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SHORT COMMUNICATIONS

Identification and Stability of a 30-kDa Nonstructural Protein Encoded by mRNA 2 of Mouse Hepatitis Virus in Infected Cells

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A bacterial expression vector encoding a fusion protein containing almost the entire first open reading frame (ORF1) of mRNA 2 of MHV-A59 has been constructed. The purified fusion protein was used to raise antibodies to the protein encoded by mRNA 2 ORF1. Specificity of the antibodies was verified by immunoprecipitation of the in vitro translation product of ORF1, which was reconstructed downstream of a T7 promoter. In vivo the antiserum reacted specifically with a 30-kDa protein synthesized in MHV-A59- and MHV-JHM-infected cells. This 30-kDa protein could not be identified in purified virions and is therefore a nonstructural viral protein. The expression pattern of this 30-kDa nonstructural viral protein in infected cells was shown to be identical to that of the viral structural proteins. However, in comparison to the nucleocapsid protein pulse-chase studies revealed a relative short half life for this 30-kDa protein in vivo.

The murine hepatitis virus strain A59 (MHV-A59) is a member of the Coronaviridae, a family of enveloped positive-stranded RNA viruses. The helical nucleocapsid consists of a linear RNA of about 30 kb (1) and nucleocapsid protein (N). In addition, a virus-encoded transmembrane glycoprotein (M) and a large spike glycoprotein (S) are present in the virion (2).

The MHV genome is organized into seven regions each containing one or more open reading frames (ORF) which are separated by junction sequences that contain the signals for transcription of the subgenomic mRNAs. The viral mRNAs form a 3' coterminally nested set, each RNA having a different “unique” region at its 5' end. Apart from sequence homology at their 3' ends all viral mRNAs have a common leader sequence of about 72 nucleotides (reviewed in (2)). This common leader sequence is a result of a discontinuous transcription mechanism (3, 4). Although the viral mRNAs are in principal polygenic, in vitro translation studies have shown that only the unique region of each mRNA is translationally active (reviewed by (2)).

In vitro translation of MHV mRNA 2 resulted in the synthesis of a 30- to 35-kDa protein (5, 6) but an unambiguous identification of this protein in infected cells was not possible due to the lack of a monospecific antiserum. Recently, we have described that the unique region of mRNA 2 of MHV-A59 contains two ORFs (7). The first ORF potentially encodes a protein of 261 amino acids (30.3 kDa). The second ORF has the potential to encode a 43-kDa protein, but it is unlikely that this protein is expressed in MHV-A59 since an AUG codon is lacking in the first 109 codons.

To obtain mRNA 2 ORF1-specific antibodies an 857-bp AatII fragment (positions 105 to 962 according to the nomenclature of (7)) which covers almost the entire ORF1 of MHV-A59 mRNA 2, was cloned in the SmaI site of the plasmid pEX2 (8). The ORF1-specific fragment was isolated from the resulting plasmid pEX2.1 by making use of the pEX2-encoded EcoRI and HindIII sites which allowed forced cloning of the mRNA 2 fragment in plasmid pEX31B. This plasmid can be used for the expression of a fusion protein in Escherichia coli containing the N-terminal part of the bacteriophage MS2 RNA polymerase (9). Transformant pEX31B.1 which expressed the expected MS2/ORF1 fusion protein was used for large-scale production of the fusion protein. After purification (9) 100 μg of the fusion protein suspended in Freund's complete adjuvant was injected subcutaneously in rabbits. Following two booster immunizations sera were collected and their specificity was tested using an in vitro transcription and translation system. To make in vitro RNA transcripts of ORF1 the full-length ORF1 was reconstructed downstream of a T7 promoter using essentially the same procedure as described previously (10). An oligonucleotide corresponding to positions -10 to +7 of the mRNA 2 ORF1 sequence (7) was used for primer extension on single-stranded M13 DNA which
Fig. 1. Identification of the mRNA 2 ORF1 product in vivo. (A) In vitro translation products of pJAP1 were analyzed directly (lane 1) or after immunoprecipitation using preimmune serum (lane 2) or anti-MS2/ORF1 antiserum (lane 3). Lysates from MHV- or mock-infected cells were immunoprecipitated using anti-ORF1 antiserum (lanes 4 and 5, respectively) or immunoprecipitated with preserum (lanes 6 and 7, respectively). (B) Immunoprecipitation of the 30-kDa ns protein from cell lysates of MHV-JHM- (lane 1) or MHV-A59- (lane 2) infected Sac(−) cells.

contained an insert covering 650 nucleotides of the 3' end of the MHV polymerase gene and the complete mRNA 2 ORF1. Sequence analysis revealed that the first AUG codon downstream of the T7 promoter of the resulting plasmid pJAP1 was identical to the proposed initiation codon for the translation of ORF1 (7). To synthesize RNA, pJAP1 was linearized with HindIII and transcribed in vitro using T7 RNA polymerase. Translation in a rabbit reticulocyte lysate of these transcripts resulted in the synthesis of a 30-kDa protein and two minor products of 28 and 27 kDa (Fig. 1 A, lane 1). The latter two products comigrated with in vitro translation products of transcripts of ORF1 deletion mutants in which the first and the second AUG codon of ORF1 (7) had been deleted (data not shown). The 47-kDa product that can be observed in lane 1 was also detected when no RNA was added to the in vitro translation system (data not shown). The anti-MS2/ORF1 antibodies immunoprecipitated the 30-kDa translation product (Fig. 1A, lane 3), whereas preimmune sera did not (Fig. 1A, lane 2). These data clearly demonstrate the presence of antibodies against the translation product of ORF1 of MHV mRNA 2.

