Volume 12 Number 22 1984 Nucleic Acids Research

Molecular cloning and sequencing of the reovirus (serotype 3) S1 gene which encodes the viral cell attachment protein σ1

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Received 31 July 1984; Revised and Accepted 18 October 1984

ABSTRACT

The complete sequence of the reovirus (serotype 3) S1 gene was obtained by using cloned cDNA derived from the RNA segment. This gene is 1416 nucleotides in length and contains two open reading frames. The first reading frame has a coding capacity of 455 amino acids, sufficient to account for the known S1 product, protein σ1 (42,000 MW). It possesses a signal peptide as well as three possible glycosylation sites. No homology could be detected when this gene sequence and the deduced amino acid sequence were compared to published sequences of the corresponding gene of a human rotavirus. The second reading frame (not in phase with the first) starts at the second ATG recently shown to be a functional initiation site. It has a coding capacity of 120 amino acids. Its outstanding feature is the highly basic amino-terminal region, a characteristic apparently shared by a number of DNA binding proteins. It is speculated that this protein, hitherto undetected, may play a role in mediating viral and/or host nucleic acid transcription.

INTRODUCTION

The genome of mammalian reoviruses consists of 10 double-stranded (ds) RNA segments, each of which appears to encode one single protein (1,2). Eight of these proteins are structural proteins (3) which are assembled in the mature virion as a double capsid (4). The outer capsid shell is composed of three proteins, namely, μ1C, σ3 and σ1. Protein μ1C and σ3 are presumably closely associated with each other (5), and they also make up over 60% of the virion mass (3). Protein σ1, on the other hand, is a minor component (about 24 molecules) (3) which is located at the 12 vertices of the viral icosahedron (6). The location and distribution of this protein on the virion surface are probably of strategic significance since this protein is used by the virus to anchor itself onto receptors of the host cell plasma membrane (6). Protein σ1 is also the reovirus hemagglutinin and is responsible for eliciting the formation of neutralizing antibody as well as for triggering host immune responses (7,8,9,10). In addition, this protein defines tissue tropism and virulence (11), and mediates the binding of virions to cellular microtubules (12), and the inhibition of host DNA synthesis (13). Protein σ1 therefore

specifies how reovirus particles interact with host cells and with the host. Another interesting feature of this protein is that it is the only reovirus protein that confers serotype specificity (5,14,15); three immunologically distinct types (serotypes 1, 2, and 3) have been identified thus far (16). That σ1 is the most type-specific of all reovirus proteins is consistent with the recent observation that of the 10 reovirus genes, the gene encoding the σ1 protein, gene S1, is the gene that has undergone the most marked evolutionary divergence (maximum of 10% homology between serotypes) (17).

Little is known concerning the molecular anatomy of protein σ1. At the present time, the major domains of interest on this protein are the ones involved in interaction with cell surface structures. Using a number of monoclonal antibodies directed against the σ1 protein, Fields and co-workers (18, 19) have identified at least three distinct functional domains on this protein and concluded that the hemagglutination and the neutralization domains correspond to discrete antigenic regions on the protein. Since anti-σ1 antibodies neutralize reovirus by preventing viral adsorption (6), these observations strongly suggest that different recognition mechanisms are involved in reovirus hemagglutination and host cell attachment.

In view of the various functions displayed by protein σ1 and the significant role it plays in viral pathogenesis (20), we have cloned and sequenced the S1 gene of reovirus serotype 3. Two open reading frames were found: one spans 455 codons, the other 120 codons. The predicted features of these two proteins are discussed in light of the deduced amino acid sequences.

MATERIALS AND METHODS

Virus

The virus used was reovirus serotype 3 (strain Dearing). It was propagated in suspension cultures of L-929 strain of mouse fibroblasts and purified by the method described by Smith *et al.* (3).

Cloning of Reovirus Genes

The procedure used for the isolation of genomic dsRNA, the preparation of ds cDNA, and the cloning of cDNA into the Pst1 site of the plasmid pBR322 was basically the same as described previously (21), with the exception that in place of DMSO, methylmercuric hydroxide (10 mM) was used to denature the dsRNA (10 min at room temperature) prior to 3' termini polyadenylation and reverse transcription.

