Duck Hepatitis B Virus Polymerase Acts as a Suppressor of Core Protein Translation

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Nucleocapsid assembly in hepadnavirus replication requires selective encapsidation of the pregenomic RNA template and the viral polymerase by the core proteins. It has been shown that an encapsidation signal located at the 5′ end of the pregenomic RNA is responsible for its interaction with the polymerase. In the present study, we have shown that a region located at the 3′ periphery of the core open reading frame may interact with the viral polymerase in duck hepatitis B virus. By using an in vitro rabbit reticulocyte lysate translation system, we found that interaction of the polymerase with this region resulted in selective suppression of core mRNA translation. Insertion of this putative inhibitory sequence into the CD4 gene also led to a selective inhibition of CD4 mRNA translation in the presence of polymerase. Specific inhibition of core protein synthesis was observed in a chicken hepatoma cell line (LMHI) cotransfected with core and polymerase plasmid DNA.

The hepadviruses are a family of small, enveloped, hepatotropic DNA viruses. To date, five members of this virus family have been identified: the hepatitis B viruses of humans (HBV) (16), woodchucks (43), ducks (DHBV) (33), ground squirrels (34), and herons (42). All hepadnaviruses have similar genome sizes (approximately 3.0 to 3.2 kb), morphological structures, and replication strategies (for reviews, see references 39, 41, and 48). Viral DNA replication takes place in a replicating core which consists of an RNA template, a viral polymerase, and core protein molecules (3, 7, 24, 30). Encapsulation of the RNA template is under stringent regulatory control. Several species of the genome length RNA transcripts have been identified in HBV- and DHBV-infected cells (5, 50). All these transcripts have colinear 3′ ends but heterogeneous 5′ ends (4, 15) (Fig. 1A). The long-genome-length transcripts, which are referred to as precore (pre-C) RNAs, encompass both the pre-C and the core (C) open reading frames (pre-C ORF and C ORF, respectively) at the 5′ end, whereas the shortest-genome-length transcript, which is generally known as pregenomic RNA, contains only the C ORF at the 5′ end. The pregenomic RNA transcript is selectively encapsidated.

Recently, several laboratories have identified a region (termed ε) in the pregenomic RNA transcript which is essential for packaging and DNA replication (6, 9, 10, 18, 21, 24). This encapsidation region is characterized by the presence of a stem-loop structure which is believed to serve as a docking site for the binding of the polymerase (2, 18, 24, 38). In HBV, the encapsidation region contains a 99-nucleotide (99-nt) sequence near the 5′ end of the pregenomic RNA (10, 24), whereas in DHBV, a large region of approximately 1,200 nt, located approximately 35 nt downstream from the cap site of the pregenomic RNA, is required for encapsidation (18) (Fig. 1A). Since mutant DHBV genomes bearing insertions within this region are packaged efficiently, it is believed that the actual recognition elements for encapsidation contain discontinuous sequences located within this region (18). A more detailed analysis by Calvert and Summers (6) has shown that two regions, both of which are within this 1,200-nt stretch in DHBV, are required in cis for encapsidation.

It is interesting that both the pre-C and the pregenomic RNAs contain the stem-loop structure (see Fig. 1A) yet only the pregenomic RNA transcript is encapsidated. The discrimination against the pre-C RNA has been attributed to translational inactivation (35), a process in which translating ribosomes that advance into the encapsidation sequence prevent polymerase or the polymerase-core complex from binding to the stem-loop structure. In contrast, scanning ribosomes advancing on the pregenomic RNA are believed to be inhibited by the stable secondary structure present in the encapsidation region (25, 28, 35), which leaves the stem-loop structure available for binding to the polymerase. Although this model explains the selective advantage of the pregenomic RNA over the pre-C RNA with respect to encapsidation, it does not explain how encapsidation of the pregenomic RNA can take place in the midst of translation, during which the translation initiation site and C ORF (located within the encapsidation region) are presumably loaded with translating ribosomes (27, 49). Using this model, one would predict that the presence of translating ribosomes on the pregenomic RNA would interfere with the encapsidation process.

