JOURNAL OF VIROLOGY, Dec. 1997, p. 9392–9399 Vol. 71, No. 12
0022-538X/97/$04.00+0

Lack of Effect of Antiviral Therapy in Nondividing Hepatocyte Cultures on the Closed Circular DNA of Woodchuck Hepatitis Virus

GLORIA MORALEDA,[1] JEFFRY SAPUTELLI,[1] CAROL E. ALDRICH,[1] DEVRON AVERETT,[2] LYNN CONDREAY,[2] AND WILLIAM S. MASON[1]*

Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111,[1] and Glaxo Wellcome Company, Research Triangle Park, North Carolina 27709[2]

Received 22 May 1997/Accepted 3 September 1997

The template for synthesis of hepadnaviral RNAs is a covalently closed circular (ccc) DNA located in the nucleus of the infected hepatocyte. Hepatocytes are normally long-lived and nondividing, and antiviral therapies in chronically infected individuals face the problem of eliminating not only the replicative forms of viral DNA found in the cytoplasm but also the cccDNA from the nucleus. Because cccDNA does not replicate semiconservatively, it is not an obvious target for antiviral therapy. However, elimination of cccDNA might be facilitated if its half-life were short in comparison to the generation time of hepatocytes and if new cccDNA formation were effectively blocked. We have therefore measured cccDNA levels in woodchuck hepatocyte cultures following in vitro infection with woodchuck hepatitis virus and treatment with inhibitors of viral DNA synthesis. The viral reverse transcriptase inhibitors lamivudine (3TC) [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine), FTC (5-fluoro-2′,3′-dideoxy-3′-thiacytidine) and ddC (2′,3′-dideoxycytidine) were added to the cultures beginning at 4 days postinfection. Treatment for up to 36 days with 3TC reduced the amount of cccDNA in the cultures not more than twofold compared to that of an untreated control. Treatment with ddC for 36 days and with FTC for 12 days resulted in effects similar to that of treatment with 3TC. Moreover, the declines in cccDNA appeared to reflect the loss of hepatocytes from the cultures rather than of cccDNA from hepatocytes. These results emphasize the important role of the longevity of the infected hepatocytes in the persistence of an infection.

Hepadnaviruses have a relaxed circular, partially double-stranded DNA genome of about 3 kbp. This DNA is converted to a covalently closed circular (ccc) DNA during initiation of infection (17, 27). cccDNA is localized to the nucleus of the infected hepatocyte (18), where it serves as the template for transcription of all of the viral RNAs (19). Reverse transcription, to produce new relaxed circular viral DNA, takes place in viral nucleocapsids in the cytoplasm of the infected cell (24). During infection, some of this newly made DNA is transported to the nucleus and converted to additional cccDNA, which reaches a final in vivo copy number of about 5 to 50 per cell (9, 10, 18, 29). cccDNA accumulation is regulated by viral envelope proteins in duck hepatitis B virus (DHBV) infections (14, 25, 26) and, presumably, in all hepadnavirus infections. These proteins interact with viral-DNA-containing nucleocapsids to produce virus particles, which are then secreted by the infected hepatocyte.

With the availability of new inhibitors of hepadnaviral reverse transcription, it is possible to treat chronic carriers and obtain a rapid decline in virus production. Once viral DNA synthesis is stopped, DNA synthesis intermediates, which are precursors to cccDNA, will gradually disappear from the hepatocyte as a result of virus assembly and/or degradation. However, the cccDNA may remain. Though it can be assumed that this species would ultimately be lost as a consequence of hepatocyte turnover, this process may be very slow in healthy carriers. Even in patients with very active liver disease, the contribution of this process will still depend on the half-life of the hepatocytes, which has been estimated to range from 10 to 100 days (20). Similarly, in most woodchucks chronically infected with woodchuck hepatitis virus (WHV), there appears to be a 10- to 20-fold increase in cell proliferation (16), as assessed by an increase in the number of hepatocytes reactive with an antibody to proliferating cell nuclear antigen (6). If hepatocyte life times were 6 to 12 months in uninfected woodchucks, this observation would imply that lifetimes were still 3 to 5 weeks in carriers. Thus, elimination of cccDNA would be a slow process if it were solely dependent on the death of infected cells; for example, 6 to 12 months of complete inhibition of new cccDNA synthesis would be required to obtain a 1,000-fold decline, which would still leave a substantial pool of infected hepatocytes.

At least two processes might, however, accelerate cccDNA loss. First, it is possible that cccDNA is intrinsically unstable. For example, Civitico and Locarnini (4) reported that DHBV cccDNA has a half-life of 3 to 5 days in infected cultures of primary duck hepatocytes, which are nearly nonproliferating. Alternatively, cccDNA may be stable within a nondividing hepatocyte but may be lost during cell division, with new cccDNA normally being formed after mitosis from cccDNA precursors present in the cytoplasm. Thus, the death of an infected hepatocyte may destroy not only the resident cccDNA but also the cccDNA that was present in the hepatocyte that divided to replace the dying cell.

The present experiments were done to test an implication of the first hypothesis, that even in nondividing hepatocytes, inhibition of new cccDNA synthesis would lead to a rapid depletion of this species. These experiments were carried out by treating woodchuck hepatocytes with various reverse transcrip-

* Corresponding author. Mailing address: Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Phone: (215) 728-2402. Fax: (215) 728-3616. E-mail: ws_mason@fccc.edu.

tase inhibitors. In our experiments, inhibition of viral reverse transcription by various nucleoside analogs did not have a major effect on the retention of cccDNA in the cultures. In particular, the half-life in treated cultures appeared at least as long as estimates for hepatocyte half-life in chronic carriers. These findings support the idea that the efficacy of antiviral therapy in eliminating infected hepatocytes in vivo will depend on cell death rather than cccDNA loss within nondividing cells. These findings are consistent with antiviral trials, which suggested a correlation between the loss of cccDNA and the amount of hepatocyte turnover during therapy (5, 15).

