Incorporation of Eukaryotic Translation Initiation Factor eIF4E into Viral Nucleocapsids via Interaction with Hepatitis B Virus Polymerase[▽]

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The DNA genome of hepatitis B virus (HBV) replicates via reverse transcription within capsids following the encapsidation of an RNA template, the pregenomic RNA (pgRNA). We previously demonstrated that the 5' cap proximity of the stem-loop structure (ϵ or epsilon), an encapsidation signal, is critically important for the encapsidation of the pgRNA (J. K. Jeong, G. S. Yoon, and W. S. Ryu, *J. Virol.* 74:5502–5508, 2000). Therefore, we speculated that the viral polymerase (Pol), while bound to the 5' ϵ stem-loop structure, could recognize the cap via its interaction with eIF4E, a eukaryotic translation initiation factor. Our data showed the direct interaction between HBV Pol and eIF4E, as measured by coimmunoprecipitation. Further, we demonstrated that eIF4E interacts with the Pol- ϵ ribonucleoprotein complex (RNP) rather than Pol alone, resulting in eIF4E-Pol- ϵ RNP complex formation. In addition, we asked whether eIF4E remains engaged to the Pol- ϵ RNP complex during nucleocapsid assembly. Density gradient analysis revealed that eIF4E indeed was incorporated into nucleocapsids. It is of great importance to uncover whether the incorporated eIF4E contributes to viral reverse transcription or other steps in the HBV life cycle.

Worldwide, an estimated 350 million persons are persistently infected with hepatitis B virus (HBV) (12). A significant subset of these HBV carriers progress to severe liver disease, such as hepatocellular carcinoma, which is assumed to cause up to one million deaths per year. Current treatment regimens for chronic HBV infections have limitations, so there is a clear medical need for new therapeutic strategies.

HBV is a prototype of the hepadnavirus family, which includes duck hepatitis B virus and woodchuck hepatitis virus (21). Although they contain a DNA genome, hepadnaviruses replicate via the reverse transcription of an RNA template, the pregenomic RNA (pgRNA) (11). The pgRNA serves not only as the RNA template for viral reverse transcription but also as the mRNA that is used to encode two viral proteins required for viral genome replication: the core (capsid, or C) protein and the polymerase (Pol, or reverse transcriptase). It is not clear, however, whether and how the dual functions of the pgRNA are coordinated. Recently, we demonstrated that HBV Pol directs genome replication by suppressing the translation of the pgRNA (19). Therefore, we speculated that the translation suppression of the pgRNA could precede encapsidation. Importantly, the recognition of the 5' stem-loop structure (Fig. 1), called the encapsidation signal (ϵ or epsilon), by HBV Pol is critical for the translation suppression (19). In addition, the ability of Pol to specifically recognize the 5' ϵ also is critical for the initiation of viral reverse transcription as well as the assembly of replication-competent nucleocapsids (11, 15, 23). The epsilon sequence, initially defined as the RNA packaging signal that directs the specific encapsidation of the pgRNA into nucleocapsids, subsequently was found to be the

origin of reverse transcription as well (20). Taking these findings together, the binding of Pol to ϵ thus triggers three critical steps in hepadnaviral replication, (i) the initiation of reverse transcription (23), (ii) translation suppression (19), and (iii) nucleocapsid assembly (11, 15), leading to the selective incorporation of both Pol and pgRNA into the viral nucleocapsids.

In fact, the epsilon elements are present twice on the pgRNA owing to terminal redundancy: one is near the 5' terminus, and another is at the 3' terminus (Fig. 1). It was shown that only the 5' ϵ , but not the 3' ϵ , is functional for encapsidation, a phenomenon termed position-effect (7, 11). We previously demonstrated that besides the 5' ϵ , the cap structure at the 5' end also is required for encapsidation, a finding that provides a molecular explanation for the position-effect (10). However, it remains unclear how the cap structure is recognized by HBV Pol. Based on the observation that the 5' cap is required for the encapsidation of pgRNA, we speculated that HBV Pol recognizes the cap structure via its interaction with eIF4E, a eukaryotic translation initiation factor (10). In accordance with this speculation, the data presented here demonstrated that HBV Pol directly interacts with eIF4E. We also found that eIF4E is incorporated into viral nucleocapsids, presenting the possibility that the eIF4E-HBV Pol interaction contributes to viral capsid assembly.