In this communication, we present evidence that the DHBV polymerase interacts with a sequence located at the 3′ periphery of the C ORF. Interaction of the polymerase with this region suppresses core protein synthesis. This observation suggests that translating ribosomes on the C ORF might be displaced by the polymerase-RNA interaction, and the encapsidation signal in the pregenomic RNA is exposed for interaction of the polymerase or polymerase-core protein complexes during the process of nucleocapsid assembly.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Gibco BRL (Burlington, Ontario, Canada) and Boehringer Mannheim (Laval, Quebec, Canada) and were used according to the manufacturers’ protocols. [35S]Methionine was purchased from Du Pont (Mississauga, Ontario, Canada). The MEGAscript in vitro transcription kit was purchased from Ambion Inc. (Austin, Tex.). The rabbit reticulocyte lysate in vitro translation kit was purchased from Promega Co. (Madison, Wis.).
DHBV DNA fragment was subsequently cloned into pTZ19R which had been previously cut with PstI and Smal. pcDNA-CORE was constructed by ligating a 1.8-kbp fragment of the DHBV sequence (nt 2632 to 960) into the pcDNA-Ampl vector. The DHBV fragment was generated by PCR with primers Bam HI-DHBV2632 (TCT GAC CAT GGA TTC GAT TCT TGG TTA TAT AGT) and Bst EI (nt 2647 and AH960). This amplified DNA fragment was digested with EcoRI and ligated with vector pcDNA-Ampl which had been previously cut with the same enzymes. The DHBV core gene mRNA was transcribed from the CMV promoter in pcDNA-CORE.

pSP72Tat and pCEP4-Tat, which contain the human immunodeficiency virus (HIV) tat open reading frame cloned downstream from a T7 promoter and a CMV promoter, respectively, were kindly provided by J.-J. Chang (University of Alberta, Edmonton, Alberta, Canada). pSP72-T4, which contains the entire human CD4 sequence under the control of a T7 promoter, was also provided by J.-J. Chang. pCD4BstII and pCD4BX are two DHBV-CD4 recombinant constructs in which a 471-bp fragment of the DHBV sequence was cloned in frame at the BstEI site within the CD4 ORF or at the BamHI site downstream from the CD4 ORF, respectively. pCD4BstEI was constructed by amplifying a DHBV fragment (nt 401 to 872) with primers DHBV401BstEI (5'-GTA AAA TGG GTT ACC CGC TTA GGA AAA AAA TTA CCT-3') and DHBV872BstEI (5'-AGG CTG CTG GAT AAC CCA AGT TGG TTT ACC AGT ATT TCT-3'). The amplified fragment was digested with BstEI, gel purified, and ligated with pSP72-T4 which had been linearized with BstEI and dephosphorylated with calf intestine alkaline phosphatase according to standard procedures (40). For pCD4BX, the DHBV fragment was amplified by PCR with primers DHBV401BstEI (5'-ACA CTT GGA TCC GGC TAG GAA ATG AT TAC CTG GCG TGG TAG) and DHBV872XhoI (5'-AAT AATTACCTG-3') and ligated with pSP72-T4 which had been previously cut with the same enzymes.

In vitro transcription and translation. Plasmid pTZ19R-BstII was linearized with PstI, purified, and transcribed in vitro by using an Ambion MEGAscript kit. Translation of the DHBV polyasome was carried out as described previously (19).

DNA templates which were intended for generation of the parental (P) and truncated (D1 to D5) core mRNAs were linearized at SalI, BglII, SpI, and AorI sites, respectively, prior to in vitro transcription. In the cases of pSP72Tat, pSP72-T4, pCD4BstII, and pCD4BX, the plasmids were linearized with SpI, BamHI, BamHI, and XhoI, respectively. In vitro transcription and translation were carried out by procedures recommended by the suppliers of the kits used (Ambion Inc. and Promega Co.).

Immunoprecipitation. Fifty microliters of each in vitro-translated product was incubated on ice with 150 μl of RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 50 mM Tris-HCl [pH 8.0]) and 2 μl of either polyclonal rabbit anti-DHBV antibody or anti-HIV type 1 (anti-HIV-1) Tat antibodies (supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases; antiserum to HIV-1 Tat was from Bryan Cullen, Albert Einstein College of Medicine). The immuno precipitated complex was washed three times with RIPA, resuspended in 50 μl of SDS sample buffer, and analyzed by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (40). After fluorography, the gel was dried and exposed to Kodak XAR film at room temperature. The typical exposure time was 12 to 16 h.