MATERIALS AND METHODS

Primary cultures of woodchuck hepatocytes. Woodchucks were purchased from Northeast Wildlife (South Plymouth, N.Y.). All animal use was reviewed and approved by the Institutional Animal Care and Use Committee. Primary hepatocyte cultures were prepared from woodchucks negative for WHV infection, as previously described (1). Hepatocytes were seeded at confluence onto 60-mm-diameter tissue culture dishes coated with rat tail collagen, and the serum-free growth medium (3 ml) was changed daily. Hepatocytes were infected with 20 μl of serum from a chronically infected woodchuck (9×10^9 virions per ml) at 2 to 3 days after seeding. WHV-containing medium was removed after a 16-h incubation period for virus uptake. Addition of the drugs to the culture medium was carried out as described in Results.

Antiviral compounds. Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine; 3TC)] was prepared by Glaxo Wellcome Company. 2′CDG (2′ carbodeoxyguanosine) was provided by ViraChem, Inc. FTC (5-fluoro-2′,3′-dideoxy-3′-thiacytidine) was provided by Burroughs Wellcome Company. ddC (2′,3′-dideoxycytidine) was purchased from Sigma Chemical Co. (St. Louis, Mo.), and suramin was obtained from Mobay Chemical Company (New York, N.Y.).

Analysis of viral nucleic acids. Hepatocyte cultures were rinsed with phosphate-buffered saline and stored at −80°C prior to isolation of intracellular DNA or RNA. Total DNA and cccDNA isolation was performed as previously described (31). To extract total DNA, hepatocytes were lysed in 1.1 ml of a solution containing 0.8 mg of pronase per ml, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 20 mM EDTA and then incubated at 37°C for 2 h. This lysate was extracted twice with phenol-chloroform, and nucleic acids were collected by ethanol precipitation. Alternatively, for the isolation of cccDNA-enriched fractions, hepatocytes were lysed in a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% SDS, followed by addition of KCl to a final concentration of 0.5 M to precipitate protein-SDS complexes. The precipitate was removed by centrifugation, the supernatant was extracted twice with phenol and once with phenol-chloroform, and the nucleic acids were precipitated with ethanol. One quarter of the preparation from each culture was subjected to electrophoresis in 1.5% agarose and transferred to nitrocellulose (30). Total DNA concentrations were estimated by the method of Labarca and Paigen (12). Unless otherwise noted, there was no significant difference in loss of cells, as reflected by a decline in total DNA recovery, in untreated versus drug-treated cultures.

Viral RNAs were isolated with the STAT-60 RNA isolation kit according to the manufacturer's instructions (TEL-TEST, Inc., Friendswood, Tex.). Hepatocytes were disrupted in lysis solution, the lysate was extracted with chloroform, and the RNA was precipitated with isopropanol. Then, the total RNA was applied to oligo(dT)-cellulose, and the polyadenylated RNA was eluted from the oligo(dT)-cellulose and precipitated with ethanol. After precipitation, one half of the polyadenylated RNA isolated from each culture was incubated with glyoxal at 50°C (2), electrophoresed on a 1% agarose gel in a solution containing 25 mM $NaHPO_4$ (pH 6.5), 2 mM EDTA, and 0.01 mM aurintricarboxylic acid, and transferred to a nylon membrane (Hybond-N) for subsequent hybridization.

In order to assay for the release of virus, 3 ml of the tissue culture medium was centrifuged through a 20% sucrose cushion containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA for 3 h at 50,000 rpm in a Beckman SW60 rotor at 4°C. The pellet was resuspended in 25 μl of a solution containing 1 mg of pronase per ml, 0.1% SDS, 10 mM Tris-HCl (pH 7.5), and 10 mM EDTA and then incubated for 1 h at 37°C, and the entire sample was subjected to 1.5% agarose gel electrophoresis and Southern blot analysis for viral DNA. We believe that this assay detects enveloped virus, not core particles released by dying cells, because predominantly double-stranded and partially double-stranded DNAs are detected.

WHV DNA and RNA were detected by hybridization with a ^{32}P-labeled probe representing the full-length viral genome. Following hybridization, bound radioactivity was determined by using either an AMBIS radioanalytic imaging system or a Fuji Image Analyzer. A full-length, linearized, cloned viral DNA served as a hybridization standard.

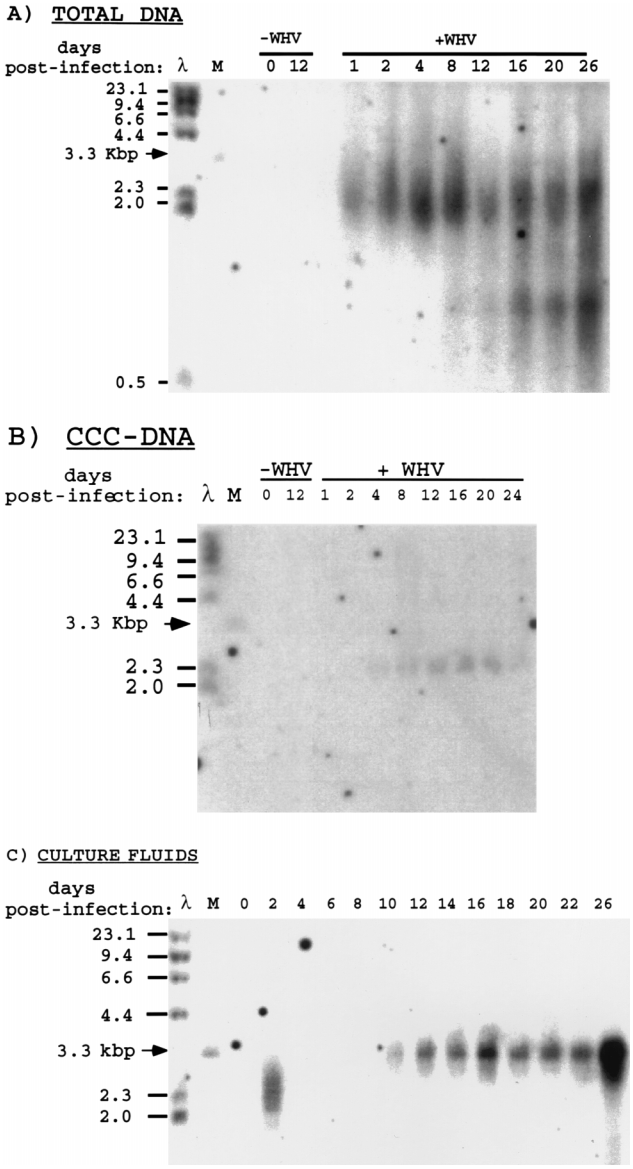

FIG. 1. Time course of viral DNA synthesis in WHV-infected hepatocyte cultures. Cultures were infected with WHV (+WHV) or not infected (−WHV) at 2 days after seeding. Total DNA (A) and cccDNA (B) were extracted from the cultures for Southern blot analysis as described in Materials and Methods. (C) The time course of production of extracellular particles was determined as described in Materials and Methods. Lanes: 0, uninfected cultures 2 days after seeding (the signal at 2 days postinfection in panel C is presumably virus from the inoculum that was absorbed to the cells and subsequently released back into the medium). Lanes M, 3.3-kbp linear WHV DNA.