MATERIALS AND METHODS

Cells, viruses, and transfection. HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 10 μ g of gentamicin per ml at 37°C in 5% CO₂ and were split every third day. A vaccinia virus, vTF7-3, that expresses T7 RNA polymerase was described previously (3). For the transfection of HEK293 and HeLa cells, a procedure involving polyethylenimine (PEI) (25 kDa; Aldrich) was performed (16). Briefly, cells were plated at a confluence of 80 to 90%. Equal amounts of plasmid DNA and PEI (30 μ g per 100-mm plate) were diluted with serum-free DMEM. Diluted DNA and PEI were mixed and incubated at room temperature for 20 min. Cells were washed with phosphate-buffered saline (PBS) and overlaid

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FIG. 1. Model illustrating how HBV Pol could recognize the cap structure. The pgRNA is shown with the stem-loop structure (epsilon or ϵ). The pgRNA harbors the epsilon sequence at the 5' end and at the 3' end. We previously established that the cap structure as well as 5' ϵ is essential for the encapsidation of the pgRNA, a finding that defines a bipartite encapsidation signal (10). Based on this finding, we hypothesized that HBV Pol recognizes the cap structure via its interaction with eIF4E, a cap binding protein.

with DNA-PEI complex solution. After 16 h, cells were rinsed and fed with fresh media.

Plasmid construction. The HBV Pol expression construct, from which HBV Pol with three copies of the Flag epitope at N terminus is expressed, was described previously (19). The pgRNA expression plasmid was derived from the pCMV-HBV/30 plasmid, the wild-type HBV replicon construct, from which both the C (core) open reading frame and the (Pol) open reading frame were inactivated by an introduction of a stop codon and a frameshift mutation, respectively, as described previously (24). The pCMV-HBV/30 plasmid was described previously (10). Note that the T7 promoter present in the pcDNA1/Amp plasmid (Invitrogen) was deleted during subcloning. pHA-Luc and pFlag-Luc constructs that express a firefly luciferase having either three copies of the hemagglutinin (HA) tag or three copies of the Flag tag at its N terminus were inserted into pcDNA3 plasmid (Invitrogen). The pT7^{max}-HBV construct that was used to generate the cap-free RNA was described previously (10). The HBV Pol-null construct that was made by an introduction of a frameshift mutation in the Pol gene was previously described (22). The plasmid constructs encoding eIF4E, eIF4E-W73A, and 4E-BP were the generous gift of N. Sonenberg (McGill University). Note that all of the expression plasmids described above were cloned into the same pcDNA3 plasmid (Invitrogen).

Coimmunoprecipitation. At 48 to 72 h posttransfection, transfected HEK293 cells were lysed in NP-40 lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA) and protease inhibitor cocktail for 30 min at 4°C. After centrifugation for removing cell debris, the supernatant was precleared with protein G-agarose beads for 30 min at 4°C. The primary antibody was added for 1 h at 4°C, and then protein G-agarose was added and the incubation was continued for 1 h at 4°C. The beads were washed five times with 1 ml of lysis buffer and examined by Western blot analysis.

Western blot analysis. Two days after transfection, cells were treated with lysis buffer (50 mM Tris-Cl [pH 7.4], 50 mM NaCl, 5 mM EDTA, 1% NP-40, EDTA-free protease inhibitor cocktail [Roche]). Equivalent amounts of samples, based on the Bradford assay (Bio-Rad), were mixed with sample buffer (100 mM Tris-Cl [pH 6.8], 4% [wt/vol] sodium dodecyl sulfate [SDS], 0.2% [wt/vol] bromophenol blue, 20% [vol/vol] glycerol, 200 mM dithiothreitol, and β -mercaptoethanol). After being boiled, the samples were loaded onto SDS-12% polyacrylamide gel electrophoresis. Following electrophoresis, the gel was blotted on a nitrocellulose membrane (Amersham). After being blocked, the membrane was incubated with a mouse anti-FLAG M2 antibody (1:5,000; Sigma) and an anti-mouse immunoglobulin G horseradish peroxidase-linked antibody (1:5,000; Amersham). The proteins were visualized by an ECL detection system (Amersham).

