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MOUSE HEPATITIS VIRUS NUCLEOCAPSID PROTEIN AS A TRANSLATIONAL EFFECTOR OF VIRAL mRNAs

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1. ABSTRACT

The mouse hepatitis virus (MHV) nucleocapsid protein stimulated translation of a chimeric reporter mRNA containing an intact MHV 5'-untranslated region and the chloramphenicol acetyltransferase (CAT) coding region. The nucleocapsid protein binds specifically the tandemly repeated -UCYAA- of the MHV leader. This RNA sequence is the same as the intergenic motif found in the genome RNA.

Preferential translation of viral mRNA in MHV infected cells is stimulated in part by this interaction and represents a specific, positive translational control mechanism employed by coronaviruses.

2. INTRODUCTION

An acute effect of mouse hepatitis virus (MHV) infection is the shut-off of host mRNA translation (Siddell et al., 1981; Hilton et al., 1986; Tahara et al., 1994). This phenomenon is observed for many cytolytic virus infections and is believed to be a means of ensuring optimum viral protein expression (Schneider and Shenk, 1987). Steady-state mRNA levels are not globally downregulated by MHV infection (Hilton et al., 1986; Kyuwa et al., 1994) so increases in viral protein expression are the sum of increased mRNA translation efficiency and increased synthesis of viral mRNAs. We reported earlier that increased translation efficiency is a property of the 5'-untranslated region UTRs of MHV mRNAs (Tahara et al., 1994). Since all MHV mRNAs of a given strain have virtually identical 5'-UTRs in an infected cell, this mechanism ensures that increased translation of viral mRNAs occurs in concert.

Coronaviruses and Arteriviruses, edited by Enjuanes *et al.*
Plenum Press, New York, 1998

We identified a 13 base sequence of the viral 5'-UTR, which includes the intergenic sequence, as the cis element primarily responsible for the increase in viral mRNA utilization (Tahara et al., 1994). The 3'-proximal region of the UTR, where this 13 nt sequence is located, was previously shown to be important for binding of MHV nucleocapsid protein to leader RNA (Stohlman et al., 1988). Thus we hypothesized that the viral nucleocapsid protein is the *trans*-acting factor responsible for increased translation efficiency of MHV mRNAs. In this report we show experimentally that it has high affinity for the intergenic sequence motif of MHV genomic and subgenomic mRNAs and acts as a *trans*-acting positive regulator of translation.

3. METHODS AND MATERIALS

3.1. Cells and Virus

Mouse DBT cells were maintained in MEM medium containing 7% newborn calf serum (heat inactivated, Gemini), 10% tryptose phosphate broth (Difco), 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Media and supplements were from Life Technologies.

The A59 strain of MHV was used.

3.2. Plasmid Constructions

Construction of pMCAT and pCAT transfection vectors was performed by insertion of CAT genes between the unique HindIII and XhoI sites of pBC/CMV/SEAP (Cullen and Malim, 1992). pCAT coding region was prepared by PCR amplification with primers containing unique HindIII and XhoI restriction sites. pMCAT was constructed by PCR amplification of the gene 6 leader sequence from phαGL-1 (Tahara et al., 1994), followed by co-amplification with CAT coding region. The resulting chimeric gene contained the MHV 5'-UTR fused directly to the start codon of the CAT reporter.

pRcCMV-N encodes the MHV-A59 nucleocapsid protein and was a gift from Dr. John Polo.

Construction of mutVI plasmid was performed by PCR methods such that an XbaI site was introduced to disrupt the intergenic sequence.

All constructs were sequenced by the dideoxynucleotide method (Sanger et al., 1977) with Sequenase II (Amersham) for verification.

3.3. DNA Transfections and CAT Assay

DBT cells were seeded at 0.5–1 X 10^6 cells per 35 mm cell culture well at least 12 h prior to transfection. Preparation of DNA-Ca-PO_4 coprecipitates, transfection conditions and glycerol shock were as described previously (Lin et al., 1986). Reporter and effector plasmids were transfected at levels indicated in the text. pCMV-SPORT-βGAL (0.5 μg/well; Life Technologies) and carrier DNA (pRcCMV; Invitrogen) were added to maintain a total DNA input of 1.7 μg/well. Cells were maintained at 37°C for 24 h prior to harvest and assay for CAT activity.

Stable transformants were prepared by cotransfecting DBT cells with 1–2 μg of pMCAT or pCAT and pSV2neo (Southern and Berg, 1982) at a 10:1 mass ratio. After transfection, cells were selected in 400 μg/ml G418 (Geneticin, Life Technologies) for 3 weeks.