Transfection. The chicken hepatoma cell line LMH was maintained in Dulbecco minimal essential medium and F12 medium (1:1 [vol/vol]) (Gibco BRL) supplemented with 10% fetal calf serum, 2 μM glutamine, and 50 μM IU of penicillin and 10 μg of streptomycin per ml. Cells were seeded at 40 to 50% confluence in a six-well tissue culture dish 24 h before transfection. A total of 10.2 μg of plasmid DNA was introduced into the cells by the calcium phosphate precipitation method as described by Kingston et al. (22). After 16 h of incubation at 37°C with 5% CO₂, the medium containing the CaPO₄-DNA precipitates was removed and replaced with fresh growth medium. Cells were harvested 72 h posttransfection and analyzed for core protein production. Mock-transfected cells were treated with CaPO₄ but without the addition of DNA.

Extraction and analysis of core protein. Cells were lysed by the addition of 200 μl of lysis buffer (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, and 1% Nonidet P-40) 72 h following transfection. Nuclei and cellular debris were removed by microcentrifuge centrifugation, and 10 μl of the supernatant was boiled in 20 μl of SDS sample treatment buffer for 5 min. Samples were analyzed by SDS-PAGE (10% polyacrylamide gels) and electroblotted to a nitrocellulose membrane by standard procedures (40). Core and Tat proteins were detected with polyclonal rabbit anti-DHBV antibody (diluted 1:500) and anti-HIV-1 Tat antibodies (diluted 1:1,000), respectively, followed by polyclonal goat anti-rabbit immunoglobulin G antibodies conjugated with alkaline phosphatase. Protein bands were visualized by development with 5-bromo-4-chloro-3-indolyl phosphate (NBT) and...
RESULTS

Inhibition of core mRNA translation in the presence of increasing amounts of polymerase protein. The pregenomic RNA of hepadnaviruses serves dual functions: it acts both as a pregenomic RNA template for viral DNA replication (35) and as an mRNA for the synthesis of both the core and polymerase proteins (36). Assembly of the virus replicating core is likely to occur in the midst of translation of the core and polymerase mRNAs. It has recently been shown that polymerase interacts with the stem-loop structure present at the 5’ end of the pregenomic RNA during nucleocapsid assembly (Fig. 1A) (3, 8, 38). It is intriguing that a few molecules of polymerase (estimated to be one to two copies per virion) are able to effectively compete with the translating ribosomes for binding to the encapsidation region. We have postulated that interaction of the polymerase with a region in the pregenomic RNA might displace the translating ribosomes, hence exposing the stem-loop structure for binding with the polymerase. If our hypothesis is correct, we would expect to see inhibition of core protein synthesis in the presence of the polymerase protein. The following experiments were designed to demonstrate the effect of the polymerase protein on the translation efficiency of core mRNA.

We have previously expressed DHBV polymerase in vitro by using a rabbit reticulocyte lysate. The in vitro-translated polymerase possesses both DNA polymerase and reverse transcriptase activities when tested on exogenous or endogenous templates (19). We have now constructed an expression plasmid, pTZ19RCore, which contains the DHBV C ORF under the control of a T7 promoter (Fig. 1B). This construct does not contain the putative stem-loop structure that is known to interact with the polymerase (45, 47). In vitro transcription and translation of this construct generated a protein of approximately 31 kDa (Fig. 2, lane 2). This protein was not present in the sample translated in the absence of core mRNA (Fig. 2, lane 1). To evaluate the effect of the polymerase protein on core mRNA translation, the polymerase protein was first synthesized in vitro by using a rabbit reticulocyte lysate. The production of the polymerase protein was verified by measuring [35S]methionine incorporation and analyzing the lysate by SDS-PAGE. The translation of the core mRNA in the presence of increasing amounts of the lysate containing the polymerase protein was examined. The final reaction products were analyzed by SDS-PAGE (12% polyacrylamide gels). With increasing amounts of the polymerase-containing lysate, a decreasing amount of core protein synthesis was observed (Fig. 2, lanes 3 to 6).

The lysate used in this study contained both the polymerase protein and its corresponding mRNA. In order to exclude the possibility that the polymerase mRNA contributes to the suppression seen in core mRNA translation, we constructed a polymerase mutant which contained double frameshifts in the P ORF. Transcription of this construct generated a mutant polymerase mRNA with two additional nucleotides (one at nt 397 and the other at nt 1657). However, translation of this mutant mRNA did not result in any protein product. We tested the translation of the core mRNA in the presence of lysate containing increasing amounts of this mutant polymerase mRNA; no inhibition of core protein synthesis was observed (result not shown). Taken together, these results suggest that the inhibition of core mRNA translation is due to the polymerase protein.