Confocal imaging. HEK293 cells were cultured on 12-well plates and transfected as described above. One day later, cells were fixed with 3.7% formaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and incubated with monoclonal anti-HA antibody and monoclonal anti-Flag antibody, followed by anti-mouse conjugated to Alexa Fluor 488 and anti-rabbit conjugated to Alexa Fluor 594 (Molecular Probes). Coverslips were mounted onto glass slides with a ProLong Antifade kit (Molecular Probes). Images were examined by confocal microscopy (LSM 510 Meta; Carl Zeiss, Germany). The quantification of the degree of colocalization was processed with associated software. These images are representative of most cells, and five cells were selected for quantitative colocalization analysis.

Density gradient analysis. Density gradient analysis was performed in a OptiPrep (60% [wt/vol] iodixanol; Axis-Shield) velocity gradient essentially as described previously (24). OptiPrep density gradients were prepared in lysis

buffer as five steps in 10% increments ranging from 10 to 50%. An aliquot of 200 μ l cell lysate was carefully loaded onto the top of the gradient and centrifuged for 45 min at 55,000 rpm in a TLS 55 swing-out rotor (Beckman Instruments) at 20°C. Fourteen 80- μ l fractions from the top were collected, and the protein content of each fraction was examined by immunoblot analysis. When indicated, cell lysates were treated with RNase A at a concentration of 100 μ g/ml for 1 h at 37°C, proteinase K at a concentration of 60 μ g/ml for 1 h at 37°C, or both.

RESULTS

HBV Pol binds to eIF4E. We hypothesized that HBV Pol interacts with eIF4E, which also is known as a cap binding protein. To test this possibility, we carried out the coimmunoprecipitation of HBV Pol with eIF4E. To facilitate coimmunoprecipitation, HBV Pol was tagged with three copies of the Flag epitope at its N terminus, while eIF4E was tagged with three copies of the HA epitope at its N terminus. Cells were transfected as indicated in Fig. 2A; immunoprecipitation was carried out with anti-HA antibody, and immunoblotting was performed with anti-Flag antibody. As shown in Fig. 2A, immunoprecipitation with anti-HA antibody pulled down HBV Pol (lane 2), indicating that HBV Pol interacts with eIF4E. The interaction appeared to be specific, since no luciferase protein was brought down with anti-Flag antibody (Fig. 2A, lane 1). Reciprocal immunoprecipitation with anti-Flag antibody further corroborated this finding (Fig. 2B, lane 2). The results supported the notion that HBV Pol recognizes the 5' cap structure via its interaction with eIF4E.

Given that HBV Pol recognizes the cap structure, most likely while bound to the 5' ϵ stem-loop structure (Fig. 1), we considered that the RNA harboring the epsilon sequence enhances the interaction between HBV Pol and eIF4E. To test this possibility, cells were cotransfected with the pgRNA expression plasmid along with the eIF4E and Pol expression plasmids. Coimmunoprecipitation was carried out as described in Materials and Methods (Fig. 2A, lanes 3 and 4). When pgRNA was coexpressed, the amount of Pol coimmunoprecipitated was increased by twofold. Reciprocal immunoprecipitation confirmed this finding (Fig. 2B, lanes 3 and 4). The data suggested that pgRNA significantly enhanced the Pol-eIF4E interaction, although a modest level of interaction still occurred in the absence of the pgRNA.

