JOURNAL OF VIROLOGY, Apr. 1982, p. 186–193
0022-538X/82/040186-08$02.00/0

Vol. 42, No. 1

Influenza B Virus Genome: Sequences and Structural Organization of RNA Segment 8 and the mRNAs Coding for the NS_1 and NS_2 Proteins

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Received 21 September 1981/Accepted 2 December 1981

Double-stranded DNA derived from influenza B virus genome RNA segment 8, which codes for the NS_1 and NS_2 proteins, was constructed by hybridization of full-length cDNA copies of RNA segment 8 and of the NS_1 mRNA. This DNA was cloned in plasmid pBR322 and sequenced. The NS_1 mRNA (~1,080 viral nucleotides) contains nonviral nucleotides at its 5′ end and is capable of coding for a protein of 281 amino acids. Sequencing of the NS_2 mRNA has shown that it contains an interrupted sequence of 655 nucleotides and is most likely synthesized by a splicing mechanism. The first ~75 virus-specific nucleotides at the 5′ end of the NS_2 mRNA are the same as are found at the 5′-end of the NS_1 mRNA. This region contains the initiation codon for protein synthesis and coding information for 10 amino acids common to the two proteins. The ~350-nucleotide body region of the NS_2 mRNA can be translated in the +1 reading frame, and the sequence indicates that the NS_1 and NS_2 protein-coding regions overlap by 52 amino acids translated from different reading frames. Thus, between the influenza A and B viruses, the organization of the NS_1 and NS_2 mRNAs and the sizes of the NS_2 mRNA and protein are conserved despite the larger size of the influenza B virus RNA segment, NS_1 mRNA, and NS_1 protein.

Although influenza A and B viruses differ in their epidemiology, immunological specificity, and several of their biological characteristics (15), they are in many respects biochemically similar. Like those of influenza A virus, the eight single-stranded genome RNA segments of influenza B virus are transcribed into mRNAs coding for different virus-specific polypeptides. The three largest genome RNA segments code for three polypeptides presumably associated with polymerase activity (P_{1-3}), and one each of the remaining segments codes for the hemagglutinin, the neuraminidase, the nucleocapsid protein, the membrane protein, and a nonstructural protein (NS_1) (2, 7, 10, 25, 28, 35). In addition to NS_1, the eighth and smallest RNA segment of influenza B virus, like that of influenza A (14, 18), codes also for a second nonstructural protein, NS_2, which is translated from a separate mRNA and shares no [^{35}S]methionine-containing tryptic peptides with NS_1 (7). The influenza A virus NS_1 and NS_2 polypeptides share nine amino acids at their N termini, and, after the leader sequence coding for these amino acids, the NS_2 mRNA has an interrupted sequence of 473 nucleotides before its body which is translated in the +1 reading frame (20).

We report here the results of cloning and sequencing studies designed to determine the precise location on genome RNA segment 8 of the nucleotide sequences coding for the influenza B NS_1 and NS_2 mRNAs and to investigate whether the influenza B NS_2 mRNA is analogous to that of influenza A virus in having an interrupted sequence, which would provide further evidence that the NS_2 mRNAs are produced by a splicing mechanism.

MATERIALS AND METHODS

Influenza B virus-specific RNA. Influenza B/Lee/40 virus-specific genome RNA from egg-grown virus and total cytoplasmic mRNA from virus-infected HeLa cells were purified as described previously (7).

Production of cloned influenza B virus-specific DNA. A strategy similar to that used to obtain cloned influenza A virus-specific DNA (8, 17) was used for influenza B virus. Influenza B virus-specific mRNA was transcribed with reverse transcriptase by using oligodeoxythymidylic acid$_{12-18}$ as a primer (17). cDNA copies of genome RNA were synthesized in a similar fashion after the addition of 30 to 50 adenylic acid residues to the 3′ ends of the molecules by using ATP:RNA adenyltransferase (33) in 1 min at 37°C in buffer containing 175 mM NaCl, 50 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 500 μg of bovine serum albumin per ml, 500 μM ATP, and [γ-

