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Mapping of the two overlapping genes for polypeptides NS_1 and NS_2 on RNA segment 8 of influenza virus genome

(Rna genes in different reading frames/nuclease S1 mapping/hybrid-arrested translation/cloned nonstructural genes/myxovirus gene structure)

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ABSTRACT **RNA segment 8 of the influenza virus genome is unique in coding for two polypeptides, NS_1 (M_r, ≈25,000) and NS_2 (M_r, ≈11,000). These polypeptides are synthesized from separate mRNA species. By using cloned DNA derived from RNA segment 8 (NS DNA) the two mRNAs have been mapped on segment 8 by hybridization of mRNAs with restriction endonuclease fragments of the DNA and nuclease S1 digestion methods. These data indicate that the body of the NS_1 mRNA (≈850 nucleotides) maps at 0.05–0.95 units of the cloned NS DNA and the body of the NS_2 mRNA (≈340 nucleotides) maps at 0.59–0.95 units, suggesting that the two mRNAs are 3′ coterminal and share the same poly(A) addition site. These positions of the mRNAs on the viral genome segment were confirmed in hybrid-arrested translation experiments using fragments of the cloned NS DNA to inhibit the synthesis *in vitro* of NS_1 or NS_2 polypeptides. In addition, in these translation experiments the use of certain DNA fragments resulted in premature termination of the NS_1 polypeptide. From these data, it could be estimated that the termination of translation of NS_1 is at ≈0.76 map unit. Thus, the coding regions of the two mRNAs overlap by ≈144–159 nucleotides, the equivalent of ≈48–53 amino acids. Peptide mapping experiments indicated that polypeptides NS_1 and NS_2 do not share methionine- or leucine-containing tryptic peptides. The results obtained indicate that translation of the NS_2 mRNA occurs in a reading frame different from that used for NS_1.**

The eight single-stranded RNA segments of the influenza virus genome (1–3), which are of opposite polarity to mRNA, have been shown (4–6) by various methods to contain the genetic information for the following eight virus-specific polypeptides: three polypeptides associated with RNA polymerase activity (P_1, P_2, P_3), the hemagglutinin (HA), the nucleocapsid protein (NP), the neuraminidase (NA), the membrane protein M, and a nonstructural protein (NS_1) (7–10). In addition to these polypeptides, we and others (8, 11–16) have observed a ninth polypeptide (M_r, 11,000) now designated NS_2. This polypeptide is not synthesized from primary transcripts of viral genome RNA, and early viral protein synthesis is required for its synthesis (13, 14). NS_2 was shown to be a unique ninth influenza virus polypeptide on the basis of its peptide composition, its synthesis *in vitro* using mRNAs from infected cells, the isolation of a separate mRNA for it, and strain-specific differences in its migration in polyacrylamide gels (13–15). Because of the evidence for nine virus-coded polypeptides and the existence of only eight influenza virus RNA segments, we postulated that one RNA segment must code for two polypeptides (14). It was subsequently shown that virus RNA segment 8 coded for both NS_1 and NS_2 in studies with recombinant viruses in which NS_1 and NS_2 reassorted together, and, in addition, hybridization of segment 8 to total viral mRNAs specifically prevented the synthesis of both NS_1 and NS_2 *in vitro* (15, 17).

We describe here results obtained by using cloned DNA segments (NS DNA) of the NS gene (18) to map the two mRNAs on RNA segment 8 and to attempt to distinguish between translation of NS_2 in a second reading frame and NS_1 and NS_2 existing as contiguous genes within RNA segment 8.

MATERIALS AND METHODS

Virus and Cells. Influenza A/Udorn/72 (H_3N_2) and A/WSN/33 (H_0N_1) viruses grown in embryonated eggs were used; HeLa (human) cell lines were grown as described (14).

mRNA Extraction and Protein Synthesis *In Vitro*. These were as described (15).

Isotopic Labeling of Polypeptides in Infected Cells, Polyacrylamide Gel Electrophoresis, and Peptide Maps. These were all as described (14).

Preparation of Cloned NS DNA. Clone PFV 26/NS was constructed previously (18) by using pBR322 (19) and was produced by transformation of *Escherichia coli* K-12 strain HB101. The bacteria were grown in L broth with tetracycline (20 μg/ml), and the plasmid was amplified by using chloramphenicol. The plasmid DNA was isolated by the cleared lysate technique (20) followed by centrifugation in isopycnic CsCl/ethidium bromide gradients. The NS gene was released from the plasmid DNA by digestion with *Pst* I and purification on 4% polyacrylamide gels (acrylamide/bisacrylamide, 20:1) (18). These experiments were conducted under P1 containment as prescribed in the National Institutes of Health guidelines.