RESULTS

Primary cultures of woodchuck hepatocytes support a full cycle of WHV replication. WHV infection of primary woodchuck hepatocytes develops slowly, with cccDNA appearing between 2 and 4 days and DNA replication intermediates appearing after about 1 week (1). However, it was not previously determined if such in vitro-infected cultures, like those from WHV-infected woodchucks (28), support production of new virus particles. Therefore, this analysis was carried out as a prelude to the use of these cultures in antiviral studies.

Figure 1 shows the results of an experiment in which the

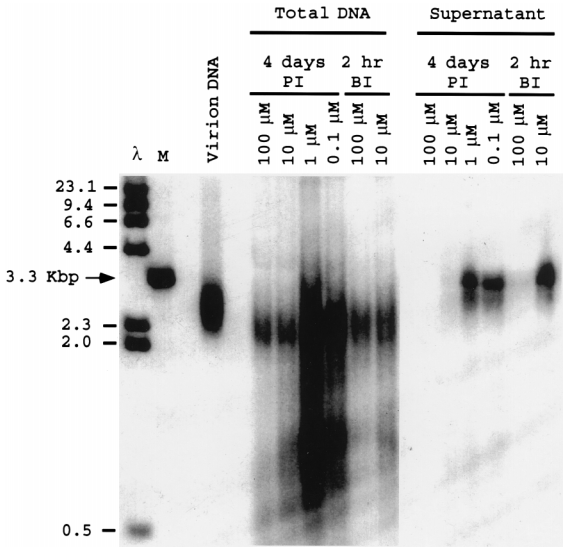

FIG. 2. 3TC inhibits release of viral particles by primary hepatocyte cultures. Two different regimens of drug were utilized. For one set of cultures, 3TC was added at 4 days postinfection (PI) and maintained in the culture medium until 16 days postinfection. In the other, drug was present only from 2 h before infection (BI) until 4 days postinfection. Again, cultures were harvested at 16 days postinfection. Total DNA and DNA in particles released into the medium were prepared and analyzed as described in the legend to Fig. 1 and in Materials and Methods. Lanes M, 75 pg of 3.3-kbp linear WHV DNA.

cultures were infected with WHV at 2 days after plating. Total DNA and cccDNA were extracted from the cultures at the indicated number of days postinfection and analyzed by Southern blotting. In this experiment, single-stranded WHV DNAs, indicative of new DNA synthesis, accumulated to detectable levels by 8 days postinfection and remained detectable until the end of the culture (26 days postinfection). cccDNA was detected at 4 days postinfection, the earliest time point examined, and did not increase greatly during the remainder of the experiment. The time course of virus release is shown in Fig. 1C. Virus was detectable by 8 days, maximal at 10 to 14 days, and declined slightly thereafter, though it was evident for as long as the cells were maintained in culture. These virus particles are most likely mature virions and not viral nucleocapsids released by dying cells, as they lack the single-stranded and smaller, partially double-stranded DNAs characteristic of the latter.

To exclude the possibility that the virions in the culture fluids were from the infecting inoculum, we asked if particle production was inhibited by 3TC, a cytosine analog previously reported to block HBV and DHBV DNA synthesis (20, 23). The appearance of particles in the culture medium at 16 days postinfection was blocked by the presence from 4 days postinfection of 100 μM 3TC, a concentration which also inhibited viral DNA synthesis in the hepatocytes (Fig. 2; also unpublished observations). There was only partial inhibition at 10 μM, and no inhibition was observed at lower concentrations of 3TC. Moreover, the appearance of extracellular particles at 16 days was partially inhibited if 3TC was present only from 2 h before to 4 days postinfection. This result again supports a role for hepatocyte infection in particle production, inasmuch as 3TC partially blocked formation of cccDNA from the infecting virus (see Fig. 4B). Thus, the primary cultures of woodchuck hepatocytes appeared to support a complete cycle of WHV replication, from infection to progeny virus production, and to be appropriate for studies of the effects of antiviral therapies on the complete cycle of virus replication.

Loss of susceptibility to infection by WHV. We previously reported that primary hepatocyte cultures retain susceptibility to WHV infection for at least the first 4 days in culture (1). However, to assess antiviral therapies, it was essential to determine if the cells remained susceptible later, since in this case recruitment might occur in the untreated but not in the treated cultures. In order to address this issue, woodchuck hepatocytes were infected at 2, 5, or 8 days after plating. Cultures were harvested 16 days postinfection and examined for the presence of cccDNA, replicative intermediate DNA, and the release of viral particles into the medium (Fig. 3). No viral DNA synthesis, no cccDNA, and no production of virus particles were detected in hepatocytes to which WHV was added at 8 days

