Coronavirus JHM: nucleotide sequence of the mRNA that encodes nucleocapsid protein

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ABSTRACT

A DNA copy of the mRNA that encodes the nucleocapsid protein of Mouse Hepatitis Virus JHM has been cloned into pAT153. The DNA copy specifically inhibited the synthesis in vitro of the nucleocapsid protein. The cDNA was subcloned into M13 vectors and the entire sequence, 1767 bases including a 15 base terminal poly (A) tract, has been determined by chain-terminator sequencing. The sequence contained an open-reading frame that could encode a basic protein of mol wt. 49700. From the predicted sequence it was apparent that the nucleocapsid protein has 5 basic regions, two of which are located near the middle of the sequence, a serine-rich region was also located, a feature which may be of functional importance as the nucleocapsid protein is phosphorylated at serine residues. The carboxy terminus of the nucleocapsid protein was found to be acidic. The 5' non-coding sequence contained a triple repeat of the pentamer AATCT, a structural feature which may play a significant role during the production of subgenomic viral mRNAs.

INTRODUCTION

Coronaviruses are enveloped, positive-stranded RNA viruses which infect vertebrates and are responsible for diseases of clinical and economic importance, in particular respiratory and gastro-intestinal disorders. The most-studied member of the group is Murine Hepatitis Virus (MHV). Depending upon the age and genetic background of the host, different MHV strains display differences in virulence, pathogenicity and in organ and cell tropisms. They therefore provide useful models for a variety of disease processes. Of particular interest is the neurotropic MHV strain, JHM, which has the ability to induce acute and subacute disorders of the central nervous system in rodents and has been used as a model for virus-induced demyelination.

The Mouse Hepatitis Virus (MHV) genome is a linear, unsegmented, infectious RNA which is about 18 kilobases long. MHV replicates in the cytoplasm of infected cells and the viral genetic information is expressed as one genome-sized and six subgenomic mRNAs. The largest (genome-sized) mRNA is termed mRNA 1 and the smaller mRNAs are numbered in order of decreasing size.
These mRNAs are synthesized in non-equimolar amounts but in constant proportions. For a review of coronavirus replication, see ref. 1. Analysis by RNase T₁ oligonucleotide fingerprinting and by hybridization to virus specific cDNA probes5-10, reveals that these mRNAs are 3'coterminal forming a "nested-set". Furthermore, analysis of RNase T₁ oligonucleotides from subgenomic mRNAs and from the equivalent regions of the genome suggests that the subgenomic mRNAs bear a leader sequence derived from the 5' end of the genome7,8. The translation of MHV mRNAs in vivo, in cell-free systems or in oocytes11-13, shows that each mRNA is translated independently to produce a single protein, the size of which corresponds to the coding capacity of the 5' sequences not found in the next smallest mRNA. The smallest and most abundant mRNA (mRNA7) encodes the virion nucleocapsid protein, a basic, phosphorylated polypeptide of 50-60000 mol.wt. The next most abundant RNA, mRNA6, encodes the virion matrix glycoprotein(s) (23000 to 25000 mol.wt.) and the third major intracellular RNA, mRNA3, encodes the virion peplomer glycoprotein(s) (90000 and 180000 mol.wt.). The remaining intracellular mRNAs, which are found in lesser amounts, are thought to encode viral non-structural proteins.

In the experiments described here, we have isolated DNA copies of MHV-JHM mRNA7 and obtained a complete sequence of the mRNA. This nucleotide sequence and the predicted sequence of the encoded protein can be compared with the equivalent sequences recently obtained for the hepatotrophic MHV strain, A5914.

MATERIALS AND METHODS

Chemicals

Avian myeloblastosis virus reverse transcriptase was supplied by Life Sciences (St. Petersburg, Florida). Radiochemicals were supplied by Amersham Buchler (Braunschweig, F.R.G.). M13 pentadecamer sequencing and hybridization probes, oligo dT₁₂-1₈ and oligo dG₁₂-1₈ were obtained from PL Biochemicals (St. Goar, F.R.G.). Escherichia coli DNA polymerase I (large fragment), S1 nuclease and terminal deoxynucleotidyl transferase were obtained from Bethesda Research Laboratories (Neu-Isenburg, F.R.G). New England Nuclear (Dreieich, F.R.G.) supplied T4 DNA ligase. Restriction enzymes were obtained from PL Biochemicals, Bethesda Research Laboratories and Boehringer Mannheim (Mannheim, F.R.G.).