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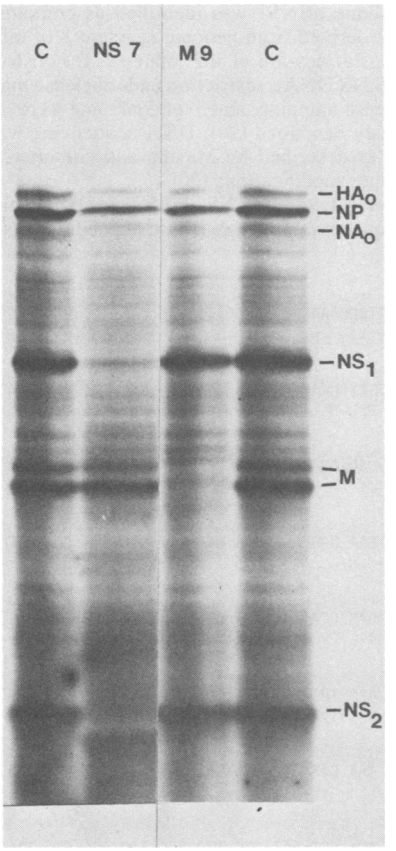

FIG. 1. Translation in vitro of total virus-specific mRNA after hybridization to virus-specific cDNA derived from individual bacterial clones. Lane C shows polypeptides synthesized from mRNA without the addition of cDNA. Lanes NS7 and M9 show polypeptides synthesized after hybridization of DNA derived from clones pBNS7 and pBM9 to mRNA. Clones pBM9 and pBNS7 were shown to have been derived from virus RNA segments 7 and 8, respectively.

[^{32}P]ATP at 0.2 Ci/mmol. RNA templates were removed by alkaline hydrolysis and subsequent neutralization. cDNA copies were extracted with phenol and desalted by gel filtration on Sephadex G-50. A total of 15 to 20 deoxycytidylyl residues were added to the 3′ ends of both the (+) and (−) cDNA copies in separate reactions with terminal transferase (31). Similarly, deoxyguanidylyl residues were added to *Pst*I-cleaved pBR322 DNA. Full-length deoxycytidylic acid-tailed (+)- and (−)-stranded cDNA copies corresponding to individual genomic RNA segments were selected by electrophoresis on 4% polyacrylamide gels containing 6 M urea, eluted from the gels (23), and hybridized at 65°C for 3 h in 300 mM NaCl–10 mM Tris-hydrochloride (pH 7.4)–2 mM EDTA.

Recombinant DNA molecules were constructed by hybridizing the deoxycytidylic acid-tailed double-stranded virus-specific cDNAs to deoxyguanidylic acid-tailed pBR322 DNA, and the resulting molecules were used to transform *Escherichia coli* K-12 strain HB101. Tetracycline-resistant colonies were screened for influenza B virus-specific sequences (13) by using individual ^{125}I-labeled influenza B virus genome RNA segments (7) as probes.

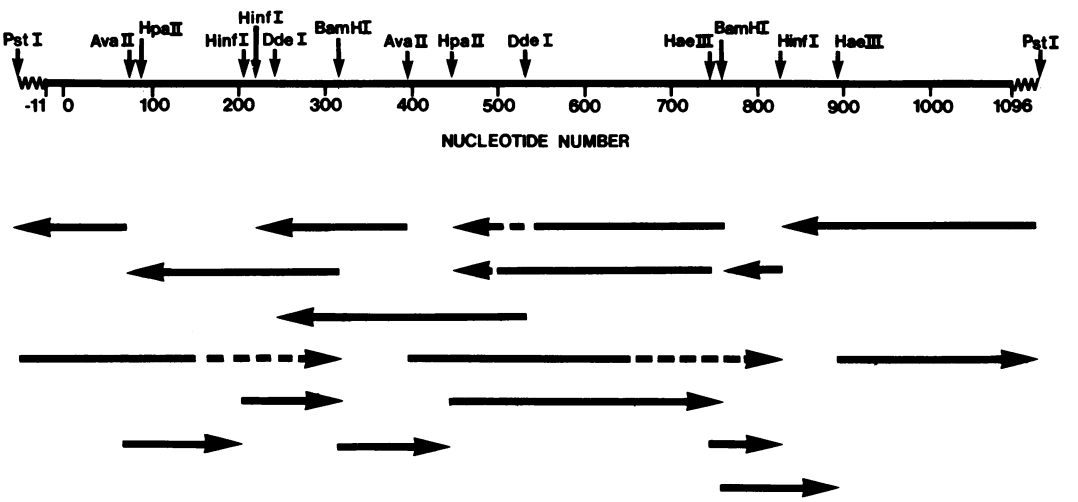

FIG. 2. Cleavage map and sequencing strategy of cloned B NS DNA. Experimentally derived restriction endonuclease cleavage sites of the B NS DNA insert from plasmid pBNS7 are shown. The direction of mRNA transcription is from left to right. The zigzag at each end represents G-C linkers. Nucleotide 1 corresponds to the 3′-terminal nucleotide of the virion RNA. Nucleotides −11 to −1 are nonviral sequences derived from the 5′ end of the cloned mRNA. The arrows represent segments used for sequencing. The foot of each arrow represents the restriction enzyme site labeled with [γ-^{32}P]ATP and polynucleotide kinase. The solid portion of each arrow represents the length of sequence information read from each segment.

