Proximity between the cap and 5′ ε stem–loop structure is critical for the suppression of pgRNA translation by the hepatitis B viral polymerase

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The pregenomic RNA (pgRNA) of hepatitis B virus (HBV) serves as an mRNA as well as an RNA template for viral reverse transcription. We previously reported that HBV Pol (polymerase) suppresses translation of the pgRNA through a mechanism involving the 5′ ε sequence [Virology 373:112–123(2008)]. Here, we found that the recognition of the 5′ ε stem–loop structure by HBV Pol is essential for the translation suppression. Intriguingly, the translation suppression was observed only when the 5′ ε sequence was positioned within approximately 60 nucleotides from the 5′ end, which is striking reminiscent of the pgRNA encapsidation. This finding implicates that the translation suppression is mechanistically linked to encapsidation of the pgRNA. However, unexpectedly, the HBV Pol–eIF4E interaction, which we reported recently [J. Virol. 84:52–58(2010)], is not required for the translation suppression. Instead, the data suggested that the cap proximity of 5′ ε sequence is necessary and sufficient for the translation suppression.

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Introduction

Human hepatitis B virus (HBV) is the prototype virus of the Hepadnaviridae family and contains a small DNA genome that is 3.2 kb in length (Seeger and Mason, 2000). Although it contains DNA genome, HBV replicates its genome through reverse transcription of an RNA template, called the pregenomic RNA (pgRNA). The pgRNA has a dual role during an HBV infection. First, it is a bicistronic mRNA encoding both core (C) and polymerase (P or Pol) ORFs. Second, the pgRNA contains all of the genetic information necessary to initiate viral genome replication and contains a cis-acting encapsidation element, called the epsilon element (ε), near the 5′ end that folds into a stem–loop structure. Thus, the pgRNA serves as a template for translation as well as a substrate for encapsidation, which is the step immediately before viral reverse transcription. As a result, HBV must streamline these two otherwise competitive processes to replicate efficiently. Recently, we showed that the dual functions of the pgRNA is, in part, regulated by suppression of translation by HBV Pol binding to the 5′ ε of the pgRNA (Ryu et al., 2008). An implication is that the HBV Pol acts as a molecular switch that regulates the transition from translation to encapsidation. However, the extent of the linkage between encapsidation and suppression of translation remains to be resolved.

Translation initiation in eukaryotes takes place through two distinct mechanisms: cap-dependent and cap-independent (Gebauer and Hentze, 2004). The binding of eIF4E to the cap structure is essential for the cap-dependent translation. Further, cap-dependent translation is regulated by eIF4E binding factors such as 4E-BP (Richter and Sonenberg, 2005). Hence, eIF4E represents a critical molecule for the regulation of cap-dependent translation. Intriguingly, the hantaviruses N protein can functionally replace eIF4F (an eIF4E, eIF4G, and eIF4A ternary complex) for cap-dependent translation (Mir and Panganiban, 2008), allowing hantavirus to carry out cap-dependent translation driven by N protein and independent of eIF4E. In contrast, eIF4E is dispensable for cap-independent translation. For this mechanism, internal ribosome entry is mediated by a RNA secondary structure, called internal ribosome entry site (IRES), that is found at the upstream of the polyprotein ORF in a few single-strand RNA viruses such as picornaviruses and flaviviruses and mammalian mRNAs as well (Jang, 2006; Martinez-Salas et al., 2008).

