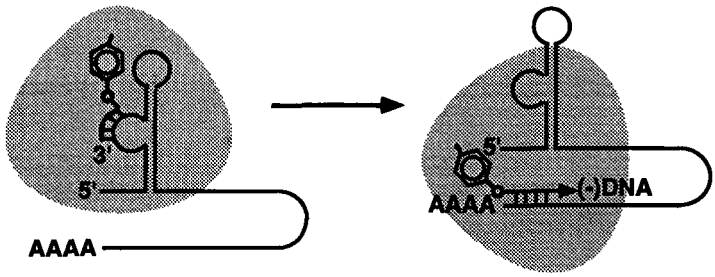

Figure 3 Model for the protein-priming reaction. Priming of DNA synthesis occurs at the RNA packaging signal ε near the 5′ end of pregenomic RNA with the help of a tyrosine residue of the polymerase polypeptide. After the polymerization of four nucleotides, the nascent DNA strand is transferred to the 3′ end of the RNA template, where minus-strand DNA synthesis continues.

RNA packaging, but actually serves as a template for the synthesis of a short 3- to 4-nucleotide-long DNA oligomer. The template for this oligomer is provided by nucleotides in the bulge region of ε (Figs. 2 and 3) (Wang and Seeger 1993). The primer for DNA synthesis is provided by a tyrosine residue located near the amino terminus of the polymerase polypeptide (Weber et al. 1994; Zoulim and Seeger 1994). As a consequence of the protein-priming mechanism, the RT remains covalently linked to the 5′ end of minus-strand DNA during all subsequent steps of viral DNA synthesis (Fig. 1). This conclusion is in good agreement with the observation that nascent DNA strands in subviral core particles, as short as 30 nucleotides in length, are covalently linked to protein (Molnar-Kimber et al. 1983).

Subsequent to the protein-priming reaction, the short DNA oligomer is transferred to the 3′ end of pregenomic RNA, where it base-pairs with a complementary sequence motif located at a 10- to 12-nucleotide-long region known as DR1 (Figs. 2 and 3). However, the 3- to 4-base acceptor site by itself is too short to specify the origin of minus-strand DNA synthesis. Genetic experiments with woodchuck hepatitis virus (WHV) and DHBV demonstrated that mutations or deletions of the natural acceptor site that prevent base-pairing with the DNA oligomer lead to the synthesis of minus-strand DNA with 5′ ends mapping to positions on pregenomic RNA that can serve as alternate acceptor sites (Condreay et al. 1992; Seeger and Maragos 1990, 1991). The selection of the natural site is most likely facilitated by the structural arrangement of pregenomic RNA in the nucleocapsid. We envision a scenario whereby the acceptor site and ε RNA are held in close physical proximity to facilitate the DNA

transfer reaction. The *cis*-acting signals on pregenomic RNA that may be required for such a structural arrangment have not yet been identified. However, deletion analysis with WHV suggested that a region extending approximately 1 kb upstream of DR1 bears the signals that specify the acceptor site (Seeger and Maragos 1991). Since polymerase requires pregenomic RNA as a vehicle for incorporation into core particles, it is likely that a single polymerase polypeptide carries out both the protein-priming and the DNA synthesis reactions (see below) (Bartenschlager and Schaller 1992).

An important prediction of the proposed model for DNA priming is that base changes in the bulge of ε should appear at the 5′ end of minus-strand DNA as a consequence of DNA replication. Transfection of tissue-culture cells with DHBV DNA that carried mutations in the ε region, which did not prevent elongation of DNA synthesis from the natural acceptor site at DR1, directly demonstrated the expected transfer of genetic information across the viral genome (Wang and Seeger 1993). Similar results were obtained with enzymatically active DHBV RT expressed with a yeast Ty-1 vector (Tavis and Ganem 1993). Insertion of a nucleotide into the bulge region of ε resulted in the synthesis of minus strands with an extra nucleotide at their 5′ ends (Tavis et al. 1994).

Following the translocation reaction, minus-strand DNA synthesis continues all the way to the 5′ end of the RNA template. RNA is concomitantly degraded by an RNase H activity on the polymerase polypeptide (Summers and Mason 1982; Radziwill et al. 1990). Because of the relative location of DR1 within the terminal redundancy on the pregenome, the completed minus-strand DNA bears a short, 9-nucleotide-long terminal redundancy, which plays a role, as described below, in the circularization of the viral genome (Seeger et al. 1986; Lien et al. 1987; Will et al. 1987).