The origin of the influenza B virus-specific cDNA sequences contained in the individual clones was identified by hybrid-arrested translation performed as described previously (19). Hybridization of an excess of plasmid DNA to total virus-specific mRNA before its translation in vitro in the wheat germ cell-free system specifically prevented synthesis of the polypeptide(s) coded for by the genome RNA segment from which the cloned DNA was derived.

Restriction analysis and sequencing of cloned B NS DNA. Clone pBNS7 was identified as containing sequences derived from genome segment 8 of influenza B virus. Purification of the influenza B nonstructural DNA (B NS DNA), restriction endonuclease mapping, S_1 nuclease mapping, and 5′ end labeling were done as previously described (20). DNA sequencing was performed as described by Maxam and Gilbert (23, 24), with minor modifications (20).

Primer extension of NS_2 mRNA for 5′ terminal sequencing. Total influenza B virus-specific mRNA was

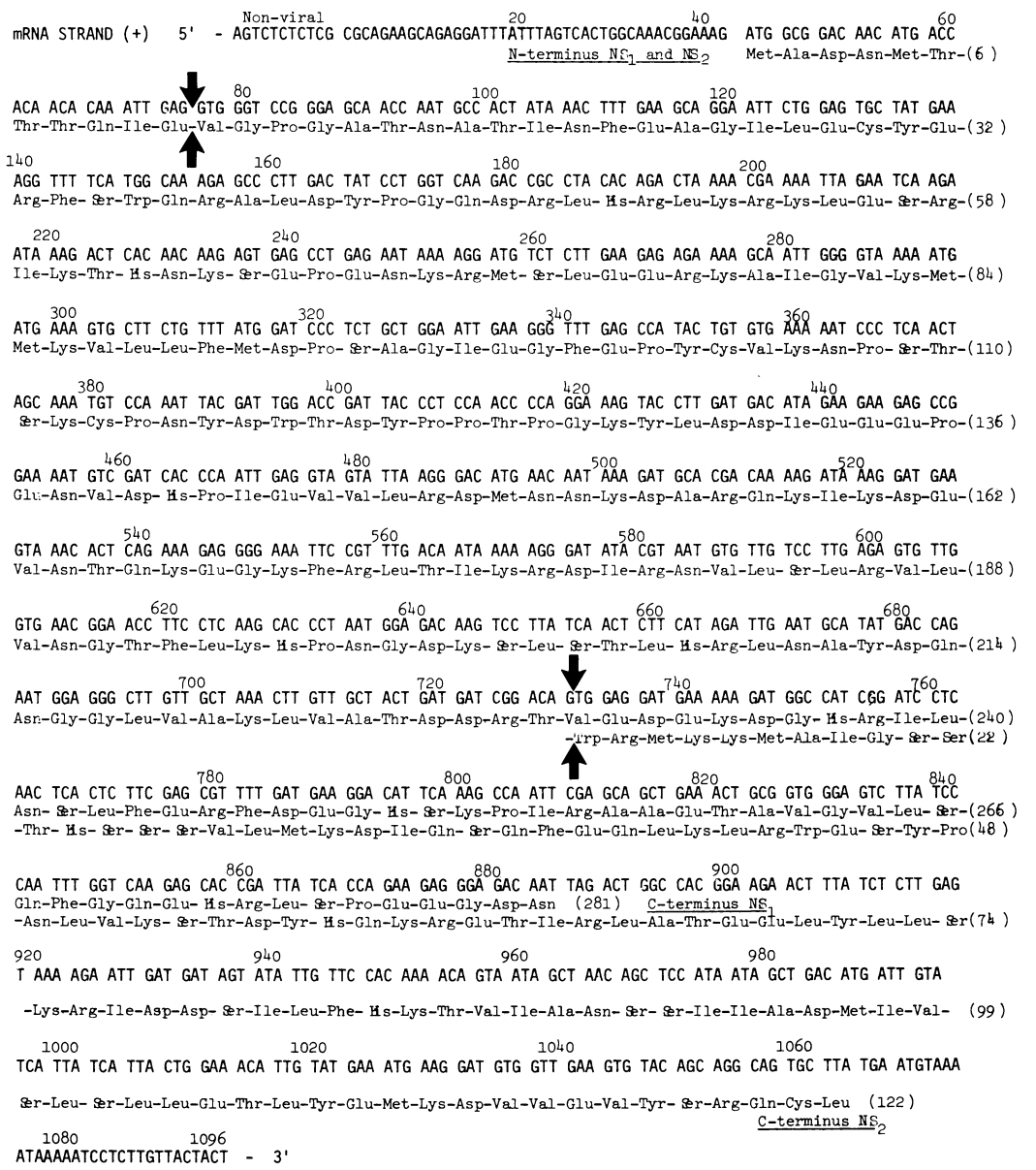

FIG. 3. Nucleotide sequence of influenza B virus RNA segment 8 derived from cloned B NS DNA. The deduced amino acid sequence of polypeptides NS_1 and NS_2 are shown. The arrows after nucleotides 75 and 730 show the probable 5′ and 3′ splice sites to form the NS_2 mRNA. Nucleotide 1 corresponds to the 3′ terminal nucleotide of the virion RNA, but in clone pBNS7 this is a C rather than an A (see text for explanation).