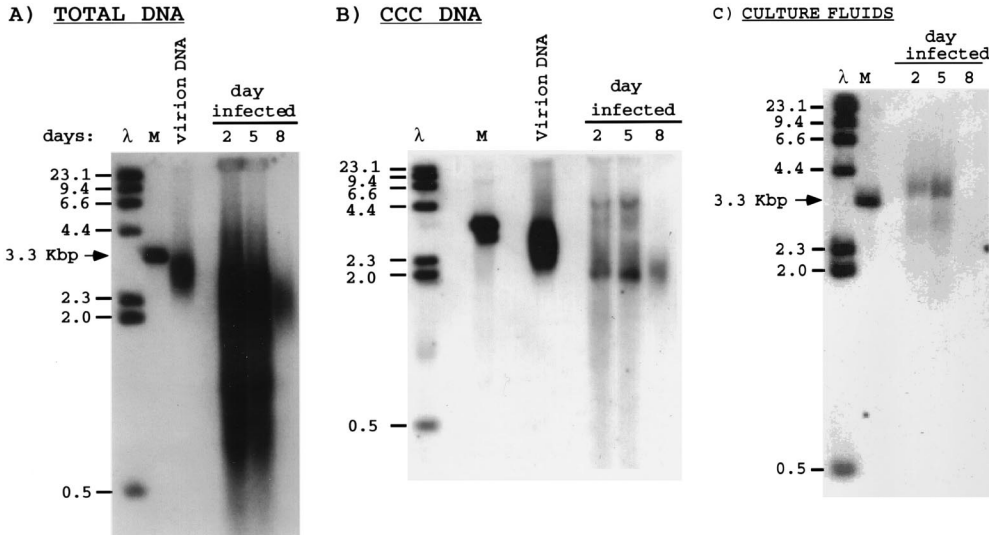

FIG. 3. Primary woodchuck hepatocytes lose susceptibility to WHV infection with time in culture. Cultures were infected with WHV at either 2, 5, or 8 days after plating, and total DNA (A) and cccDNA (B) were isolated and analyzed by 1.5% agarose gel electrophoresis. For panel C, virus particles present in the culture medium were sedimented through a sucrose gradient, and DNA was extracted for Southern blot analysis. No significant loss of total cellular DNA or deterioration of the cultures was noted, at the time of harvest, between cultures infected at 5 or 8 days after seeding. Lanes: M, 75 pg of 3.3-kbp linear WHV DNA.

after plating. (The signal seen in the cccDNA lane [8 days] in Fig. 3B has a mobility distinct from that of cccDNA and appears to be derived from the inoculum [cf. Fig. 3A and B].) Thus, the cultures appeared to lose susceptibility a few days before the detection of progeny virus in the culture fluids. Nonetheless, because of the narrow interval between loss of susceptibility and appearance of progeny virus, suramin was added to the cultures, in subsequent experiments, beginning at 24 h postinfection. Suramin blocks initiation of infection by WHV and a number of other viruses, apparently by blocking a step in virus uptake (1, 21). In separate experiments (not shown), it was found that neither suramin nor Polybrene, which also blocks initiation (1), altered the time course of infection.

cccDNA levels did not appreciably decline when hepatocytes were continuously exposed to WHV reverse transcriptase inhibitors. Using this in vitro infection system, we next assessed the effects of treatment with inhibitors of viral DNA synthesis on cccDNA levels. As shown in Fig. 1 and as previously reported (1), cccDNA levels in untreated cultures reached a level by 4 days postinfection which increased slightly (two- to threefold) over the next few weeks. Therefore, the following treatments using nucleoside analogs as inhibitors of viral replication were begun at 4 days postinfection, which was also prior to the accumulation of appreciable levels of new minus-strand DNA.

The first drugs utilized were 3TC and FTC. Either the drugs were added 2 h before infection and maintained in culture medium until 4 days after infection or they were added 4 days postinfection and remained until the end of the experiment at 16 days postinfection. Suramin (0.1 mg/ml) was also added beginning at 1 day postinfection. In preliminary studies, the antiviral activity of these two drugs was found to be similar; that is, 10 μM only partially blocked viral DNA synthesis, whereas 100 μM appeared to inhibit WHV DNA synthesis more effectively. As shown in Fig. 4, FTC at a concentration of 500 μM was similar to 3TC in its ability to inhibit WHV DNA synthesis and to partially block initiation of the infection. The final level of cccDNA in cultures to which the inhibitors were added 2 h before infection was fivefold lower than in cultures in which treatment began at 4 days postinfection. With neither drug was there an indication of cccDNA loss when viral DNA synthesis was inhibited between 4 and 16 days postinfection. Thus, the cccDNA level under these conditions appeared to be stable. In cultures in which drug was removed after 4 days, the reason why viral DNA synthesis failed to reach levels that were proportional to the cccDNA that was formed is unknown. One possibility that needs to be investigated is that intracellular pools of inhibitor remained elevated after drug was removed from the medium.

We next looked at the effect on cccDNA of treating for a longer period of time. 3TC (500 μM) was present from 4 days after infection until up to 40 days. Cells were harvested at the indicated times, and WHV DNA content was assayed by Southern blot hybridization (Fig. 5). In the 3TC-treated hepatocytes, total viral DNA levels declined slightly from the amount present at 4 days and then remained almost constant (Fig. 5A). This DNA was comprised of a mixture of cccDNA and species with an electrophoretic pattern typical of partially double-stranded, relaxed circular (RC) DNA. The presence of cccDNA was confirmed by digestion with *Hin*dIII, which cleaves just downstream from the 5′ end of the plus strand (11) and converts this species and fully double-stranded RC DNA to a 3.3-kbp linear DNA (data not shown).

A small amount of hybridization signal was also detected in the region characteristic of viral single-stranded (ss) DNAs, extending up to the full-length ss DNA. The presence of minus-strand DNA in this region was confirmed by using a strand-specific probe (data not shown). The ratio of RC DNA to ss DNA at 4 days, prior to drug addition, was about 28:1, whereas the ratio in the inoculum was greater than 110:1. This ratio increased in the treated samples between 24 and 40 days postinfection, but the signal was too close to the background level for reliable quantification. Thus, though not definitive, it is possible that a small amount of ss DNA may be produced early, prior to drug addition at 4 days postinfection, and it is also possible that some is produced in the presence of 3TC. Thus, compared to the untreated cells, the inhibition of ss DNA accumulation by 3TC at 40 days was at least 40-fold, if the amount present at 4 days was subtracted out, or 30-fold if it was not.

