Regulation of Mouse Hepatitis Virus RNA synthesis by Heterogeneous Nuclear Ribonucleoprotein A1

[1]STEPHANIE T. SHI, [1]PEIYONG HUANG, HSIN-PAI LI, AND [1,2]MICHAEL M. C. LAI

[1]Department of Molecular Microbiology and Immunology and [2]Howard Hughes Medical Institute, University of Southern California School of Medicine, Los Angeles, CA

1. INTRODUCTION

Mouse hepatitis virus (MHV) RNA synthesis, including replication of viral genome and transcription of subgenomic mRNAs, has been shown to be regulated by several viral RNA elements, including 5'-untranslated region (5'-UTR), *cis*- and *trans*-acting leader RNA, intergenic (IG) sequence, and 3'-UTR (Lai and Cavanagh 1997). Biochemical evidence suggests that these regulatory sequences likely interact with each other either directly or indirectly, probably through protein-RNA and protein-protein interactions involving both viral and cellular proteins (Zhang and Lai 1995). Several cellular proteins have been found to bind these regulatory sequences by UV-crosslinking experiments (Furuya and Lai 1993, Huang and Lai 1999); three of these proteins have been identified to be heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Li *et al* 1997), polypyrimidine-tract-binding protein (PTB) (Huang and Lai 1999, Li *et al* 1999), and poly(A)-binding protein (PABP) (Spagnolo and Hogue 2000).

hnRNP A1 is involved in pre-mRNA splicing and transport of cellular RNAs (Dreyfuss *et al* 1993). It is predominantly a nuclear protein, but also shuttles between the nucleus and the cytoplasm (Piñol-Roma and Dreyfuss 1992). The cytoplasmic hnRNP A1 has been implicated in the regulation of mRNA stability (Hamilton *et al* 1993, Henics *et al* 1994) and translation (Svitkin *et al* 1996). hnRNP A1 binds MHV (-)-strand leader and IG sequences (Fig. 1) (Furuya and Lai 1993). The extent of binding of hnRNP A1 to the (-)-strand IG sequences correlates with the efficiency of

The Nidoviruses (Coronaviruses and Arteriviruses).
Edited by Ehud Lavi *et al.*, Kluwer Academic/Plenum Publishers, 2001.

transcription from the IG site (Zhang and Lai 1995). In MHV-infected cells, hnRNP A1 relocates to the cytoplasm, where viral RNA synthesis occurs (Li *et al* 1997). hnRNP A1 also interacts with the MHV nucleocapsid (N) protein (Wang and Zhang 1999), which is required for MHV RNA synthesis (Compton *et al* 1987). Furthermore, hnRNP A1 mediates the formation of a ribonucleoprotein complex containing the MHV (-)-strand leader and IG sequences (Zhang *et al* 1999). hnRNP A1 and PTB bind to the precisely complementary sites on the (-)- and (+)-strand RNA, respectively, of the leader region of MHV RNA (Li *et al* 1997, Li *et al* 1999), and also the 5'- and 3'-ends of both the (+)- and (-)-strand RNAs (Huang and Lai 1999, unpublished) (Fig. 1). The complementarity of their binding sites on both ends of MHV RNA suggests that hnRNP A1 and PTB may mediate the interaction between these regions to form a ribonucleoprotein complex, which functions in MHV RNA synthesis.

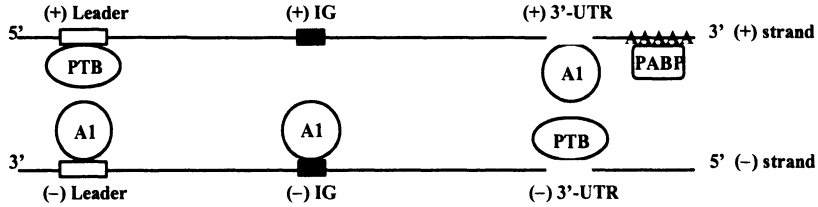

Figure 1. Schematic drawings of the cellular proteins that interact with MHV RNA.