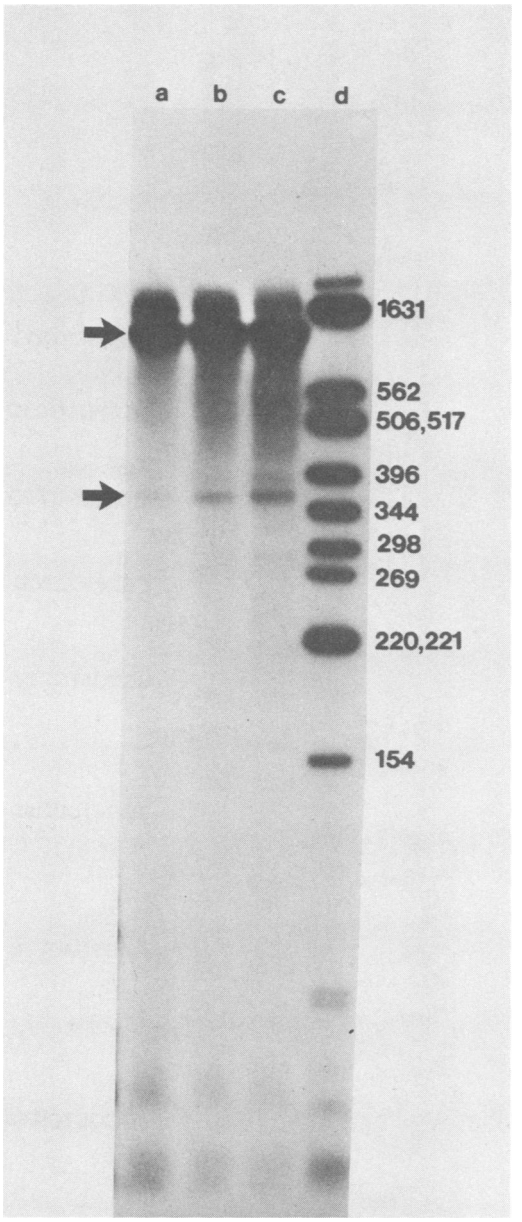

FIG. 4. Analysis of the influenza B virus NS_1 and NS_2 mRNAs by the S1 nuclease technique. Polyadenylated mRNAs from influenza B virus-infected HeLa cells were denatured and allowed to hybridize with ^{32}P-labeled B NS DNA, and the hybridization mixtures were treated with S1 nuclease. S1 nuclease-resistant single-stranded DNA fragments were analyzed on a 4% polyacrylamide gel containing 9 M urea after alkali denaturation. Lanes a, b, and c represent increasing amounts of total mRNA hybridized with a constant amount of ^{32}P-labeled B NS DNA. Lane d shows ^{32}P-labeled DNA size markers of the nucleotide lengths noted. The arrows point to S1 nuclease-resistant DNA segments corresponding to NS_1 and NS_2 mRNA body regions ~1,100 and 360 nucleotides in length, respectively.

fractionated on sucrose gradients (7). Fractions containing NS_2 mRNA were identified by in vitro translation, pooled, and hybridized to a 5′ singly end-labeled restriction enzyme fragment of B NS DNA. This primer was extended with reverse transcriptase, and the resulting product was purified and sequenced as described previously (20, 21).