The epsilon-containing RNA modulates the HBV Pol-eIF4E interaction. To account for the enhanced Pol-eIF4E interaction, we considered three possibilities: (i) Pol has greater affinity for eIF4E when it is bound to the epsilon RNA, since the epsilon RNA acts as an allosteric regulator of the Pol structure; (ii) the stability of the Pol protein is enhanced upon binding to the epsilon RNA; and (iii) the epsilon-containing RNA simply acts to tether the two proteins via its cap structure and the epsilon sequence. The second possibility was largely excluded, since the Pol protein level was not significantly increased when pgRNA was coexpressed (Fig. 2A, lanes 3 and 4).

To assess the extent of a tethering effect on the enhancement of the Pol-eIF4E interaction, we generated cap-free pgRNA by transfecting cells with a cap-free RNA (cfRNA) construct and then infecting them with vTF7-3, a recombinant vaccinia virus expressing T7 RNA polymerase, as performed previously in our laboratory (10). This cap-free pgRNA contains the epsilon sequence but lacks the cap structure; any tethering effect would be precluded. Cells were transfected and

FIG. 2. HBV Pol interacts with eIF4E. (A) HEK293 cells were transfected with each plasmid as indicated. Anti-HA antibody was used to immunoprecipitate proteins from the cell lysates. The immunoprecipitated proteins (IP) were blotted with anti-HA antibody to detect HA-tagged proteins and anti-Flag antibody to detect coimmunoprecipitated HBV Pol. Cell lysates (input; 2%) were analyzed in parallel by immunoblotting (IB). The pgRNA encoding the 5' ϵ stem-loop structure was provided by transfecting cells with the HBV C-null, Pol-null construct that can express the pgRNA but not the viral proteins. Data are normalized to results shown in lane 2 (no RNA control) and are presented as the means $\pm$ standard deviations in triplicate, and the results are representations of three separate experiments. Luc, luciferase. (B) Reciprocal coimmunoprecipitation of the samples described for panel A.

infected as indicated, and coimmunoprecipitation was carried out (Fig. 3A). It was noted that the vaccinia virus infection led to the significant overexpression of eIF4E as well as HBV Pol and luciferase, perhaps due to the fact that T7 RNA polymerase drives transcription off of the T7 promoter present in the pcDNA3 plasmid (8). To ensure that the protein expression level derived from pcDNA3 was comparable between plates, all four plates, in addition to the plate for lane 3, were infected in parallel with the vaccinia virus. Coimmunoprecipitation data consistently showed that the cap-free RNA similarly enhanced the Pol-eIF4E interaction compared to that with pgRNA (Fig. 3A, lanes 3 and 4). Reciprocal immunoprecipitation confirmed

FIG. 3. HBV Pol and eIF4E interact in a cap-independent manner. (A) HEK293 cells were transfected with each plasmid as indicated. Anti-HA antibody was used to immunoprecipitate proteins from the cell lysates. The immunoprecipitated proteins (IP) were blotted with anti-HA antibody to detect HA-tagged proteins and anti-Flag antibody to detect coimmunoprecipitated HBV Pol. Cell lysates (input; 2%) were analyzed in parallel by immunoblotting (IB). cfRNA was generated by infecting cells with the vaccinia virus vT7-3, which expresses T7 RNA polymerase, followed by transfection with the pT7^m-HBV construct, as previously described (10). Other symbols are the same as those described for Fig. 2. Data are normalized to results shown in lane 2 (no RNA control) and are presented as the means $\pm$ standard deviations in triplicate, and the results are a representation of three separate experiments. Luc, luciferase. (B) Reciprocal coimmunoprecipitation of samples described for panel A.

this finding (Fig. 3B, lanes 3 and 4). The observation that the cap-free pgRNA enhanced the Pol-eIF4E interaction argues that pgRNA enhanced the Pol-eIF4E interaction in the absence of a tethering effect. One interpretation is that the epsilon RNA confers enhanced eIF4E binding ability to the HBV Pol.