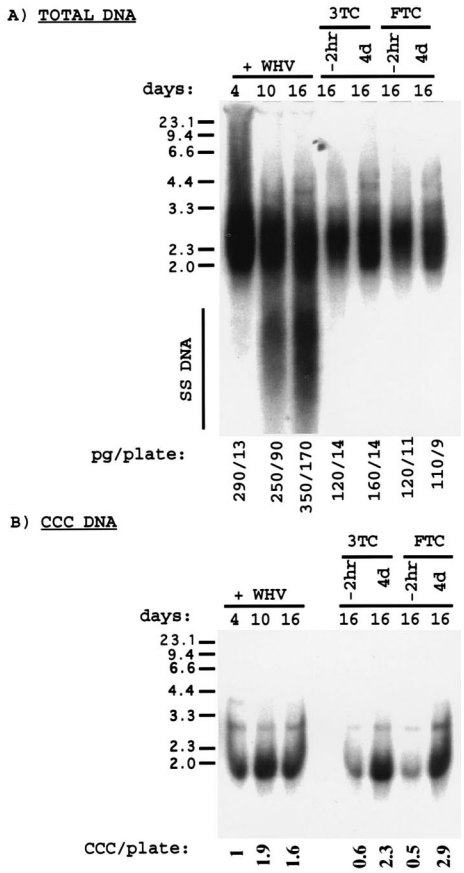

FIG. 4. Short-term inhibition of viral DNA synthesis with 3TC and FTC did not lead to loss of WHV cccDNA. DNA was prepared as described in Materials and Methods. Nucleic acids recovered from each tissue culture dish at 16 days postinfection were subjected to gel electrophoresis. (A) The total amount of viral DNA and the amount of ss DNA recovered from each dish were determined and are shown below the gels. The vertical bar marks the region of the gel containing ss viral DNAs. (B) The amounts of cccDNA, relative to the control at 4 days, which contained 60 pg of cccDNA per dish, are shown below the gels. Abbreviations: + WHV, infected with WHV; −2hr, 2 h before infection; 4d, 4 days.

The effects of 3TC treatment on cccDNA synthesis are shown in Fig. 5B. During the treatment period, we observed a twofold decline in the amount of cellular DNA in the cultures. Likewise, the survival of cccDNA during this period was about 50%. Thus, after accounting for cell loss, there appeared to be little or no loss of cccDNA from surviving cells (Table 1). The amount of cccDNA was similar in the control cultures until about 24 days, after which time there was an increase, which may be analogous to the late copy number amplification ob-

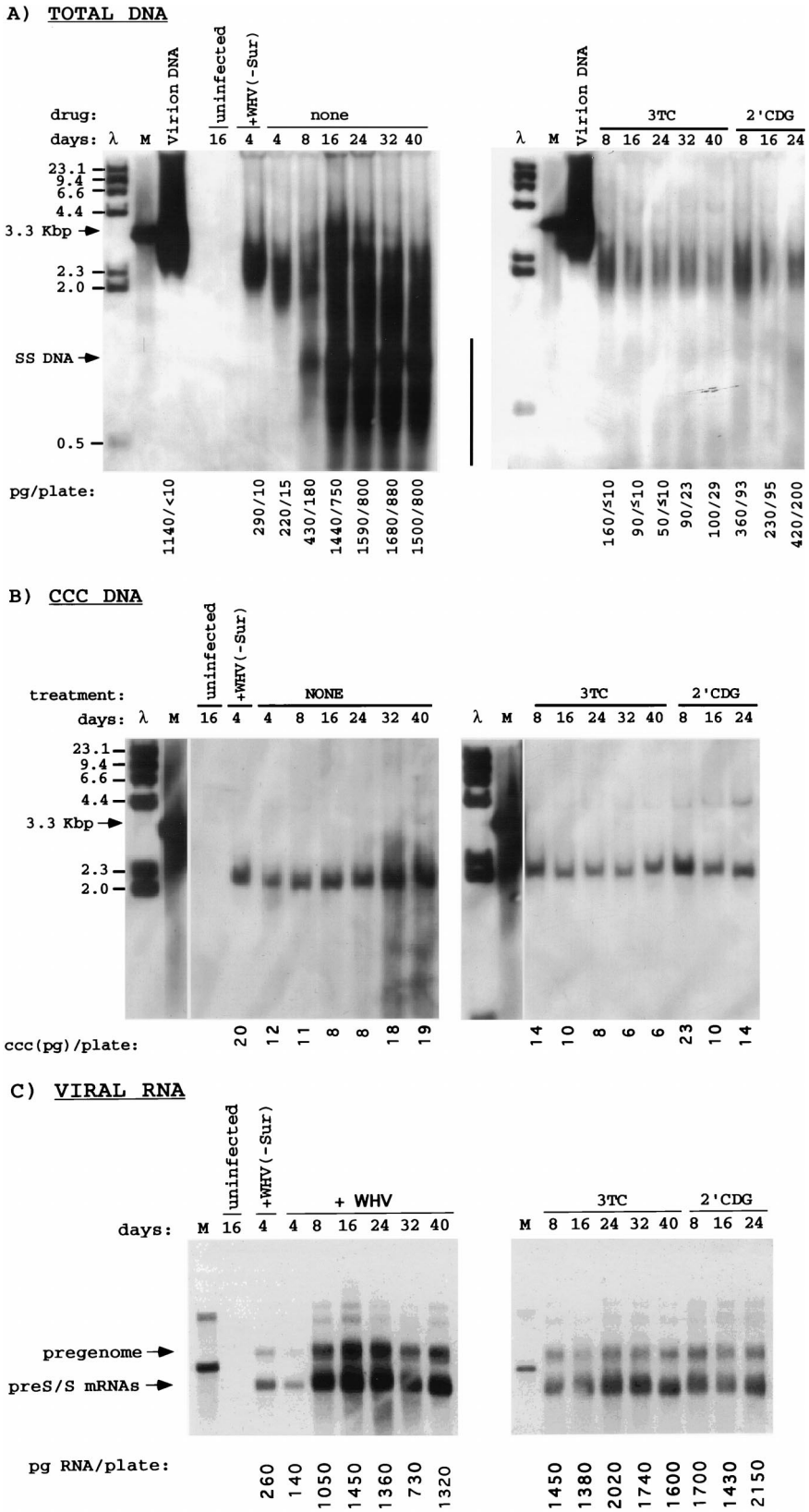

FIG. 5. Long-term inhibition of viral DNA synthesis with 3TC did not lead to loss of WHV cccDNA. Hepatocytes were treated with 3TC (500 μM) and 2′CDG (10 ng/ml). Drugs were added 4 days after infection and remained in culture medium throughout. Cells were harvested at the days indicated. Total DNA (A), cccDNA (B), and RNA (C) were analyzed by agarose gel electrophoresis and hybridized with a WHV DNA probe. The total amount of viral DNA in each lane and the amount of ss DNA are indicated below the gels. The vertical bar marks the region of the gel containing ss viral DNAs. Lanes: M, 3.3-kbp linear WHV DNA (75 pg for DNA gels; 200 pg for Northern blots). +WHV, infected with WHV; −Sur, no suramin.