2. MATERIALS AND METHODS

2.1 Cells

Permanent DBT cell lines were established by transfecting pcDNA3.1 alone or the plasmid containing an open reading frame for the Flag-tagged hnRNP A1 or hnRNP A1ΔC, under the control of a CMV immediate-early gene promoter, into DBT cells and selected with 0.5 mg/ml G418.

2.2 Electrophoretic mobility shift and UV-crosslinking assays

Different amounts of GST-fusion proteins and the ^{32}P-labeled (-)-strand MHV 5'-end RNA (182 nt) were incubated for 10 min at 30°C. The protein-

RNA complexes were then separated on a 4% nondenaturing polyacrylamide gel according to the published procedures (Furuya and Lai 1993). UV-crosslinking assay was performed as described (Huang and Lai 1999).

3. RESULTS

3.1 The effects of the wt and mutant hnRNP A1 on MHV RNA synthesis

We established murine DBT cell lines stably expressing the Flag-tagged wt hnRNP A1 (DBT-A1) or a mutant hnRNP A1, which has a 75-amino acid deletion from the C terminus (DBT-A1ΔC) (Fig. 2). This mutant lacks part of the glycine-rich domain and the M9 sequence responsible for shuttling hnRNP A1 between the nucleus and the cytoplasm (Siomi and Dreyfuss 1995, Weighardt *et al* 1995). The Flag-tagged wt hnRNP A1 was localized almost exclusively in the nucleus, whereas the mutant hnRNP A1 was localized predominantly in the cytoplasm (data not shown) as predicted.

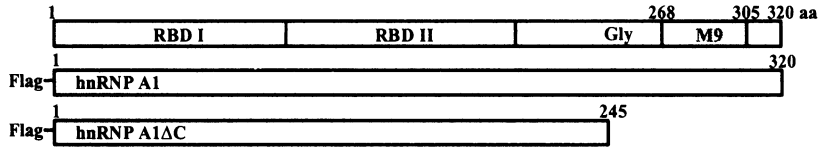

Figure 2. Diagrammatic structure of the wt and mutant hnRNP A1. RBD, RNA-binding domain; Gly, glycine-rich region.

We assessed the production of genomic and subgenomic MHV RNAs in these cell lines by Northern blot analysis. At 8 h p.i., there were significantly higher steady-state levels of all of the viral RNA species in DBT-A1 cells than in DBT-VEC cells, which were transfected with the empty vector (Fig. 3A). In contrast, no viral RNA was detected in DBT-A1ΔC cells at that time point. At 16 h p.i., viral RNA levels of DBT-VEC and DBT-A1 cells decreased generally because of the loss of dead cells, while the smaller subgenomic RNAs became detectable in DBT-A1ΔC cells (data not shown). By 24 h p.i., most viral RNA species became detectable in DBT-A1ΔC cells, while most of the DBT-A1 cells had already been completely lysed. Therefore, the wild-type hnRNP A1 accelerated MHV RNA synthesis, whereas its mutant significantly delayed it.

We also detected a defective-interfering (DI) RNA species (arrow in Fig. 3A), which appeared to be inhibited to a greater extent than other RNA

species in DBT-A1ΔC cells. To confirm this result, we further studied replication of another DI RNA. DBT cells were infected with MHV-A59 and transfected with DIssE RNA of JHM (Makino and Lai 1989); the virus released was passaged twice in DBT cells and used to infect various cell lines. Similar to the A59 DI RNA, the replication of DIssE RNA was much more strongly inhibited than that of MHV genomic and subgenomic RNAs in DBT-A1ΔC cells (Fig. 3B). Our results thus suggest that MHV DI RNA replication is more dependent on the function of cytoplasmic hnRNP A1.