HBV Pol interacts with eIF4E at a site distinct from the YX₄L motif. To gain more insight into the Pol-eIF4E interaction, we wanted to define the eIF4E binding site within the Pol

FIG. 4. HBV Pol interacts with eIF4E at a site distinct from that of the YX₄L motif. (A) Effect of eIF4E-W73A mutant. Cells were transfected with the eIF4E-W73A mutant plasmid as indicated; otherwise, the experiment was carried out as described for Fig. 3. IP, immunoprecipitate; IB, immunoblot; α -HA, anti-HA antibody; α -Flag, anti-Flag antibody; Luc, luciferase. (B) 4E-BP failed to compete off the eIF4E-Pol interaction. Cells were transfected with 4E-BP expression plasmid as indicated; otherwise, the experiment was carried out as described in the legend to Fig. 3.

protein. It is well established that many known eIF4E-binding proteins, which include eIF4G, 4E-BP (eIF4E binding protein), Maskin, and Cup, interact with eIF4E via their YX₄L motifs (5). To determine whether HBV Pol interacts with eIF4E via its yet-to-be-identified YX₄L motif, we employed an eIF4E mutant, W73A, in which the critical tryptophan residue recognizing the YX₄L motif was replaced with alanine. It previously was shown that the eIF4E mutant lacked the ability to interact with eIF4E binding proteins (17). For the present study, cells were transfected as indicated (Fig. 4A). To maintain comparable levels of the expression of the transfected

expression plasmids, all three plates were transfected with the cfRNA construct and infected with vTF7-3, as described for Fig. 3. The data showed that eIF4E-W73A remained to interact with HBV Pol (Fig. 4A, lanes 2 and 3), suggesting that HBV Pol interacts with eIF4E via a site distinct from that of the YX₄L motif. Reciprocal coimmunoprecipitation with anti-Flag antibody yielded a similar finding (data not shown). In addition, it might be inferred that the eIF4E-Pol interaction occurs in the absence of an eIF4E-eIF4G interaction, since the eIF4E mutant excludes the interaction with eIF4G. This interpretation excludes the possibility that the Pol protein interacts with eIF4E indirectly via its interaction with eIF4G.

One interpretation of the observation that HBV Pol could interact with eIF4E independently of eIF4G is that 4E-BP, an eIF4E binding protein, could not compete with the Pol-eIF4E interaction. Thus, we tested whether 4E-BP could diminish the Pol-eIF4E interaction (Fig. 4B). Cells were transfected as indicated, and coimmunoprecipitation was carried out. The data showed that the cotransfection of the 4E-BP expression construct did not affect the extent of the Pol-eIF4E interaction, indicating that 4E-BP failed to compete with the Pol-eIF4E interaction (Fig. 4B, lanes 2 and 3). However, cotransfection with the 4E-BP construct resulted in a decreased eIF4E-eIF4G interaction, indicating that 4E-BP fully competed with the eIF4E-eIF4G binding, as shown previously (6 and data not shown). Therefore, the data indicated that the HBV Pol protein interacts with eIF4E via a site distinct from that of the YX₄L motif. Overall, we concluded that HBV Pol interacts with eIF4E via a mechanism not involving eIF4G.

Subcellular colocalization of HBV Pol and eIF4E. We next examined the interaction between HBV Pol and eIF4E *in vivo* by determining the subcellular colocalization using immunofluorescence confocal microscopy (Fig. 5). HEK293 cells were cotransfected with the Pol and eIF4E expression constructs, and the subcellular localization of these two proteins was examined by indirect immunofluorescence using anti-Flag and anti-HA antibodies, respectively. In parallel, an HA-Luc construct was employed as a negative control. The results showed that both HA-Luc and HBV Pol were dispersed throughout the cytoplasm and only minimally overlap each other (Fig. 5A). In contrast, when HA-eIF4E and HBV Pol were coexpressed, both eIF4E and HBV Pol were localized primarily to the nuclear periphery of the cytoplasm in punctate structures (Fig. 5B). In other words, it appeared that the ectopic expression of eIF4E facilitated the condensation of HBV Pol to the nuclear periphery. In the merged image, the coincidence of red and green fluorescence indicated a significant overlapping distribution of eIF4E and HBV Pol (Fig. 5B). Hence, immunofluorescence data support the interaction between HBV Pol and eIF4E.

eIF4E is copackaged with HBV Pol within the nucleocapsid.