Suppression of translation mediated by the DHBV polymerase protein is specific and exclusive to DHBV core mRNA. In order to show that the inhibition of core mRNA translation in the presence of the polymerase protein was not a general inhibitory effect seen with coexpression of proteins in this system, we performed a similar experiment with increasing amounts of HIV Tat protein added to the reticulocyte lysate. The results, shown in Fig. 3A, demonstrate that core protein synthesis was not inhibited by increasing amounts of lysate containing Tat protein (lanes 2 to 4). A smaller species (~27 kDa), which might have resulted from the internal initiation of the C ORF during translation, was also detected. The presence and quantity of this species varied among the different batches of the reticulocyte lysate. No inhibition of core mRNA translation was seen when an in vitro-translated lysate containing proteins produced from five different species of brome mosaic virus mRNA was used (results not shown). These results suggest that the inhibition of core mRNA translation was not due to nonspecific inhibition by mRNAs or proteins. We therefore attributed the observed arrest of core mRNA translation to specific inhibition mediated by the polymerase protein.

We have also considered the possibility that the specific inhibition of core mRNA translation mediated by the polymerase protein could be due to binding of the polymerase to translational factors rather than direct interaction with the core mRNA. To distinguish between these two possibilities, we translated the human CD4 mRNA in the presence and absence of the polymerase protein. If polymerase protein interacts with translational factors, suppression of CD4 mRNA translation would be expected. No inhibition of CD4 translation was observed in the presence or absence of polymerase (Fig. 3B, lanes 1 and 2). In addition, in samples which contained both core and CD4 mRNAs, only the core mRNA translation was inhibited in the presence of the polymerase protein (Fig. 3B, lanes 3 and 4). We have also examined the effect of the polymerase on translation of the core mRNA in the presence of increasing amounts of polymerase protein. First, 2 μg of polymerase mRNA was translated in 100 μl of rabbit reticulocyte lysate in the presence of [35S]methionine at 30°C for 1 h as described previously (19). This lysate, now containing polymerase protein, is identified as Polymerase. Core represents a solution containing 2 μg of core mRNA mixed with 100 μl of rabbit reticulocyte lysate containing [35S]methionine and a methionine-free amino acid mixture. Lysate represents rabbit reticulocyte lysate plus [35S]methionine and a methionine-free amino acid mixture. Twenty microliters of lysate containing core mRNA (Core) was translated in the presence of increasing amounts of lysate containing polymerase protein (Polymerase) as indicated. The final volume of the reaction mixture was brought up to 60 μl by the addition of lysate. Lane 1, lysate alone; lane 2, core mRNA translated in the absence of polymerase; lanes 3 through 6, core mRNA translated in the presence of 5, 10, 20, and 40 μl of lysate containing polymerase protein, respectively. In vitro-translated products (1.5[S]methionine labeled) were electrophoresed on SDS–10% polyacrylamide gels and detected by autoradiography. The positions of molecular mass markers are indicated to the right of the figure.
protein on the translation of *Xenopus* elongation factor 1 mRNA and did not observe any inhibition (result not shown).

**DHBV polymerase interacts with the 3' periphery of the C ORF.** We attempted to define the region in core mRNA which is responsible for binding the polymerase protein. The expression plasmid pTZ19RCore, which was used in the previous experiments, was constructed by inserting a PCR fragment of the DHBV sequence starting at nt 2632 (17 nucleotides upstream from the AUG codon of the C ORF) and ending at nt 960. The transcript generated from this construct will not contain the putative stem-loop structure described by Hirsch et al. ([Fig. 1B](#)) (18). Since the encapsidation sequence in DHBV was found to consist of an extensive region (18), it is probable that the polymerase interacts with the regions downstream from the cap site. We constructed a series of 3'-truncated core mRNAs (D1 to D5) ([Fig. 4A](#)) and compared their translation efficiencies in the presence and absence of the polymerase protein. The translation products were immunoprecipitated and ana-