Translation in eukaryotes can also be regulated by many distinct stimuli to meet physiological needs. Translation regulation of the ferritin mRNA is one of the best characterized translation regulatory mechanisms in eukaryotes (Gebauer and Hentze, 2004). Translation of the ferritin mRNA is mediated by iron regulatory protein (IRP) that recognizes the iron responsive element (IRE) stem–loop structure located upstream of the ferritin ORF (Muckenthaler et al., 1998). Interestingly, it was found that the proximity of the 5′ cap to the IRE (<60 nt) is critical for the suppression of ferritin translation and underlies the molecular mechanism of the ‘position effect’ of the IRE element for the translation suppression (Goossen et al., 1990; Goossen and Hentze, 1992). The translation suppression of the HBV pgRNA by Pol is reminiscent of ferritin mRNA regulation in two respects. First, a stem–loop structure (IRE and 5′ ε) located upstream...
to the ORF is required for the translation suppression (Ryu et al., 2008). Second, an RNA-binding protein (IRP and HBV Pol) that specifically recognizes the stem–loop structure is also required for the translation suppression (Ryu et al., 2008). These striking similarities led us to determine if the translation suppression of the pgRNA by HBV Pol is mechanistically related to that of ferritin mRNA by IRP.

To relate the suppression of HBV pgRNA translation to the mechanism that regulates ferritin mRNA translation, we asked whether proximity of the cap to the 5′ε stem–loop structure is required for the translation suppression. We found that the cap-proximity of the 5′ε stem–loop structure is critically important for the translation suppression of the pgRNA by the HBV Pol, a finding that parallels the ‘position-effect’ of the IRE on the translation of ferritin mRNA (Goossen et al., 1990; Goossen and Hentze, 1992). In addition, since HBV Pol was shown to interact with eIF4E, a cap binding translation initiation factor, we asked whether the interaction between HBV Pol and eIF4E is essential for the translation suppression (Kim et al., 2010). Unexpectedly, the data revealed that the HBV Pol–eIF4E interaction is not required for the suppression of pgRNA translation. Instead, the data suggest that the recognition of the 5′ε by HBV Pol is both necessary and sufficient for the translation suppression by HBV Pol.

Results

Recognition of 5′ stem–loop structure by HBV Pol precedes suppression of translation

Although we have previously shown that HBV Pol suppresses translation of the pgRNA in the 5′ε sequence-dependent manner (Ryu et al., 2008), it remained uncertain whether recognition of 5′ε by HBV Pol is required for the suppression. Thus, we investigated whether the interaction between HBV Pol and the 5′ε is critical for suppression of pgRNA translation. In previous studies using an Escherichia coli expressed recombinant HBV Pol GST-fusion protein, it was shown that the bulge region, not but apical loop region, of the stem–loop structure is important for the binding with HBV Pol in vitro (Hu and Boyer, 2006) (Fig. 1A). We then sought to determine how the in vitro binding of HBV Pol to the ε stem–loop structure is related to the suppression of translation. Two previously described deletion mutants were made (Hu and Boyer, 2006): (i) Δbulge (ΔB) and (ii) Δapical loop (ΔL). The Δ5′B-WT and Δ5′B-P-null constructs were generated by deleting the bulge part of 5′ε in WT or P-null constructs, respectively (Fig. 1B). In parallel, the loop region on the 5′ε was deleted to generate the Δ5′L-WT and Δ5′L-P-null constructs (Fig. 1B).