Synthesis and Cloning of cDNA

Polyadenylated RNA was isolated from Sac(-) cells that had been infect-
ed with MHV-JHM, as previously described. Double-stranded cDNA was prepared from 10 μg of polyadenylated RNA, using oligo dT and oligo dG to prime first and second strand synthesis, respectively, then oligo dC-tailed, double-stranded cDNA was annealed to PstI-cleaved, oligo dG-tailed pAT153, according to protocols described by Land et al. Escherichia coli HB101 was transformed with the annealed plasmid/cDNA using the method of Dagert and Ehrlich. Tetracycline-resistant, ampicillin-sensitive bacterial clones, from two independent cDNA cloning experiments, were screened for JHM-specific sequences by hybridization with a single-stranded cDNA probe, containing \( ^{32}P \), that had been copied from genome RNA isolated from purified virions.

Characterization of cloned cDNA

The size of inserts in plasmids from strongly-hybridizing clones was determined by gel electrophoresis of DNA extracted by the method of Holmes and Quigley. Plasmids containing the largest inserts were prepared from 1 litre cultures and were purified by equilibrium centrifugation in ethidium bromide/caesium chloride. Inserts were excised from the plasmids using PstI and were recovered from agarose gels by electroelution. The inserts were mapped by partial digestion with restriction enzymes of DNA that had been labelled using \( ^{32}P \) cordycepin triphosphate and terminal deoxynucleotidyl transferase.

Nucleotide sequencing

Fragments of two cDNA inserts were generated by a variety of restriction enzymes and cloned into the M13 vectors mp 8 and mp 9. The fragments were sequenced using the chain terminator method of Sanger et al. 77 % of the cDNA was sequenced on both strands, a further 14 % on different but overlapping fragments of the same strand and the remainder was sequenced at least twice. Towards the end of the sequencing project specific clones were identified by their hybridization to a panel of characterized M13 clones. M13 hybridization probes were prepared by the method of Hu and Messing.

Translation in vitro, Hybrid-arrested translation and polyacrylamide gel electrophoresis

Polyadenylated RNA from cells infected with MHV-JHM was translated in a rabbit reticulocyte lysate as previously described. Hybrid-arrested translation experiments, using purified cDNA insert and polyadenylated RNA from cells infected with MHV-JHM, were performed according to the method of Paterson and Kuff, using a rabbit reticulocyte lysate. Translation products were analysed on 15 % polyacrylamide gels.
Fig. 1
Hybrid-arrested translation of MHV-JHM mRNA7
 Autoradiograph of the $^{35}$S methionine-labelled products synthesized in a rabbit reticulocyte lysate and separated on a 15% polyacrylamide-SDS gel. The samples translated were: (a) no added RNA, (b-f) 250 ng of polyadenylated, cytoplasmic RNA from cells infected with MHV-JHM, and either 500 ng of the insert DNA (c and d) or 1000 ng of the insert DNA (e and f). Samples (c) and (e) were in the hybrid conformation and samples (d) and (f) were heated to melt the hybrids. The major products of 60000 and 23000 mol.wt. have been identified as the nucleocapsid and matrix proteins respectively. Sample (m) contained $^{14}$C-labelled molecular weight markers (CFA626, Amersham Buchler, Braunschweig, F.R.G.).

RESULTS
Identification of cDNA clones as copies of mRNA7

The largest JHM-specific cDNA clones from each of two cloning experiments (those within pMS38 and pSS38), were analyzed by digestion with restriction enzymes, showing that together they contained enough sequence to represent a full-length copy of mRNA7 (about 1800 bases$^{24}$). The insert in pSS38 was about 1700 base pairs and was therefore sufficient to account for most of mRNA7. The insert in pMS38 contained 830 base pairs. Mapping of both inserts, by partial digestion with restriction endonucleases, showed that the insert in pMS38 contained an extra 40 base pairs at one end.