Priming of Plus-strand DNA Synthesis

Unlike the situation in retroviruses, plus-strand DNA synthesis does not initiate until minus-strand DNA synthesis has been completed. This is a direct consequence of the mechanism for the priming of plus-strand DNA synthesis. It relies on the formation of an RNA primer by the viral RNase H activity. The primer is derived from the 5′ end of pregenomic RNA, from the cap through DR1, and hence cannot be created prior to the completion of minus-strand DNA synthesis (Fig. 4) (Lien et al. 1986; Loeb et al. 1991). To prime plus-strand DNA synthesis, the RNA primer is first translocated and hybridized with sequences near the 5′ end of

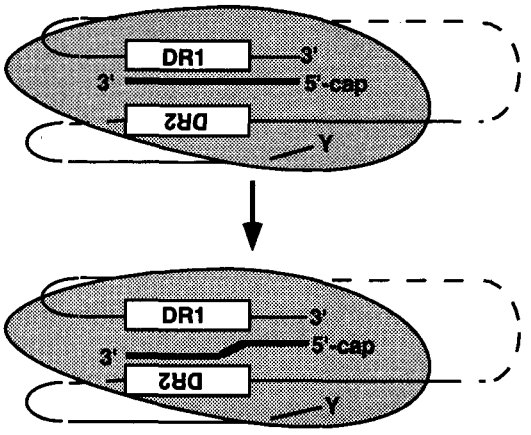

Figure 4 Model for plus-strand priming. Minus-strand DNA is arranged to facilitate the transfer of the capped RNA primer from DR1 to DR2. The polymerase is covalently linked through a tyrosine residue (Y) to the 5′ end of minus-strand DNA.

minus-strand DNA. This step is facilitated by a short 11- to 12-nucleotide-long sequence homology between DR1 and DR2. DR2 is located, depending on the species of hepadnavirus, about 50–200 bases downstream from the 5′ end of the minus-strand DNA (Figs. 2 and 4). The exact details of this reaction, which is reminiscent of the translocation reaction that occurs during the priming of minus-strand DNA synthesis, are not known, but there is ample biochemical and genetic evidence to support it. Biochemical analysis of the 5′ end of plus-strand DNA revealed the presence of an 18-nucleotide-long RNA primer of the expected sequence with a cap structure, and genetic experiments revealed that mutations introduced into the 5′ copy of DR1 appear on the RNA primer at the 5′ end of plus-strand DNA as a consequence of viral DNA replication (Lien et al. 1986; Seeger et al. 1986; Strapans et al. 1991). It is likely that the RNA transfer reaction is facilitated through the spatial arrangement of the minus-strand DNA; i.e., through the juxtaposition of the regions encompassing DR1 and DR2 on minus-strand DNA (Fig. 4). Such a structure may invoke forces that help to stabilize the 3′-OH group of the RNA primer in a position that allows the polymerase to initiate DNA synthesis at DR2 rather than at DR1. In fact, disruption of the homology between DR1 and DR2 favors an in situ DNA-priming reaction at DR1 that leads to the synthesis of double-stranded linear genomes as compared to the relaxed circular DNA species, which is the predominant species produced by wild-type virus (Strapans et al. 1991; Condreay

et al. 1992). However, preparations of wild-type virus do contain a small fraction of particles with linear genomes. Although the latter are not known to play an important role in the hepadnavirus life cycle, they are infectious, as evidenced by the formation of CCC DNA and subsequent viral DNA synthesis (Yang and Summers 1995).