RESULTS

Preparation of cloned B NS DNA. cDNA strands synthesized separately from genome RNA and from virus-specific mRNA were hybridized to form influenza B virus-specific DNA

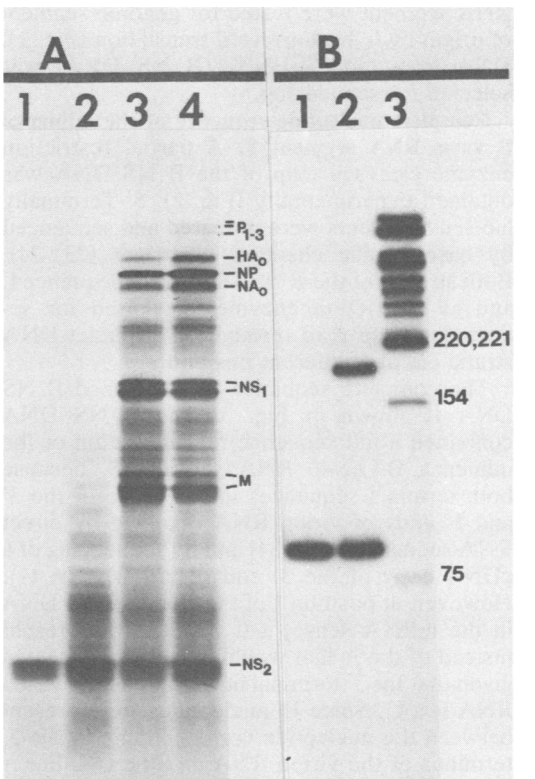

FIG. 5. (A) Polypeptides synthesized in vitro from virus-specific mRNAs after separation on a sucrose gradient. Lanes 1, 2, and 3 show polypeptides synthesized from mRNAs from, respectively, the top, middle, and bottom of the gradient. Lane 4 shows polypeptides synthesized from total virus-specific mRNA. Polypeptides were analyzed on a 17.5% polyacrylamide gel. (B) Primer extension of the NS_2 mRNA. A DNA restriction fragment extending from nucleotide 750 to nucleotide 833 and uniquely 5′ end labeled at nucleotide 833 was hybridized to mRNA from gradient fraction 1 and extended with reverse transcriptase. Lane 1 shows unextended primer. Lane 2 shows primer after extension. Lane 3 shows ^{32}P-labeled marker DNA of the nucleotide lengths noted. Samples were analyzed on an 8% polyacrylamide gel containing 9 M urea.

duplexes. Full-length cDNA copies were selected by gel electrophoresis before hybridization and cloning at the single *Pst*I site of plasmid pBR322. Plasmid DNA from positive colonies was purified, influenza B virus-specific sequences were excised with *Pst*I, and the lengths of the DNA inserts were analyzed on 4% acrylamide gels. The length of the influenza B virus genome RNA segment 8 has been estimated to be ~1,100 nucleotides. All presumptive segment 8-derived cloned DNA inserts contained 1,100–1,150 nucleotide base pairs including G-C linkers, indicating the usefulness of the selection process for full-length cDNA copies. The longest DNA inserts corresponding to each genome RNA segment were tested for genome segment of origin by hybrid-arrested translation (Fig. 1). DNA from clone pBNS7 (B NS DNA) was selected for sequencing.

Complete nucleotide sequence of the influenza B virus RNA segment 8. A partial restriction enzyme cleavage map of the B NS DNA was obtained experimentally (Fig. 2). 5′-Terminally labeled fragments were prepared and sequenced by base-specific chemical cleavages (23, 24). Both strands of the B NS DNA were sequenced, and all restriction enzyme sites used for sequencing were read through on an intact DNA strand cut at a different position.

The complete sequence of the cloned B NS DNA is shown in Fig. 3. The B NS DNA contained a full sequence representation of the influenza B/Lee/40 RNA segment 8, because both terminal sequences match those of the 3′ and 5′ ends of virion RNA obtained by direct RNA sequencing (11, 34) and by sequencing of a cDNA copy of the 3′ end of virion RNA (1). However, at position 1 of the cloned B NS DNA in the mRNA sense, a C residue was present instead of the A that would have been expected given that the 3′ terminal nucleotide of the virion RNA is a U. Since 11 nucleotides were present between the nucleotide corresponding to the 3′ terminus of the virion RNA and the G-C linker region, it seems likely that transcription of influenza B virus RNA occurs in a manner similar to that of influenza A. Transcription of influenza A virion RNA is primed by cellular mRNAs which donate their caps and approximately 7 to 15 nucleotides to the 5′ ends of the nascent viral mRNAs, probably initiating transcription at the second rather than the first nucleotide at the 3′ end of the virion RNA strand and leading to some heterogeneity among viral mRNAs in the nucleotide corresponding to that at the 3′ end of virion RNA (5, 6, 9, 12, 16, 21, 26, 29).

