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Influenza C Virus RNA 7 Codes for a Nonstructural Protein

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The complete nucleotide sequence of RNA segment 7 of influenza C/California/78 virus was determined by using cloned cDNA derived from viral RNA. The gene is 934 nucleotides long and possesses a long open reading frame which can code for a protein of 286 amino acids. Hybrid arrest translation experiments with the cloned cDNA fragment and poly(A)-containing RNA isolated from virus-infected cells showed that a 28,500-molecular-weight protein is coded for by RNA 7. Comparison of the proteins induced in the cell-free system and in virus-infected cells with those found in purified virus suggests that the 28,500-molecular-weight protein is a nonstructural protein.

The genome of influenza C viruses most likely consists of only seven single-stranded RNA segments of negative polarity (for a review, see references 1, 6, and 18). In contrast, influenza A and B viruses both have genomes of eight segments (for a review, see reference 12). The virus-coded polypeptides of influenza C viruses include three minor polypeptides designated P1, P2, and P3, the hemagglutinin (HA), the nucleoprotein (NP), a matrix (M) protein, and a nonstructural (NS) protein (1, 5, 9, 19). Recent studies have indicated that RNA segments 4 and 5 of influenza C viruses code for the HA and NP, respectively (16, 17, 20). However, coding assignments for the other genes of the C-type influenza viruses have not been established.

We report here the complete nucleotide sequence of the smallest RNA, segment 7, of the influenza C/California/78 virus. This gene codes for an NS protein found in influenza C virus-infected cells.

MATERIALS AND METHODS

Viruses. Influenza C/California/78 (C/Cal/78) and C/JJ/50 viruses were grown in the amniotic sacs of embryonated chicken eggs. Virus purification and RNA extraction have been described previously (21).

Cloning of virus-specific DNA and identification of clones. We synthesized double-stranded cDNA from influenza C/Cal/78 virus RNA by using reverse transcriptase and synthetic dodecamer nucleotide primers as described previously (2, 16). Double-stranded cDNA was ligated into the *Eco*RI site of plasmid pBR322 after addition of synthetic *Eco*RI linkers to the virus-specific DNA (2, 16). *Escherichia coli* C600 cells were transformed with the plasmid, and clones containing RNA 7-specific sequences were identified by Northern blot analysis with viral RNA as the hybridization probe as described previously (16, 28, 29). The plasmid (pC374) containing the longest insert was used for further studies.

Sequencing and computer analysis. The sequence of the cloned cDNA was determined by the Maxam and Gilbert chemical modification method (15). The 5′ end sequence of the viral RNA was determined by direct RNA sequencing with the chain termination procedure (11, 24) and a restriction enzyme fragment (nucleotides 730 to 807 of pC374) as the primer. Nucleotide and amino acid sequence data were stored and edited in an Amdahl 470/V6 computer at the university computer center of the City University of New York with published programs (25–27).

The nucleotide and amino acid sequences of the C/Cal/78 virus RNA 7 were compared with those of the A/PR/8/34 (2) and B/Lee/40 (3) virus NS genes by an algorithm performed on an IBM 3033 computer (10, 16, 17). We analyzed the relative hydrophilicities of the influenza C/Cal/78 virus RNA 7 product and the NS1 proteins of A and B viruses by using a relative hydrophilicity value for each amino acid as described previously (relative hydrophilicity = total hydrophilicity of decapeptide/10) (8, 16).

Analysis of viral proteins. Confluent monolayers of MDCK cells in 35-mm dishes were infected with different influenza C viruses grown in embryonated chicken eggs (0.1 ml of undiluted amniotic fluid) at 33.5°C. Cells were pulse-labeled for 1 h at 7.5 to 8.5 h postinfection with [^{35}S]methionine (200 μCi/ml) as described previously (22). For purification of [^{35}S]methionine-labeled virus, the cells were pulse-labeled for 2 h at 6.5 to 8.5 h postinfection with [^{35}S]methionine and incubated again for 47 h at 33.5°C. Purified virus was obtained from cell supernatants by centrifugation onto the interface of a 20 to 60% discontinuous sucrose gradient (SW41 rotor; Beckman Instruments, Inc.; 3 h at 35,000 rpm) (21).