Once plus-strand DNA synthesis has progressed from DR2 to the 5′ end of minus-strand DNA, a template switch (i.e., circularization) is required for the continuation of DNA synthesis. The terminally redundant sequences of minus-strand DNA are believed to promote the strand-transfer reaction from the 5′ to the 3′ end. The structural requirements for this reaction must be complicated, since the polymerase has to accommodate both ends of minus-strand DNA in close proximity while it is still covalently attached to the 5′ end. The template transfer then leads to the circularization of the viral genome. In mammalian hepadnaviruses, the RT then extends the plus-strand DNA to approximately half the genome length, but in avian viruses, the polymerase elongates plus strands to nearly full length (Robinson et al. 1974; Summers et al. 1975; Lien et al. 1987). It is notable, however, that the polymerase does not displace the RNA primer and the 5′ end of plus-strand DNA (i.e., the RNA primer from DR2, Fig. 1), an event that would lead to the formation of a terminally redundant, linear genome. Linear genomes are observed in pools of viral DNA, but these arise as a consequence of the in situ plus-strand-priming event described above. The cause and significance of the premature termination of plus strands synthesized in the mammalian viruses remain obscure. It is conceivable that the steric factors imposed by the capsid and by the RT prevent the completion of this reaction during virion morphogenesis. Alternatively, capsids with incomplete plus strands may be more readily packaged into viral envelopes by the mammalian than by the avian viruses. This latter possibility may be supported by the observation that the polymerase activity present in intact virus cores can repair the single-stranded region in an in vitro reaction (Kaplan et al. 1973; Summers et al. 1975). However, core particles assembled from capsid proteins with truncated carboxyl termini lead to the accumulation of virion DNA with incomplete plus strands, indicating that capsid structure can influence elongation of plus-strand DNA (Yu and Summers 1991; Nassal 1992).

Formation and Amplification of CCC DNA from Viral DNA

Following maturation of viral DNA within cytoplasmic core particles, these particles may enter one of two pathways (Fig. 1). In the first, they interact with viral envelope proteins to form virions. In the second, they

or their content of viral DNA is transported to the nucleus, where this DNA is processed to amplify the copy number of CCC DNA (Tuttleman et al. 1986; Wu et al. 1990). Entry into the second pathway only occurs if there is an inadequate cytoplasmic concentration of viral envelope proteins. Thus, CCC DNA amplification normally occurs only during the first few days of an infection, prior to the initiation of virus assembly and release. The final copy number per nucleus, in vivo, is usually between 5 and 30 (Jilbert et al. 1992; Kajino et al. 1994).

Transport to the nucleus is not mediated by viral envelope proteins, since amplification occurs when hepatocytes are infected with viral mutants that are unable to synthesize these proteins (Summers et. al. 1990, 1991). The route and mechanism of transport are unknown. It appears, however, that this route is regulated by the signals generated on core particles during their maturation, perhaps the same signals that facilitate differential assembly of nucleocapsids containing mature DNA into virions (Yu and Summers 1991, 1994a,b; Guidotti et al. 1994). The carboxyl terminus of the core protein of cytoplasmic nucleocapsids can be phosphorylated at up to four sites (Schlicht et al. 1989a; Machida et al. 1991; Yeh and Ou 1991; Yu and Summers 1994b), whereas virion nucleocapsids are probably only phosphorylated at one of these sites (Pugh et al. 1989), suggesting that differential phosphorylation may signal intracellular trafficking of nucleocapsids.

What remains completely unclear is how and when viral DNA is processed to form CCC DNA. This step requires removal of the RT from the 5′ end of minus-strand DNA, trimming of the 3′ repeat from the terminus of minus-strand DNA, and ligation of the two ends. In addition, plus-strand DNA synthesis must be completed, RNA removed from the 5′ end, and the ends ligated. It seems probable that the viral DNA polymerase could be involved in plus-strand elongation up to the primer-binding site, DR2. However, the polymerase does not appear capable of displacing this primer in intact nucleocapsids (Lien et al. 1987), suggesting that elongation through DR2 is mediated subsequent to the release of viral DNA from core particles. An important issue is the role of the viral polymerase in the cleavage and joining reactions that are involved in formation of CCC DNA versus the role of cellular enzymes, such as that proposed for topoisomerase I (Rogler 1991). Current knowledge of the functional capacity of the polymerase polypeptide is discussed below.