Identification of Cloned S1 Gene

Of all transformed bacterial colonies (selected by screening for resis-

tance to tetracycline and sensitivity to ampicillin), 20 were randomly chosen and their plasmids isolated. Plasmid DNA was then ^{32}P-labelled by nick translation (22) and hybridized to reovirus dsRNA which had been resolved in a 7.5% polyacrylamide gel (in which the S1 RNA segment is well separated from the other segments), and subsequently transferred to nitrocellulose paper (NEN Genescreen) as described previously (21). The marker RNA pattern on such paper was identified by hybridizing a strip to ^{32}P-labelled DNA transcripts of total reovirus dsRNA using the random primer method of Taylor et al. (23).

DNA Sequencing and Computer Analysis

Recombinant plasmids containing inserts from the S1 gene were isolated using the method described by Birnboim and Doly (24). Restriction digests of whole plasmids or of inserts (obtained by Pst1 digestion of plasmids followed by their isolation from agarose gels) were then subcloned in various vectors of the M13 mp series (25,26). Sequencing was done by the dideoxy chain termination method of Sanger (27).

Analyses of nucleotide and amino acid sequences were done using published algorithm (28) programmed in Fortran on the Honeywell DPS-870 computer. Graphic comparison matrices were obtained using a Calcomp 1051 plotter.

RESULTS AND DISCUSSION

Identification of Clones Derived from Reovirus S1 Gene

Of the twenty cloned plasmids randomly chosen, 3 were found to contain S1-derived inserts using the Northern blotting technique. Two such plasmids (pL662 and pL676) are shown in Fig. 1. To size the inserts, the two plasmids were digested with Pst1 and analyzed by polyacrylamide gel electrophoresis (Fig. 2). Clone pL662 was found to contain an insert approximately 740 base pairs in length. The insert of clone pL676 has an internal Pst1 site, thus yielding two S1-specific segments, one of about 870, the other of about 430 base pairs in length. Upon sequencing, clones pL662 and pL676 were found to contain, respectively, sequences from the 3' and 5' end of the S1 gene as deduced previously from RNA sequencing of the termini of reovirus genes (29, 30). Since the S1 RNA segment was estimated by polyacrylamide gel electrophoresis to be between 1400 and 1500 base pairs long, the two plasmids presumably span the entire sequence of the S1 gene.

Gene S1 Sequence

Inserts from the two plasmids (pL662 and pL676) were sequenced according to the strategy shown in Fig. 3. The majority of the sequence (90%) was determined in both directions and unambiguous data were obtained for the

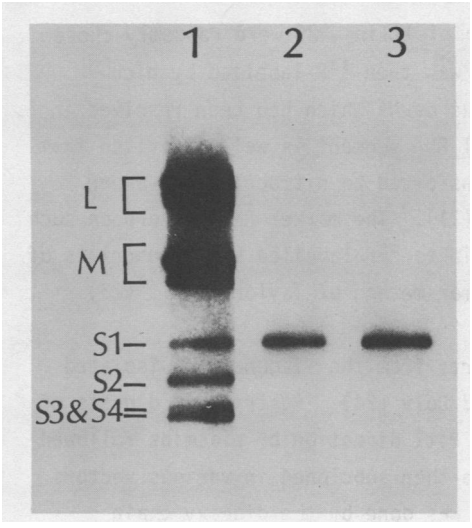

Fig. 1. Identification of clones derived from the S1 gene. Total reovirus genomic RNA was resolved in a 7.5% polyacrylamide gel and transferred to nitrocellulose paper. Transblotted RNA was then hybridized to nick translated plasmids isolated from transformed bacterial colonies. Lane 1, RNA hybridized to ^{32}P-labelled DNA transcripts of total genomic RNA. Lanes 2 and 3, RNA hybridized to nick translated recombinant plasmids pL676 and pL662, respectively.