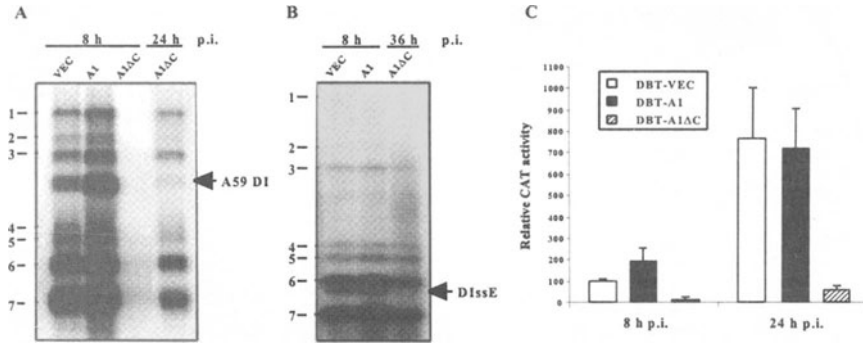

Figure 3. MHV RNA replication (A, B) and transcription (C) in DBT cells. Northern blot analysis of MHV RNA (A) or DIssE RNA (B). (C) CAT assay of A59-infected DBT cells transfected with the 25CAT DI RNA. The values represent averages of three independent experiments.

3.2 hnRNP A1ΔC inhibits transcription of MHV DI RNAs

To demonstrate that MHV RNA transcription machinery is defective in cells expressing the mutant hnRNP A1, we studied transcription of an MHV DI RNA, 25CAT (Liao and Lai 1994). The 25CAT RNA was transfected into MHV-A59-infected cells 1 h after infection. At 8 h p.i., CAT activity in DBT-A1 cells was higher than that in DBT-VEC cells (Fig. 3C). On the other hand, the CAT activity was very low in DBT-A1ΔC cells. At 24 h p.i., CAT activity in DBT-A1 cells became less than that in DBT-VEC cells because of the loss of dead DBT-A1 cells. CAT activity in DBT-A1ΔC remained significantly lower than that in DBT-VEC cells. Thus, there was an inhibition of mRNA transcription from the DI RNA, consistent with the results observed for the mRNA transcription from the wt viral genome.

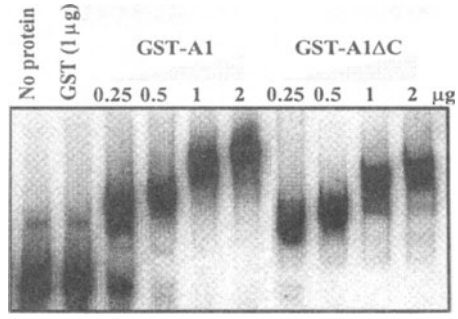

Figure 4. Electrophoretic mobility shift assay for the RNA-binding properties of the wt and mutant hnRNP A1 using the ^{32}P-labeled (-)-strand MHV leader RNA.

3.3 Characterization of the interaction between hnRNP A1 and MHV RNA

To explore the mechanism of the inhibitory effects of hnRNP A1ΔC, we examined the MHV RNA-binding ability of this mutant by electrophoretic mobility shift assay. GST-hnRNP A1 protein, but not GST, efficiently binds the (-)-strand MHV leader RNA to form an RNA-protein complex (Fig. 4). The complex increased in size with the increasing amounts of GST-hnRNP A1 (Fig. 4). A similar RNA-binding pattern was also observed for hnRNP A1ΔC. Furthermore, UV-crosslinking experiments showed that increasing amounts of purified GST-hnRNP A1ΔC efficiently competed with the cellular endogenous hnRNP A1 for the binding of (-)-strand MHV leader RNA (data not shown), indicating that the binding of hnRNP A1ΔC to RNA was not affected. Also, the ability of hnRNP A1ΔC to bind viral proteins N and gene 1 protein was not affected; however, this mutant protein failed to bind a cellular protein, p250, which was associated with the wild-type hnRNP A1 (data not shown).

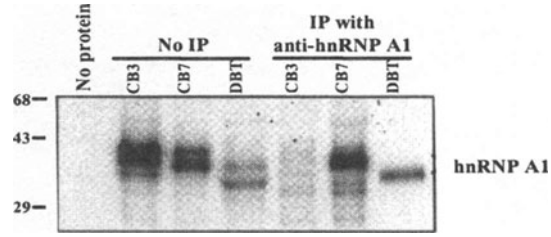

Figure 5. Detection of MHV RNA-binding proteins in CB3 and CB7 cells by UV-crosslinking.