Since the pgRNA serves as the mRNA, many host factors engage the pgRNA during translation, including translation initiation factors such as eIF4E. These translation factors most likely are dissociated from the pgRNA prior to encapsidation. In this regard, an intriguing question was whether eIF4E remain bound to the 5' end of the pgRNA and subsequently is incorporated into nucleocapsids (Fig. 6A) or whether eIF4E is disengaged upon capsid assembly (Fig. 6B). To address this issue, we attempted to detect the incorporated eIF4E through

FIG. 5. Subcellular localization of eIF4E and HBV Pol. HEK293 cells were cotransfected with either (A) HA-Luc and Flag-Pol constructs or (B) HA-eIF4E and the Flag-Pol expression constructs. Cells were fixed and stained sequentially with mouse anti-HA antibody and rabbit anti-Flag antibody, as detailed in Materials and Methods. Yellow color indicates the overlap of green (eIF4E) and red (HBV Pol). Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). On the right are merged images showing superimposed eIF4E, HBV Pol, and DAPI results. Luc, luciferase.

density gradient analysis (Fig. 7A). Cells were transfected with the HBV Pol-null replicon construct, along with the Pol expression plasmid and eIF4E expression plasmid. Three days following transfection, cell lysates were prepared and subjected to OptiPrep density gradient analysis, as described previously (24). In parallel, to detect molecules incorporated into the capsids, cell lysates were pretreated with RNase or RNase plus proteinase K prior to density gradient analysis to remove unencapsidated proteins (24). Each fraction was examined for HBV Pol, eIF4E, and the core protein by immunoblotting. As shown in panel a of Fig. 7A, core proteins were detected

mainly in fractions 8 to 11, which represent the nucleocapsid fractions. Pol was detected in fractions 4 to 10, partly overlapping the capsid fractions, while eIF4E was detected mostly in the upper fraction and, to a lesser extent, in fractions 6 to 10, perhaps representing eIF4E associated with polysomes (Fig. 7A, panel a). On the other hand, when cell lysates were pretreated with RNase, eIF4E was more abundantly detected in the upper fractions (Fig. 7A, panel b). Furthermore, when cell lysates were pretreated with both RNase and proteinase K, both eIF4E and Pol were undetectable in the upper fractions, whereas both remained detectable in the capsid fractions, although the levels were substantially reduced (Fig. 7A, panel c). Thus, the resistance of eIF4E to proteinase K treatment implied that eIF4E indeed is incorporated into nucleocapsids.

To substantiate this finding, we attempted to detect the encapsidated eIF4E following the immunoprecipitation of nucleocapsids, as described previously (24). We reasoned that if eIF4E is incorporated into capsids, then eIF4E should be detectable following the immunoprecipitation of capsid particles using anti-core antibodies. As shown in Fig. 7A, the HBV Pol-null replicon was complemented with an HBV Pol expression construct. Thus, HEK293 cells were transfected with the HBV Pol-null, HBV Pol, and HA-eIF4E expression constructs. Immunoprecipitation was carried out, and HBV Pol, eIF4E, and the core protein were detected by immunoblotting with their respective antibodies (Fig. 7B). Immunoprecipitation with anti-Flag or anti-HA antibody showed that the HBV Pol-eIF4E interaction occurs in the context of the replicon system, as anticipated (Fig. 7B, lanes 1 and 2). In contrast, the lack of the detection of the core protein suggested that measurable Pol-core or eIF4E-core interactions did not occur (Fig. 7B, lanes 1 and 2). However, both HBV Pol and eIF4E were modestly detected following immunoprecipitation with the anti-core antibody in a manner that depends on HBV Pol expression (Fig. 7B, lanes 3 and 5). Our interpretation is that the modest detection of both Pol and eIF4E following immuno-

FIG. 6. Models illustrating the HBV nucleocapsid assembly process with respect to the incorporation of eIF4E into nucleocapsids. (A) A schematic model illustrating that eIF4E remains bound to the cap of the pgRNA during the encapsidation process and thereby becomes incorporated into the nucleocapsid. (B) A schematic model illustrating that eIF4E becomes dissociated from the cap of the pgRNA, resulting in a failure of the incorporation of eIF4E.