TABLE 1. cccDNA levels during 5 weeks of treatment with 3TC and ddC

No. of days postinfection	cccDNA level[a] in culture				
	Not treated		Treated with 3TC		Treated with ddC, expt 2
	Expt 1	Expt 2	Expt 1	Expt 2	
4	100	100			
8	92 (108)	130 (141)	117 (133)	208 (288)	169 (164)
16	67 (75)	113 (206)	83 (108)	122 (206)	122 (201)
24	67 (92)	104 (194)	67 (92)	74 (137)	78 (176)
32	150 (216)	83 (232)	50 (83)	65 (142)	57 (133)
40	158 (258)	57 (161)	50 (116)	49 (139)	57 (175)

[a] The cccDNA data are taken from Fig. 5B and 6B and are calculated as percentages of the amount at 4 days, which is set at 100%. The numbers in parentheses show cccDNA levels corrected to the amount of total cell DNA at 4 days postinfection.

served in DHBV-infected primary duck hepatocyte cultures (29). Viral RNA (Fig. 5C) was detectable in the cultures at 4 days postinfection, increased ca. 5- to 10-fold between 4 and 8 days, and remained relatively constant thereafter. Release of virus particles from the control cultures continued for the course of the experiment, whereas no viral-DNA-containing particles were detected in culture fluids from the treated cultures (data not shown).

In parallel with 3TC, we also analyzed the effect of 2′CDG administration (Fig. 5). This drug, at a dose of 10 ng per ml, caused toxic effects which resulted in deterioration of the cultures after 24 days postinfection. Moreover, at the dose that was used, this drug was a less effective inhibitor of viral DNA synthesis than 3TC.

We next asked if ddC, used at concentration of 500 μM, would have effects similar to those of 3TC in long-term treatment. ddC was previously found to be a highly effective inhibitor of WHV DNA synthesis at this dose (1). Again, drug was added to the cultures beginning at 4 days postinfection. In this experiment, we were unable to detect ss DNAs as discrete electrophoretic species in DNA extracted from the treated cultures (Fig. 6A), though some WHV-specific signal could be detected in this region. By 16 days, inhibition of minus-strand DNA accumulation was at least 15-fold if the signal in this region at 4 days was not subtracted or 40-fold if it was.

cccDNA levels declined about twofold during treatment with ddC, roughly comparable to the amount of cell loss from the cultures. In the 3TC-treated cultures, loss of cccDNA, after correction for cell loss, was somewhat less than twofold (Table 1). With respect to the presence of viral RNAs (Fig. 6C), the levels of viral transcripts declined with time in both the treated and untreated cultures. This decline was in general agreement with the declines in cell number and in total cccDNA.

DISCUSSION

The experiments in the current study were designed to evaluate the role of intrinsic cccDNA instability as a factor in resolution of transient infections and as a potential factor in the treatment of chronic infections with viral DNA synthesis inhibitors. We have found that cccDNA levels did not change appreciably when viral DNA synthesis was inhibited for up to 36 days. In addition, levels of viral RNA remained relatively constant. Moreover, in the untreated cultures, there was only about a twofold increase in cccDNA levels during the course of the experiment, relative to the amount present at 4 days postinfection, indicating intracellular restraints on production of new cccDNA from RC DNA precursors, possibly mediated by viral envelope proteins (13, 26). At present, it is not known if cccDNA formation in these cultures during the first 4 days and later occurs entirely from infecting virus, or if there is also a contribution from the intracellular cccDNA amplification pathway described by Tuttleman et al. (29). This distinction cannot be readily made by using inhibitors of viral DNA synthesis, since for WHV, substantial plus-strand synthesis is apparently needed during initiation of infection. Thus, inhibitors should have substantial effects on initiation of infection and on the intracellular amplification pathway.

A half-life of 3 to 5 days for cccDNA was suggested from experiments of DHBV-infected cultures of primary duck hepatocytes (4). Such a half-life in our experiments would have produced, in the absence of any replacement synthesis, a 100- to 500-fold drop in cccDNA concentration within the hepatocytes. Our results do not support the idea that cccDNA has such a high rate of turnover in primary cultures of woodchuck hepatocytes. However, we cannot rule out the possibility that cccDNA turnover did occur and was compensated by residual viral DNA synthesis in the presence of the drugs. Whatever the case, inhibition of viral DNA synthesis with these drugs was insufficient to significantly reduce cccDNA levels.

The results of antiviral therapy in DHBV-infected ducks have also been consistent with the idea that cccDNA levels are relatively stable in the presence of inhibitors of viral DNA synthesis (5, 15) and that cccDNA elimination is dependent on cell death. In particular, a substantial decline in cccDNA within the liver during 3 to 6 months of therapy was seen only with 2′CDG, which was also toxic to hepatocytes, and not with two other DNA synthesis inhibitors, which were not toxic.

Nonetheless, hepadnaviruses, including WHV, can cause not only chronic infections but also transient infections. During transient infections, there is often a viremia of several weeks which is associated with infection of virtually the entire hepatocyte population. This is followed by a rapid clearance of virus from the blood and liver. While the mechanism of clearance is unknown, it is clear that conversion from complete infection of the hepatocyte population to a virus-free state can occur in less than 4 weeks (9, 10, 22). It is possible that a rapid clearance may also occur during spontaneous resolution of the viremic phase of chronic infections, which is sometimes associated with an acute exacerbation of liver disease (3).