Radioactively labeled protein samples were separated by electrophoresis on a 7 to 14% linear gradient sodium dodecyl sulfate-polyacrylamide gel as described previously (31).

In vitro translation of virus-specific mRNA and hybrid arrest translation with segment 7 cDNA. Cytoplasmic RNA was isolated from influenza C virus-infected MDCK cells (5×10^8) at 7.5 h postinfection by a method described previously (4). After incubation, virus-infected cells were suspended in 5 ml of guanidinium thiocyanate solution (50% [wt/vol] guanidinium thiocyanate, 0.5% sodium *N*-lauroylsarcosine, 25 mM EDTA [pH 7.8], 10 mM β-mercaptoethanol) and homogenized with 30 to 40 strokes in a Dounce homogenizer. The homogenates were centrifuged for 10 min at 600 × *g*. The supernatant, to which 2 g of CsCl was added, was layered on top of 2 ml of a 5.7 M CsCl solution (in 0.1 mM EDTA [pH 7.0]). After centrifugation at

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30,000 rpm and 20°C for 16 h in a Beckman SW41 rotor, RNA pellets were suspended in 0.3 M sodium acetate, precipitated with ethanol three times, and then dissolved in water at a concentration of 2 μg/μl.

The poly(A)-containing RNA fraction was selected by oligo(dT) cellulose column chromatography (7) and used for translation in an in vitro rabbit reticulocyte system (no. 8110SB; Bethesda Research Laboratories, Inc.). For hybrid arrest translation (13), 0.6 μg of poly(A) RNA was mixed with 0.2 μg of a cloned cDNA fragment containing RNA 7-specific nucleotides (positions 15 to 898) obtained from plasmid pC374. The mixture (in 10 μl of H_2O) was heated at 100°C for 2 min, rapidly chilled in an ethanol-dry ice bath, and lyophilized. The dried nucleic acids were dissolved in 50 μl of hybridization solution containing 80% (vol/vol) deionized formamide, 0.4 M NaCl, 10 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) hydrochloride (pH 6.4), and 2 mM EDTA and incubated at 44°C for 3 h (14). The hybrids were diluted with 130 μl of H_2O and ethanol precipitated. They were recovered by centrifugation, dissolved in 100 μl of 0.3 M sodium acetate and ethanol precipitated. We repeated this process three times to remove residual formamide. Finally, the hybrids were dissolved in 3 μl of H_2O and used to prime the in vitro translation system.

RESULTS

Identification of a plasmid insert derived from influenza C/Cal/78 virus RNA 7. We analyzed recombinant pBR322 plasmids derived from transcribed influenza C/Cal/78 virus RNA by hybridizing nick-translated insert DNA to influenza C virus RNAs which were electrophoretically separated and transferred to nitrocellulose membrane (16, 28, 29). This Northern blot analysis identified a virus-specific cDNA clone (pC639) which derived from RNA 7 (Fig. 1, lane 3). In the same experiment, an NP-specific clone (17) was used to demonstrate the position of the NP RNA on the gel (Fig. 1, lane 1). Since the insert of pC639 was only 300 base pairs long, this plasmid was used in identifying additional recombinant clones carrying RNA 7-specific sequences. As a result of this search, a clone (pC374) possessing an insert of approximately 900 base pairs was obtained.

Nucleotide sequence of RNA 7. Initial end sequencing of the insert of clone pC374 revealed the presence of sequences previously found at the 3′ terminus of different influenza C virus RNAs (6). This suggested that the insert most likely started at the 3′ end of RNA 7 and could encode for the entire gene. It was then decided to obtain a complete sequence of the insert. Approximately 97% of the DNA was sequenced on both strands by Maxam-Gilbert sequencing (15). We confirmed the positions of all restriction enzyme sites used in the preparation of fragments by sequencing overlapping fragments. Since clone pC374 lacked sequences previously found at the 5′ ends of the C virus RNAs (6), the 5′ terminal nucleotides of viral RNA 7 were determined directly by dideoxy sequencing (11, 24) of purified virion RNA with a restriction enzyme fragment (nucleotides 730 to 807 of pC374) as the primer. It was found that the insert of clone pC374 was lacking 36 nucleotides at the 5′ terminus of viral RNA 7.