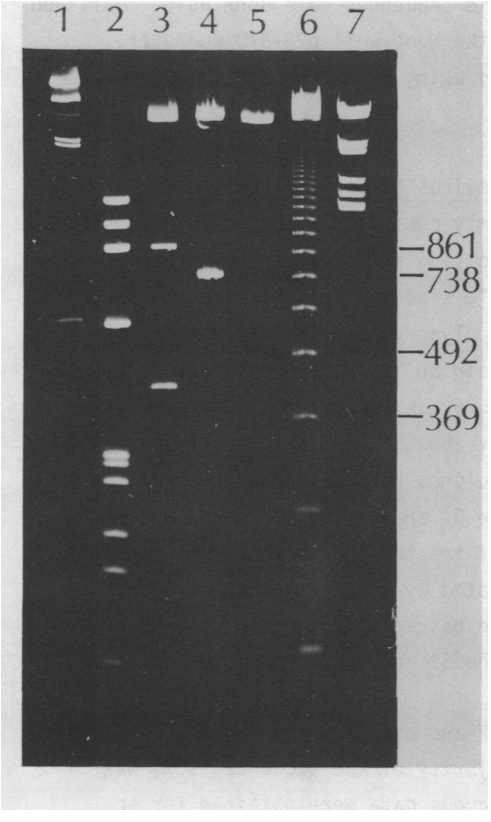

Fig. 2. Sizing of S1-derived inserts in a 7.5% polyacrylamide gel. The gel was stained with ethidium bromide after electrophoresis. Lane 1, HindIII fragments of λDNA (size markers). Lane 2, HaeIII fragments of øX174 DNA (size markers). Lane 3, plasmid pL676 restricted with Pst1. Lane 4, plasmid pL662 restricted with Pst1. Lane 5, pBR322 restricted with Pst1. Lane 6, 123 base pair ladder marker (from BRL). Lane 7, reovirus genomic dsRNA. The numbers on the right designate size (base pairs) of the 123 base pair ladder marker.

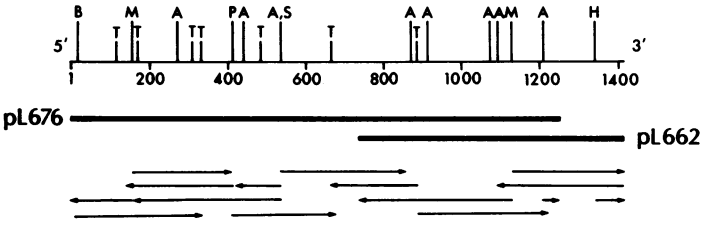

Fig. 3. Strategy of sequencing S1-derived inserts in pL676 and pL662 containing overlapping S1 sequences. Restriction sites are indicated: A, AluI; B, BamHI; H, HaeIII; M, MspI; P, PstI; S, SstI; T, TaqI. The arrows indicate the direction and extent of sequence analysis performed on each of the restriction fragments.

remaining regions. All restriction sites used for the generation of fragments for subcloning into M13 were covered by overlapping sequences. The complete nucleotide sequence of the positive strand of the S1 gene is presented in Fig. 4. The 5' and 3' terminal sequences were in excellent agreement with those previously determined by RNA sequencing techniques (30).

The entire S1 sequence is 1416 nucleotides long. The quantitative distribution of the four bases was found to be relatively even: 27% A, 21% C, 26% G, and 26% T. There is an open reading frame of 1365 bases (455 codons) which starts at nucleotide 13 with the first possible initiation codon and terminates at nucleotide 1377. The 5'- and 3'-noncoding sequences are 12 and 39 nucleotides long, respectively. The absence of adenine-rich strings of nucleotides at the 3'-noncoding region is consistent with the lack of polyadenylation of reovirus mRNAs. There is a second open reading frame which starts at nucleotide 71 and extends for 120 codons until a termination codon at nucleotide 431 is reached (Fig. 4). The detection of two open reading frames of significant length (over 100 amino acids) is in accord with the previous observation that the two initiation sites in the reovirus S1 mRNA are both recognized by ribosomes (31), and with the more recent finding that both sites are functional (32). In this regard, it is interesting to note that a mRNA of Influenza B virus was recently found to be bicistronic (33). No additional reading frames longer than 100 amino acids beginning with a methionine were detected on either the plus or minus sense strand.

A computer analysis comparing nucleotide sequences of the S1, S2 and S3 genes revealed no homologous region of statistical significance (data not shown).