It is evident, therefore, that a better understanding of cccDNA stability and of how cccDNA is lost during recovery from transient infections would have direct implications for treatment of chronic infections, which in some patients involve lifelong viremia. Models for viral clearance during resolution of transient infections might involve several different steps. The process presumably begins with the induction of a T-cell response to viral antigens, leading to an inflammatory response in the liver. Evidence for the next step was obtained by Guidotti and colleagues (7, 8). Using a transgenic mouse which produced HBV, they found that the inflammatory response leads to the production of cytokines, including tumor necrosis factor alpha and alpha and gamma interferons, which can induce degradation of viral RNAs as well as viral proteins and DNAs within the cytoplasm of the infected cell. These cytokines may also induce the turnover of the cccDNA in the nucleus, which would cure the cell and, ultimately, the liver. However, this can only be determined by appropriate studies of natural infections, since cccDNA is not produced in the transgenic mice (8). Alternatively, cccDNA may be intrinsically unstable and spontaneously disappear after the precursor pool in the cytoplasm is destroyed (7). However, unless elimination

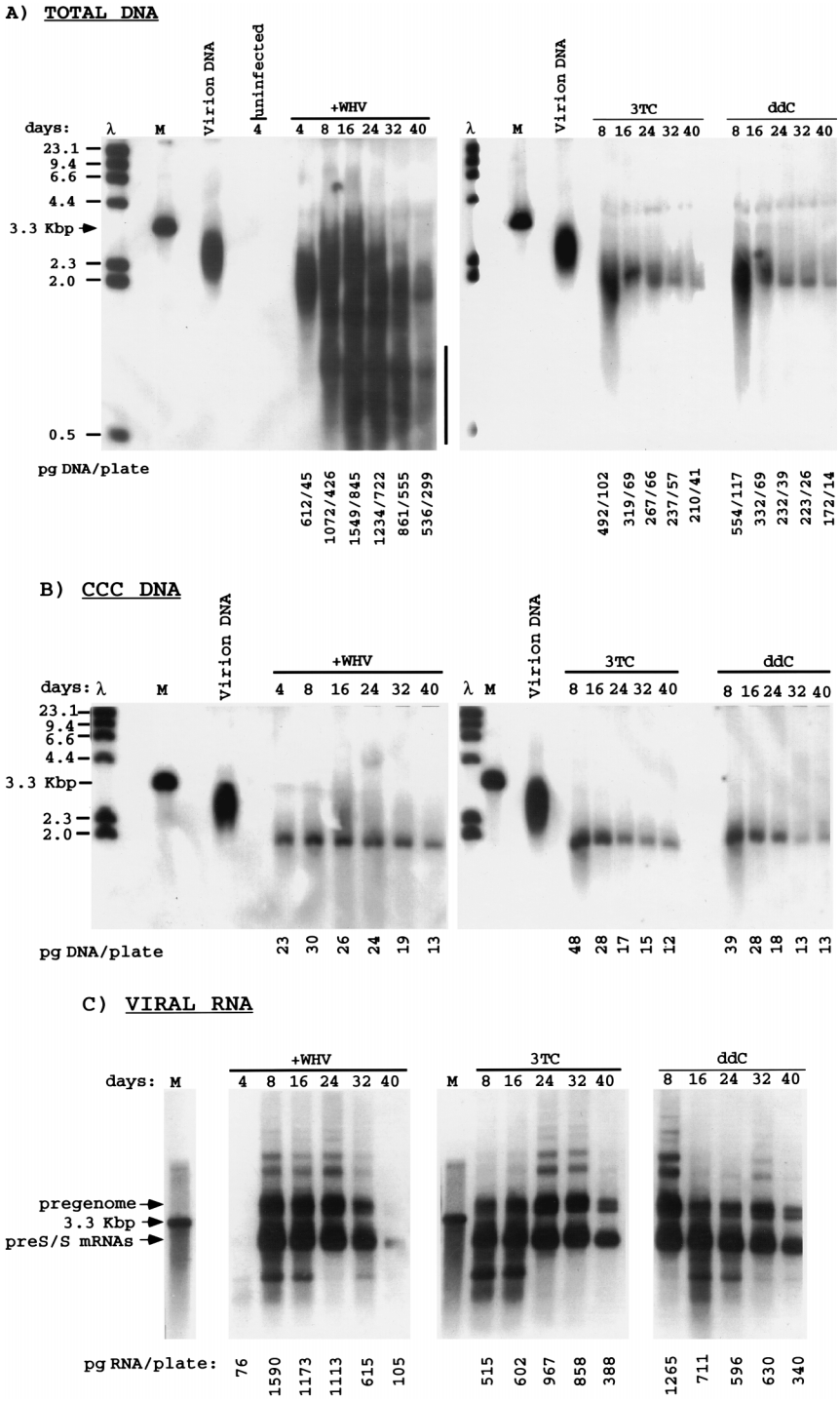

FIG. 6. Long-term inhibition of viral DNA synthesis with ddC did not lead to loss of WHV cccDNA. Antiviral effects of 3TC and ddC in woodchuck hepatocyte cultures were determined. Cells were treated with 3TC (500 μM) or ddC (500 μM) from 4 days after WHV addition (+WHV) until the end of the culture. Total DNA (A), cccDNA (B), and RNA (C) were extracted at the days indicated and subjected to Southern or Northern blot analysis. The total amount of viral DNA in each lane and the amount of ss DNA are indicated below the blots or gels. The vertical bar marks the region of the gel containing ss viral DNAs. Lanes: M, 3.3-kbp linear WHV DNA (75 pg for DNA gels; 200 pg for Northern analysis).

of replicative intermediate DNAs from the cytoplasm was complete and sustained over many weeks, it is difficult to understand, from the results reported here and elsewhere (4, 5, 15), how this would lead to cccDNA clearance. Another possibility is that cccDNA is too stable to be rapidly eliminated from nonproliferating hepatocytes and other processes are needed. For example, destruction of infected hepatocytes would directly eliminate cccDNA. Likewise, the surviving cccDNA might be lost as the remaining hepatocytes are induced to proliferate to maintain liver cell mass. The specula-

tion here is that cccDNA is lost during cell division because of its dispersal following breakdown of the nuclear membrane.