STRUCTURE AND FUNCTION OF RT

Hepadnaviral RTs are encoded by the viral polymerase (*pol*) gene and have an approximate molecular mass of 90 kD. Synthesis of polymerase

polypeptides occurs through internal initiation of translation from an AUG codon, which is located near the 3′ end of the core gene, allowing for a short overlap between the two open reading frames. Because of amino acid homologies with retroviral RTs, it has been possible to identify the DNA polymerase and RNase H domains encoded by hepadnaviral *pol* genes (Fig. 5) (Toh et al. 1983; Radziwill et al. 1990). Alignment of conserved residues between the polymerase polypeptides of the two virus families shows that the hepadnaviral polymerase polypeptides bear an amino-terminal domain, also referred to as the terminal protein (TP) region (Fig. 5) (Bartenschlager and Schaller 1988; Radziwill et al. 1990). This domain bears the tyrosine residue utilized for the protein-priming reaction. It is separated from the RT domain by a spacer region that is not essential for any of the known activities of the RT, as indicated by observations that RT functions are refractory to mutagenesis of this region (Bartenschlager and Schaller 1988; Chang et al. 1990).

Genetic analyses of the RT of DHBV expressed either in reticulocyte lysates, in frog oocytes, or with the help of the transposon Ty-1 in yeast showed that the polymerase can exhibit protein-priming and DNA-polymerization activities in the absence of other viral polypeptides, most notably the viral capsid proteins (Wang and Seeger 1992; Seifer and Standring 1993; Tavis and Ganem 1993). Furthermore, mutations altering residues in the catalytic site of the polymerase abolish the protein-priming activity, demonstrating that an enzymatically active RT domain is required for this reaction (Wang and Seeger 1992). Interestingly, unlike the situation with other systems where a protein is known to act as a primer for DNA synthesis (i.e., adenovirus and bacteriophage ϕ29 [Salas 1991]), in hepadnaviruses the protein primer and the DNA polymerase reside on the same polypeptide. As suggested from genetic analyses of the DHBV polymerase expressed in vitro, it appears that both the TP and RT domains are required for the interaction of the polymerase with ε RNA (Pollak and Ganem 1994; Wang et al. 1994). Although it is likely that the polymerase binds directly to ε, it is conceivable that host factors are required to support this interaction, perhaps similar to the scenario proposed for the binding of HIV Tat to the TAR response element (Marciniak et al. 1990; Madore and Cullen 1993). Mutational analysis of ε suggested that binding of the RNA hairpin to the polymerase alone may not be sufficient for RNA packaging and that one or more cellular proteins may indeed be required for this reaction (Pollak and Ganem 1994). The observation that the DHBV RT expressed in wheat germ extracts fails to bind to ε RNA, unless supplemented with a factor(s) present in rabbit reticulocyte lystates, also supports the view that host fac-

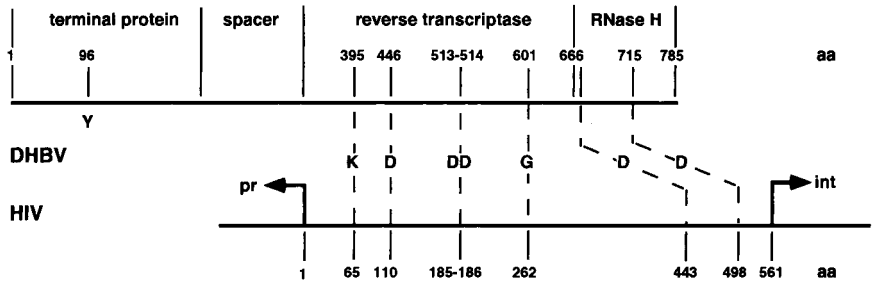

Figure 5 Structural comparison of hepadnaviral and retroviral reverse transcriptases. Shown are the linear maps of the DHBV and HIV *pol* gene products. The maps were aligned with the help of amino acids that are conserved among RTs. Position 1 on the DHBV polymerase corresponds to the AUG codon at position 170 on the DHBV genome (Mandart et al. 1984; Chang et al. 1989; Schlicht et al. 1989b). Amino acid positions on the HIV RT map were adapted from Jacobo-Molina et al. (1993). (pr) Protease, (int) integrase.

tor(s) may be involved in the expression of enzymatically active enzyme (Hu and Seeger 1996).

It is not known whether the polymerase functions within cores as a monomer or as a dimer and whether more than one functional polymerase molecule is required for viral DNA synthesis. However, it is likely that a single polymerase polypeptide may catalyze one complete round of the DNA replication cycle. This view is consistent with the observation that assembly of the RT into core particles depends on its interaction with ε sequences on pregenomic RNA, which would indicate that polymerase and RNA templates may be present at equimolar amounts in subviral particles (Bartenschlager and Schaller 1992). Quantitation of radioactively labeled RT of HBV present in core particles revealed the molar ratio of 0.7 polymerase molecule per virion DNA, which is in good agreement with the proposed mechanism for particle assembly and DNA synthesis (Bartenschlager and Schaller 1992; Bartenschlager et al. 1992).

