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Relationship between Viral DNA Synthesis and Virion Envelopment in Hepatitis B Viruses

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While the intracellular pool of encapsidated hepatitis B viral DNA contains genomes in all stages of DNA replication, serum-derived virions contain predominantly mature, partially duplex, circular DNA genomes. To account for this finding, Summers and Mason proposed in 1982 that virion envelopment is somehow linked to the state of genomic maturation (J. Summers and W. S. Mason, Cell 29:403–415, 1982). Core gene mutations with phenotypes consistent with this concept have previously been identified in the duck hepatitis B virus (DHBV). Here we show that DHBV polymerase mutants with altered DNA synthesis also display defects in envelopment, and we provide quantitative estimates of the magnitude of the preference for the envelopment of mature DNA. In cells transfected with wild-type DHBV DNA, immature minus-strand DNA represents 18% of the intracellular pool but only 4% of extracellular virion DNA. A point mutation in the C-terminal domain of the polymerase strongly and selectively impairs plus-strand synthesis; in this mutant, the ratio of immature to mature DNA in the intracellular pool rises to 6:1 but is reduced to 1.5:1 in released virions. A missense mutation in the polymerase active site inactivates all viral DNA synthesis but still allows efficient RNA encapsidation; in this mutant, no detectable viral nucleic acid is enveloped and released. Thus, viral DNA synthesis is absolutely required for envelopment and export, and a strong further bias exists in favor of the export of genomes that have completed minus-strand synthesis and at least initiated plus-strand synthesis. These results imply that events within the interior of the nucleocapsid can powerfully influence its interactions with external viral envelope glycoproteins.

Hepatitis B viruses (hepadnaviruses) are small, enveloped DNA viruses that replicate within cells via reverse transcription of RNA intermediates (14). The mature viral genome is a partially duplex, relaxed circular species of 3 kb (12, 14, 15). Upon delivery of this DNA to the host nucleus, it is converted to a superhelical form that serves as a template for host RNA polymerase II. Transcription of the genome generates genomic and subgenomic mRNAs that encode the following key viral proteins: C, the core (nucleocapsid) protein; P, the polymerase (reverse transcriptase); and pre-S/S, the surface (envelope) proteins. In the cytoplasm, assembling C subunits assimilate complexes of P protein and genomic RNA into icosahedral nucleocapsids (1, 2, 5, 8, 9), within which most viral DNA synthesis occurs (14). Synthesis of the two viral DNA strands is sequential. Minus-strand DNA synthesis occurs first, and following the largely contemporaneous removal of the RNA template from this product (via an RNase H activity encoded within the P protein; see references 3 and 11), plus-strand synthesis then occurs (14). Free minus strands are linear; circularization of the viral genome results from a template transfer that occurs during plus-strand synthesis (for details, see reference 4).

It has long been known that the forms of viral DNA detected intrahepatically differ markedly from those found within progeny virions in the serum. As outlined above, the intracellular pool contains viral DNA corresponding to all stages of genomic replication, including abundant free minus strands (and partial RNA-DNA hybrids), rare linear duplexes, and substantial numbers of mature, partially duplex relaxed circles (8). Exported virions, by contrast, contain predominantly the partially double-stranded circles corresponding to the most mature elements of the intracellular population (12, 14, 15). In 1982, Summers and Mason (14) proposed that only nucleocapsids whose genomes have nearly completed replication can be enveloped by the surface proteins, that is, that envelopment is somehow coupled to genomic maturation. To further explore this possibility, we have examined the envelopment of mutant duck hepatitis B virus (DHBV) genomes with lesions in the viral polymerase that affect the structure and maturation of viral DNA. The mutants we chose for study virtually ablate the formation of mature (partially duplex) viral genomes, thereby allowing assessment of the efficiency of envelopment of immature capsids in a setting in which no mature capsids are present to compete for interactions with envelope proteins. Our results indicate that there is a relative but not absolute bias against the envelopment of immature DNA, that competition with mature capsids may contribute significantly to the underrepresentation of immature DNA in virus stocks, and that capsids bearing only viral RNA cannot be enveloped at all.