3.4 hnRNP A1-related proteins bind to MHV RNA in CB3 cells

A mouse erythroleukemia cell line, CB3, was reported to lack detectable hnRNP A1 expression (Ben-David *et al* 1992). However, MHV was still able to replicate efficiently in these cells. Since hnRNP A1 protein is involved in a variety of important cellular functions, it is conceivable that other gene products may substitute for the function of hnRNP A1 in CB3 cells. We performed UV-crosslinking assays to detect MHV RNA-binding proteins present in CB3 and its parental cell line, CB7. hnRNP A1 was present only in DBT and CB7 cells as determined by immunoprecipitation with the monoclonal antibody against hnRNP A1 (Fig. 5). Three proteins comparable to hnRNP A1 in size that could interact with the MHV (-)-strand leader RNA were detected in CB3 cells, but none of them reacted with the antibody against hnRNP A1. The size of these proteins was slightly larger than hnRNP A1 in the DBT cell extracts. These proteins may represent hnRNP A1-related proteins. Therefore, multiple cellular proteins may have the capacity to be involved in MHV RNA synthesis.

4. DISCUSSION

There is increasing evidence that RNA viruses subvert cellular factors for replication and transcription of viral RNAs (Lai 1998). In the present study, we showed that MHV RNA transcription and replication were enhanced by the overexpression of the wt hnRNP A1 protein, but inhibited by the expression of a dominant-negative hnRNP A1 mutant in DBT cell lines. Our results suggest that hnRNP A1 is a host protein involved in the formation of a cytoplasmic transcription/replication complex for viral RNA synthesis.

hnRNP A1ΔC caused a preferential inhibition of at least two DI RNA species, suggesting that the inhibition of MHV replication by the hnRNP A1 mutant was most likely a direct effect on viral RNA synthesis rather than an indirect effect on other aspects of cellular or viral functions. Since hnRNP A1 binds directly to the *cis*-acting MHV RNA sequences critical for MHV RNA transcription and replication, it is most likely that hnRNP A1 may participate in the formation of the transcription/replication complex. hnRNP A1 may modulate MHV RNA transcription or replication by participating in the processing, transport and controlling the stability of viral RNAs. Alternatively, hnRNP A1 may participate more directly in viral RNA synthesis in a similar role to that of transcription factors in DNA-dependent RNA synthesis, such as maintaining favorable RNA conformation for RNA synthesis.

The mechanism of the dominant-negative effects of hnRNP A1ΔC is still not clear. hnRNP A1ΔC retains the MHV RNA-binding and self-association ability (Fig. 4) and is capable of binding the viral proteins that are required for RNA replication. Preliminary data, however, suggest that the mutant protein is unable to bind certain cellular proteins, which may be involved in MHV RNA synthesis.

In summary, our data provide evidence that hnRNP A1 is directly or indirectly involved in MHV RNA transcription and replication. Our results also suggest that other related cellular proteins may substitute for the role of hnRNP A1 in MHV RNA synthesis. For example, other hnRNP proteins, such as hnRNP A2/B1 and hnRNP B2, are potential candidates because of their similarity in size, sequence (especially in RNA-binding domains), structure and function to hnRNP A1 (Dreyfuss *et al* 1993). Identification of the proteins in hnRNP A1-deficient CB3 cells will unveil more cellular factors that regulate MHV RNA synthesis.

ACKNOWLEDGMENTS

This work was partially supported by a National Institutes of Health research grant (to MMCL) and a postdoctoral fellowship (to STS). MMCL is an investigator of the Howard Hughes Medical Institute.

REFERENCES

Ben-David, Y., Bani, M.R., Chabot, B., De Koven, A., and Bernstein, A., 1992, Retroviral insertions downstream of the heterogeneous nuclear ribonucleoprotein A1 gene in erythroleukemia cells: evidence that A1 is not essential for cell growth. *Mol Cell Biol* **12:** 4449-4455.

Compton, S.R., Rogers, D.B., Holmes, K.V., Fertsch, D., Remenick, J., and McGowan, J.J., 1987, In vitro replication of mouse hepatitis virus strain A59. *J Virol* **61:** 1814-1820.