Foci of G418 resistant cells were trypsinized and cloned separately in multi-well culture dishes. Continued selection in G418 was performed for an additional 3 weeks. All drug resistant clones were assayed for presence of CAT gene expression prior to any further use.

3.4. Enzyme Assays

Cell pellets were lysed by four, freeze-thaw cycles; cell-free extracts were assayed for CAT activity as described earlier (Gorman et al., 1982). Acetylated products were resolved by thin-layer chromatography and dried chromatograms were quantitated in a radiographic plate scanner (AMBIS). All conditions were assayed in duplicate.

β-Galactosidase activity was measured by a chemiluminescence assay (Galacto-Light, Tropix, Inc.) using conditions recommended by the vendor. Luminescence was measured in a Berthold luminometer. β-galactosidase activities for each transfectant were used to normalize transfection efficiency and observed CAT activity.

Protein measurements were determined by the method of Bradford using bovine gamma globulin as the standard (Bradford, 1976).

3.5. RNA Binding Assay

Construction of recombinant glutathione-S-transferase (GST) fusion proteins of JHMV nucleocapsid protein and fragments was as described earlier (Nelson, 1996). Data in Fig. 1A were obtained with N protein without GST fusion protein. There was no difference in kinetic constants for the GST fusion proteins compared to their counterparts without GST (Nelson, 1996).

RNA binding assays were performed with ^{32}P-labeled, *in vitro* transcribed pBSL (Nelson and Stohlman, 1993) as described previously (Nelson, 1996). Transcription of pBSL after linearization with HindIII using T7 RNA polymerase yields a 153 nt RNA molecule which has the first 114 bases of mRNA 6 of JHMV.

Binding data were analyzed using the EZ-Fit program, (written by Dr. Frank Perrella, E. I. DuPont de Nemours & Co.). Data were fitted to a single substrate binding curve.

4. RESULTS

Using cell-free translation extracts, we previously established that reporter mRNAs with intact MHV 5'-UTRs were specifically stimulated in cell-free extracts prepared from MHV infected cells (Tahara et al., 1994). In order to determine whether this type of stimu-

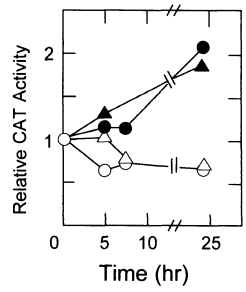

Figure 1. MHV infection of stably transformed DBT cells expressing MHV-CAT and CAT. Cell lines expressing pMCAT or pCAT were infected with MHV-A59 at an m.o.i.~1 using standard conditions. Cells were harvested at the indicated times and assayed for CAT activity. Relative CAT expression is shown in the figure. pCAT: ○, clone A1; △, clone B6. pMCAT: ●, clone A5; ▲, clone B6.

lation was physiologically relevant in the intact cell we continued these studies using a transfection approach in DBT cells. We established stable transformants expressing either a control CAT gene or an intact MHV 5'-UTR fused to the CAT reporter. These stable transformants were in turn infected with MHV-A59 and assayed for CAT expression vs. time. We expected that only CAT mRNAs with an MHV 5'-UTR (MHV-CAT) would show increased activity, based on our previous *in vitro* studies (Tahara et al., 1994). The results of such an experiment are shown in Fig. 1. Infection of clonal lines expressing MHV-CAT genes showed a general two-fold stimulation of CAT expression over a 24 hour period of infection. By contrast, MHV infection of cell lines expressing the control CAT gene showed no such increase in CAT activity. Indeed, a decline in activity was observed which was consistent with the general shut-off of translation due to MHV infection. This result confirmed the previous result obtained *in vitro* and demonstrated that the effect of MHV leader was independent of the reporter gene.

Previous experiments implicated the MHV nucleocapsid gene product as the viral gene product which binds leader RNA. We examined the biochemical properties of JHMV N protein interactions with viral leader RNA. In these studies, recombinant N protein was synthesized as a GST fusion protein in *E. coli* and assayed for binding activity with the synthetic MHV leader RNA as ligand. We observed that the N protein exhibited saturable binding kinetics when assayed against the full length leader RNA molecule. The apparent K_d for this reaction was 14 nM. Subfragments of N protein were also prepared as GST fusions consisting of N^{1-168} (A), $N^{176-230}$ (B1) and $N^{309-454}$ (C). These were tested for leader RNA binding. As shown in Fig. 2B only the GST-B1 fusion protein showed RNA binding activity.