Experimental strategy. We studied two mutant DHBV genomes that affect the extent of genomic replication. These mutants bear lesions in the P gene and thus encode wild-type C proteins. The first mutant, G2312A, is a missense substitution (G to A at nucleotide residue 2312) in the C-terminal (RNaseH) domain of the polymerase (3, 11). Figure 1 shows the forms of viral DNA produced by this mutant examined by Southern blotting of intracellular core DNA with strand-specific probes. As shown in Fig. 1A, while this mutation globally reduces viral DNA synthesis, it also selectively impairs plus-strand DNA synthesis, leading to a strong reduction of duplex relaxed circles and a relative accumulation of immature forms. The various immature species in the G2312A mutant probably represent free minus strands and RNA-DNA hybrids of various extent. When these forms are examined under denaturing

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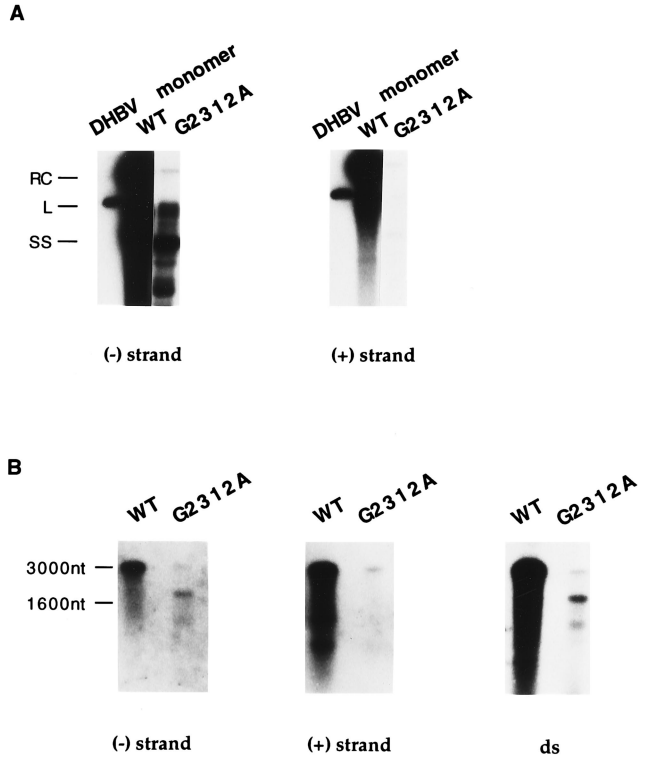

FIG. 1. DHBV P mutation G2312A selectively impairs plus-strand DNA synthesis. LMH cells were transfected with WT and G2312A mutant DNA. Three days after transfection, nucleic acids were extracted from intracellular cores. The resulting DNAs were electrophoresed through either standard agarose gels (A) or alkaline agarose gels (50 mM NaOH, 1 mM EDTA) (B), transferred to nylon membranes (Hybond N^+), and hybridized with a ^{32}P-labelled riboprobe specific for detection of minus strand [(−) strand blots]. After autoradiography, the probe was stripped from the filters by incubation in 0.4 M NaOH. The blots were then probed with ^{32}P-labelled ribonucleotides specific for detection of plus strand [(+) strand blots]. The blot in panel B (center) was then incubated in 0.4 M NaOH to strip the plus-strand-specific probe and rehybridized with ^{32}P-DHBV DNA (ds blot). RC, relaxed circle; L, linear duplex; SS, single strand; ds, double strand.

(alkaline) conditions (Fig. 1B), the hybridization pattern of nucleic acid species was significantly simplified. Interestingly, most of the minus-strand DNA migrated as a relatively discrete band of ca. 2,000 nucleotides (Fig. 1B, left blot). Since all of this DNA is protein linked (not shown), it therefore presumably initiates at the known origin of viral DNA synthesis (16); this suggests that lesions in the RNase H domain affect the processivity of minus-strand synthesis (see also references 3 and 11) and may promote termination in a particular region of the viral genome. The relative simplicity of this DNA pattern may also suggest that the greater complexity of the mobilities of the undenatured species (Fig. 1A) is due to variation in the length of the associated RNA.

The second mutant we examined bears two lesions in the conserved YMDD motif at the catalytic center of the reverse transcriptase (YMDD to YMHA mutation); as previously described (3, 5), this mutant encapsidates viral RNA normally but makes no viral DNA whatsoever.