Fig. 1. The bulge but not the loop of the stem–loop structure is essential for suppression of translation. (A) A schematic diagram showing the epsilon stem–loop structure. Two regions of the stem–loop structure that are deleted in this work are denoted: bulge loop and apical loop. (B) Map of the two pairs of the epsilon mutants. A schematic diagram of the pgRNA transcribed from each constructs is shown. The C and P ORFs, which are in different reading frames, are indicated by black and gray boxes, respectively. The P-null counterpart of the WT construct is shown below, where a frame-shift mutation introduced in the P ORF is denoted. The deleted part of the stem–loop structure is denoted by dotted lines: ΔB (a deletion of the bulge), and ΔL (a deletion of the apical loop). (C) Western blot analysis. Cells were transfected with the indicated constructs. Core protein levels were assayed by using anti-HBc antibody. The core level from transfected cells by Δ5′B-WT or Δ5′L-WT were set to 100%. Data are presented as the mean±standard deviations of experiments preformed in triplicate. Actin served as loading control. (D) RNase protection assay. HBV RNA (closed arrow) was analyzed using a core-specific riboprobe. The RNA levels from cells transfected with Δ5′B-WT or Δ5′L-WT were set to 100%. Data are presented as mean±standard deviations of experiments performed in triplicate. Yeast RNA, which served as a negative control, was analyzed with (+) and without (−) RNase A/T1 treatment. As control, GAPDH mRNA (open arrow) was detected by a GAPDH-specific riboprobe.
Cells were transfected as indicated, and Western blot analysis was performed to assess the level of core protein produced, as a measure of translation from the pgRNA (Fig. 1C). The data showed that the core (C) protein level in ΔS'-B-P-null transfected cells was comparable to that in the ΔS'-B-WT-transfected cells (Fig. 1C, lane 2 versus 1), indicating that translation suppression was not detectable. This result suggested that the bulge is important for the suppression of pgRNA translation. In contrast, the core protein level in ΔS'-L-P-null transfected cells was approximately 3-fold greater than in the ΔS'-L-WT-transfected cells (Fig. 1C, lane 4 versus 3), indicating the apical loop is dispensable for the suppression of translation. RPA analysis demonstrated that the pgRNA levels were comparable for each construct, confirming the effects seen were related to the suppression of pgRNA translation (Fig. 1D). Based on the correlation between the published in vitro binding data and the translation suppression data observed in these studies, we concluded that the recognition of the 5' stem-loop structure by HBV Pol is a prerequisite for the suppression of pgRNA translation.

To gain further insight into the Pol–5′ε interaction for the translation suppression, we carried out deletion analysis of the HBV Pol. HBV Pol can be divided into four subdomains, which are (from N-terminus) the terminal protein (TP), spacer (SP), reverse transcriptase (RT), and RNase H (RH) subdomains (Nassal, 2008) (Fig. 2A). By using in vitro reconstitution, it was shown that TP and RT domain is indispensable for the binding to an RNA containing the ε stem–loop structure, whereas the SP and RH domain is largely dispensable (Hu and Boyer, 2006). Based on the in vitro result, we generated three deletions of HBV Pol, which lacks each individual subdomain: (i) ΔTP-P, (ii) ΔRT-P, and (iii) ΔRH-P (Fig. 2A). To avoid the compounding effect of nucleocapsid assembly, nucleocapsid formation was precluded by employing an assembly defective C allele (i.e., Δε42 or deletion of the 42nd leucine of core protein), as described previously (Ryu et al., 2008). Cells were transfected as indicated, and Western blot analysis was performed (Fig. 2B). As anticipated, the data showed that C protein level in the wild-type Pol expression plasmid-transfected cells was significantly reduced to that in P-null-ΔL42 only transfected cell (Fig. 2B, lane 3 versus 2). Importantly, the data indicated that ΔTP-P and ΔRH-P suppressed translation of C protein, to the extent similar to that of the wild type (Fig. 2B, lanes 4 and 6), whereas ΔRT-P failed to suppress translation of the C protein (Fig. 2B, lane 5). As above, RPA analysis confirmed that the pgRNA levels were comparable (Fig. 2C). Thus, we concluded that both the TP and RH subdomains are dispensable for the translation suppression. An interpretation is that both the TP and RH domains are dispensable for the binding of the HBV Pol to the ε stem–loop structure. The dispensability of the TP domain for the translation suppression is inconsistent with what was expected from the result of the in vitro reconstitution experiment (see Discussion). Overall, the data shown in Fig. 2, together with the result shown in Fig. 1, suggested that the recognition of 5'ε stem–loop structure by the HBV Pol is critical for the translation suppression.