FIG. 7. Incorporation of eIF4E into nucleocapsids depends on HBV Pol. (A) HEK293 cells were cotransfected with the HBV Pol-null construct and the Flag-Pol and Flag-eIF4E expression constructs. Three days following transfection, the cells were lysed with NP-40 lysis buffer and subjected to a 10 to 50% OptiPrep density gradient after no treatment (a), after RNase A treatment (b), and after RNase A and proteinase K treatment (c). The overexposed image of fraction 10 is shown in the insert of panel a. OptiPrep density gradient analysis was performed as detailed in Materials and Methods. The HBV Pol, eIF4E, and core proteins were detected by Western blot analysis with anti-Flag (α -Flag) and anti-core (α -core) antibodies, respectively. IP, immunoprecipitate; IB, immunoblot; α -HA, anti-HA antibody. (B) Cells were transfected as indicated above each lane, and cell lysates were immunoprecipitated with the antibodies as indicated above each lane. The immunoprecipitated proteins were detected by immunoblotting using the antibodies denoted to the left. A fraction of total lysate (input; 2%) was analyzed in parallel by immunoblotting.

precipitation with anti-core antibody was consistent with the incorporation of one or a few of these molecules into nucleocapsids. Overall, the data presented here indicate that eIF4E is incorporated into capsid particles in an HBV Pol-dependent manner.

DISCUSSION

We tested the hypothesis that HBV Pol recognizes the 5' cap structure via its interaction with eIF4E. Here, we showed that

HBV Pol directly interacts with eIF4E using coimmunoprecipitation (Fig. 2), which demonstrated that the eIF4E-Pol interaction is enhanced significantly by ϵ RNA, and implicating the formation of an eIF4E-Pol- ϵ ribonucleoprotein (RNP) complex prior to nucleocapsid assembly (Fig. 2). Further, we showed that eIF4E was incorporated into the nucleocapsid along with HBV Pol, indicating that the eIF4E interacts with the Pol- ϵ RNP complex during nucleocapsid assembly (Fig. 7). To our knowledge, it is unprecedented that a translation factor such as eIF4E is incorporated into viral nucleocapsid particles.

The pgRNA could enhance the HBV Pol-eIF4E interaction as a consequence of tethering, since both HBV Pol and eIF4E could bind to the pgRNA independently via their respective binding to epsilon and cap structure, respectively (Fig. 1). To preclude the tethering effect, we utilized the cap-free pgRNA, as shown in Fig. 3. The result indicated that the eIF4E-Pol interaction still occurred when the cap-free pgRNA was provided (Fig. 3). Further, pgRNA enhanced the Pol-eIF4E interaction (Fig. 2), suggesting that the 5' ϵ stem-loop structure of the pgRNA acted as an allosteric regulator modulating the protein-protein interaction. Overall, our interpretation of this result is that the Pol protein could undergo a subtle conformational change upon its binding to the 5' ϵ RNA, with the resulting 5' ϵ -Pol RNP complex as an entity that binds to eIF4E.

The eukaryotic translation initiation factor eIF4F is composed of three factors: eIF4E, eIF4G, and eIF4A (RNA helicase) (5). In particular, eIF4G is an eIF4E binding protein that also is known as an adaptor protein linking the 5' to 3' termini of the mRNAs via its interaction with eIF4E and poly(A) binding protein. Two independent results supported the direct interaction of the HBV Pol protein with eIF4E: (i) the eIF4E-W73A mutant that harbors an alteration at the critical binding site for eIF4G remained to interact with HBV Pol (Fig. 4A), and (ii) 4E-BP failed to compete with the eIF4E-HBV Pol interaction (Fig. 4B). Overall, the data are consistent with an interpretation that HBV Pol interacts with eIF4E at a site distinct from that of the YX₄L motif.