Each mutant was cloned into a 1.5-mer of the viral genome and transfected into permissive LMH chicken hepatoma cells; wild-type (WT) DHBV DNA transfected in parallel served as a control. Three days later, cytoplasmic nucleocapsids were prepared as previously described (9, 10) and lysed in 1% sodium dodecyl sulfate (SDS)–500-μg/ml proteinase K; their nucleic acids were then extracted with a phenol-chloroform mixture (1:1) and precipitated with 70% ethanol. At the same time, extracellular viral particles from the same transfections were harvested from the medium by polyethylene glycol precipitation and their nucleic acids were extracted by a modification of the method of Lenhoff and Summers (6). Particles were first digested with pronase (750 μg/ml) at 37°C for 1 h and then exposed to DNase I (500 μg/ml) at 37°C for 0.5 h; under these conditions, plasmid DNA and DNA within free cores (e.g., those released by lysis of transfected cells) should be completely degraded (6), leaving only authentic virions still intact. From these virions, nucleic acid was then extracted by exposure to 1% SDS and 500 μg of proteinase K per ml (1 h at 37°C); this was followed by phenol-chloroform extraction and ethanol precipitation. The resulting DNAs were then examined by standard agarose gel electrophoresis and Southern blot hybridization with ^{32}P-DHBV DNA as previously described (7, 13). To validate this method of virion preparation, we undertook control experiments in which we extracted the DNA of both DHBV virions (from LMH culture media) and, in parallel, a 10-fold excess of naked intracellular cores (derived by Nonidet P-40 extraction of transfected LMH cell extracts) by this procedure. As shown in Fig. 2, core DNA was completely degraded under these conditions, while virion DNA was spared.

Encapsidated mature viral DNA is preferentially enveloped. Figure 3A shows the nature of intracellular core and extracellular virion DNA produced by LMH cells transfected with WT DHBV DNA (lanes 1 and 3) and the G2312A mutant (lanes 2 and 4). For WT DHBV infection, quantitative phosphorimager analysis of the mature (relaxed circle) and immature (free minus strand) DNA reveals that immature DNA represents only 4% of the DNA of the enveloped Dane particles (Fig. 3A, lane 3), while at least 18% of the intracellular pool (lane 1) is composed of this species. This confirms and quantitates the known bias against the envelopment and release of immature DNA. Figure 3B shows that virtually identical ratios obtain

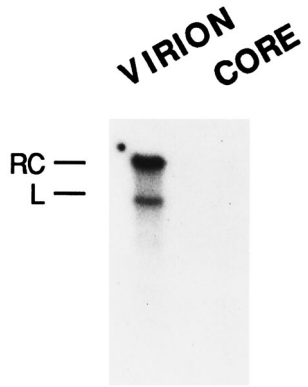

FIG. 2. Validation of the virion detection method. LMH cells were transfected with a 1.5-mer of cloned WT DHBV DNA. Three days later, intracellular cores were prepared from Nonidet P-40-treated cytoplasmic extracts (7); in addition, virus particles were concentrated by polyethylene glycol precipitation from 10 ml of culture medium from these cells. Samples of the two preparations were taken for further analysis; control experiments showed that the intracellular sample contained 10 times as many viral genome equivalents as the extracellular sample (data not shown). Each of the two samples was then treated with pronase and DNase I as described in the text; this was followed by SDS-proteinase K treatment and phenol extraction. Resulting viral nucleic acids were examined by 1.25% agarose gel electrophoresis, transfer to nylon membranes, hybridization to ^{32}P-DHBV DNA, and autoradiography as previously described (7). Left lane, DNA from extracellular virions; right lane, DNA from Nonidet P-40-treated intracellular cores; RC, relaxed circle; L, linear duplex.