Dreyfuss, G., Matunis, M.J., Piñol-Roma, S., and Burd, C.G., 1993, hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem* **62:** 289-321.

Furuya, T., and Lai, M.M.C., 1993, Three different cellular proteins bind to complementary sites on the 5'- end-positive and 3'-end-negative strands of mouse hepatitis virus RNA. *J Virol* **67:** 7215-7222.

Hamilton, B.J., Nagy, E., Malter, J.S., Arrick, B.A., and Rigby, W.F.C., 1993, Association of heterogeneous nuclear ribonucleoprotein A1 and C proteins with reiterated AUUUA sequences. *J Biol Chem* **268:** 8881-8887.

Henics, T., Sanfridson, A., Hamilton, B.J., Nagy, E., and Rigby, W.F.C., 1994, Enhanced stability of interleukin-2 mRNA in MLA 144 cells. Possible role of cytoplasmic AU-rich sequence-binding proteins. *J Biol Chem* **269:** 5377-5383.

Huang, P., and Lai, M.M.C., 1999, Polypyrimidine tract-binding protein binds to the complementary strand of the mouse hepatitis virus 3' untranslated region, thereby altering RNA conformation. *J Virol* **73:** 9110-9116.

Lai, M.M.C., and Cavanagh, D., 1997, The molecular biology of coronaviruses. *Adv Virus Res* **48:** 1-100.

Lai, M.M.C., 1998, Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. *Virology* **244:** 1-12.

Li, H.P., Zhang, X., Duncan, R., Comai, L., and Lai, M.M.C., 1997, Heterogeneous nuclear ribonucleoprotein A1 binds to the transcription- regulatory region of mouse hepatitis virus RNA. *Proc Natl Acad Sci U S A* **94:** 9544-9549.

Li, H.P., Huang, P., Park, S., and Lai, M.M.C., 1999, Polypyrimidine tract-binding protein binds to the leader RNA of mouse hepatitis virus and serves as a regulator of viral transcription. *J Virol* **73:** 772-777.

Liao, C.L., and Lai, M.M.C., 1994, Requirement of the 5'-end genomic sequence as an upstream cis-acting element for coronavirus subgenomic mRNA transcription. *J Virol* **68:** 4727-4737.

Makino, S., and Lai, M.M., 1989, High-frequency leader sequence switching during coronavirus defective interfering RNA replication. *J Virol* **63:** 5285-5292.

Piñol-Roma, S., and Dreyfuss, G., 1992, Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355:** 730-732.

Siomi, H., and Dreyfuss, G., 1995, A nuclear localization domain in the hnRNP A1 protein. *J Cell Biol* **129:** 551-560.

Spagnolo, J.F., and Hogue, B.G., 2000, Host protein interactions with the 3' end of bovine coronavirus RNA and the requirement of the poly(A) tail for coronavirus defective genome replication. *J Virol* **74:** 5053-5065.

Svitkin, Y.V., Ovchinnikov, L.P., Dreyfuss, G., and Sonenberg, N., 1996, General RNA binding proteins render translation cap dependent. *EMBO J* **15:** 7147-7155.

Wang, Y., and Zhang, X., 1999, The nucleocapsid protein of coronavirus mouse hepatitis virus interacts with the cellular heterogeneous nuclear ribonucleoprotein A1 in vitro and in vivo. *Virology* **265:** 96-109.

Weighardt, F., Biamonti, G., and Riva, S., 1995, Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. *J Cell Sci* **108:** 545-555.

Zhang, X., and Lai, M.M.C., 1995, Interactions between the cytoplasmic proteins and the intergenic (promoter) sequence of mouse hepatitis virus RNA: correlation with the amounts of subgenomic mRNA transcribed. *J Virol* **69:** 1637-1644.

Zhang, X., Li, H.P., Xue, W., and Lai, M.M.C., 1999, Formation of a ribonucleoprotein complex of mouse hepatitis virus involving heterogeneous nuclear ribonucleoprotein A1 and transcription- regulatory elements of viral RNA. *Virology* **264:** 115-124.