RNA segment 8 of influenza B/Lee/40 virus is 1,096 nucleotides long. There is a noncoding region of 42 viral nucleotides from the 5′ end of the (+) sense DNA strand. The first open read-

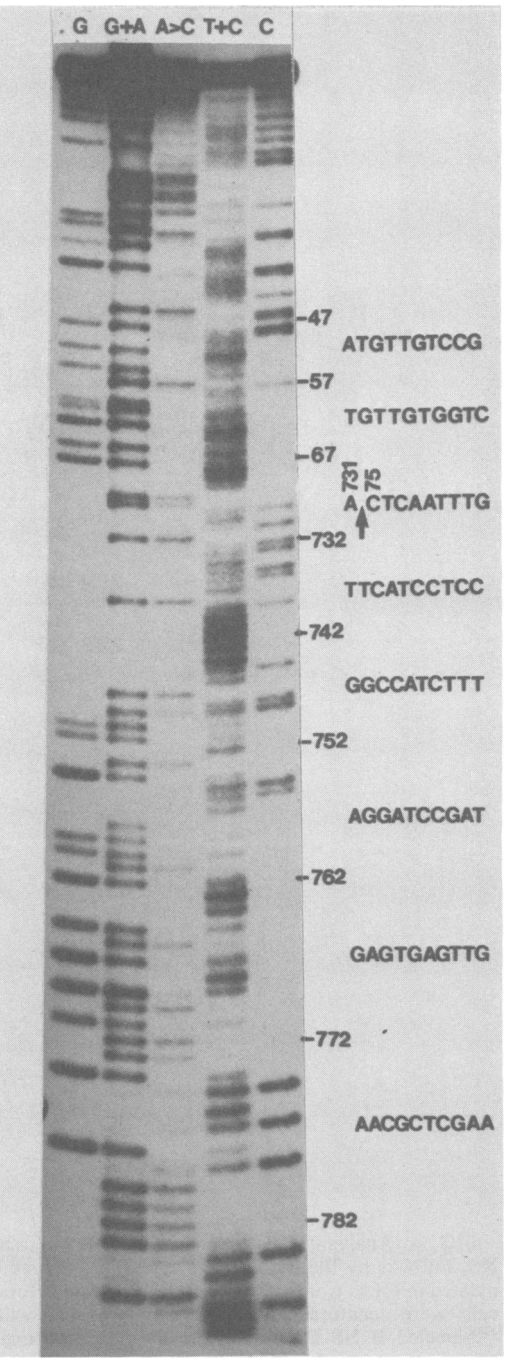

FIG. 6. Sequence of DNA complementary to the 5′ terminal region of the NS_2 mRNA. Base-specific chemical cleavages are indicated over each lane. The sequence obtained from the gel is interpreted with the nucleotides numbered as in Fig. 3. The probable 5′ and 3′ sites (nucleotides 75 and 731) at which the interrupted sequence is excised are indicated by an arrow. Sequence here is complementary to mRNA sense.

ing frame begins with the initiation codon at nucleotides 43 through 45 and continues until a termination codon at nucleotides 886 through 888. A tract of nine adenine residues interrupted by one thymidine occurs at nucleotides 1,072 through 1,081. By analogy with influenza A virus (30), this probably represents the polyadenylation site for the virus mRNA. Polyadenylation at this site would yield an mRNA of ~1,080 viral nucleotides capable of coding for a protein of 281 amino acids including the initial methionine. Although this protein size is somewhat smaller than would have been expected from the M_r of 40,000 estimated for NS_1 on the basis of mobility on polyacrylamide gels, the size of the mRNA is compatible with the estimated size of the NS_1 mRNA as determined by S1 nuclease mapping experiments (Fig. 4). From nucleotide 672 there is a second protein-coding region in the +1 reading frame extending until a termination codon at nucleotides 1,064 through 1,066, which could code for a protein of 131 amino acids.

