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Infection Initiated by the RNA Pregenome of a DNA Virus

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We describe experiments demonstrating that after transfection into permissive cells, the RNA pregenome of an avian hepadnavirus, the duck hepatitis B virus, is infectious. Using a Sindbis virus expression vector, we showed that cytoplasmic synthesis of the pregenome resulted in hepadnaviral DNA synthesis. Moreover, complete infectious virus was produced from cells transfected with hepadnaviral pregenomic RNA. We conclude that the pregenome of hepadnaviruses can express all the proteins required for DNA synthesis as well as serve as a template for reverse transcription and that DNA resulting from pregenome expression can be utilized to establish a productive infection in pregenome-transfected cells.

Many positive-strand RNA viruses contain RNA genomes that are infectious when they are isolated from viral structural proteins and introduced into the cell by artificial techniques. Such viral RNAs are infectious because they can be translated directly to produce the enzymes required for the initial replication and transcription of the viral genome as well as serve as templates for genome replication (1). In this report, we describe experiments that demonstrate that the RNA pregenome of an avian hepadnavirus, a DNA-containing virus, is likewise infectious. This result implies that, like the genomes of positive-strand RNA viruses, the hepadnavirus pregenome can be translated to produce all the replication proteins as well as serve as a template for viral DNA synthesis by reverse transcription (22). The biological activity of the pregenome that we report here confirms certain models of the hepadnavirus replication cycle that have been derived from molecular studies of the separate steps in virus replication.

In a normal cycle of hepadnavirus infection (Fig. 1), pregenomes and envelope mRNAs are transcribed (step a) from a molecule of covalently closed circular DNA (cccDNA) (16, 19, 26) that is contributed by the infecting virus. Pregenomes are packaged into nucleocapsids along with a virus-encoded multifunctional protein which contains a reverse transcriptase activity (P protein). After assembly, relaxed circular viral DNA is synthesized by reverse transcription of the pregenome within the nucleocapsid (step c). The capsid protein is thought to be produced by translation (step b) of pregenome transcripts (3, 17), but it is uncertain whether the P protein is also produced by translation of the pregenome (4, 11, 17) or by translation of other minor RNA species (5, 21, 25). If the pregenome were to serve as an mRNA for both capsid and P proteins, then intracellular production of the pregenome in the absence of any other viral transcripts would result in the synthesis of viral DNA. If, however, other minor or spliced transcripts are required for P-protein expression, viral DNA should not result from the expression of the pregenome alone.

We used an expression vector based on a cytoplasmic RNA virus to express the pregenome of the duck hepatitis B virus (DHBV) in the absence of any transfected DNA. Under these conditions, DHBV DNA was synthesized in the transfected cell. We also introduced a facsimile of the DHBV pregenome, synthesized in vitro, into a chicken hepatoma cell line permissive for DHBV replication and synthesis of cccDNA by using lipofectin-mediated transfection. The synthesis of intracellular DHBV DNA and the production of extracellular infectious virus by these cells indicate that the hepadnavirus pregenome is infectious.

MATERIALS AND METHODS

Plasmids. The recombinant plasmid pTRDHBV-62-ts6 (labeled pTRDHVB) was produced from elements of two Sindbis virus cDNA-containing plasmids, pTRCAT-62 and pTSCAT-ts6 (30). pTRCAT-62 contains DNA encoding the 5′ end of Sindbis virus genomic RNA, which contains the nonstructural genes required for RNA replication, and 62 nucleotide pairs derived from the 3′ end of Sindbis virus-encoding sequences, which are required in *cis* for RNA replication. A temperature-sensitive mutation (*ts*6 [8]) derived from pTSCAT-ts6 was introduced into the nonstructural genes by fragment replacement, and DHBV sequences cloned into the region encoding the subgenomic transcript were derived from the DHBV clone sequenced by Mandart et al. (15). The pTRDHBV-62-ts6 plasmid thus contained an SP6 promoter, cDNA encoding the nonstructural proteins of Sindbis virus, the transcriptional start site for the Sindbis virus subgenomic mRNA, DHBV pregenome-encoding sequences, 62 bp which encode the Sindbis virus 3′ end, and 30 bp encoding a poly(A) stretch. Some 3,294 bp of DHBV DNA from nucleotides 2530 through 2802 (*Afl*II to *Dra*III) were positioned so that the first base of the pregenome coincided with the Sindbis virus subgenomic transcriptional start site. Two nucleotides in the pregenome were mutated to preserve the translational stop codon for the Sindbis virus nonstructural proteins, which overlaps the subgenomic mRNA. The plasmid map is shown (Fig. 2a) linearized at the unique *Mlu*I site used for runoff transcription, and the in vitro transcription product [TRDHBV(+)] and the products produced in vivo, the Sindbis virus minus strand [TRD HBV(−)] and its transcription products, are indicated below. The plasmid pSPDHBV-polyA contained the SP6 promoter upstream of 3,294 bp of DHBV DNA from nucleotides 2530 through 2802, followed by 30 bp which encode a poly(A) stretch and a *Sal*I site used for runoff transcription. The DHBV DNA was positioned so that the SP6 transcriptional start site coincided with the first nucleotide of the pregenome. The plasmid is shown (Fig. 2b) linearized by