To identify the mRNA 2 ORF1 encoded protein in vivo, monolayers of Sac(−) cells were infected with MHV-A59 (m.o.i., 30) and labeled with [35S]cysteine (50 μCi/ml in cysteine-free medium containing 2% dialyzed fetal calf serum) for 4 hr beginning at 4 hr postinfection (h p.i.). The cells were lysed (11) and the lysate was used for immunoprecipitation with the anti-MS2/ORF1 antibodies (12). A 30-kDa protein precipitated by the antibody was detected (Fig. 1A, lane 4). This 30-kDa protein comigrated with the in vitro translation product of pJAP1 transcripts and was neither recognized by the preimmune serum (Fig. 1A, lane 5) nor detected after immunoprecipitations of lysates from mock-infected cells (Fig. 1A, lanes 6 and 7). Some spike protein (180 kDa) and nucleocapsid protein (55 kDa) was precipitated nonspecifically since both proteins were also precipitated with preimmune serum.

The 28- and 27-kDa proteins which resulted from initiation at the second and third AUG codon of ORF1 in vitro, respectively (data not shown), could not be detected in the lysates of MHV-infected cells. This demonstrates that only the first AUG codon which is in a preferred context (AAAAUGG) for the initiation of protein synthesis (7, 13) is functional in vivo.

A 30-kDa protein could also be immunoprecipitated in lysates of MHV-JHM-infected Sac(−) cells (Fig. 1B). During the preparation of this manuscript the nucleotide sequence of the unique region of mRNA 2 of MHV-JHM was reported to be nearly identical to the MHV-A59 sequence and to encode a 30.9-kDa protein (14). Using the anti-fusion protein antibodies we were unable to demonstrate the 30-kDa protein in purified virions indicating that this viral-encoded protein is most likely a nonstructural (ns) protein.

The kinetics of the expression of the 30-kDa ns protein during viral infection was studied and compared to the expression of the structural proteins. MHV-A59-infected Sac(−) cells (m.o.i., 30) were labeled with [35S]cysteine (50 μCi/ml) for periods of 1 hr at the times indicated in the legend to Fig. 2. Cell lysates were prepared and immunoprecipitated with anti-30-kDa antibodies or polyclonal anti-MHV serum. The 30-kDa protein was detectable 5 hr p.i. (Fig. 2, lane 5); longer exposure of the gel demonstrated that a small amount of the 30-kDa protein was synthesized as early as 4 hr p.i. (data not shown). The amount of 30-kDa protein synthesized during the progression of the infection increased significantly (Fig. 2, 4–7 hr), reaching a maximal level between 6 and 8 hr p.i. The sharp decrease in the amount of 30-kDa protein synthesized late in infection is partly due to virus-induced cytopathic effect resulting in cell death. Similar kinetics were observed for the expression of the structural proteins (Fig. 2) although their synthesis seemed to decrease somewhat earlier. These results clearly show that the 30-kDa ns
protein is synthesized continuously during viral infection. To investigate the turnover of the 30-kDa ns protein in vivo, monolayers of MHV-A59-infected Sac(-) cells (m.o.i., 30) were pulse labeled ([35S]cysteine; 50 μCi/ml) for 15 min beginning at 4.75 hr p.i. and chased for various times as indicated in the legend to Fig. 3. Cell lysates were prepared and analyzed for the presence of 30-kDa ns protein and the nucleocapsid protein. After labeling for 15 min the 30-kDa ns protein can readily be seen. No change in the molecular weight was observed during the chase periods. However, in contrast to the nucleocapsid protein the 30-kDa protein was shown to have a fast turnover (Fig. 3). Densitometry of the autoradiogram using a scanning densitometer revealed that the 30-kDa protein had a half life of approximately 40 min. The S and M protein were excluded from this analysis since these proteins are rapidly incorporated into virions, whereas a large pool of intracellular nucleocapsid protein remains (15; data not shown).

It has been suggested previously that a strong translational control is exerted on the synthesis of the 30-kDa protein in infected cells (16). However, comparison of the kinetics of the expression of the 30-kDa ns protein and the three structural proteins N, M, and S does not support this hypothesis. In fact, the 30-kDa protein is expressed continuously during viral infection. The relative low amount of 30-kDa protein present in infected cells merely seems to reflect the low relative molality of mRNA 2 in infected cells (17). In addition, pulse-chase experiments revealed a relatively fast turnover of the 30-kDa protein in infected cells. Therefore in contrast to the structural proteins there is hardly any accumulation of the 30-kDa protein during infection.

Although the function of the 30-kDa ns protein encoded by mRNA 2 is not yet known, it is likely to be specific to coronaviruses belonging to the same antigenic cluster as MHV since sequence analysis demonstrated that this or a similar gene is absent in IBV (18) and FIPV (19) which are representatives of other antigenic clusters. Complementation studies using MHV ts mutants and a recombinant 30-kDa protein will yield information about the role of this protein in viral replication.

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