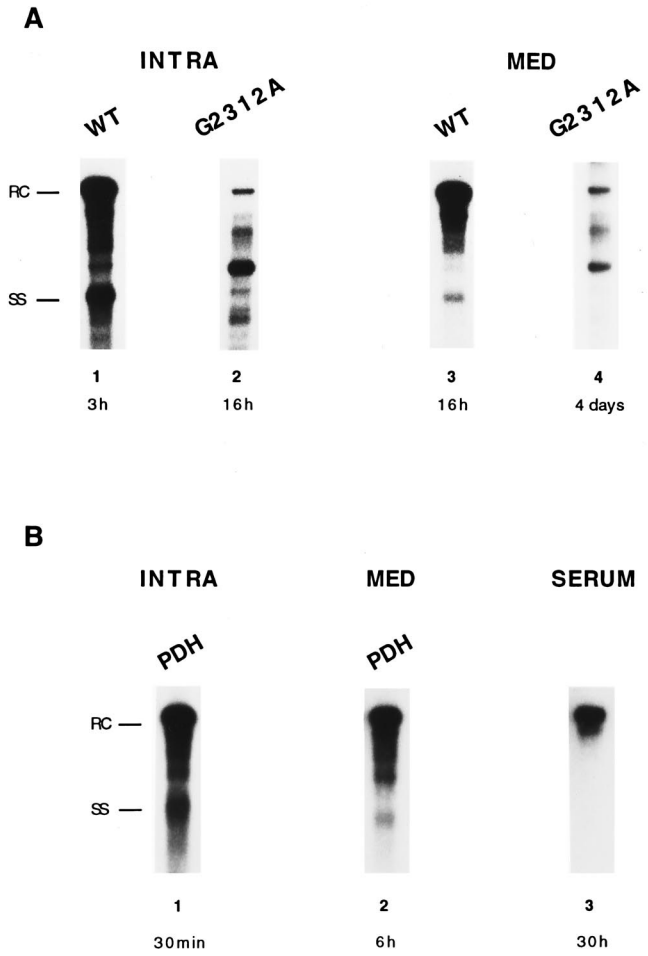

FIG. 3. Envelopment and release of WT and mutant DHBV genomes. (A) LMH cells were transfected with WT or G2312A mutant DHBV DNA; 3 days later, cells and media were collected. DNA was extracted from intracellular cores (blots 1 and 2) and extracellular virions (blots 3 and 4) as described in the text, and viral DNAs were examined by agarose gel electrophoresis, Southern hybridization with ^{32}P-DHBV DNA, and autoradiography. The autoradiographic exposure time of each sample is indicated below each lane. (B) Envelopment of viral DNA in infected primary hepatocytes and animal hosts. Primary duck hepatocytes were infected with DHBV, and 17 days later, DNAs from intracellular cores (blot 1) and exported virions (blot 2) were examined as described above. Blot 3 represents virion DNA extracted from virus particles concentrated from the serum of a viremic duck. Autoradiographic exposure time is indicated below each lane. INTRA, intracellular cores; MED, media; PDH, primary duck hepatocytes; RC, relaxed circle; SS, single strand.

when primary duck hepatocytes infected with WT DHBV virions are similarly examined (lanes 1 and 2), although examination of DHBV virions from the serum of viremic birds (Fig. 3B, lane 3) suggests that liver cells in the intact animal host appear to discriminate even more stringently against immature DNA.

As previously noted, the G2312A mutation globally depresses overall viral DNA synthesis (compare lanes 1 and 2 of Fig. 3A) but has in addition a distinctive block to plus-strand DNA synthesis (Fig. 1). In G2312A intracellular viral cores, there is about a sixfold excess of immature DNA strands over mature, partially double-stranded circular molecules (Fig. 3A, lane 2). However, in released virions (Fig. 3A, lane 4) the two species are present in nearly equimolar ratios, once again betokening a relative (but not absolute) bias against the envelopment of immature minus strands. The larger proportion of virion DNA in G2312A that is immature (ca. 50%) compared with that in WT virions (ca. 4%) may indicate that competition for envelope interactions with capsids bearing mature DNA may contribute significantly to the underrepresentation of immature DNA in stocks of WT virions.

Viral DNA synthesis is required for envelopment. Since capsids bearing free minus strands appeared to have some ability to be enveloped, we were interested in whether capsids bearing only pregenomic RNA could likewise be enveloped. Accordingly, we examined LMH cells transfected with DHBV genomes bearing the YMDD→YMHA mutation in the active site of the viral reverse transcriptase. WT DHBV DNA transfected in parallel served as a control. Following the extraction of the viral nucleic acids from the intracellular or extracellular particles, the nucleic acids were electrophoresed through 2.2 M formaldehyde–1% agarose gels, transferred to nylon membranes, and then examined by hybridization to ^{32}P-DHBV DNA (i.e., by Northern [RNA] blotting) (Fig. 4). As expected, WT genomes gave rise to extracellular particles bearing DNA