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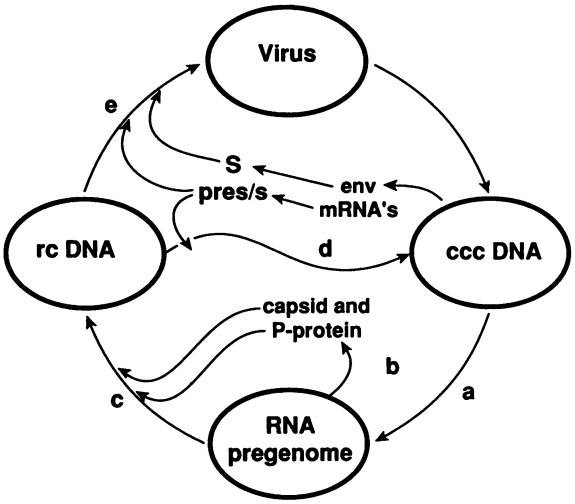

FIG. 1. Replication cycle of hepadnaviruses. See text for discussion of the steps in virus replication. rc, relaxed circular.

*Sal*I, and the in vitro transcription product, the pregenome, is shown below. Plasmids were constructed by standard recombinant DNA techniques. In vitro mutagenesis was performed by the method of Kunkle et al. (13) on single-stranded DNA-containing fragments subcloned into pUC119 (28).

In vitro RNA synthesis and transfection. For the synthesis of Sindbis virus-DHBV recombinant RNA, 1 μg of pTRD HBV-62-ts6 DNA linearized by digestion with *Xmn*I was incubated in the 10-μl reaction mixture containing 40 mM Tris-HCl (pH 7.4), 6 mM Mg acetate, 10 mM NaCl, 2 mM spermidine, 10 U of RNasin, 1 mM each UTP, ATP, and CTP, 0.5 mM GTP, 0.5 mM GpppG (cap analog), and 2 U of SP6 RNA polymerase (Promega). After 4 h at 37°C, the reaction was digested with RNase-free DNase (RQ1 DNase; Promega) and phenol extracted, and the RNA was recovered by ethanol precipitation. The RNA from the reaction was mixed with 10 μg of lipofectin in 0.2 ml of phosphate-buffered saline and applied to a layer of 2×10^6 BHK-21 cells seeded the day before in a 35-mm plastic tissue culture dish. After 10 min at room temperature, the lipofectin was removed, 2 ml of culture medium (F12 medium plus Dulbecco modified Eagle medium) containing 10% fetal bovine serum was added to the dish, and the cells were incubated at either the permissive (30°C) or the nonpermissive (40°C) temperature. For the production of DHBV pregenomic RNA, 1 μg of pSPDHBV-polyA was transcribed as described above and used to transfect 2×10^6 LMH cells seeded the previous day on a 35-mm dish. LMH cells were incubated at 37°C.