Restriction Enzyme Analysis. Uniformly ^{32}P-labeled NS gene DNA, electrophoretically eluted from polyacrylamide gels, was digested with restriction enzymes and the products were analyzed on 4% polyacrylamide gels. ^{32}P-Labeled simian virus 40 DNA segments obtained by digestion with *Hin*dII and *H*indIII were used as markers.

Analysis of mRNA by Nuclease S1 Digestion. The technique of Berk and Sharp (21), modified as described (22, 23), was used to map the template locations of the NS_1 and NS_2 mRNAs. Total influenza virus-infected HeLa cell poly(A)-containing mRNA was mixed with a 5- to 10-fold molar excess of various restriction endonuclease-cleaved [^{32}P]DNA probes from PFV 26/NS in 80% formamide, denatured at 68°C for 10 min, and annealed for 3 hr at 48°C. Nuclease S1 treatment and analysis by alkaline agarose gel electrophoresis were as described (22).

Hybrid-Arrested Translation. This was done by using the method of Paterson and co-workers (24). A molar excess of various restriction endonuclease fragments of PFV 26/NS DNA were hybridized to poly(A)-containing mRNA from influenza virus-infected HeLa cells in 80% (vol/vol) deionized and recrystallized formamide/0.4 M NaCl/0.01 M Pipes-HCl, pH 6.4/2 mM EDTA at 44°C (25) for 2 or 5 hr, with a total volume of 50 μl in Eppendorf 1.5-ml polypropylene test tubes. Then, 20 μg of wheat germ tRNA in 130 μl of H_2O was added fol-

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Abbreviation: NS DNA, cloned DNA derived from RNA segment 8.

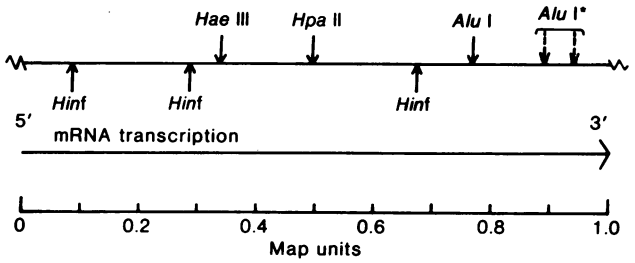

FIG. 1. Detailed cleavage map of NS DNA obtained with several endonucleases, and strand orientation with respect to mRNA transcription. The sites of cleavage by *Alu* I* to yield three fragments of 110, 70, and 56 base pairs located between 0.77 and 1.00 units have not been ordered.

lowed by 700 μl of ethanol and storage at −80°C for 2 hr. The hybrids were collected by centrifugation and resuspended in 200 μl of 0.1 M NaOAc at pH 5.5 and reprecipitated with ethanol at −20°C overnight. For translation in wheat germ extracts, hybrids were redissolved in 45 μl of H_2O, and 20 μl was used.

Chemicals and Isotopes. [^{35}S]Methionine was purchased from Amersham; nuclease S1 was from Worthington; and restriction enzymes were from New England BioLabs or Bethesda Research Laboratories (Rockville, MD).

RESULTS

Analysis of Restriction Enzyme Cleavage Sites. DNA sequences that code for the nonstructural proteins NS_1 and NS_2 (PFV 26/NS) were constructed from cDNA copies derived from virus RNA segment 8 and from its corresponding cytoplasmic mRNA. The constructed DNA duplexes were cloned in plasmid pBR322, and the NS-specific sequences contained ≈950 base pairs including ≈20–30 linker G/C sequences (18). The NS DNA was cleaved once at 0.34 map unit by *Hae* III and at 0.49 map unit by *Hpa* II, and these map positions were oriented with respect to the direction of mRNA transcription (18). In order to obtain more detailed information on the gene structure, other restriction enzyme cleavage sites were determined. *Hin*fI cleaved the *Hae* III B fragment into two segments of ≈190 and ≈80 base pairs and the *Hae* III A fragment into two segments of ≈325 and ≈305 base pairs. From a digestion of total cloned NS gene with *Hin*fI, the order of the segments could be deduced. Digestion of cloned NS gene with *Alu* I yielded a fragment of ≈730 base pairs, and the cleavage site was determined to be at 0.77 map unit. In addition, *Alu* I yielded three other fragments of ≈110, ≈70, and ≈56 base pairs whose order between 0.77 and 1.0 map units has not been determined. The detailed map of these restriction enzyme cleavages with respect to the direction of mRNA transcription is shown in Fig. 1.