**Close proximity of the cap to the 5'ε is required for the suppression of translation and pgRNA encapsidation.**

To gain further insight into the translation suppression of the pgRNA, we asked whether the proximity of the cap to the 5'ε stem–loop structure is critical for the suppression of pgRNA translation. We generated a luciferase reporter construct that places the 5′ε stem–loop structure 30 nt from the cap (ε-FL-30), which mimics the wild-type pgRNA (Fig. 3A). In addition, we made three variants in which the 5′ε sequence is placed 55, 82, or 298 nt from the 5′ cap (ε-FL-55, ε-FL-82, and ε-FL-298, respectively) (Fig. 3A). To assess the effect of increasing the distance between the cap and the 5′ε stem–loop has on translation, HEK293 cells were transfected with each construct along with an increasing amount of a HBV Pol expression plasmid, and luciferase levels were measured. As expected, luciferase activity in the cells transfected with the ε-FL-30 and ε-FL-55 reporter constructs decreased with increasing HBV Pol expression in a dose-dependent manner (Fig. 3B), demonstrating that HBV Pol suppressed translation of the luciferase ORF, as previously reported (Ryu et al., 2008). Further, the
magnitude of the reduction of luciferase activity for ε-FL-30 construct was comparable to previous reports (Ryu et al., 2008). In contrast, luciferase activity from cells transfected with the ε-FL-82 and ε-FL-298 constructs did not change upon overexpression of HBV Pol, indicating that translation was unaffected (Fig. 3B). This finding indicates that the proximity of the cap to within less than 60 nt of the 5' ε stem–loop structure is critical for the suppression of pgRNA translation. This result was also confirmed by measuring the level of luciferase protein by Western blot (data not shown).

Next, to directly relate the above findings to pgRNA encapsidation, we measured the encapsidation of the RNA derived from the four reporter constructs by RPA. Consistent with our previous findings using similar constructs (Jeong et al., 2000), RNA encapsidation was proportionally reduced as the 5' ε stem–loop structure was placed...
farther from the 5′ end (Fig. 3C). Thus, since the distance between the cap and the 5′ ε stem–loop structure has a strikingly similar effect on both the suppression of translation and RNA encapsidation, this led us to speculate that the suppression of pgRNA translation is mechanistically linked to RNA encapsidation. Similar results were obtained using HepG2 cells, a human hepatoma cell line (Fig. S1A and Fig. S1B).

HBV Pol fails to suppress both cap-dependent and cap-independent translation of nonviral mRNA

Recently, we reported that HBV Pol interacts with eIF4E, a translation initiation factor (Kim et al., 2010). This interaction led us to speculate that HBV Pol may not affect the cap-independent translation, since eIF4E is dispensable for IRES-mediated translation [Martinez-Salas et al., 2008]. To assess this prediction, we generated a bicistronic mRNA, in which the translation of the second ORF is initiated by an internal ribosome entry site (IRES). Two IRES-containing bicistronic constructs were constructed (Fig. 4A): one having the IRES derived from the encephalomyocarditis virus (EMCV) and another being derived from hepatitis C virus (HCV) (Kim et al., 2004). In the RL-EI-FL construct, an EMCV IRES was inserted between the renilla luciferase and firefly luciferase ORFs. Similarly, the HCV IRES was inserted between the two luciferase ORFs in the RL-HI-FL construct. Cells were transfected with these bicistronic plasmids and luciferase activity was measured. As anticipated, firefly luciferase activities were not altered by coinexpression of HBV Pol, indicating that IRES-dependent translation was unaffected by HBV Pol regardless of the origin of the IRES (Fig. 4B). Further, since IRES-mediated translation was not regulated by HBV Pol, we could eliminate the possibility that translation initiation factors other than eIF4E were involved in the suppression of translation by HBV Pol, but suggested that eIF4E was a potential target for the regulation of pgRNA translation. Finally, since renilla luciferase activities were also unaffected by HBV Pol expression, we concluded that cap-dependent translation in the absence of 5′ ε stem–loop structure was unaffected (Fig. 4B). These results substantiated that HBV Pol suppresses cap-dependent mRNA translation only in the presence of a cap-proximal 5′ ε stem–loop structure.