A key question in resolving these various issues is whether hepatocytes can recover from an infection without going through one or more rounds of cell division. Such a demonstration will require methods for monitoring individual cells within the liver. Though theoretically possible, there are formidable difficulties in achieving this goal, especially with the outbred hosts which are naturally infected with the known hepadnaviruses. A more practical goal may be to determine how the immune response changes to initiate the rapid clearance of transient infections after many weeks of massive infection of the liver. Such knowledge might be directly applicable to treatment of chronic infection, even if the underlying mechanisms were not known.

ACKNOWLEDGMENTS

We are grateful to J. Taylor, C. Seeger, and J. Summers for a critical review of the manuscript.

This work was supported by USPHS grants AI-18641, CA-57425, CA-40737, CA-06927, GM-35535, and RR-05509 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania. G. Moraleda received a Fellowship from the Ministerio de Educacion y Ciencia (Spain).

REFERENCES

1. **Aldrich, C. E., L. Coates, T. T. Wu, J. Newbold, B. C. Tennant, J. Summers, C. Seeger, and W. S. Mason.** 1989. In vitro infection of woodchuck hepatocytes with woodchuck hepatitis virus and ground squirrel hepatitis virus. Virology **172:**247–252.
2. **Carmichael, G. G., and G. K. McMaster.** 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enmyol. **65:**380–391.
3. **Chu, C. M., Y. F. Liaw, C. C. Pao, and M. J. Huang.** 1989. The etiology of acute hepatitis superimposed upon previously unrecognized asymptomatic HBsAg carriers. Hepatology **9:**452–456.
4. **Civitico, G. M., and S. A. Locarnini.** 1994. The half-life of duck hepatitis B virus supercoiled DNA in congenitally infected primary hepatocyte cultures. Virology **203:**81–89.
5. **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 duck hepatitis B virus during antiviral therapy of chronically infected ducks. J. Virol. **68:**8321–8330.
6. **Galand, P., and C. Degraef.** 1989. Cyclin/PCNA immunostaining as an alternative to tritiated pulse labelling for marking S phase cells in paraffin sections from animal and human tissues. Cell Tissue Kinet. **22:**383–392.
7. **Guidotti, L. G., T. Ishikawa, M. V. Hobbs, B. Matzke, R. Schreiber, and F. V. Chisari.** 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity **4:**25–36.
8. **Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari.** 1995. High-level hepatitis B virus replication in transgenic mice. J. Virol. **69:**6158–6169.
9. **Jilbert, A. R., T.-T. Wu, J. M. England, P. de la 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.
10. **Kajino, K., A. R. Jilbert, J. Saputelli, C. E. Aldrich, J. Cullen, and W. S. Mason.** 1994. Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. J. Virol. **68:**5792–5803.
11. **Kodama, K., N. Ogasawara, H. Yoshikawa, and S. Murakami.** 1985. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutional relationship between hepadnaviruses. J. Virol. **56:**978–986.
12. **Labarca, D., and K. Paigen.** 1980. A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem. **102:**344–352.
13. **Lenhoff, R. J., and J. Summers.** 1994. Construction of avian hepadnavirus variants with enhanced replication and cytopathogenicity in primary hepatocytes. J. Virol. **68:**5706–5713.
14. **Lenhoff, R. J., 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.
15. **Luscombe, C., J. Pedersen, E. Uren, and S. Locarnini.** 1996. Long-term ganciclovir chemotherapy for congenital duck hepatitis B virus infection in vivo: effect on intrahepatic-viral DNA, RNA, and protein expression. Hepatology **24:**766–773.
16. **Mason, W., D. Averett, J. Pugh, K. Kajino, J. Cullen, I. Fourel, and A. Jilbert.** 1996. Antiviral therapy for chronic hepadnavirus infections. Antiviral Ther. **1:**129–141.
17. **Mason, W. S., M. S. Halpern, J. M. England, G. Seal, J. Egan, L. Coates, C. Aldrich, and J. Summers.** 1983. Experimental transmission of duck hepatitis B virus. Virology **131:**375–384.
18. **Miller, R. H., and W. S. Robinson.** 1984. Hepatitis B virus DNA forms in nuclear and cytoplasmic fractions of infected human liver. Virology **137:**390–399.
19. **Newbold, J. E., H. Xin, M. Tencza, G. Sherman, J. Dean, S. Bowden, and S. Locarnini.** 1995. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J. Virol. **69:**3350–3357.
20. **Nowak, M. A., S. Bonhoeffer, A. M. Hill, R. Boehme, H. C. Thomas, and H. McDade.** 1996. Viral dynamics in hepatitis B virus infection. Proc. Natl. Acad. Sci. USA **93:**4398–4402.
21. **Petcu, D. J., C. E. Aldrich, L. Coates, J. M. Taylor, and W. S. Mason.** 1988. Suramin inhibits in vitro infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus. Virology **167:**385–392.
22. **Ponzetto, A., P. J. Cote, E. C. Ford, R. H. Purcell, and J. L. Gerin.** 1984. Core antigen and antibody in woodchucks after infection with woodchuck hepatitis virus. J. Virol. **52:**70–76.
23. **Severini, A., X.-Y. Liu, J. S. Wilson, and D. L. J. Tyrrell.** 1995. Mechanism of inhibition of duck hepatitis B virus polymerase by (−)-β-L-2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **39:**1430–1435.
24. **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.
25. **Summers, J., P. M. Smith, and A. L. Horwich.** 1990. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J. Virol. **64:**2819–2824.
26. **Summers, J., P. M. Smith, M. J. Huang, and M. S. Yu.** 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. J. Virol. **65:**1310–1317.
27. **Tagawa, M., M. Omata, O. Yokosuka, K. Uchiumi, F. Imazeki, and K. Okuda.** 1985. Early events in duck hepatitis B virus infection. Sequential appearance of viral deoxyribonucleic acid in the liver, pancreas, kidney, and spleen. Gastroenterology **89:**1224–1229.
28. **Theze, N., P. Gripon, I. Fourel, O. Hantz, C. Trepo, C. Guguen-Guillouzo.** 1987. Maintenance of woodchuck hepatitis virus activity in woodchuck hepatocyte primary culture. J. Gen. Virol. **68:**1029–1039.
29. **Tuttleman, J., C. Pourcel, and J. Summers.** 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus infected cells. Cell **47:**451–460.
30. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76:**3683–3687.
31. **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 replication is amplified by an intracellular pathway. Virology **175:**255–261.