The complete nucleotide sequence of RNA 7 of the influenza C/Cal/78 virus is shown in Fig. 2. The segment contains 934 nucleotides. The first initiation codon in this gene is found at nucleotides 27 to 29, and the reading frame remains open until a termination codon at nucleotides 885 to 887. The adenosine-rich area at nucleotides 914 to 918 probably represents the polyadenylation site for the virion

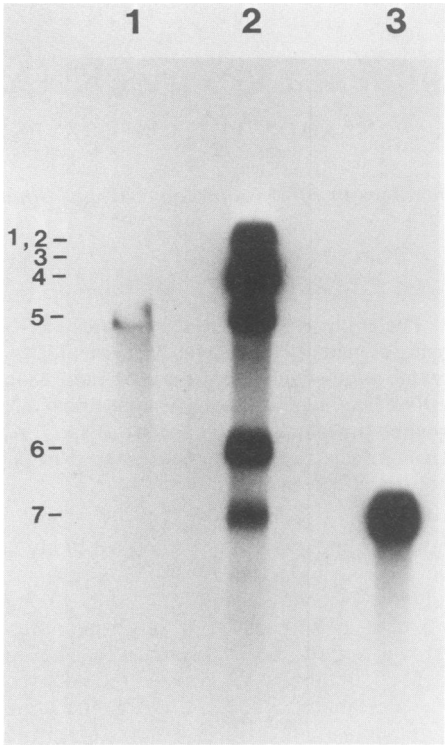

FIG. 1. Identification of plasmid containing cDNA derived from influenza C/Cal/78 virus RNA 7. Glyoxalated total viral RNA was electrophoretically separated on 2.8% polyacrylamide gels and transferred to nitrocellulose membranes for visualization with specific hybridization probes (18, 28, 29). Lanes: 1, hybridization with nick-translated DNA insert derived from pC708 corresponding to the NP gene of influenza C/Cal/78 virus (17); 2, hybridization with radioactively labeled cDNA prepared from total viral RNA (16); 3, hybridization with nick-translated insert DNA derived from pBR322 recombinant plasmid pC639. Numbers on the left indicate RNA segments.

mRNA as has been reported for influenza A virus mRNA (23). The nucleotides at positions 22, 913, and 929 in the mRNA sense of C/Cal/78 virus RNA 7 differ from those in the partial sequences published for a different strain of influenza C virus, C/Johannesburg/1/66 virus (6). These changes would appear to be strain related.

Analysis of the deduced amino acid sequence of RNA 7. The deduced amino acid sequence corresponding to the long open reading frame in the influenza C/Cal/78 virus RNA 7 is also shown in Fig. 2. Preliminary comparison of this sequence with those of the M and NS proteins of influenza A and B viruses showed insufficient homology to permit an unequivocal identification of the RNA 7 gene product as an analog of the M or NS protein. Furthermore, a dot matrix analysis comparing the C/Cal/78 RNA 7 sequences with those of other known A and B virus genes also did not reveal significant sequence homologies, either on the nucleotide level with a stringency condition of 80% homology in a window 10 nucleotides long or on the amino acid level with a stringency condition of 60% homology in a window of five amino acids (data not shown) (11, 16). There were, however, two short peptide stretches found in the influenza C virus RNA 7 sequence which shared homologies with sequences found in the influenza B virus NS1 protein (but they were not conserved in the NS1 proteins of influenza A viruses). The first peptide (positions 54 to 61) shared four of eight amino

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                                    MET SER ASP LYS THR VAL LYS SER THR ASN LEU MET ALA PHE VAL ALA THR LYS MET LEU GLU ARG     22
      AGCAGAAGCAGGGGTACTTTTCCAAA ATG TCC GAC AAA ACA GTC AAA TCA ACA AAT TTA ATG GCA TTT GTA GCC ACA AAA ATG TTA GAG AGA     92