Suppression of pgRNA translation by HBV Pol occurs by a mechanism that does not require direct interaction with eIF4E

Since a close proximity between the cap and the 5′ ε stem–loop structure is crucial for suppression of translation suppression, we investigated the possibility that the HBV Pol−eIF4E interaction is essential for the suppression of pgRNA translation. Since eIF4E is central to the regulation of mRNA translation by a diverse set of eIF4E binding factors (Richter and Sonenberg, 2005), we reasoned that the eIF4E engaged by pgRNA could be regulated by HBV Pol as well.

To test whether the interaction between HBV Pol and eIF4E is essential for the suppression of translation, we utilized the hantavirus N protein as a cap binding factor, instead of eIF4E. Mir and Panganiban (2008) demonstrated that hantavirus N protein could replace eIF4F for cap-dependent translation of the hantavirus mRNA. Thus, the hantavirus N protein can functionally substitute for eIF4E as a cap binding factor, as well as other translation initiation factors crucial for translation initiation, such as eIF4A and eIF4G. By utilizing the hantavirus N protein for translation initiation, we asked whether the HBV pgRNA is subjected to translation suppression by HBV Pol. If the HBV Pol−eIF4E interaction is essential for the suppression of translation, then N protein-driven translation would not be affected by HBV Pol. To test this hypothesis, an 8 nt hantavirus sequence, which is minimally required for hantavirus N protein binding (Mir and Panganiban, 2008), was substituted into the 5′ end of the pgRNA (Figs. 5A and B).

Fig. 4. HBV Pol does not suppress IRES-dependent translation. (A) A schematic diagram illustrating two IRES-encoding bicistronic constructs. The RL-EI-FL encodes the renilla luciferase (RLuc) ORF followed by firefly luciferase (FLuc) ORF, in which the EMCV IRES is inserted in front of the second ORF. Similarly, the RL-HI-FL construct encodes the renilla luciferase (RLuc) ORF followed by firefly luciferase (FLuc) ORF, in which the HCV IRES is inserted in front of the second ORF. (B) Reporter assay. Cells were transfected with each bicistronic construct, along with an increasing amount of a HBV Pol expression plasmid. The luciferase activity assays were performed according to manufacturer’s instruction.
Cells were transfected with the P-null* and HBV Pol expression constructs, along with an increasing amount of a hantavirus N protein expression construct (pT-N). Three days following transfection, HBV core protein was detected by Western blot analysis (Fig. 5C). Remarkably, the data showed that the core protein level increased upon expression of the N protein, in a dose-related manner (Fig. 5C, lanes 1–4). The observation that core protein levels increased linearly with the amount of the N protein construct used to transfect the cells indicated that HBV core protein synthesis was primarily driven by the N protein and not by endogenous eIF4E. This result confirmed previous findings that the N protein can functionally replace eIF4F for the cap-dependent translation (Mir and Panganiban, 2008). Unexpectedly, core protein levels were decreased upon HBV Pol expression, indicating that binding of the 5′ ε stem–loop structure by HBV Pol is both necessary and sufficient for the suppression of pgRNA translation.

**Discussion**

We previously demonstrated that translation of the pgRNA is suppressed by HBV Pol (Ryu et al., 2008). Here, we sought to gain further insights into the mechanism by which HBV Pol suppresses the pgRNA translation. We found that close proximity of the 5′ cap to the 5′ ε stem–loop structure of the pgRNA is required for the suppression of translation (Fig. 3), and this close proximity was also required for efficient RNA encapsidation (Jeong et al., 2000). These results indicated that suppression of pgRNA translation precedes encapsidation. Importantly, by substituting eIF4E with the hantavirus N protein, as a cap binding translation factor, we demonstrated that the HBV Pol–eIF4E interaction is not essential for translation suppression (Fig. 5), and instead, the data indicated that proximity between the