Confirmation that the cDNA insert in pSS38 represented the body of mRNA7
Fig. 2
Diagram showing those restriction endonuclease sites, in the DNA copy of MHV-JHM mRNA 7, used for subcloning into M13 vectors. Solid arrows show the direction and extent of sequence obtained from each clone. Broken lines indicate the probable extent of each clone. Restriction endonuclease cleavage sites are denoted by the symbols: † HaeIII, ▲ MspI, ◇ BalI, ☆ AluI, ▼ Sau3A and ◆ PvuII. The box at the 3' terminus represents the poly(A) tract.

was obtained by hybrid-arrested translation (Fig. 1). When hybridized to cytoplasmic, polyadenylated RNA from cells infected with JHM, the insert specifically inhibited the translation of a 60000 mol.wt. polypeptide that has been previously identified as the intracellular precursor of the virion nucleocapsid protein produced from mRNA15. Melting of the hybrids before translation restored the synthesis of nucleocapsid protein.

Nucleotide sequence of cloned cDNA

Fragments of both cDNA inserts were generated by various restriction enzymes (Fig. 2), cloned into M13 vectors and their sequences were determined. The combined sequence of 1767 base pairs (contained between the oligo dC/dG tails made during the process of cloning the cDNA) is presented in Fig. 3. A 5' non-coding sequence of 83 base pairs preceded the first AUG codon, which initiated an open reading frame (1365 bases long) with the potential to encode a basic polypeptide of 455 amino acids (49700 mol.wt.). The first putative termination codon in this reading frame was found at nucleotide 1449 and was followed by 301 base pairs of 3' non-coding sequence. A polyadenylate tract was found beginning at nucleotide 1753. The only other open-reading frame of greater than 100 bases was from nucleotides 361-771 (inclusive), which is potentially able to encode 137 amino acids. Within the 5' non-coding
Fig. 3
Complete nucleotide sequence of the DNA copy of MHV-JHM mRNA7 (1767 nucleotides), including a 15 base long, terminal poly (A) tract. The predicted sequence of the encoded protein is also depicted.

sequence, two large, RNase T1-resistant oligonucleotides were found (at positions 26-50 and 54-82), the compositions of which were very similar to those of the MHV-A59 oligonucleotides 10 and 19, respectively. Strikingly, the larger oligonucleotide contained a triple repeat of the pentamer AATCT and this sequence was found within both, independently generated clones.
DISCUSSION

Analysis of the sequence of MHV genome RNA and subgenomic mRNAs is a way of predicting the primary structure of encoded polypeptides and, at the same time, identifying non-coding sequences which may be relevant to the regulation of the viral genes. A comparative analysis of the sequences of different MHV strains, or mutants with altered phenotypes, will also be needed as a basis for further studies on MHV replication and pathogenicity.

Three criteria define the cloned sequences we have described here as representing the MHV-JHM nucleocapsid gene. Firstly, the 3' terminal location of this sequence on the genome (unpublished data) is consistent with the known gene order of MHV-JHM. Secondly, the cloned DNA has the ability to specifically arrest the translation in vitro of mRNA7 and, thirdly, the characteristics of the predicted polypeptide as a basic protein of 49700 mol.wt. are consistent with the electrophoretic properties of the nucleocapsid protein in two-dimensional, non-equilibrium, pH gradient, gel electrophoresis. We believe that the cDNA sequence represents a complete copy of mRNA7 because it extends from a polyadenylate tract to a 5' terminal sequence UAUAAG, which is very similar to the cap-NUAAG sequence found by direct sequencing at the 5' terminus of mRNAs of the related MHV-A59.

Examination of the sequence of MHV-JHM mRNA7 reveals several interesting features. The presence of the two large RNase T1-resistant oligonucleotides (with similar base compositions to oligonucleotides 10 and 19 of A59) within the 5' non-coding sequence supports the proposal that MHV subgenomic mRNAs contain a leader sequence. The A59 oligonucleotide 10 (equivalent to the JHM oligonucleotide at position 26-50) is found in all subgenomic mRNAs and in genomic RNA. It is not, however, found in 3' terminal fragments of the genome corresponding to mRNA7 and so must be nearer the 5' end of the genome. A59 oligonucleotide 19 (equivalent to the JHM oligonucleotide at position 54-82) is found only in mRNA7, but not in the equivalent region of the genome, and related oligonucleotides are found in other mRNAs. It has therefore been suggested that oligonucleotide 10 is contained within a leader sequence derived from the 5' end of the genome and that oligonucleotide 19 is formed by fusion of the leader to the body of mRNA7. The relative positions of the equivalent JHM oligonucleotides are consistent with this proposal. It is interesting to note that the JHM equivalent of oligonucleotide 19 contained the triple repeat of the pentamer AATCT. It is likely that such a striking feature has a significant functional role in the production of subgenomic mRNAs. MHV is known to replicate in the absence of
the host cell nucleus\textsuperscript{27,28}. It has also been shown that the target size for
the UV inactivation of the synthesis of each MHV mRNA is identical to the
physical size of the mRNA\textsuperscript{29}. Thus the mRNAs cannot be produced by splicing of
larger precursors. It has been suggested\textsuperscript{7} that the mRNAs could be produced by
extension of a virus-encoded RNA primer or by a previously undescribed,
unusually-specific polymerase jumping mechanism.