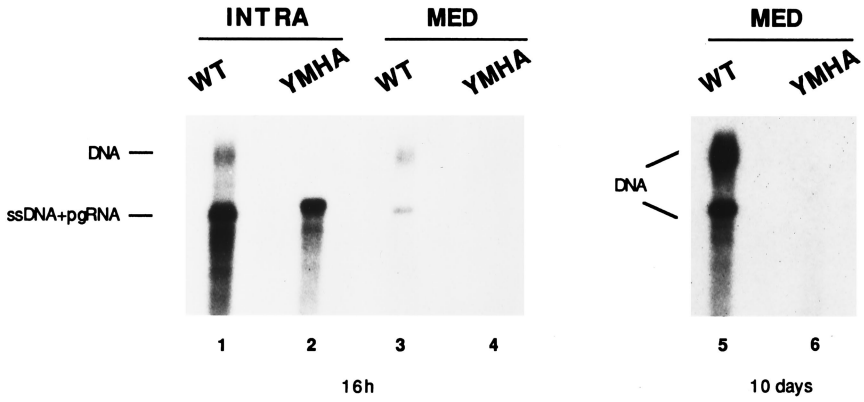

FIG. 4. DNA synthesis is required for nucleocapsid envelopment and export. LMH cells were transfected with WT or reverse transcriptase– (YMHA) mutant DHBV DNA; 3 days later, viral nucleic acid was prepared from intracellular cores (lanes 1 and 2) and extracellular virions (lanes 3 to 6). Nucleic acid from equivalent numbers of infected cells was then electrophoresed through denaturing formaldehyde-agarose gels, transferred to nylon membranes, and hybridized to^{32}P-DHBV DNA. The signal in lanes 3 and 5 is entirely sensitive to DNase (data not shown) and thus represents viral DNA; input viral RNA has been degraded by RNaseH during reverse transcription. The signal in lane 2 is fully RNase sensitive (data not shown) and represents encapsidated pregenomic RNA. Lanes 5 and 6 represent a long exposure of the samples in lanes 3 and 4, respectively; exposure times are indicated below each panel. ssDNA, single-stranded DNA; pgRNA, pregenomic RNA; INTRA, intracellular cores; MED, media.

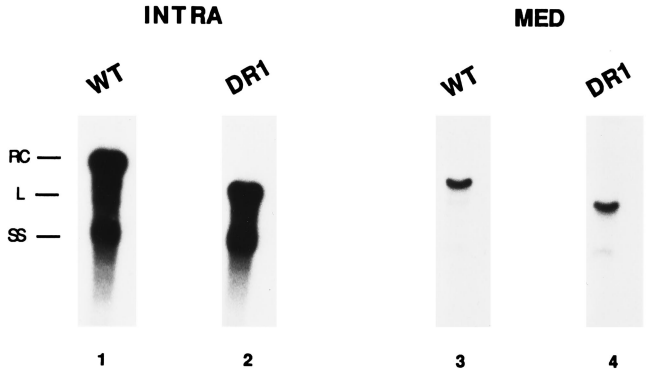

FIG. 5. Circularization of the viral genome is not required for envelopment. LMH cells were transfected with 1.5-mer constructs bearing WT or DR1 5/12 mutant DHBV genomes (7). Three days after transfection, DNA was extracted from intracellular cores (lanes 1 and 2) and extracellular virions (lanes 3 and 4) as described in the legend for Fig. 3 and examined by agarose gel electrophoresis, Southern hybridization with ^{32}P-DHBV DNA, and autoradiography. INTRA, intracellular cores; MED, media; RC, relaxed circle; L, linear duplex; SS, single strand.

(lanes 3 and 5). For the reverse transcriptase-deficient mutant, however, although particles bearing RNA were present in the cytosol, none of these particles were detectably enveloped and exported (lanes 4 and 6). Thus, some viral DNA synthesis must occur before nucleocapsids can be enveloped.