**Fig. 5.** Evidence that HBV Pol suppresses translation through a mechanism independent of its interaction with eIF4E. (A) A schematic diagram illustrating the P-null* construct encoding the hantavirus N protein binding site. The position of the inserted N protein binding site is denoted by an arrow. It is important to note that the HBV 5′ ε stem–loop structure is present in the pgRNA used in this experiment, unlike the experiment shown in Fig. 3. (B) The nucleotide sequence of the 5′ end of the pgRNA transcribed from the P-null* construct. The nucleotide sequence of the pgRNA transcribed from HBV WT replicon construct is shown on the top for comparison: the transcription start site (nt. 1820) is indicated by right-hand arrow and an 8 nt sequence substituted in the P-null* was underlined. The 8-nt hantavirus N protein binding site introduced by substitution mutation into the P-null* construct is highlighted by bold font. (C) Western blot analysis. Cells were transfected with the P-null* and P (Pol) expression constructs, along with an increasing amount of the hantavirus N protein expression construct, pT-N (Mir and Panganiban, 2008). Data presented as the mean ± standard deviations of experiments performed in triplicate. Actin served as loading control. Symbols are as shown in Fig. 1C.
cap and 5′ ε stem–loop structure is necessary and sufficient for the suppression of the pgRNA translation.

Evidence that binding of HBV Pol to the 5′ ε stem–loop structure precedes translation suppression was demonstrated by two different experiments: (i) deletion analysis of the 5′ ε stem–loop structure (Fig. 1) and (ii) deletion analysis of HBV Pol subdomains (Fig. 2). Previously, Hu and Boyer (2006) demonstrated that a recombinant HBV Pol GST-fusion protein fails to bind to the stem–loop structure lacking the bulge region in vitro, suggesting that the bulge region is crucial for the interaction between HBV Pol and the pgRNA. Here, we showed that the bulge, but not apical loop, of the 5′ ε stem–loop structure is indispensable for the translation suppression (Fig. 1C).

In addition, they also showed that the TP and RT subdomains are essential for the RNA binding, but the SP and RH domains are largely dispensable, evidenced by gel mobility shift assay (Hu and Boyer, 2006). Consistently, our result showed that ΔTP-P failed to induce translation suppression, whereas the ΔRH-P did (Fig. 2B). Correlation between the ability of HBV Pol to bind to the 5′ ε stem–loop structure in vitro and the ability of HBV Pol to inhibit translation of the pgRNA led us to conclude that the binding of 5′ ε stem–loop structure by HBV Pol is critical for the suppression of pgRNA translation.

On the other hand, some discrepancy was evident with respect to the TP subdomain. Since the TP subdomain is essential for the RNA binding in vitro, it was expected that the ΔTP-P fails to induce the translation suppression. In contrast, the ΔTP-P suppressed translation of C protein to the extent comparable to that of wild type (Fig. 2B, lanes 4 versus 3), implicating that the recombinant HBV Pol GST-fusion protein lacks the bulge region in vivo, suggesting that the bulge region is crucial for the interaction between HBV Pol and the pgRNA. Here, we showed that the bulge, but not apical loop, of the 5′ ε stem–loop structure is indispensable for the translation suppression (Fig. 1C). In addition, they also showed that the TP and RT subdomains are essential for the RNA binding, but the SP and RH domains are largely dispensable, evidenced by gel mobility shift assay (Hu and Boyer, 2006). Consistently, our result showed that ΔTP-P failed to induce translation suppression, whereas the ΔRH-P did (Fig. 2B). Correlation between the ability of HBV Pol to bind to the 5′ ε stem–loop structure in vitro and the ability of HBV Pol to inhibit translation of the pgRNA led us to conclude that the binding of 5′ ε stem–loop structure by HBV Pol is critical for the suppression of pgRNA translation.