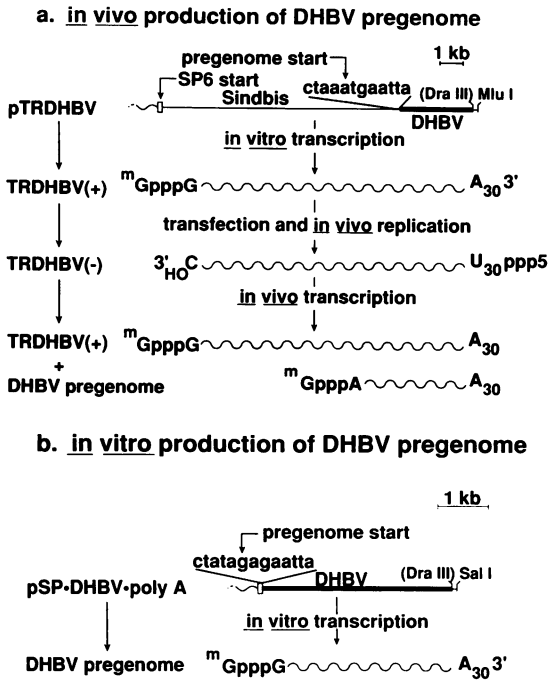

FIG. 2. Recombinant plasmids and the pregenomes produced in vivo and in vitro.

Analysis of transfected cells and supernatants. The methods used for the extraction and assay of intracellular DHBV DNA and RNA, for the assay of enveloped DNA-containing particles, and for the assay of infectious DHBV by using primary duck hepatocytes have been previously described (23, 24).

RESULTS

DHBV DNA synthesis results from the cytoplasmic expression of the RNA pregenome by a Sindbis virus vector. DHBV DNA sequences encoding the pregenomic transcript were cloned into a Sindbis virus vector cDNA positioned downstream of a bacteriophage SP6 promoter (30). Cloning was done so as to substitute the DHBV DNA for the region that normally encodes the Sindbis virus structural proteins. This region is expressed as a subgenomic mRNA during Sindbis virus replication. Transcription of the plasmid in vitro with SP6 RNA polymerase yielded a Sindbis virus recombinant RNA which was capable of replication and the production of large amounts of subgenomic mRNA, in this case the DHBV pregenome, after transfection into susceptible cells. The structures of the plasmid we constructed, the recombinant vector produced in vitro, and the pregenome produced in vivo are summarized in Fig. 2a.

Runoff RNA transcripts of the recombinant Sindbis virus plasmid pTRDHBV-62-ts6 were introduced into BHK21 cells by transfection, and total RNA isolated from the transfected cell cultures was analyzed by RNA blot hybridization with a riboprobe specific for the detection of DHBV plus strands. Within 1 day after transfection at 30°C, two species of DHBV-specific RNAs were detected (Fig. 3, upper panel). The large transcript of 11 kb was of the size expected for genomic recombinant Sindbis virus RNA, while the size (3.4 kb) of the smaller species corresponded to that expected for the DHBV pregenome produced as a Sindbis virus subgenomic transcript. The absence of these species in cells incubated at 40°C, a temperature that is nonpermissive for replication of the temperature-sensitive Sindbis virus vector that we utilized (8), confirmed that these species were generated by Sindbis virus replication and not by retention or processing of the input transfected RNA.

Total cytoplasmic DNAs from the transfected cultures incubated at 30°C contained DHBV species migrating at the position expected for single-stranded DHBV minus strands and for double-stranded relaxed circular DHBV genomes (Fig. 3, lower panel). The absence of these species in transfected cultures incubated at 40°C confirmed that the production of these viral DNAs was dependent on the