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5'-GCTATTGGTCGG ATG GAT CCT CGC CTA CGT GAA GAA GTA GTA CGG CTG ATA ATC GCA TTA ACG AGT GAT AAT GGA     75
                met asp pro arg leu arg glu glu val val arg leu ile ile ala leu thr ser asp asn gly     21
                                                                                    met glu      2

GCA TCA CTG TCA AAA GGG CTT GAA TCA AGG GTC TCG GCG CTC GAG AAG ACG TCT CAA ATA CAC TCT GAT ACT ATC    150
ala ser leu ser lys gly leu glu ser arg val ser ala leu glu lys thr ser gln ile his ser asp thr ile     46
 his his cys gln lys gly leu asn gln gly ser arg arg ser arg arg arg leu lys tyr thr leu ile leu ser     27

CTC CGG ATC ACC CAG GGA CTC GAT GAT GCA AAC AAA CGA ATC ATC GCT CTT GAG CAA AGT CGG GAT GAC TTG GTT    225
leu arg ile thr gln gly leu asp asp ala asn lys arg ile ile ala leu glu gln ser arg asp asp leu val     71
 ser gly ser pro arg asp ser met met gln thr asn glu ser ser leu leu ser lys val gly met thr trp leu     52

GCA TCA GTC AGT GAT GCT CAA CTT GCA ATC TCC AGA TTG GAA AGC TCT ATC GGA GCC CTC CAA ACA GTT GTC AAT    300
ala ser val ser asp ala gln leu ala ile ser arg leu glu ser ser ile gly ala leu gln thr val val asn     96
 his gln ser val met leu asn leu gln ser pro asp trp lys ala leu ser glu pro ser lys gln leu ser met     77

GGA CTT GAT TCG AGT GTT ACC CAG TTG GGT GCT CGA GTG GGA CAA CTT GAG ACA GGA CTT GCA GAC GTA CGC GTT    375
gly leu asp ser ser val thr gln leu gly ala arg val gly gln leu glu thr gly leu ala asp val arg val    121
 asp leu ile arg val leu pro ser trp val leu glu trp asp asn leu arg gln asp leu gln thr tyr ala leu    102

GAT CAC GAC AAT CTC GTT GCG AGA GTG GAT ACT GCA GAA CGT AAC ATT GGA TCA TTG ACC ACT GAG CTA TCA ACT    450
asp his asp asn leu val ala arg val asp thr ala glu arg asn ile gly ser leu thr thr glu leu ser thr    146
 ile thr thr ile ser leu arg glu trp ile leu gln asn val thr leu asp his                              120

CTG ACG TTA CGA GTA ACA TCC ATA CAA GCG GAT TTC GAA TCT AGG ATA TCC ACG TTA GAG CGC ACG GCG GTC ACT    525
leu thr leu arg val thr ser ile gln ala asp phe glu ser arg ile ser thr leu glu arg thr ala val thr    171

AGC GCG GGA GCT CCC CTC TCA ATC CGT AAT AAC CGT ATG ACC ATG GGA TTA AAT GAT GGA CTC ACG TTG TCA GGG    600
ser ala gly ala pro leu ser ile arg asn asn arg met thr met gly leu asn asp gly leu thr leu ser gly    196

AAT AAT CTC GCC ATC CGA TTG CCA GGA AAT ACG GGT CTG AAT ATT CAA AAT GGT GGA CTT CAG TTT CGA TTT AAT    675
asn asn leu ala ile arg leu pro gly asn thr gly leu asn ile gln asn gly gly leu gln phe arg phe asn    221

ACT GAT CAA TTC CAG ATA GTT AAT AAT AAC TTG ACT CTC AAG ACG ACT GTG TTT GAT TCT ATC AAC TCA AGG ATA    750
thr asp gln phe gln ile val asn asn asn leu thr leu lys thr thr val phe asp ser ile asn ser arg ile    246

GGC GCA ACT GAG CAA AGT TAC GTG GCG TCG GCA GTG ACT CCC TTG AGA TTA AAC AGT AGC ACG AAG GTG CTG GAT    825
gly ala thr glu gln ser tyr val ala ser ala val thr pro leu arg leu asn ser ser thr lys val leu asp    271