Another pertinent question is whether the interaction between HBV Pol and eIF4E was required for the translation suppression. We recently reported that HBV Pol interacts with eIF4E, resulting in the incorporation of eIF4E into nucleocapsid particles (Kim et al., 2010). Thus, the HBV Pol–eIF4E interaction could be directly involved in translation suppression. Unexpectedly, however, our data show that the HBV Pol–eIF4E interaction was not required for translation suppression, since hantavirus N protein-driven translation was still suppressed by HBV Pol (Fig. 5C). Instead, we speculate that the recognition of the 5′ ε stem–loop structure by HBV Pol is necessary and sufficient for translation suppression. Nonetheless, a possibility that endogenous eIF4E present in the experiment shown in Fig. 5C somehow contributes to the translation suppression has not been formally excluded. Intriguingly, hantavirus N protein, which appears somehow contributes to the translation suppression has not been formally excluded. Intriguingly, hantavirus N protein, which appears to remain bound to the 5′ end of the pgRNA in the place of eIF4E, still supported pgRNA encapsidation (data not shown). Whether nucleocapsid-incorporated eIF4E has any function in viral life cycle remains to be seen.

It is not unprecedented that a viral polymerase plays a role in translation regulation in addition to its primary role in viral genome replication. For instance, the genome of single-strand RNA viruses are templates for translation and replication, so these viruses must resolve the inherent competition between translation (viral protein synthesis) and genome replication. In case of picornaviruses, it was shown that the recognition of a cloverleaf structure located at 5′ end of the RNA genome by 3CD, a precursor of viral RNA-dependent RNA polymerase, is critical for the switch from translation to RNA genome replication.
replication (Gamarnik and Andino, 1998). Further, translation suppression induced by the 3CD/cloverleaf structure interaction is essential for RNA genome replication. We have previously shown that the HBV Pol/5′-ε interaction could regulate the switch from translation to pgRNA encapsidation (Ryu et al., 2008). Although hepadnaviruses contain a DNA genome, they share this inherent competition problem with single-strand RNA viruses because both translation and RNA encapsidation share the pgRNA template. To coordinate these two processes, hepadnaviruses have evolved the 5′-ε stem–loop structure, positioned within close proximity to the 5′ cap structure, which associates with the viral Pol to suppress translation and simultaneously triggering encapsidation.

The hepadnaviral Pol has multiple functional roles in viral genome replication as well as capsid assembly. Here, we explored the novel regulatory role of HBV Pol in the inhibition of pgRNA translation and provided experimental evidence that links suppression of translation to encapsidation. More in-depth studies of how HBV Pol regulates translation and encapsidation should provide us with valuable information for exploiting HBV Pol as an antiviral target for the development of effective drugs to treat HBV-associated diseases.

Materials and methods

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 10 μg of gentamycin per milliliter at 37 °C in 5% CO₂ and were subcultured every third day. HEK293 cells were transfected using polyethylenimine (PEI) (25 kDa; Aldrich) as described before (Ryu et al., 2008). Briefly, cells were plated at a confluity of approximately 50% one day before transfection. Equal amounts of plasmid DNA and PEI (4 μg per 35-mm plate, 12 μg per 60-mm plate) were diluted with serum-free DMEM. DNA and PEI were mixed and incubated for 10 minutes at room temperature. Cells were washed with DMEM and then overlaid with the DNA–PEI complex solution. After 3 h, cells were rinsed and fed with fresh media. Transfection efficiencies of 40%–50% routinely were obtained.