GLN GLU ASP LEU ASP THR CYS THR GLU MET GLN VAL GLU LYS MET LYS THR SER THR LYS ALA ARG LEU ARG THR GLU SER SER PHE ALA     52
CAA GAA GAT TTA GAC ACA TGC ACT GAA ATG CAA GTA GAA AAA ATG AAA ACA TCA ACA AAA GCC AGG CTG AGA ACA GAA TCC TCT TTT GCA    182

PRO ARG THR TRP GLU ASP ALA ILE LYS ASP GLY GLU LEU LEU PHE ASN GLY THR ILE LEU GLN ALA GLU SER THR THR MET THR PRO ALA     82
CCT AGA ACA TGG GAA GAT GCA ATA AAA GAT GGT GAG CTT CTA TTC AAC GGA ACG ATT CTG CAA GCA GAG TCT ACT ACA ATG ACG CCA GCA    272

SER VAL GLU MET LYS GLY LYS LYS PHE PRO ILE ASP PHE VAL PRO SER ASN ILE ALA PRO ILE GLY GLN ASN PRO ILE TYR LEU SER PRO    112
TCC GTA GAA ATG AAG GGG AAG AAA TTT CCT ATT GAT TTT GTT CCA AGC AAC ATA GCA CCA ATT GGG CAA AAT CCA ATA TAT TTG TCA CCA    362

CYS ILE PRO ASN PHE ASP GLY ASN VAL TRP GLU ALA THR MET TYR HIS HIS ARG GLY ALA THR LEU THR LYS THR MET ASN CYS ASN CYS    142
TGT ATT CCT AAC TTT GAT GGA AAC GTC TGG GAA GCA ACG ATG TAT CAT CAT CGT GGA GCA ACT TTG ACA AAG ACA ATG AAT TGC AAC TGT    452

PHE GLN ARG THR ILE TRP CYS HIS PRO ASN PRO SER ARG MET ARG LEU SER TYR ALA PHE VAL LEU TYR CYS ARG ASN THR LYS LYS ILE    172
TTT CAA AGA ACA ATT TGG TGC CAT CCA AAT CCT TCA CGT ATG AGA TTG AGC TAT GCA TTT GTT TTG TAT TGC AGA AAT ACT AAG AAG ATC    542

CYS GLY TYR LEU ILE ALA LYS GLN VAL ALA GLY ILE GLU THR GLY ILE ARG LYS CYS PHE ARG CYS ILE LYS SER GLY PHE VAL MET ALA    202
TGT GGA TAC CTC ATC GCT AAA CAA GTG GCC GGA ATT GAA ACA GGA ATT AGA AAA TGT TTC AGA TGC ATT AAA AGC GGA TTC GTT ATG GCT    632

THR ASP GLU ILE SER LEU ILE ILE LEU GLN SER ILE LYS SER GLY ALA GLN LEU ASP PRO TYR TRP GLU MET LYS HIS GLN ILE LEU THR    232
ACC GAT GAA ATC TCT CTC ATT ATA CTC CAG AGT ATC AAA TCA GGA GCC CAG CTC GAT CCC TAT TGG GAA ATG AAA CAC CAG ATA TTG ACA    722

ARG LEU LYS LEU ILE CYS SER ARG LEU GLU LYS LEU ASP LEU ASN LEU SER LYS ALA VAL LEU GLY ILE GLN ASN SER GLU ASP LEU ILE    252
AGA CTG AAG CTT ATA TGC TCT CGC TTA GAG AAG CTG GAC CTT AAC CTG AGT AAA GCA GTC TTA GGA ATC CAA AAT TCT GAA GAT CTT ATT    812

LEU ILE ILE TYR ASN ARG ASP ILE CYS LYS ASN THR ILE LEU MET ILE LYS SER LEU CYS ASN SER LEU ILE ***                        286
TTG ATC ATA TAT AAC AGA GAT ATT TGT AAA AAC ACT ATA TTG ATG ATA AAA TCT CTG TGC AAT TCA CTT ATA TAA TTGTTTTAAGTTGTTATTC    906