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**FIG. 3.** Specificity of inhibition of core mRNA translation by polymerase. (A) Translation of core mRNA in the presence of HIV Tat protein lysate. One microgram of *tat* mRNA was translated in vitro at 30°C for 1 h in the presence of 50 µl of rabbit reticulocyte lysate and [35S]methionine. One microgram of core mRNA was mixed with 0 µl (lane 1), 5 µl (lane 2), 7.5 µl (lane 3), or 12.5 µl (lane 4) of in vitro-translated HIV Tat protein lysate. Each reaction mixture was brought up to a final volume of 25 µl by the addition of rabbit reticulocyte lysate translation mix. Translated products were labeled with [35S]methionine and immunoprecipitated with anti-DHBV cAg and anti-HIV-1 Tat specific antibodies and analyzed by SDS–12% PAGE. (B) Effect of polymerase on the translation of core and CD4 mRNAs. Translation reaction mixtures containing rabbit reticulocyte lysate translation mix (total volume, 50 µl) were incubated with 1.5 µg of CD4 mRNA (lanes 1 and 2) or a mixture of 1.5 µg of core mRNA and 0.8 µg of CD4 mRNA (lanes 3 and 4). Translation was carried out in the presence or absence of lysate containing polymerase protein (Pol) (10 µl). The [35S]methionine-labeled reaction products were electrophoresed on SDS–12% polyacrylamide gels and detected by autoradiography. The positions of molecular mass markers are indicated to the left of panel A and to the right of panel B.

**FIG. 4.** Inhibition of translation of truncated core mRNAs by polymerase lysate. (A) Schematic diagram of the truncated core mRNAs. The locations of the ORFs of the core (Core) and polymerase (Pol) proteins are shown as open boxes. D1 to D5 are the 3'-truncated core mRNAs transcribed from pTZ19RCore which had been linearized at the positions indicated. P represents core mRNA generated from pTZ19RCore. Core proteins translated from truncated core mRNAs were quantified with a PhosphorImager. The means of the results obtained from four experiments are summarized on the far right. Symbols: −, <5% inhibition; +, 5–25% inhibition; ++, >25% inhibition; ++++, >50% inhibition; ++++, >95% inhibition. (B) Translation of truncated core mRNAs in the absence or presence of polymerase. The translation products (final volume, 25 µl) containing 1 µg of parental (P) or 3'-truncated core RNAs (D1 to D5) were incubated in the absence or presence of 12.5 µl of lysate containing polymerase protein (Pol). The [35S]methionine-labeled translation products were immunoprecipitated with polyclonal rabbit anti-DHBcAg antibodies, analyzed by SDS–12% PAGE, and detected by autoradiography. The positions of molecular mass markers are indicated to the right of each gel.
lyzed by SDS-PAGE (10% polyacrylamide gels). As shown in Fig. 4B, translation of the core mRNA transcribed from the parental construct was inhibited in the presence of the polymerase protein (lanes 1, 2, 9, and 10). No inhibition was found with D1 (Fig. 4B, lanes 3 and 4), indicating that sequences upstream from nt 140 may not contain the region responsible for binding the polymerase protein. However, inhibition of translation of truncated mRNAs D2 to D4, which contained increasing lengths of the 3' downstream sequence, in the presence of polymerase began to appear (Fig. 4B, lanes 5 to 8, 11, and 12). With the D5 truncated mRNA, translation was inhibited to the same extent as that of the parental core mRNA (Fig. 4B; compare lanes 13 and 14 with lanes 9 and 10). The reduced amounts of translation products obtained with D5 truncated mRNA (Fig. 4B, lane 13) relative to those obtained with the other mRNAs (P to D4) (Fig. 4B, lanes 1, 3, 5, 7, 9, and 11) were not seen in repeat experiments (data not shown).

In summary, results from the translation of the 3'-truncated core mRNAs in the presence of the polymerase protein identified a region of about 700 nt (nt 140 to 828) at the 3' periphery of the C ORF which may be responsible for binding the polymerase protein. With increasing lengths of the sequence within this region, progressive inhibition of core mRNA translation was observed.