In order to test the hypothesis that the N protein recognizes 3'-proximal sequences of the MHV leader, specifically the intergenic motif, we assayed ligands which had one to three copies of the pentamer repeat sequence. As shown in Table 1, the binding of these three ligands to GST-N was compared. We found that there was no difference in the affin-

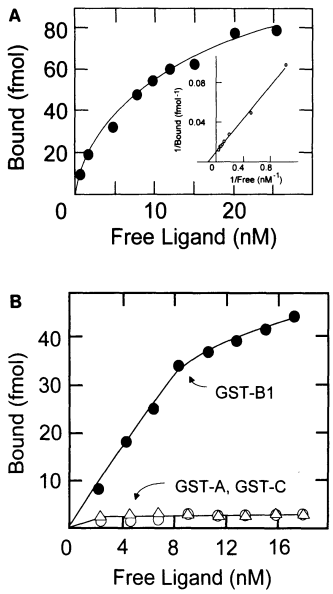

Figure 2. Leader RNA binding activity of GST-N protein. RNA binding was assayed for GST-N protein as indicated in Materials and Methods. A. Primary plot and double-reciprocal plot (inset) of leader RNA binding activity of N protein. B. Binding activity of N protein subfragments to leader RNA.

Table 1. Effect of UCYAA copy number on N protein binding

Ligand[a]	Apparent K_d
-UCUAA-	14.7 ± 3.1 nM
-UCUAAUCCAA-	18 ± 5.4 nM
-UCUAAUCUAAUCUAA-	14 ± 4 nM

[a] RNA ligands containing one, two or three copies of the UCYAA motif were tested as for binding to N protein. For the two copyligand, RNA from pBSL was used. K_d values were determined as in Fig. 2.

ity of N protein for the multimers of the UCUAA pentamer. All of the UCUAA repeat ligands showed the same experimental K_d for binding to N protein. This was a somewhat surprising result because a change in the number of tandem repeats is observed to alter the rates of viral mRNA transcription (Zhang et al., 1994; van Marle et al., 1995). Our results indicate then that the interaction between N protein and the UCUAA repeat is not capable of distinguishing between tandem copies of this sequence, thus N protein likely is not involved in this type of discrimination.

In order to demonstrate a trans effect of the JHMV nucleocapsid protein on translation of 5'-leader containing mRNA, we performed a cotransfection experiment using a nucleocapsid expression plasmid as the effector with reporter plasmids containing the CAT gene and either an intact JMHV leader sequence (...*UCUAAUCCAA*C...; MHV-CAT) or a mutated intergenic sequence (...*UCU*UCUAGA*AA*C...; mutVI). As shown in Table 2, inclusion of the effector plasmid in the transfection experiment resulted in an increase in CAT expression from MHV-CAT in a dose-dependent fashion. Expression of the MHV-CAT reporter was consistently two-fold higher than the activity of mutVI which showed at best modest increases in activity. This was the first clear indication that the nucleocapsid protein stimulates translation of mRNAs which contain the viral 5'-UTR. It is important to note that the effector to reporter ratios varied from 1:20 to 1:10 in this experiment. The results are indicative that N protein was acting in a rate limiting process, *e.g.* initiation of translation, rather than in a process requiring stoichiometric ratios of mRNA and protein.

5. DISCUSSION

Identification of the MHV nucleocapsid protein as a factor important for translation of MHV mRNAs underscores its multifunctionality. This protein is thought to have roles in tran-

Table 2. Co-transfection of nucleocapsid protein gene with chimeric MHV-CAT reporter

	Relative reporter activity[a]	
Nucleocapsid DNA	mutVI	MHV-CAT
none	1	1
50 ng	0.86	1.81
100 ng	1.79	4.45

[a]Activity is normalized to control CAT reporter plasmid which completely lacks viral 5'-UTR sequences.

scription and replication in addition to encapsidation; to this list we propose to add its function in translation of MHV mRNAs. All of these processes are tied to N protein via its RNA binding activity. Our identification of the RNA sequence bound by N protein as well as the corresponding domain of the N protein itself clearly establishes this RNA-protein interaction. This information can now be used as a starting point for an investigation into N protein domains which are responsible for the effector activities in the processes listed above.

The RNA binding domain of nucleocapsid protein was identified in earlier studies as domain B ($N^{169-308}$) (Nelson and Stohlman, 1993) or domain II ($N^{163-380}$) . In this report we show that the B1 region ($N^{176-230}$) of the nucleocapsid protein is responsible for binding to the intergenic region of the viral leader RNA. Our results do not address a mechanism of N protein as a translational activator, *i.e.* whether it has intrinsic activity or whether it facilitates the activity of the canonical initiation factors. Regardless of its mechanism this phenomenon represents one of few examples of positive regulators of translation activity and as such deserves further study.

ACKNOWLEDGMENTS

This work supported by grants from the Public Health Service: NS30880 (SMT), NS18146 (SAS). DJM was supported by training grant NS07149.

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