Structure of the NS_2 mRNA. Because the influenza A NS_2 mRNA contains an interrupted sequence, experiments were performed to determine more precisely the nucleotide sequence at the 5' end of the influenza B NS_2 mRNA. S1 nuclease mapping experiments revealed that the body of the mRNA was ~360 nucleotides in length (Fig. 4). A restriction enzyme fragment of the B NS DNA extending from nucleotide 750 (*Hae*III site) to nucleotide 833 (*Hin*fI site) and uniquely 5' end labeled at nucleotide 833 was prepared and hybridized to sucrose gradient-purified NS_2 mRNA and extended with reverse transcriptase (Fig. 5). The single-stranded DNA product of ~190 nucleotides was sequenced (Fig. 6). From the end of the primer fragment at nucleotide 750, the sequence of the extended DNA followed that of the genome segment and the NS_1 mRNA until nucleotide 731. At that point the sequence was interrupted, rejoining the sequence of the NS_1 mRNA at nucleotide 75. The NS_2 mRNA therefore has a leader sequence of ~75 viral nucleotides which appear to be identical with those at the 5' end of the NS_1 mRNA, followed by an interrupted sequence of 655 nucleotides and then a body of 350 nucleotides. The sizes would indicate that the NS_1 and NS_2 mRNAs are probably 3' coterminal. The exact nucleotide at which the 5' terminal leader sequence is joined to the body of the NS_2 mRNA could not be unequivocally demonstrated by this experiment because of the repetition of the nucleotides AG at positions 74 through 75 and 729 through 730. The junction is likely to be between nucleotides 75 and 731 by analogy to the consensus sequences at the splicing sites of intervening regions of eucaryotic mRNAs (22). Whereas the AG↓GTG (↓ = cleavage) sequence at the likely 5' splice terminus is consistent with both the simple and the complicated proposed consensus sequences, the 3' nucleotides of the intervening sequence of the influenza B NS_2 mRNA (TGATCGGACAG↓T), like those of the influenza A M_2 mRNA (21), do not contain an immediately adjacent pyrimidine-rich tract.

DISCUSSION

Comparison of the organization of the nonstructural genes of influenza A and B viruses. Figure 7 shows a comparison of models for the organization of the NS_1 and NS_2 mRNAs of influenza A and B viruses. Despite the greater length of the genome RNA segment and of the protein-coding region for the influenza B NS_1 protein, the organization and size of the influenza B NS_2 mRNA and protein-coding region are remarkably similar to those of the influenza A NS_2 mRNA. This is because the greater length of the NS_1 protein-coding region in influenza B virus is compensated for by a longer interruption in the sequence of the NS_2 mRNA. The influenza B NS_2 protein-coding region is longer than that of influenza A by only a single amino acid which is coded within the 5' terminal leader sequence. The virion (−) strand of the B NS DNA does not contain an open reading frame as has been found in three of the four influenza A virus RNA segment 8 sequences which have been determined (3, 4, 20, 27). The longest open coding region of the (−) strand of the B NS DNA is only 77 amino acids long and contains no methionine residues.

Predicted NS_1 and NS_2 protein sequences. The calculated M_r for the NS_1 protein is 32,026, which is almost 25% smaller than the 40,000 estimated from the mobility of the protein on polyacrylamide gels. The NS_2 protein is predicted to have an M_r of 14,223, slightly larger than the 11,500 determined by gel mobility, but remarkably close to the M_r of 14,216 calculated for the influenza A NS_2 protein (20). After the initiation codon for protein synthesis, the NS_2 leader sequence would code for 10 amino acids which would be the same as those at the N terminus of NS_1. The body of the NS_2 mRNA would code for an additional 111 amino acids in the +1 reading frame, 52 of which would overlap the NS_1 protein-coding region (Fig. 7). Although the NS_1 and NS_2 proteins would share the same 11 amino acids including an internal methionine at their 5' termini, the initial trypsin cleavage site after the 5' terminus is different for the two proteins confirming that they would not share [^{35}S]methionine-labeled tryptic peptides as has been earlier indicated (7).