ATG CTA ATA GAC AGT TCA ACA CTT GAA ATT AAT TCT AGT GGA CAG CTA ACT GTT AGA TCG ACA TCC CCG AAT TTG    900
met leu ile asp ser ser thr leu glu ile asn ser ser gly gln leu thr val arg ser thr ser pro asn leu    296

AGG TAT CCG ATA GCT GAT GTT AGC GGC GGT ATC GGA ATG AGT CCA AAT TAT AGG TTT AGG CAG AGC ATG TGG ATA    975
arg tyr pro ile ala asp val ser gly gly ile gly met ser pro asn tyr arg phe arg gln ser met trp ile    321

GGA ATT GTC TCC TAT TCT GGT AGT GGG CTG AAT TGG AGG GTA CAG GTG AAC TCC GAC ATT TTT ATT GTA GAT GAT   1050
gly ile val ser tyr ser gly ser gly leu asn trp arg val gln val asn ser asp ile phe ile val asp asp    346

TAC ATA CAT ATA TGT CTT CCA GCT TTT GAC GGT TTC TCT ATA GCT GAC GGT GGA GAT CTA TCG TTG AAC TTT GTT   1125
tyr ile his ile cys leu pro ala phe asp gly phe ser ile ala asp gly gly asp leu ser leu asn phe val    371

ACC GGA TTG TTA CCA CCG TTA CTT ACA GGA GAC ACT GAG CCC GCT TTT CAT AAT GAC GTG GTC ACA TAT GGA GCA   1200
thr gly leu leu pro pro leu leu thr gly asp thr glu pro ala phe his asn asp val val thr tyr gly ala    396

CAG ACT GTA GCT ATA GGG TTG TCG TCG GGT GGT GCG CCT CAG TAT ATG AGT AAG AAT CTG TGG GTG GAG CAG TGG   1275
gln thr val ala ile gly leu ser ser gly gly ala pro gln tyr met ser lys asn leu trp val glu gln trp    421

CAG GAT GGA GTA CTT CGG TTA CGT GTT GAG GGG GGT GGC TCA ATT ACG CAC TCA AAC AGT AAG TGG CCT GCC ATG   1350
gln asp gly val leu arg leu arg val glu gly gly gly ser ile thr his ser asn ser lys trp pro ala met    446

ACC GTT TCG TAC CCG CGT AGT TTC ACG TGA GGA TCA GAC CAC CCC GCG GCA CTG GGG CAT TTC ATC-3'           1416
thr val ser tyr pro arg ser phe thr                                                                  455
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Fig. 4. Sequence of the S1 gene written in the mRNA sense. The two predicted amino acid sequences are shown below the gene sequence: the first reading frame starts at nucleotide 13 and terminates at nucleotide 1377; the second reading frame starts at nucleotide 71 and terminates at nucleotide 431. Possible glycosylation sites are underlined.

Deduced Amino Acid Sequences and Their Features

First Open Reading Frame. The deduced amino acid sequence of the polypeptide encoded by the first reading frame is shown in Fig. 4. Its length (455 amino acid residues) is sufficient to account for the size of the known S1 gene product, σ1, which has an estimated molecular weight of 42,000 (3). The calculated net charge of this protein at pH 7.0, assuming glutamic and aspartic acid are each -1, and arginine and lysine each +1, histidine 0.5 and the remaining amino acids are neutral (charge 0) at this pH (34), is -6.5. This is in agreement with the relatively low pI value of 5.5 to 5.9 previously reported for protein σ1 (35). There are three potential glycosylation sites (Asn-X-Thr/Ser) (Fig. 4), consistent with previous glycosylation studies which suggest that σ1 may be a glycoprotein (36). There are also indications that one or more of these sites may be involved in the attachment of σ1 (and hence virus particles) to cellular receptors (Paul and Lee, unpublished data).

An examination of the amino-terminal region of the deduced sequence revealed the presence of a hydrophobic core (residues 9 through 16). Using the rules proposed by Perlman and Halvorsen (37), a signal peptide comprised of the first 18 amino acids (2,109 MW) can be identified. Cleavage after the serine residue would then result in an amino-terminal aspartic acid residue of the protein. While it is likely that the presumptive signal peptide is cleaved off before the incorporation of σ1 in the mature virion, the possibility remains that this short hydrophobic region may be used for interacting with host cell plasma membrane during viral attachment. It would therefore be of interest to identify the amino-terminal residue of σ1 in the mature virion. However, this may prove difficult since it has been shown that most of the proteins in reovirions contain a blocked amino terminus (38).