CAAAGTTAAAAATCCCCTTGCTCCTGCT                                                                                            934
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FIG. 2. Complete nucleotide sequence of influenza C/Cal/78 virus RNA 7 and deduced amino acid sequence of the long open reading frame. The sequence data were obtained following Maxam and Gilbert sequencing of a DNA clone (pC374) and direct RNA sequencing of the 5′ end of the virion RNA. The nucleotide sequence is presented in the mRNA sense, and the amino acid sequence corresponds to the long open reading frame starting at nucleotides 27 to 29 and terminating at positions 885 to 887. Asterisks indicate the termination codon.

acids, and the second peptide (positions 111 to 118) shared five of eight amino acids with sequences found in the influenza B/Lee/40 virus NS1 protein (positions 53 to 60 and 111 to 117, respectively). This allows for a one-amino-acid insertion between amino acid positions 113 and 114.

Since previous comparative analyses of influenza A, B, and C virus HAs suggested that structural features of homologous proteins can be retained even though evolutionary divergence eliminated all primary sequence homology, we proceeded to compare structural parameters of the RNA 7 product and those of other proteins of A and B viruses. For example, the relative hydrophilicity pattern of the RNA 7 protein of influenza C/Cal/78 virus is shown in comparison with those of the NS1 polypeptides of A/PR/8/34 (2) and B/Lee/40 (3) viruses (Fig. 3). However, all three patterns were dissimilar. Comparisons of the hydrophilicity curve of RNA 7 with those of other influenza A and B virus proteins also failed to show a conserved pattern.

In vitro translation of virus-specific mRNA and hybrid arrest translation with using cDNA derived from RNA 7. To confirm the coding assignment for influenza C virus RNA 7, we isolated messenger RNA from virus-infected cells, hybridized it to RNA 7-specific cDNA, and translated it in a rabbit reticulocyte in vitro translation system. C/JJ/50 virus-infected MDCK cells were used for mRNA preparations, since in vivo labeling of virus-specific polypeptides and the yield of virus-specific mRNA were much greater with C/JJ/50 virus than with C/Cal/78 virus. The results obtained with C/JJ/50 virus should also be valid for C/Cal/78 virus, since there is a high level of homology (>95%) of segment 7 RNA sequences among several influenza C viruses which have been sequenced (D. Buonagurio, S. Nakada, M. Krystal, and P. Palese, unpublished data).

C/JJ/50 virus-specific polypeptides synthesized in MDCK cells and in the in vitro translation system were separated on polyacrylamide gels as indicated in the legend to Fig. 4. The HA, NP, and M proteins were clearly seen in the lanes containing infected cell lysate and purified virus (lanes 2 and 6), although the three polymerase (P) polypeptides could not be distinguished under the electrophoresis conditions used. In the lane containing the virus-infected cell lysate (lane 2), a strong band of estimated molecular weight of 28,500 (28.5K) was detected. This protein was absent from the mock-infected MDCK lysate and the purified virus preparation (lanes 1 and 6) but was present in the lane containing the in vitro translation products (lane 3). When the mRNA preparation was hybridized to an RNA 7-specific cDNA fragment before translation, the synthesis of this translation product was completely blocked, (lane 4). This result indicates that RNA 7 of influenza C virus codes for the 28.5K product.

DISCUSSION

A cDNA clone derived from the shortest RNA of influenza C/Cal/78 virus was obtained, and its sequence was determined. Analysis of the complete sequence of RNA 7 revealed a long open reading frame which could code for a protein of 286 amino acids. Attempts were made to identify the gene product coded by RNA 7, but nucleotide and amino acid sequence comparison of this gene with those of known influenza A and B virus genes did not result in unambiguous gene assignment. However, there were two short peptide regions in the deduced amino acid sequence of the long open reading frame which shared four of eight and five of eight amino acids, respectively, with sequences found in the NS1 polypeptide of influenza B/Lee/40 virus (3). (It should be noted that homologous sequences are not present in the known NS1 polypeptides of influenza A viruses [2].) A definitive coding assignment of this gene to an NS protein was subsequently achieved by hybrid arrest cell-free translation with influenza C virus-specific mRNA preparations and cloned cDNA of RNA 7 (Fig. 4). This NS protein migrates as a 28.5K band on polyacrylamide gels under our gel conditions and most likely corresponds to the 24K NS protein described previously by Petri et al. (19) and the 29.5K C1 band defined by Yokota et al. (30). Our data are thus compatible with the suggestion that RNA 7 of influenza C viruses codes for an NS protein and that the 28.5K NS polypeptide is translated from an mRNA containing the long open reading frame covering 286 codons (Fig. 5).