Given that eIF4E is a cap binding protein that plays a central role in translation regulation (18), the question arose as to whether the HBV Pol-eIF4E interaction affects translation. All known eIF4E binding proteins, including eIF4G, 4E-BP, Maskin, and Cup, were shown to inhibit translation initiation via their binding to eIF4E (18). In line with these observations, we recently reported that HBV Pol suppresses the translation of the pgRNA via its interaction with 5' ϵ (19). However, we considered it unlikely that the Pol-eIF4E interaction per se contributes to the translation suppression, since the overexpression of Pol did not diminish the eIF4E-eIF4G interaction (data not shown), which is essential for efficient translation (4).

HBV Pol has a multitude of critical functions in viral assembly as well as viral genome replication. The ability of HBV Pol to recognize the 5' stem-loop structure is critical for multiple events that precede viral genome replication, including (i) translation suppression (19), (ii) the initiation of viral reverse transcription (23), and (iii) the replication-competent capsid assembly (11, 15). It is intriguing to speculate that HBV Pol undergoes a conformational change upon eIF4E binding such that one of the activities of Pol (e.g., the initiation of viral reverse transcription) could be manifested only upon eIF4E

binding. A critical question is to what extent the Pol-eIF4E interaction impacts on these multiple events? One approach to address this question would be to define the eIF4E contact sites on HBV Pol and then examine whether the Pol mutants defective for eIF4E binding could support the three processes mentioned above. To this end, we attempted to define the eIF4E binding domain of HBV Pol. The analysis of HBV Pol mutants lacking one of four subdomains revealed that deletion mutants lacking either the TP or RT domain failed to interact with eIF4E, while mutants lacking the RNase H domain were able to interact with eIF4E (data not shown). Given that (i) both TP and RT domains are minimally required for ϵ RNA binding (9) and (ii) the Pol-eIF4E interaction is enhanced by ϵ RNA (Fig. 2), it was not possible to isolate a Pol mutant out of the four subdomain deletion mutants that is defective in eIF4E binding but retains its ϵ RNA binding ability. Our failure to dissect genetically the eIF4E binding domain from RNA binding domain prevented us from resolving the issue.

Little is known about the molecular mechanism that underlies viral capsid assembly, in particular with respect to a molecular determinant that recruits the core protein (Fig. 6A). It was thought that the Pol molecule of the Pol- ϵ RNP complex recruited the core protein to initiate capsid assembly, since (i) Pol, but not the core protein, directly recognizes the viral pgRNA (1), (ii) HBV Pol was shown to bind the core protein (14), and (iii) Pol cannot assemble into capsid particles in the absence of the pgRNA (2). One intriguing possibility is that Pol that has undergone conformational alteration upon eIF4E binding recruits core proteins to initiate capsid assembly. Our ongoing studies are directed to address this issue.

Another emerging issue is whether eIF4E incorporated into particles contributes to viral reverse transcription. Of relevance, the residual 18-nucleotide RNA molecule resulting from the cleavage of the pgRNA mediated by RNase H during minus-strand DNA synthesis serves as the RNA primer for the plus-strand DNA synthesis of viral reverse transcription (13). It is most likely that the incorporated eIF4E remains bound to the cap structure of the RNA primer during viral reverse transcription. Thus, it is tempting to speculate that eIF4E that is engaged in the cap structure of the RNA primer contributes to the plus-strand DNA synthesis.

HBV Pol is a central viral protein that executes multiple biochemical functions related to viral genome replication and capsid assembly. It is likely that such multiple biochemical activities are regulated upon interaction with viral or host factors. In this regard, the interaction of eIF4E with HBV Pol represents an important part of viral genome replication. The further delineation of the eIF4E-Pol interaction will provide insight into viral genome replication and reveal novel therapeutic targets for anti-HBV drug development.

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