The hydrophilicity profile of the entire sequence was then deduced using the method described by Kyte and Doolittle (39), and is shown in Fig. 5A. A highly hydrophobic amino-terminal region corresponding to the presumptive signal peptide is clearly discernible. Clusters of hydrophobic regions are also found in the carboxyl-terminal half of the molecule. The carboxyl terminus, however, is relatively hydrophilic. The three glycosylation sites, clustered around the middle section of the molecule, do not appear to be located in regions of marked hydrophilicity. A second hydrophilicity plot [Hopp and Woods method (40)], based on a slightly different set of hydrophilicity values assigned to the amino acids, is shown in Fig. 5B. Such a plot has been found to be useful for the prediction of antigenic determinants since they correspond to highly hydrophilic sites. Two such sites were found, one around residue 56,

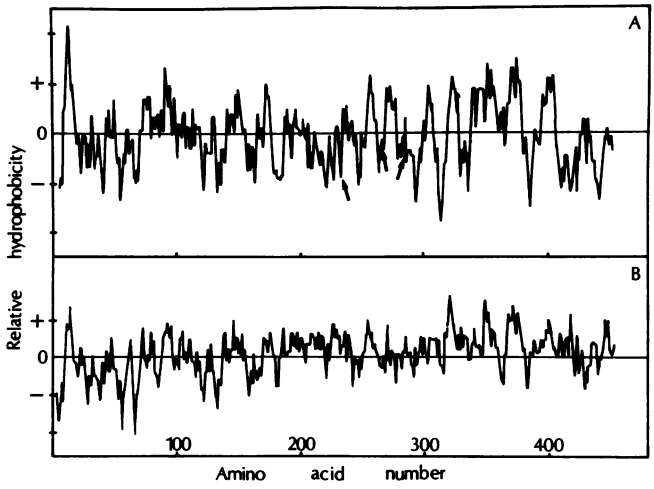

Fig. 5. Relative hydrophobicity plots of the reovirus (serotype 3) σ1 protein. (A) Kyte and Doolittle (39) plot. The arrows indicate potential glycosylation sites. (B) Hopps and Woods (40) plot of relating hydrophilic sites to antigenic determinants.

and the other around residue 66. Since a library of monoclonal antibodies directed against protein σ1 is now available (5,18) and σ1 which retains cell-binding capacity has recently been isolated from intact virions (Yeung and Lee, unpublished data), it should now be feasible to pinpoint such sites (including neutralization sites) on the protein.

The deduced amino acid sequence yields limited information on the secondary structure of the protein. Using the rules proposed by Chow and Fasman (41), most α-helical regions were found to be confined to the amino-terminal third of the molecule (data not shown). In this regard it is interesting to note that 13 of the 14 proline residues which do not favor α-helix formation reside in the latter two-thirds of the sequence. The presence of a single cysteine residue also precludes the formation of intramolecular disulfide bonds.

Comparison of Deduced σ1 Sequence with Sequence of Rotavirus Type-Specific Protein. As mentioned above, protein σ1 is the type-specific protein of reovirus and the genes (S1) encoding this protein of the three reovirus serotypes are related to the extent of not more than 1-10% (17). The corresponding gene of mammalian rotaviruses, however, appears to be highly conserved: both the nucleotide and amino acid sequences of human, simian and bovine rotaviruses were found to be closely related (approximately 80%) (42). These findings are therefore consistent with earlier observations that rotaviruses

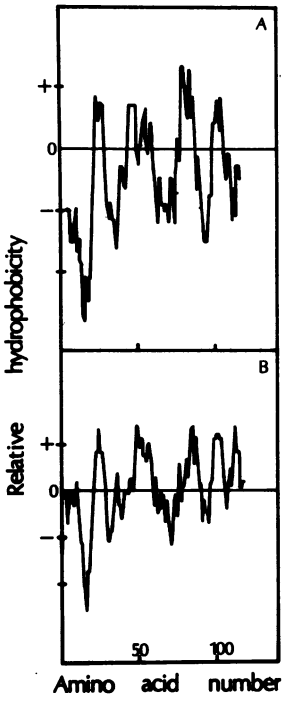

Fig. 6. Relative hydrophobicity plots of the second deduced amino acid sequence of gene S1. (A) Kyte and Doolittle (39) plot. (B) Hopps and Woods (40) plot.

and reoviruses are not antigenically related (43,44), although both viruses belong to the same family (Reoviridae) and have similar morphology and structural composition (45).