Insertion of the DHBV sequence into a heterologous RNA resulted in translation inhibition. To confirm that this region causes the downregulation of core mRNA translation, presumably by binding the polymerase, we examined the possibility that addition of this sequence to a heterologous mRNA would render it susceptible to inhibition by the polymerase. Translation of CD4 was found to be unaffected by the presence of the polymerase protein (Fig. 3B). We inserted the DHBV sequence (nt 401 to 870) into plasmid pSP72-T4, which contains a human CD4 coding sequence cloned downstream from a T7 promoter. Two recombinant constructs were made (Fig. 5A). One construct (pDCD4BstEII) contains the DHBV sequence inserted in frame 877 nt downstream from the translation initiation site. Transcription and translation of this construct will produce a fusion protein of approximately 72 kDa. Another construct (pDCD4BX) contains the DHBV sequence cloned immediately downstream from the CD4 ORF. Transcription and translation of this construct will produce a CD4 protein (~58 kDa) similar to that produced by the parental plasmid, pSP72-T4.

Figure 5B shows the translation products obtained from these DHBV-CD4 recombinant constructs. A DHBV-CD4 fusion protein (~72 kDa) and a CD4 protein (~58 kDa) were produced from pDCD4BstEII and pDCD4BX, respectively (Fig. 5B, left panel). The constructs produced proteins of the predicted molecular weights. Cotranslation of mRNAs generated from pSP72-T4 and pDCD4BstEII yielded the corresponding proteins (Fig. 5B, right panel, lane 1), indicating that these two mRNAs together can be translated efficiently. However, cotranslation of these two mRNAs in the presence of the polymerase resulted in the production of only the 58-kDa species (Fig. 5B, right panel, lane 2), indicating that translation of the pDCD4BstEII mRNA was selectively inhibited. Likewise, the presence of the DHBV sequence in pDCD4BX also resulted in suppression of CD4 protein synthesis in the presence of the DHBV polymerase protein (Fig. 5B, right panel, lanes 3 and 4). Since the DHBV sequence was inserted downstream from the CD4 ORF in pDCD4BX, this result suggests that the interaction of the polymerase with an RNA sequence outside the ORF can mediate inhibition of translation of the mRNA. In this study, we have demonstrated that the presence of the DHBV sequence (nt 401 to 870) is sufficient to cause...
which the DHBV DNA fragment containing the C ORF was removed and subsequently cloned downstream from a CMV promoter for eukaryotic expression. pcDNA-POL contains a replication-competent DHBV polymerase gene which is expressed by a CMV promoter. To evaluate the effect of the polymerase on core gene expression within cells, we transfected the LMH cells with 2 μg of pcDNA-CORE and increasing amounts of pcDNA-POL (2 to 8 μg). The total amount (10 μg) of plasmid DNA used for the transfection was kept constant by adding the plasmid pcDNAI-Amp. Cells were harvested 3 days posttransfection, and core protein was analyzed by immunoblotting with anti-DHBV cAg antibodies. Transfection of LMH cells with pcDNA-CORE and its vector, pcDNAI-Amp, resulted in the production of a core protein of approximately 31 kDa (Fig. 6A, lane 6), whereas no core protein was detected in mock-transfected cells (Fig. 6A, lane 1).

Cotransfection of LMH cells with pcDNA-CORE and increasing amounts of pcDNA-POL led to a gradual decrease in core protein synthesis (Fig. 6A, lanes 2 to 5). In contrast, the expression of human growth hormone was not affected in the presence of the polymerase (Fig. 6A, transfection efficiency). This suggests that the inhibition of core mRNA translation by polymerase is specific. These observations are consistent with those observed in vitro (Fig. 2) and suggest that the presence of the polymerase does interfere with core protein synthesis in vivo.

In another experiment, we demonstrated that the selective inhibition of core protein synthesis in vivo is specific to the polymerase. LMH cells were cotransfected with core and HIV Tat expression plasmids. As shown in Fig. 6B, transfection with increasing amounts of pCEP4-Tat resulted in corresponding increases in the production of Tat protein (lanes 2 to 5), the presence of which, however, did not affect core protein synthesis (compare lanes 2 to 5 with lane 6). The reduced amount of protein in samples cotransfected with pcDNA-CORE and pCEP4-Tat was due to lower transfection efficiency (lanes 2 to 5). After normalization, the amount of core protein produced in samples cotransfected with the pcDNA-CORE and pCEP4-Tat plasmids was comparable to that of the control. This observation provides evidence that polymerase selectively inhibits the translation of core mRNA in vivo, which is consistent with the results observed in vitro. We have also examined the effect of Escherichia coli β-galactosidase on core expression in LMH cells. No inhibition was observed in this cotransfection study (results not shown).