Given this stoichiometry, it is still possible to envision, at least in a schematic sense, how the RT, RNase H, and protein-primer domains might function in minus-strand synthesis. What is more difficult to understand is how the polymerase facilitates elongation of the plus strand, prior to the strand switch, virtually all the way to the point of attachment to the protein primer (Fig. 4). A priori, this would seem to require either multiple copies of the polymerase polypeptide within virions or an unusual amount of flexibility and accessibility between different domains

of the protein. It is also difficult to understand how the primer is removed by cleavage of the phosphotyrosine bond during formation of CCC DNA from virion DNA. Both these considerations, and particularly the latter, suggest that there may be additional functional domains or activities of the polymerase polypeptide that remain to be identified.

SUMMARY AND PERSPECTIVES

It has been little more than a decade since the discovery that hepadnaviruses replicate via reverse transcription. In that time, there has evolved a highly detailed model for the major steps in viral DNA synthesis. It has also become clear that reverse transcription is involved not only in the production of progeny virus DNA, but also, through a highly regulated process, in the synthesis and maintenance of CCC DNA, a species which, despite its nuclear location, lacks the regulatory sequences that would facilitate its reproduction via semi-conservative DNA synthesis. As should be clear from the discussion above, the major emphasis of most investigations has been on those steps of reverse transcription that lead to the creation of the mature virion DNA. The process of CCC DNA synthesis has been characterized; however, the molecular details underlying this process are not at all understood. Moreover, the connection between the various viral DNA forms that are associated with productive infections and the integrated DNA that accumulates in the liver and, at least in some instances, plays a major role in hepatocellular carcinogenesis via oncogene activation through region-specific integration, is completely unexplored. Finally, the role of host proteins in viral DNA synthesis, CCC DNA amplification, and virion assembly are only beginning to be addressed. These issues will define important avenues of future research, and it is hoped they will lead not only to a better understanding of how to control and eliminate chronic infections, but also to an understanding of how virus replication is spontaneously shut down both in chronically and in transiently infected individuals.

ACKNOWLEDGMENTS

The authors acknowledge support from the National Institutes of Health and the Commonwealth of Pennsylvania.

REFERENCES

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.

———. 1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. *EMBO J.* **11:** 3413–3420.

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.

Bartenschlager, R., C. Kuhn, and H. Schaller. 1992. Expression of the P-protein of the human hepatitis B virus in a vaccinia virus system and detection of the nucleocapsid-associated P-gene product by radiolabelling at newly introduced phosphorylation sites. *Nucleic Acids Res.* **20:** 195–202.

Blumberg, B.S., B.J.S. Gerstley, D.A. Hungerford, W.T. London, and A.I. Sutnik. 1967. A serum antigen (Australai antigen) in Down's syndrome, leukemia and hepatitis. *Ann. Intern. Med.* **66:** 924–931.

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.

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.

Chang, L.J., P. Pryciak, D. Ganem, and H.E. Varmus. 1989. Biosynthesis of the reverse transcriptase of hepatitis B viruses involves de novo translational initiation not ribosomal frameshifting. *Nature* **337:** 364–368.

Chen, Y., W.S. Robinson, and P.L. Marion. 1992. Naturally occurring point mutation in the C terminus of the polymerase gene prevents duck hepatitis B virus RNA packaging. *J. Virol.* **66:** 1282–1287.

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.

Crowther, R.A., N.A. Kiselev, B. Bottcher, J.A. Berriman, G.P. Borisova, V. Ose, and P. Pumpens. 1994. Three-dimensional strucuture of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell* **77:** 943–950.

Fourel, I., J.M. Cullen, J. Saputelli, C.E. Aldrich, P. Schaffer, D. Averett, J. Pugh, and W.S. Mason. 1994. Evidence that hepatocyte turnover is required for rapid clearance of DHBV during antiviral therapy of chronically infected ducks. *J. Virol.* **68:** 8321–8330.