Studies to compare the level of homology

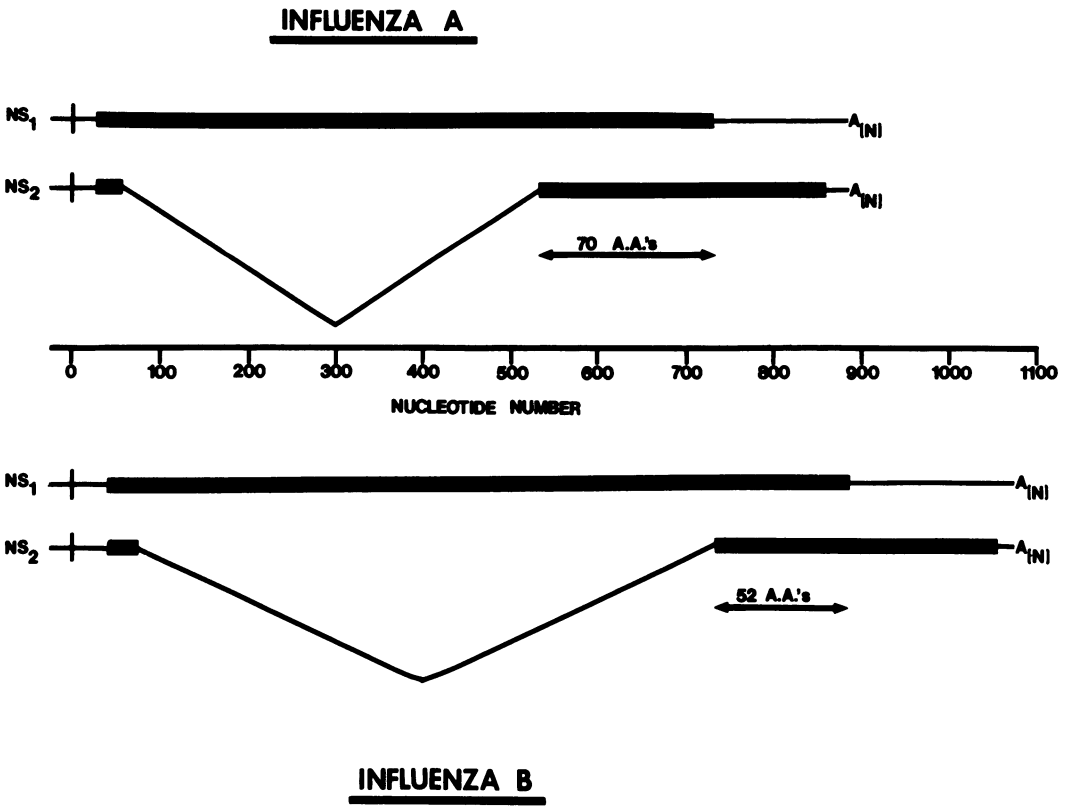

FIG. 7. Comparison of models for the arrangement of the NS_1 and NS_2 mRNAs of the influenza viruses A/Udorn/72 (20) and B/Lee/40. The thick lines represent protein coding regions, whereas the thin lines represent 5′ and 3′ noncoding regions. The line and bar before nucleotide 1 at the 5′ termini represent heterogeneous nucleotides derived from cellular mRNAs. The V-shaped lines in the NS_2 mRNAs represent the interrupted sequences in the translated regions of the NS_2 mRNAs. In both cases, the regions of the NS_2 mRNAs after the interruption are translated in reading frames different from those used for NS_1. After the interruption, the coding regions for NS_1 and NS_2 overlap by 70 and 52 amino acids, respectively, for influenza A and B viruses.

between the nucleotide sequence or the amino acid sequence of influenza A and B virus NS_1 and NS_2 proteins are in progress. It has been found experimentally by hybridization studies that there is 42% base sequence homology between RNA segments 8 of influenza A (fowl plague) and B (Mass/71) viruses (32). By using complex algorithms we will be able to compute the degree of homology between the A and B viruses, but this is complicated by the differing lengths of the segments and the decision as to the number of nucleotides to place in a group and the amount of mismatching permissible when making comparisons. However, despite the absence of detailed homology studies, the similarity in structural arrangement of the influenza A and B virus NS_1 and NS_2 mRNAs argues for a definite relationship between the two viruses. An interrupted region with nucleotides at the 5′ and 3′ junctions similar to those of the consensus sequences at the splicing sites of eucaryotic mRNAs (22) is conserved within the NS_2 mRNAs of these distantly related viruses. This would seem to make more likely a splicing mechanism for the genesis of these mRNAs rather than such hypothetical mechanisms as a (−)-stranded RNA segment 8 with an internal deletion.

ACKNOWLEDGMENTS

We thank Nigel Fraser and Jeffrey Faust of the Wistar Institute, Philadelphia, for the gift of ATP:RNA adenyltransferase enzyme and Mary Conners for excellent technical assistance.

This work was supported by Public Health Service research grant AI-05600 from the National Institute of Allergies and Infectious Diseases. D.J.B. was a Medical Research Council of Canada Fellow, and R.A.L. is an Irma T. Hirschl Career Scientist Awardee.

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