行政院國家科學委員會專題研究計畫　成果報告

D 型肝炎病毒 mRNA 體外轉錄系統製備：細胞 RNA 聚合胺之鑑定與病毒抗原的功能分析

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ABSTRCT.

Hepatitis delta virus (HDV) is a single-stranded RNA virus that encodes two viral nucleocapsid proteins named small and large form hepatitis delta antigen (SHDAg and LHDAg). The SHDAg is essential for viral RNA replication while the LHDAg is required for viral assembly. We had identified HDAg as an acetylated protein and Lys-72 of SHDAg as one of the acetylation sites. Substitution of Lys-72 to Arg modulated HDAg subcellular localization and might participate in viral RNA nucleocytoplasmic shuttling and replication. In the following study, overexpression of SIRT, the type III histone deacetylase, translocated HDAg from mainly nucleolar distribution to nucleoplasmic or even cytoplasmic distribution. To elucidate the correlation between acetylation and nucleolar location of HDAg, putative acetylation motif (K/R)XKK of nuclear receptor and the nucleolar localization motif (R/K)(R/K)X(R/K) was found between 38KKKLKK43 of SHDAg. SHDAg mutant with Lys-to-Arg substitutions of 38KKKLKK43 (SHDAg-(K38-43R)) localized outside the nucleoli and could not facilitate the replication of HDV RNA. We added a heterologous nucleolar localization sequence to this mutant to restore its nucleolar localization. This will disclose the role of nucleolar localization for HDV replication. The functions and mechanisms of nucleolus-localization of SHDAg on HDV RNA replication are under investigation. In another part of our study, we also analyzed the cellular machinery for HDV RNA replication. By ultracentrifugation, nuclear extracts from HDV cDNA transfected cells were fractionated and the distribution of HDV RNA and HDAg were analyzed. RNA smearing in fraction 8-11 was observed, suggesting replication intermediates were produced during HDV replication cycle. These fractions were perforned in vitro transcription with ^{32}P labeling and the products then hybridized with cold HDV genomic or antigenomic RNA probe in slot blot. Unfortunately, the signal in slot blot was not strong enough to show the

replication activity in these fractions. Optimization of the system is proceeding.

Keywords: HDV, nucleolus, NoLS, acetylation, in vitro transcription

Hepatitis delta virus (HDV) contains a 1.7-kb circular RNA genome. It is a satellite virus that requires its helper virus, hepatitis B virus (HBV), to supply the envelope proteins for viral assembly. However, HDV is capable of replicating its genome in the absence of HBV. A double rolling-circle mechanism has been proposed for HDV RNA replication. Host cellular transcription machinery is believed to be responsible for this process. There are two viral proteins encoded by HDV named large and small delta antigens (HDAg), respectively. These two proteins are identical in sequence, except that the large HDAg (LHDAg) contains an additional 19 amino acids at its C-terminus resulting from RNA editing of the termination codon. Although the similarity in amino acid sequences, each protein has distinct functions. The small HDAg (SHDAg) is essential for HDV replication, while the LHDAg is necessary for virion assembly. We had identified HDAg as an acetylated protein and Lys-72 of SHDAg as one of the acetylation sites. Substitution of Lys-72 to Arg modulated HDAg subcellular localization and might participate in viral RNA nucleocytoplasmic shuttling and replication. In the following study, overexpression of SIRT, the type III histone deacetylase, translocated HDAg from mainly nucleolar distribution to nucleoplasmic or even cytoplasmic distribution, implicating acetylation may play an important role in modulating the subcellular localization of HDAg.

Putative acetylation motif (K/R)XKK of nuclear receptor and the nucleolar localization motif (R/K)(R/K)X(R/K) was found between ^{38}KKKLKK^{43} of SHDAg. To elucidate the correlation between acetylation and nucleolar location of HDAg, SHDAg mutant with Lys-to-Arg substitutions of ^{38}KKKLKK^{43} (SHDAg-(K38-43R)) was created and characterized.As shown in Fig.2, wild-type SHDAg localized within nucleus and nucleolus, while SHDAg-(K38-43R) localized outside the nucleolus. This result implicated ^{38}KKKLKK^{43} is crucial on SHDAg nucleolar localization. To further characterize the its importance, the trans-activation activity of s-HDAg on HDV

replication was assessed. As shown in Fig. 3, when co-transfected with HDV genomic RNA template, Lys-to-Arg substitutions of ^{38}KKKLKK^{43} on S-HDAg (SHDAg-(K38-43R)) impairs its ability to facility HDV RNA replication. These results imply nucleolar localization of SHDAg is correlated with HDV RNA accumulation.

To elucidate the importance of nucleolar localization of SHDAg on HDV RNA replication, the nucleolar localization sequence (NoLS) of HIV Rev protein was used to restore the nucleolar distribution of SHDAg-(K38-43R). Rev NoLS-fused SHDAg-(K38-43R) (NoLS-SHDAg-(K38-43R)) could localize in nucleoli, while with or without HDV genomic RNA template. To further characterize the its trans-activation activity on HDV replication, HDV genomic RNA template with the NoLS-fused SHDAg or SHDAg-(K38-43R) mutant was co-transfected and analyzed. It shown that Rev NoLS-fused S-HDAg-(K38-43R) partially restored its ability to facilitate HDV RNA replication.

Taken together, these results implicate nucleolar localization of S-HDAg is crucial for its trans-activation activity on HDV replication. However, its functions and mechanisms and correlation with acetylation are still obscure and need more study for elucidation.

In another part of our study, we also analyzed the cellular machinery for HDV RNA replication. It was acceptable that HDV RNA replication was through double rolling circle machinery, but there were still no strong evidences to support the model. Here, we attempted to purify the replication intermediates to clarify the issue. It was known HDV replication occurred in the nucleus. Therefore, nuclear extract of HDV transfected cells was purified and performed glycerol gradient sedimentation. After ultra-centrifugation, each fraction was analyzed by Northern and Western blot to

verify the presence of HDV RNA and HDAg. In Northern blot, HDV RNA smearing was observed in fraction 8-11. This suggested replication intermediates were produced during HDV replication cycle. We assumed these fractions containing replication intermediates might possess replication activity. To demonstrate our hypothesis, these fractions were detected by in vitro transcription. The products of in vitro transcription then hybridized with cold HDV genomic or antigenomic RNA probe in slot blot. Unfortunately, the signal in slot blot was too weak to distinguish the replication activity in these fractions. This might be due to the enzymatic activity was lost in our system or the condition of in vitro transcription was not optimal to HDV RNA replication. In the future, the system will be optimized to support our hypothesis.