Gerlich, W. and W. S. Robinson. 1980. Hepatitis B virus contains protein covalently attached to the 5′ terminus of its complete DNA strand. *Cell* **21:** 801–809.

Grisham, J.W. 1962. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; Autoradiography with thymidine-H3. *Cancer Res.* **22:** 842–849.

Guidotti, L.G., V. Martinez, Y.-T. Loh, C.E. Rogler, and F.V. Chisari. 1994. Hepatitis B virus nucleocapsid particles do not cross the hepatocyte nuclear membrane in transgenic mice. *J. Virol.* **68:** 5469–5475.

Hatton, T., S. Zhou, and D.N. Standring. 1992. RNA- and DNA-binding activities in hepatitis B virus capsid protein: A model for their roles in viral replication. *J. Virol.* **66:** 5232–5241.

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.

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.

Hu, J.-M. and C. Seeger. 1996. Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc. Natl. Acad. Sci.* (in press).

Huang, M. and J. Summers. 1991. Infection initiated by the RNA pregenome of a DNA virus. *J. Virol.* **65:** 5435–5439.

Jacobo-Molina, A., J. Ding, R.G. Nanni, A.D. Clark, Jr., X. Lu, C. Tantillo, R.L. Williams, G. Kramer, A.L. Ferris, P. Clark, A. Hizi, S.H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci.* **90**: 6320–6324.

Jilbert, A.R., T.-T. Wu, J.M. England, P.M. Hall, N.Z. Carp, A.P. O'Connell, and W.S. Mason. 1992. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. *J. Virol.* **66:** 1377–1388.

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.

Kajino, K., A.R. Jilbert, J. Saputelli, C.E. Aldrich, J. Cullen, and W.S. Mason. 1994. A study of woodchuck hepatitis virus infections revealed very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. *J. Virol.* **68:** 5792–5803.

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.

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.

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.

Lien, J.-M., 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.

Loeb, D.L., 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.

MacDonald, R.A. 1960. "Lifespan" of liver cells. *Arch. Intern. Med.* **107:** 335–343.

Machida, A., H. Ohnuma, F. Tsuda, A. Yoshikawa, Y. Hoshi, T. Tanaka, S. Kishimoto, Y. Akahane, Y. Miyakawa, and M. Mayumi. 1991. Phosphorylation in the carboxyl-terminal domain of the capsid protein of hepatitis B virus: Evaluation with a monoclonal antibody. *J. Virol.* **65:** 6024–6030.

Madore, S.J. and B.R. Cullen. 1993. Genetic analysis of the cofactor requirement for human immunodeficiency virus type 1 tat function. *J. Virol.* **67:** 3703–3711.

Mandart, E., A. Kay, and F. Galibert. 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: Comparison with woodchuck and human hepatitis B virus sequences. *J. Virol.* **49:** 782–792.

Marciniak, R.A., B.J. Calnan, A.D. Frankel, and P.A. Sharp. 1990. HIV-1 tat protein *trans*-activates transcription in vitro. *Cell* **63:** 791–802.

Marion, P.L., L.S. Oshiro, D.C. Regnery, G.H. Scullard, and W.S. Robinson. 1980. A virus in Beechey ground squirrels which is related to hepatitis B virus of man. *Proc. Natl. Acad. Sci.* **77:** 2941–2945.

Mason, W.S., G. Seal, and J. Summers. 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J. Virol.* **36:** 829–836.

Molnar-Kimber, K., 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.

Nassal, M. 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J. Virol.* **66:** 4107–4116.

Nassal, M. and H. Schaller. 1993. Hepatitis B virus nucleocapsid assembly. In *Virus strategies* (ed. W. Doerfler and P. Bohm), pp. 42–74. VCH, Weinheim, Germany.

Onodera, S., H. Ohori, M. Yamaki, and N. Ishida. 1982. Electron microscopy of human hepatitis B virus cores by negative staining-carbon film technique. *J. Med. Virol.* **10:** 147–155.

Pollack, J.R., and D. Ganem. 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. *J. Virol.* **67:** 3254–3263.

———. 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.

Pugh, J., A. Zweidler, and J. Summers. 1989. Characterization of the major duck hepatitis B virus core particle protein. *J. Virol.* **63:** 1371–1376.

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.