Plasmid construction

All HBV constructs were derived from a wild-type HBV expression clone, pCMV-HBV/30. The nucleotide sequence of the HBV genome was numbered starting from the unique EcoRI site of the HBV ayw subtype (Galibert et al., 1979). All substitution and deletion mutants were generated by overlap extension PCR protocols as previously described (Lee et al., 2004). All mutants were sequenced to confirm the base change. The details of the molecular cloning of any plasmid construct described in this report will be provided upon request. To generate Δ5′-B-WT construct, we deleted six nucleotides (nucleotide 1862–1867, CTGTGC) of 5′-ε bulge region of pCMV-HBV/30. In parallel, the fragment having the identical mutation was introduced into the P-null construct (Ryu et al., 2008) to create Δ5′-B-P-null counterpart. The Δ5′-L-WT and Δ5′-P-null constructs were made by deleting six nucleotides (nucleotide 1878–1884, CTGTGC) of the 5′ apical loop, respectively. To generate epsilon position mutants, Pml I-ε-Luc was made by inserting a Pml I restriction enzyme site upstream of the ε sequence of ε-Luc (i.e., ε-FL-30) (Ryu et al., 2008), in which epsilon is located 30 nucleotides from 5′ cap. The PCR fragments derived from pLZ-ε-55, pLZ-ε-82, and pLZ-ε-298 (Jeong et al., 2000) were introduced into Pml I-ε-Luc to create ε-FL-55, ε-FL-82, and ε-FL-298, respectively. The helper plasmid (pCH3142) provides the viral core and Pol proteins as previously described (Jeong et al., 2000). Two IRES constructs, RL-EL-FL (pREF) and RL-HI-FL (pRH402F), were generous gifts from Dr. S.K. Jang (POSTECH, Korea). HBV P-null construct was made by replacing HBV sequence (nucleotide 1920–1927) of the P-null construct with hantavirus sequences (TAGTAGTA). The pT-N (pSNV N TriEx 1.1) was a gift from Dr. A.T Panganiban (University of New Mexico, USA).

Western blot analysis

Three 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, and EDTA-free protease inhibitor cocktail [Roche]). The samples were electrophoresed through 12% SDS–polyacrylamide gels. Proteins then were transferred to a PVDF membrane (Immobilon-P; Millipore). After blocking, the membrane was incubated with a rabbit anti-core antibody (DAKO, 1:2000) or a mouse anti-FLAG M2 antibody to detect HBV Pol (Sigma, 1:5000) followed by anti-rabbit or anti-mouse IgG horseradish peroxidase–linked antibody (Amersham, 1:5000), respectively, β-Actin was detected by using a rabbit anti-actin antibody (Sigma, 1:5000). The proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer). Images were quantified using LAS-3000 (Fujifilm).

Reporter assay

Three days after transfection, cells were harvested and assayed as the manufacturer's instruction using Luciferase Reporter Assay System (Promega) and a TD 20/20 Luminometer (Turner Design Inc.).

Encapsulation assay

RNA was extracted as previously described (Jeong et al., 2000) and analyzed by RNase protection analysis (RPA) according to the manufacturer's instructions (Ambion). Briefly, cells were lysed with lysis buffer (10 mM Tris–Cl [pH 7.9], 1 mM EDTA, 1% NP-40, 50 mM NaCl). The lysate was centrifuged for 2 min at 12,000 rpm to pellet nuclei. To isolate core-associated RNA, the supernatant was treated with DNase I (10 U/ml; Sigma) and microccocal nuclease (30 U/ml; USB) for 15 min at 37 °C. The sample was precipitated with PNE buffer (26% polyethylene glycol, 1.4 M NaCl, 40 mM EDTA) for 1 h on ice. Followed by centrifugation at 12,000 rpm for 15 min, RNA was extracted by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Total cell RNA was extracted by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Core RNA and total cell RNA were loaded at 1:1 ratio and assayed by RNase protection analysis as described below.

RNase protection analysis

RNase protection analysis (RPA) was carried out according to the manufacturer's instructions (Ambion). The core region or luciferase-specific riboprobes were employed as previously described (Ryu et al., 2008). Briefly, each sample of RNA was hybridized with 10⁶ cpm of [α-32P] UTP (3000 Ci/mmol; Amersham)–labeled probe for 16 h at 42 °C. RNase digestion was carried out with a mixture of RNase A/T1 for 30 min at 37 °C. The digested fragments were separated in a 5% acrylamide–8 M urea gel. The gel was dried and exposed on the imaging plate for 16 h at 25 °C. Radiographic phosphorimages were analyzed using a Bio-Imaging Analyzer (BAS-2500; Fujifilm).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.07.005.

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