DISCUSSION

Regulation of translation is a common mechanism for controlling gene expression in both prokaryotes and eukaryotes (26, 29). Autosuppression of translation was first characterized in bacteriophage R17, in which the coat protein binds to the RNA synthetase mRNA and suppresses its translation (23).

Since then, similar mechanisms have been described in eukaryotic systems, such as the mRNAs for yeast ferritin, human thymidylate synthase, and human dihydrofolate reductase (11, 12, 23). We have demonstrated here that DHBV may also use suppression of translation as a means of regulating core protein synthesis.

In this report, we have shown that the DHBV polymerase serves as a suppressor of core mRNA translation. We have demonstrated that suppression of the translation is not due to any systematic effect, such as competition among the mRNAs for the translating ribosomes, interference with translation by an exogenous protein, or interaction of the polymerase protein with the translational factors or ribosomes (Fig. 3A and B). We attribute the translation suppression to direct interaction of the polymerase with the core mRNA. This hypothesis is supported by the observation that insertion of the putative binding sequence of DHBV into or downstream from the CD4 coding sequence leads to an inhibition of the recombinant CD4
mRNA translation in the presence of the DHBV polymerase protein (Fig. 5B). Furthermore, we have localized the binding region of the core mRNA to the 3' end, downstream from the C ORF (Fig. 4B). More importantly, the selective inhibition of core mRNA translation by the polymerase that was seen in vitro was also observed in cells cotransfected with core and polymerase expression plasmids (Fig. 6A and B). This suggests that the polymerase may play a role in regulating core protein synthesis in the replication cycle of hepadnaviruses. We believe this regulation of core protein synthesis is the result of binding of the polymerase protein to core mRNA, resulting in inhibition of translation. We have not demonstrated direct binding of the polymerase to the core mRNA, nor can we exclude other possibilities, such as increased degradation of core mRNA after interaction with the polymerase protein.

It is interesting that the putative inhibitory region resides at the 3' end and is downstream from both the C ORF in DHBV (Fig. 4A) and the CD4 ORF in PDCDBX (Fig. 5A). The translational arrest seen with these mRNAs may be a consequence of binding of the polymerase protein at this region, which leads to bending of the pregenomic RNA by the polymerase, causing interference with translation initiation or elongation. Such changes in RNA conformation have been found to result from other RNA-protein interactions, such as HIV TAR-Tat and RRE-Rev and alfalfa mosaic virus RNA4-CP (1, 13, 44). However, the data currently available are not sufficient to define the mechanism by which the polymerase interferes with the translation process.

The results of the present study contribute important insights to an understanding of the mechanism of the hepadnaviral nucleocapsid assembly. During the process of nucleocapsid formation, there is a cis preference for encapsidation of the nascent mRNA (17). A stem-loop structure present at the 5' end of the HBV and DHBV pregenomic RNAs has been identified as the recognition site for the polymerases (37, 45–47). The results of our study, however, suggest that there is a second region in HBV which may also serve as a docking site for the polymerase. With the data presently available, we favor the model of coordinate control of translation and encapsidation of the pregenomic RNA. In this model, binding of the polymerase may displace the ribosomes from the entrance at the 5' end of the pregenomic RNA, hence exposing the 5' stem-loop structure and facilitating the selective uptake of the pregenomic RNA into the nucleocapsid. The region for suppression of translation identified in our study coincides with the region II suggested by Calvert and Summers (6). They reported that deletion of this region abrogates nucleocapsid assembly in transfected cells (6).

In keeping with the above-described concept, the binding of the polymerase to this region may also interfere with its own translation. The scarcity of the polymerase protein in HBV-infected cells has often been attributed to the infrequent events of ribosomal leaky scanning or internal ribosomal entry to the P ORF during translation (14, 20, 31). However, in vitro translation of the polymerase transcripts consistently resulted in a substantially lower yield than that of an equimolar amount of the core mRNAs (result not shown). In this system, both the mRNAs were generated from constructs in which the translation initiation codon of the corresponding ORF was the first AUG that was encountered by the ribosomes. We speculate that the polymerase protein may inhibit its own translation by binding to one or more mRNA sequences. Autoregulatory control of polymerase protein translation may account for the difficulties in cloning and expression of the polymerase protein which have been encountered in our laboratory and others and the low copy numbers of polymerase in the virion.

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