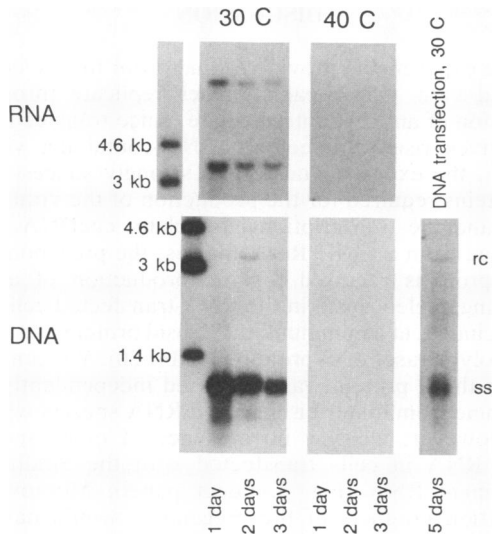

FIG. 3. DHBV-specific RNA and DNA produced after transfection of a Sindbis virus-DHBV recombinant RNA. Upper panel: Northern (RNA) blot of total RNA extracted from BHK21 cells transfected with TRDHBV-62-ts6 RNA. At 1, 2, and 3 days posttransfection, RNA was purified from the cell layer by the acid phenol-guanidinium thiocyanate method (6). RNA was glyoxylated and electrophoresed through a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with a riboprobe specific for the detection of DHBV plus strands (26). The leftmost lane contains glyoxylated DNA molecular size markers. Lower panel: Southern blot analysis of DNA extracted from BHK21 cells transfected with Sindbis virus-DHBV recombinant RNA. At 1, 2, and 3 days posttransfection, the cells were lysed with transfection lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Nonidet P-40), and the cytoplasmic supernatant fraction was recovered after a brief microcentrifuge centrifugation. Viral DNA was purified from this fraction by pronase digestion, phenol extraction, and ethanol precipitation (23). Viral DNA was analyzed by electrophoresis through a 1% agarose gel, transfer to a nylon filter, and hybridization with a riboprobe specific for the detection of DHBV minus strands (26). The leftmost lane contains double-stranded DNA molecular size markers. Lower right: DHBV-specific DNA in BHK21 cells after transfection with a DHBV-containing plasmid DNA. pUC119-DHBV-CMV (3 μg) was transfected into BHK21 cells as described above, and at 3 and 5 days postinfection, cytoplasmic DNA was recovered and analyzed as above except that the cytoplasmic fraction was first adjusted to 6 mM Mg acetate and treated with 100 μg of DNase I per ml for 30 min at 37°C before pronase digestion. The migration positions of relaxed circular (rc) and single-stranded (ss) DHBV DNA are indicated.

replication of the Sindbis virus vector and the consequent production of the subgenomic RNA transcripts. In a parallel experiment, BHK21 cells were transfected with an infectious DHBV-containing plasmid DNA in which production of the RNA pregenome was driven by the cytomegalovirus immediate-early promoter (24). The viral DNA pattern detected in these cells after incubation at 30°C for 5 days was similar to that seen in the cells transfected with recombinant DHBV-Sindbis virus RNA (Fig. 3, bottom right panel). The results demonstrated that expression of the DHBV pregenome alone was sufficient for the synthesis of viral DNA.

Transfection of pregenomic RNA results in production of cccDNA and infectious virus. We tested whether the viral DNA synthesized in RNA-transfected cells could be utilized to initiate a complete cycle of virus replication. DNA-containing nucleocapsids, early during an infectious cycle, are utilized for the production of more cccDNA molecules (Fig. 1, step d) which then serve as additional templates for transcription of pregenomes and envelope mRNAs (23). The conversion of relaxed circular viral DNA to nuclear cccDNA may occur independently of the expression of viral genes, as in the infecting virus. Alternatively, conversion to cccDNA during amplification could occur through a different pathway, one that depends on viral functions expressed from cccDNA that is derived directly from the infecting genome. In the former case, nucleocapsids synthesized as a result of pregenome expression in the RNA-transfected cell should be converted to cccDNA and should initiate a complete infectious replication cycle. In the latter case, nucleocapsids alone would be unable to generate cccDNA and an infectious cycle.

To distinguish between these possibilities, we produced a close facsimile of pregenomic RNA by transcription in vitro of a plasmid containing the DHBV pregenome-encoding sequences alone positioned downstream of an SP6 promoter (Fig. 2b). The purified transcript was then transfected into a chicken hepatoma cell line, LMH (12), previously shown to be permissive for DHBV cccDNA amplification (7). Analysis of the transfected cells revealed that DHBV replicative intermediates and relaxed circular DNA accumulated over a period of at least 7 days posttransfection (Fig. 4a). To determine whether the production and amplification of cccDNA in the RNA-transfected cells could contribute to the accumulation of replicative intermediates, we assayed for the presence of cccDNA using a selective extraction (23) and Southern blot hybridization. In addition, we assayed cells transfected with pregenomes containing a mutation in the pre-S envelope protein, which causes the infected cell to produce high levels of cccDNA (24). cccDNA could be easily detected in both sets of transfected cells, with higher levels occurring as a consequence of the envelope gene mutation (Fig. 4b). This result indicated not only that cccDNA was produced but that the pre-S envelope mRNA was also expressed, presumably from the cccDNA. In a separate experiment, we showed that RNase but not DNase destroyed the infectivity of the nucleic acid in LMH cells (Fig. 4c).