Analysis of the other two reading frames in Fig. 5 does not reveal open reading frames longer than 68 codons. Future

examinations will be necessary to demonstrate whether influenza C viruses—like influenza A and B viruses (3, 12–14)—direct the synthesis of spliced mRNAs, which may result in the usage of these short open reading frames observed in the gene. Petri et al. (19) had reported the presence of a second NS protein which migrates as a 14K band. However, Yokota et al. (30) were not able to confirm this result. Rather, these authors suggest that a series of small proteins (C3, C4, and C5) observed in influenza C virus-infected cells are breakdown products of the M protein. Our data do not allow a distinction of these two possibilities. It should also be noted that, in addition to the short open reading frames mentioned above (Fig. 5), an open reading frame of 135 codons is located on one of the frames in the virion sense of RNA 7 (data not shown).

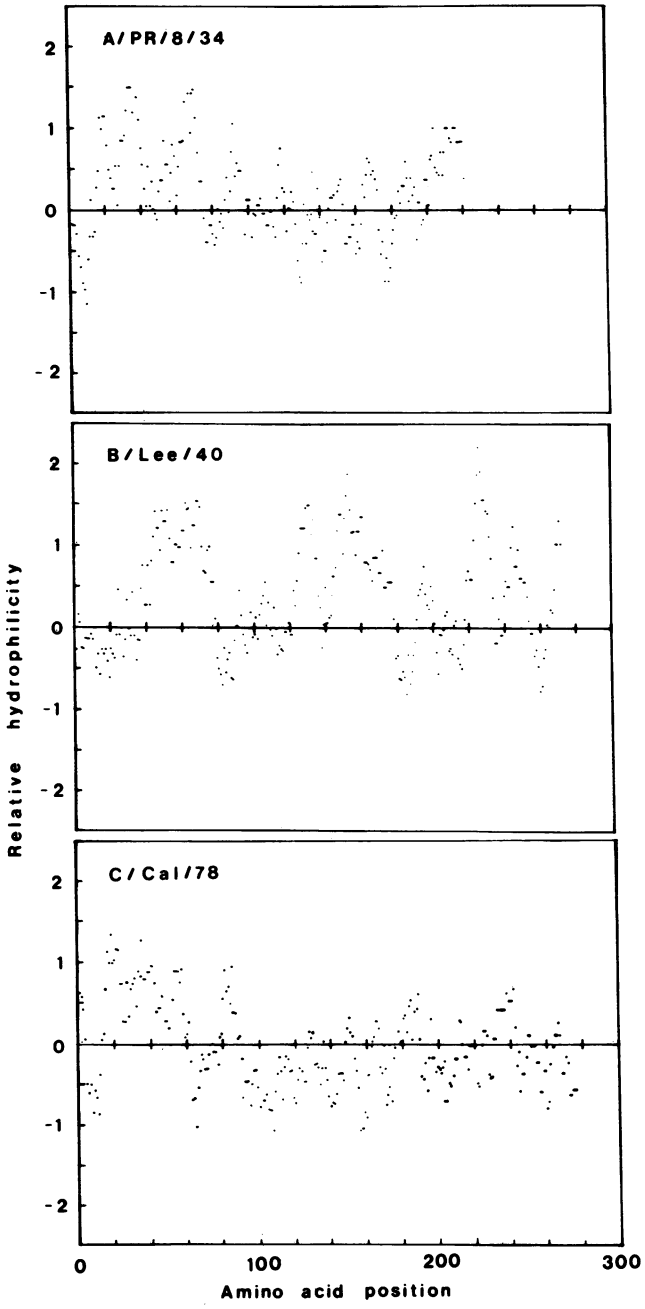

FIG. 3. Relative hydrophilicity plots of influenza A/PR/8/34 (2) and B/Lee/40 (3) virus NS1 proteins and of the RNA 7 protein of influenza C/Cal/78 virus. The plots were generated as described previously (8, 16).