Rekosh, D.M.K., W.C. Russell, A.J.D. Bellet, and A.J. Robinson. 1977. Identification of a protein linked to the ends of adenovirus DNA. *Cell* **11:** 283–295.

Robinson, W.S., D.A. Clayton, and R.L. Greenman. 1974. DNA of a human hepatitis B virus candidate. *J. Virol.* **14:** 384–391.

Rogler, C.E. 1991. Cellular and molecular mechanisms of hepatocarcinogenesis associated with hepadnavirus infection. *Curr. Top. Microbiol. Immunol.* **168:** 104–140.

Roychoudhury, S., A.F. Faruqi, and C. Shih. 1991. Pregenomic RNA encapsidation analysis of eleven missense and nonsense polymerase mutants of human hepatitis B virus. *J. Virol.* **65:** 3617–3624.

Salas, M. 1991. Protein-priming of DNA replication. *Annu. Rev. Biochem.* **60:** 39–71.

Schlicht, H.J., R. Bartenschlager, and H. Schaller. 1989a. The duck hepatitis B virus core protein contains a highly phosphorylated C terminus that is essential for replication but not for RNA packaging. *J. Virol.* **63:** 2995–3000.

Schlicht, H.J., G. Radziwill, and H. Schaller. 1989b. Synthesis and encapsidation of duck hepatitis B virus reverse transcriptase do not require formation of core-polymerase fusion proteins. *Cell* **56:** 85–92.

Seeger, C. and J. Maragos. 1990. Identification and characterization of the woodchuck hepatitis virus origin of DNA replication. *J. Virol.* **64:** 16–23.

———. 1991. Identification of a signal necessary for initiation of reverse transcription of the hepadnavirus genome. *J. Virol.* **65:** 5190–5195.

Seeger, C., D. Ganem, and H.E. Varmus. 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. *Science* **232:** 477–484.

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.

Sprengel, R., E.F. Kaleta, and H. Will. 1988. Isolation and characterization of a hepatitis

B virus endemic in herons. *J. Virol.* **62:** 932–937.

Strapans, 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.

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.

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.* **72:** 4597–4601.

Summers, J., P.M. Smith, and A.L. Horwich. 1990. Hepadnaviral envelope proteins regulate covalently closed circular DNA amplification. *J. Virol.* **64:** 2819–2824.

Summers, J., J.M. Smolec, and R. Snyder. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc. Natl. Acad. Sci.* **75:** 4533–4537.

Summers, J., P.M. Smith, M. Huang, and M. Yu. 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. *J. Virol.* **65:** 1310–1317.

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.* **90:** 4107–4111.

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.

Toh, H., H. Hayashida, and T. Miyata. 1983. Sequence homology between retroviral reverse transcriptase and putative polymerases of hepatitis B virus and cauliflower mosaic virus. *Nature* **305:** 827–829.

Tuttleman, J.S., C. Pourcel, and J. Summers. 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* **47:** 451–460.

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.

———. 1993. Novel mechanism for reverse transcription in hepatitis B viruses. *J. Virol.* **67:** 6507–6512.

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.

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.

Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Buscher, R. Sprengel, R. Cattaneo, and H. Schaller. 1987. Replication strategy of human hepatitis B virus. *J. Virol.* **61:** 904–911.

Wu, T.-T., L. Coates, C.E. Aldrich, J. Summers, and W.S. Mason. 1990. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. *Virology* **175:** 255–261.

Yang, W. and J. Summers. 1995. Illegitimate replication of linear hepadnaviral DNA through non-homologous recombination. *J. Virol.* **69:** 4029–4036.

Yeh, C.T. and J.H. Ou. 1991. Phosphorylation of hepatitis B virus precore and core proteins. *J. Virol.* **65:** 2327–2331.

Yu, M.-S. and J. Summers. 1991. A domain of the hepadnavirus capsid protein is specifi-

cally required for DNA maturation and virus assembly. *J. Virol.* **65:** 2511–2517.

———. 1994a. Multiple functions of capsid protein phosphorylation in duck hepatitis B virus replication. *J. Virol.* **68:** 4341–4348.

———. 1994b. Phosphorylation of the duck hepatitis B virus capsid protein associated with conformational changes in the C terminus. *J. Virol.* **68:** 2965–2969.

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.