Culture fluids were harvested from RNA-transfected LMH cells, and particles were concentrated by polyethylene glycol precipitation and analyzed for the presence of DNA-containing enveloped particles by fractionation in an isopycnic cesium chloride gradient (24). The fractions of the gradient contained particles with mature DHBV relaxed circular DNA banding at the buoyant density expected of enveloped virus (Fig. 4d). Since both DHBV envelope proteins are required for the production of enveloped DNA-containing particles (24), this result indicated that envelope mRNAs were present inside the RNA-transfected cells. The combined results suggest that envelope mRNAs were produced by transcription of cccDNA.

Concentrated particles from culture fluids were also assayed for the presence of infectious DHBV by infection of primary cultures of duck hepatocytes (18, 27). These cultures were found to contain replicative intermediates of DHBV DNA at 6 and 12 days postinfection, indicating the presence of infectious DHBV in the culture fluids of RNA-transfected LMH cells (Fig. 4e). Therefore, DHBV nucleocapsids produced within the cell as a result of RNA transfection were able to initiate a complete cycle of infection.

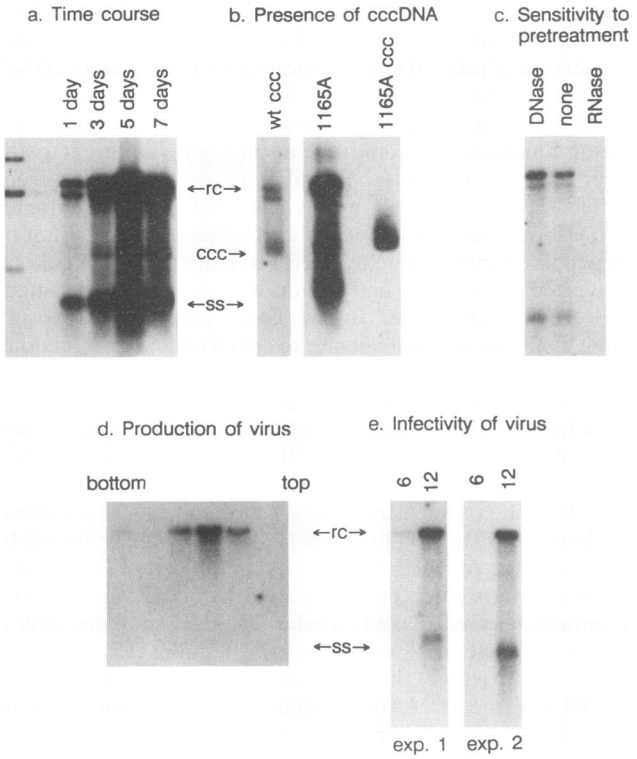

FIG. 4. Replication of DHBV in LMH cells after transfection with in vitro-transcribed pregenome RNA. (a) DHBV DNA synthesis in transfected LMH cells. RNA prepared by runoff transcription of plasmid pSPDHBV-polyA was transfected into 2×10^6 LMH cells seeded the day before in 35-mm tissue culture dishes, as described in the legend to Fig. 3. Cells were maintained for the indicated times at 37°C, after which cytoplasmic viral DNA was purified and analyzed by Southern blot hybridization. The leftmost lanes contain molecular size markers of 4.6, 3.0, and 1.4 kp. (b) cccDNA synthesis in RNA-transfected cells. LMH cells were transfected with either the wild-type pregenome or the mutant 1165A pregenome and incubated at 37°C for 5 days. cccDNA (wt ccc and 1165A ccc) or replicative intermediates (1165A) were extracted and assayed by blot hybridization. The replicative intermediate pattern for the wild-type transfected cells is shown in panel a in the lane labeled 5 days. (c) Sensitivity of transfecting nucleic acid to RNase. Wild-type pregenome RNA (3 μg) was incubated for 15 min at 37°C with DNase I, RNase A, or no addition. The surviving nucleic acid was used for transfection of LMH cells, and the cells were incubated at 37°C for 4 days. Cytoplasmic viral DNA was extracted and assayed by agarose gel electrophoresis and blot hybridization. (d) Enveloped DNA-containing particles released from wild-type pregenome-transfected LMH cells. Culture fluids were collected between 5 and 9 days posttransfection and concentrated by polyethylene glycol precipitation (24). The concentrated particles were centrifuged to equilibrium in an isopynic gradient of cesium chloride (24) (starting density, 1.21 g/cm^3), and fractions of the gradient were analyzed for DHBV DNA by Southern blot hybridization. Bottom and top fractions of the gradient are indicated. (e) Replicative intermediates in duck hepatocyte cultures infected with wild-type pregenome-transfected LMH culture fluids. Particles prepared by polyethylene glycol precipitation as described above were used to infect a culture of primary duck hepatocytes. After 6 and 12 days, cytoplasmic viral DNA was extracted from the infected cells (18) and analyzed for DHBV replication by Southern blot hybridization. The results of two separate transfection experiments are shown. Migration positions of relaxed circular (rc), ccc double-stranded (ccc), and single-stranded (ss) DHBV DNAs are indicated.