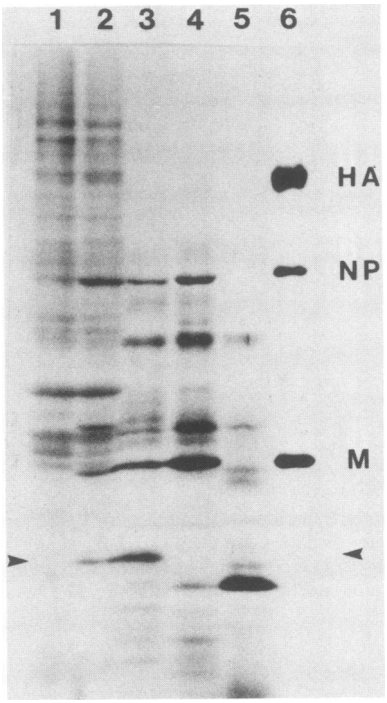

FIG. 4. Hybrid arrest in vitro translation of the 28.5K polypeptide with the cDNA fragment derived from RNA 7 of influenza C/Cal/78 virus. [^{35}S]methionine-labeled virions and virus-infected cell lysate were obtained as described in the text. Translation of infected cell mRNAs in a reticulocyte lysate containing [^{35}S]methionine and hybrid-arrested translation were also done as described in the text. The samples were analyzed on a 7 to 14% polyacrylamide gel (31). Lanes: 1, mock-infected MDCK cells; 2, C/JJ/50 virus-infected MDCK cells; 3, in vitro translation products of mRNA preparation without prior hybridization to RNA 7-specific cDNA; 4, in vitro translation products after hybridization of mRNA preparation to a cDNA copy derived from segment 7 of influenza C/Cal/78 virus; 5, in vitro translation products obtained without addition of influenza C virus-specific mRNA preparation; 6, labeled proteins of purified C/JJ/50 virus. Arrowheads indicate 28.5K bands.

The function of the 28.5K NS protein of influenza C virus remains unknown. Also, we do not know whether the NS protein of influenza C viruses is equivalent to the NS1 (NS2) of influenza A or B viruses, and no attempts have been made to identify the cellular localization of the influenza C virus NS protein during virus replication. If the influenza C virus NS protein corresponds functionally to the NS1 of influenza A or B viruses, it is not reflected in the conservation of the hydrophilicity patterns of these proteins (Fig. 3). Rather, all three NS proteins have different structural features when hydrophilicity is used as the parameter. In this context it should be noted, however, that the HA molecules of A, B, and C viruses retain similar hydrophilicity patterns even though their primary amino acid sequences share limited or no sequence homologies.

Influenza C viruses have been shown to share many features with influenza A and B viruses, and the evolutionary relatedness of influenza type A, B and C viruses is now

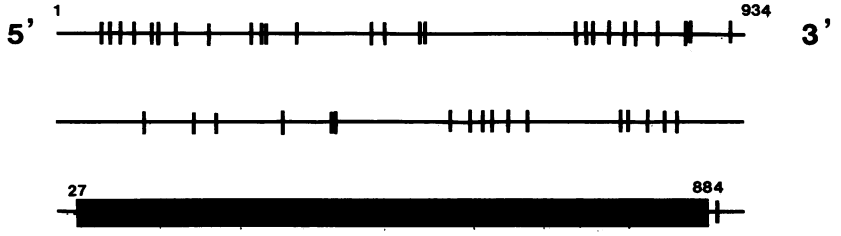

FIG. 5. Open reading frames in the m sense of the RNA 7 gene of influenza C/Cal/78 virus. Vertical lines indicate the positions of termination codons, and the long, dark rectangle represents the long open reading frame.

unquestioned. Clearly, a structural and functional conservation was observed among the HA molecules of these viruses (10, 16) and, more recently, sequence conservation among the NP genes of influenza A, B, and C viruses has been reported (12, 17). Although the present study showed a lack of significant sequence and structural homology between the C virus NS protein and the NS proteins of A and B viruses, it is notable that the shortest of the influenza virus genes in viruses belonging to all three types codes for an NS protein(s).

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