From the sequence alone, it is difficult to speculate about the impor-
tance of specific features of the nucleocapsid protein structure, at least
until more is known about the interactions between nucleocapsid protein and
genome RNA and between the ribonucleoprotein complex and other virion pro-
teins. Particularly intriguing is the specific interaction of nucleocapsid protein with genome RNA but not with subgenomic mRNAs. The MHV-JHM nucleo-
capsid protein does not contain the clusters of lysine residues seen in the
N-terminal portion of the capsid protein of Semliki Forest Virus\textsuperscript{30} so, in
this respect, it is similar to the nucleocapsid protein of influenza virus\textsuperscript{31}. Some regions enriched in basic amino acids are however apparent (e.g. arg-43
to lys-50, lys-101 to lys-113, arg-196 to lys-230, arg-264 to arg-290 and
lys-392 to lys-405) and, as in the nucleocapsid protein of Semliki Forest
Virus, the carboxy terminus is acidic. The region ser-194 to ser-220 contains
9 serine residues, or 24 % of the total serine content within 6 % of the
coding sequence. Moreover, this is a basic region of the protein. Another 4
serines are found in the five residues from ser-12 to ser-16. This clustering
of serine residues may be significant in view of the fact that JHM nucleo-
capsid protein is phosphorylated specifically at serine residues\textsuperscript{32}.

The sequence of MHV-JHM mRNA\textsuperscript{7} can also be compared with the recently
published sequence of the nucleocapsid gene of MHV-A59\textsuperscript{14}. The major dif-
fferences between the JHM coding sequence reported here and the sequence
reported for MHV-A59\textsuperscript{14} are that the JHM sequence contains an additional base
(nucleotide 408) and lacks a base (after nucleotide 725). However, it is now
clear (J. Armstrong, personal communication) that the A59 sequence should be
identical to the JHM sequence at these positions. Therefore, the two sequen-
ces are very similar, with a 94 % overall homology within the coding
sequence. This finding is in accord with the degree of homology based on
hybridization kinetics of cDNA, copied from MHV-A59 mRNA\textsuperscript{7}, with mRNA from
cells infected with MHV-JHM\textsuperscript{28}. Although the sequence homology is so high
overall, it is not constant throughout the length of the coding sequence.
Between nucleotides 497 and 569 the homology falls to 63 % and in a sequence
of 23 bases (nucleotides 1271-1293) near the 3' end of the coding sequence,
only 9 bases are common to both strains. Also, an extra glutamine codon is found in the JHM sequence at nucleotide 1227.

The non-coding regions of mRNA7 from MHV-JHM and those reported for MHV-A59\(^{14}\) may also be compared. The 5' non-coding sequences are identical for the 17 nucleotides immediately before the AUG codon but the rest of the JHM 5' non-coding sequence is different from the sequence reported for A59\(^{14}\). More recent studies on the sequence of A59 subgenomic messengers (J. Armstrong, personal communication) have shown that the reported 5' non-coding sequence\(^{14}\) is not derived from mRNA7 and therefore further comparison cannot be made. When the 3' non-coding sequences are compared, they are found to be highly conserved (98% homology). We believe that this conservation of sequence at the termini of MHV RNA is significant and is most likely related to the interaction of these molecules with RNA polymerase during the synthesis of negative stranded template as well as during the production of subgenomic mRNAs and the replication of genome RNA.

In conclusion, this comparison reveals that, as might have been expected, the sequence encoding the nucleocapsid protein of MHV is relatively conserved, even between strains of MHV that show differences in pathogenicity. Our immediate aim is to determine the level of sequence homology, between the same strains, for genes more directly involved in the interaction between virus and the host cells.

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