Since envelopment proceeds with maximal efficiency only when plus-strand synthesis is under way, and since genomic circularization occurs shortly after the initiation of plus-strand synthesis, one model consistent with these facts would be that circularization itself might be the trigger to efficient envelopment. If so, then 5′ DR1 mutations that interfere with plus-strand primer translocation and promote in situ priming (thereby generating duplex linear DNA) (see references 7 and 13) should impair envelopment. Accordingly, we examined the intracellular and virion DNA of a mutant (5/12 DR1) in which 5 of the 12 nucleotides of DR1 have been mutated (TACAC CCCTCTC to TATACGCCGTTA); in cells transfected with this mutant genome, 95% of the total progeny viral DNA is linear duplex (7). In agreement with recent results of Yang and Summers (17), who showed that in such a mutant linear duplex DNA can be incorporated into infectious virions, we find that double-stranded linear duplexes can be encapsidated at levels equivalent to wild-type efficiency (Fig. 5). Clearly, circularization of the genome is not the signal for envelopment.

Our observations confirm and extend previous studies on the envelopment of hepadnaviral genomes. They affirm that only mature genomes are efficiently enveloped, provide quantitation on the relative efficiency of envelopment of more immature forms, and establish for the first time that capsids bearing only RNA cannot be enveloped at all. Similar results have also recently been obtained for human HBV envelopment (2a), suggesting that these findings are generally applicable to all hepadnaviruses. In related studies, Yu and Summers (18) showed that mutations in the C terminus of the DHBV core protein that remain assembly competent displayed defects in plus-strand DNA elongation and viral DNA envelopment. Their studies are also consistent with a relationship between DNA maturation and envelopment and further suggest that a functional domain of the C protein may be required for both plus-strand elongation and envelope protein interactions.

How might the envelopment machinery sense the maturation state of encapsidated viral DNA? The simplest explanation would be that elongation of viral DNA synthesis, and particularly the priming and elongation of plus strands, triggers a subtle change in the alignment of core subunits or in their phosphorylation state (19), producing a structural alteration on the surface of the capsid. This change would then facilitate core interactions with the envelope proteins (and/or any cellular factors, e.g., molecular chaperones, that might be required for the envelopment reaction). Progress in further understanding how this process proceeds will likely require development of an in vitro system in which capsid envelopment by membrane-bound envelope precursors can be observed.

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REFERENCES

1. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of the hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol. **64:**5324–5332.
2. **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.

2a. **Bruss, V.** Personal communication.

3. **Chang, L.-J., R. Hirsch, D. Ganem, and H. Varmus.** 1990. Effects of insertional and point mutations on the functions of the duck hepatitis B virus polymerase. J. Virol. **64:**5553–5558.
4. **Ganem, D.** 1996. Hepadnaviridae: the viruses and their replication, p. 2703–2737. *In* B. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology. Raven Press, New York.
5. **Hirsch, R., J. Lavine, L. Chang, H. 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 (London) **344:**552–555.
6. **Lenhoff, R., and J. Summers.** 1994. Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. J. Virol. **68:**4565–4571.
7. **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.
8. **Mason, W. S., C. Aldrich, J. Summers, and J. M. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus strand DNA. Proc. Natl. Acad. Sci. USA **79:**3007–4001.
9. **Pollack, J., and D. Ganem.** 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. J. Virol. **67:**3254–3263.
10. **Pollack, J. R., and D. Ganem.** 1994. Site-specific RNA binding by a hepatitis B virus reverse transcriptase initiates two reactions: RNA packaging and DNA synthesis. J. Virol. **68:**5579–5587.
11. **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.
12. **Robinson, W. S., D. A. Clayton, and R. L. Greenman.** 1974. DNA of a human hepatitis B virus candidate. J. Virol. **14:**384–391.
13. **Staprans, S., 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.
14. **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.
15. **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.
16. **Wang, G.-H., and C. Seeger.** 1993. Novel mechanism for reverse transcription in hepatitis B viruses. J. Virol. **67:**6507–6512.
17. **Yang, W., and J. Summers.** 1995. Illegitimate replication of linear hepadnavirus DNA through nonhomologous recombination. J. Virol. **69:**4029–4036.
18. **Yu, M., and J. Summers.** 1991. A domain of the hepadnavirus capsid protein is specifically required for DNA maturation and virus assembly. J. Virol. **65:**2511–2517.
19. **Yu, M., and J. Summers.** 1994. Phosphorylation of the duck hepatitis B virus capsid protein associated with conformational changes in the C terminus. J. Virol. **68:**2965–2969.