DISCUSSION

These experiments provide formal proof for the currently accepted view that hepadnaviruses replicate through the production of an RNA intermediate, since transfection with RNA gives rise to infectious DNA-containing virus. In addition, the experimental results strongly suggest that all the proteins required for the production of the viral nucleocapsid and the transcriptional template, cccDNA, can be expressed from a single RNA species, the pregenome.

The proteins required for the production of a DNA-containing nucleocapsid in the RNA-transfected cells would have included, at a minimum, the capsid protein and the viral DNA polymerase, or P protein (10, 14, 20). We cannot rule out that the P protein was expressed independently of the pregenome from minor or degraded RNA species within the cells; however, we saw no evidence of other species of DHBV RNA in cells transfected with the Sindbis virus recombinant RNA (Fig. 3, upper panel). Moreover, any degradation products of the pregenome would have been poorly translated, since they would have lacked a cap structure. Assuming that intact pregenomes were responsible for the production of all the viral products seen after transfection, we could draw the conclusion that pregenomic RNA not only serves as a template for viral DNA synthesis but also can supply all the replication proteins by executing dual mRNA functions. Whether a single pregenome molecule could carry out both the template and the mRNA functions that result in DNA synthesis was not addressed directly by these experiments, since we do not know how many copies of the pregenome were sufficient to initiate an infection in one cell.

Circumstantial evidence from previous studies, however, suggests that a single pregenome molecule can have both mRNA function and template function. First, it has been shown that the viral DNA polymerase, or P protein, acts to replicate preferentially the DNA genomes of the genotype from which it was expressed (2, 9, 29). This *cis* effect could be explained if the pregenome which served as a template for DNA synthesis was the same molecule as that which acted as the mRNA for the packaged P protein. Second, the P protein has been shown to be required for packaging of the pregenome into a nucleocapsid (2, 9). Thus, close proximity of the newly synthesized P protein to its translation template may provide a mechanism by which the P protein selects in *cis* a particular pregenome for packaging.

Previous studies have indicated that the P open reading frame may be expressed through internal translational initiation at the P-protein start codon in the pregenome (4, 17). These studies have not, however, ruled out that expression of a functional P protein during virus replication might require the presence of minor spliced transcripts or transcripts initiated from a cryptic promoter upstream of the P open reading frame. The experiments reported here provide additional evidence that the expression of the proteins required for DHBV DNA synthesis does not require novel transcripts produced by splicing in the nucleus or by the action of a weak transcriptional promoter, since Sindbis virus subgenomic RNA is produced in and confined to the cytoplasm, and since the viral replication cycle could be initiated by transfection of the pregenome in the absence of any viral DNA sequences.

RNA- and DNA-containing nucleocapsids produced as a result of RNA transfection were formed in the absence of other events that normally occur early in infection, i.e., entry of virus particles, formation of cccDNA, or expression

of viral genes from the infecting DNA. These capsids nevertheless were apparently capable of initiating a complete replication cycle through the formation of cccDNA and therefore could be considered functionally equivalent to nucleocapsids contributed by infecting virions. Since infected cells contain 10^3 to 10^4 nucleocapsids per cell, these intermediates must constitute an important intracellular reservoir of viral genomes for sustaining a persistent infection.

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