The extent of such unrelatedness was then measured by comparing the deduced amino acid sequence of reovirus protein σ1 to the reported sequence of the serotype-specific protein of human rotavirus Hu/5 (42) using computer-generated comparison matrices. Although the lowest stringency condition was used (single amino acid comparison), no sequence homology was detected between these two proteins (data not shown). This was further reflected by the lack of homology between their nucleotide sequences (data not shown). These data strongly suggest that the corresponding genes of the two viruses might have evolved independently during the more recent period. It is interesting to note, however, that both proteins possess an amino-terminal hydrophobic region and a carboxyl-terminal hydrophilic region (46,47). The significance and the universality of such an arrangement among serotype-specific proteins of Reoviridae is not known at this time.

Second Open Reading Frame. Recent evidence that the second AUG of the S1

mRNA is a functional initiation codon (31,32) suggests the possible presence of another S1-specified protein, hitherto undetected, in reovirus-infected cells. Starting at nucleotide 71 and spanning 120 codons (Fig. 4), this protein has a calculated molecular weight of 13,990 and is somewhat basic, with an estimated net charge of +5 at pH 7.0.

A sequence analogous to the amino-terminal hydrophobic core found in the first reading frame is absent in this protein. In its place is an arginine-rich region (residues 14 through 19), rendering this end of the molecule highly basic. That this region is also the most hydrophilic region of the protein is clearly indicated from hydrophilicity plots (Fig. 6). It is worth noting that the concentration of basic residues at the amino end appears to be a common feature of a number of DNA binding proteins such as the Cro protein of bacteriophage 434 (48), the c1 repressor and the N protein of bacteriophage λ (49,50), and the Salmonella phage P22 c2 repressor (51). It is therefore tempting to speculate that this smaller S1-specified protein may have an analogous function of binding to viral genomic RNA and/or host DNA, thereby mediating positive and/or negative control of viral and/or host gene transcription.

Two possible glycosylation sites are also detected (Fig. 4). However, the lack of a signal peptide makes it unlikely that this protein is glycosylated. Like the σ1 protein, only one cysteine residue was found.

CONCLUDING REMARKS

It would now be of interest to clone and sequence the S1 genes of the other two reovirus serotypes (serotypes 1 and 2). Since protein σ1 is used by all three serotypes for cell attachment (6), and yet this protein is the most type-specific of all reovirus proteins, sequence analysis of the S1 genes (and σ1) of the three serotypes may distinguish functional conserved sites (e.g., sites for attachment and hemagglutination) from sites that confer diverse antigenicity. Such data may also prove helpful for the ultimate elucidiation of the nature of tissue tropism and virulence.

The detection of a second reading frame with a functional initiation site is of interest and deserves further investigation. A logical point of departure would be to identify such a protein in reovirus-infected cells or in in vitro translation products of the S1 gene or mRNA. Its isolation and characterization would then follow, and its possible function (such as the mediating of viral and/or host nucleic acid transcription mentioned above) would be probed. Undoubtedly, the implication on the functional significance

of this putative protein would be dramatically amplified should the S1 genes of the other two reovirus serotypes, upon sequencing, both be found to possess a second reading frame of comparable characteristics to the one reported here for reovirus serotype 3.

Experiments following the forementioned courses are now in progress.

ACKNOWLEDGEMENTS

We thank Ralph Paul and Tomonori Morinaga for stimulating discussions; David Pot and Michael Yeung for their assistance with computer analysis of sequences; and Shirley Eikerman for typing the manuscript.

This work was supported by the Medical Research Council of Canada and by the Alberta Heritage Foundation for Medical Research (AHFMR). L.N. and D.C.W.M. are recipients of AHFMR Studentships, and P.W.K.L. is an AHFMR Scholar.

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