Mapping the NS_2 and NS_1 mRNAs by Hybridization and by Nuclease S1 Digestion. The method of Berk and Sharp (21) was used to determine the size and genomic positions of the NS_1 and NS_2 mRNAs. Poly(A)-containing mRNA from HeLa cells infected with influenza virus was mixed with a 5- to 10-fold molar excess of U-[^{32}P]DNA of the total NS gene or segments produced by restriction enzyme digestion, denatured, and allowed to hybridize in 80% formamide at 48°C for 3 hr. Under these conditions the strands of the DNA probe were kept dissociated. The DNA·RNA hybrid molecules were digested with nuclease S1 and the digest was analyzed by alkaline agarose gel electrophoresis. As shown in Fig. 2, when the unit-length NS DNA fragment was hybridized to the mRNA preparation, two DNA bands could be identified after nuclease digestion (lane a). One band migrated at a position equivalent to single-stranded DNA of ≈860 nucleotides; the other band was

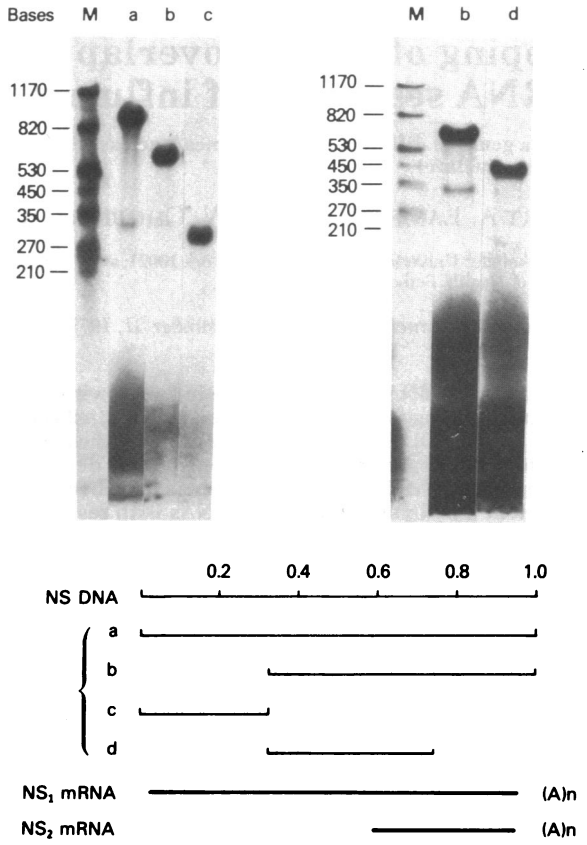

FIG. 2. Nuclease S1 analysis of the NS_1 and NS_2 mRNAs. Poly(A)-containing mRNAs from influenza virus-infected HeLa cells were denatured and allowed to hybridize in 80% formamide at 48°C for 3 hr with ^{32}P-labeled NS DNA cleaved with restriction endonucleases, and the hybridization mixtures were treated with nuclease S1. The digests were analyzed by electrophoresis on 1.4% alkaline agarose gels to determine the size of the nuclease S1-resistant DNA segments. Lane M: Marker simian virus 40 DNA digested with *Hin*dII and *Hin*dIII. Lane a: mRNAs hybridized with total NS DNA. Lane b: mRNAs hybridized with the *Hae* III A fragment of NS DNA (0.34–1.00 units). Lane c: mRNAs hybridized with the *Hae* III B fragment of NS DNA (0–0.34 units). Lane d: mRNAs hybridized with the *Hae* III/*Alu* I fragment of NS DNA (0.34–0.77 units). The diagram below shows the positions of the DNA fragments used in hybridization and the mapping of the NS_1 and NS_2 mRNAs on the cloned NS DNA.

equivalent to ≈340 nucleotides. Thus, two NS-specific mRNA species were present in the infected cells.

These findings are consistent with the idea that the large mRNA codes for NS_1 and the small mRNA codes for NS_2, because the molecular size and relative abundance of these cDNA probes correlated closely with those expected for two mRNAs for the two polypeptide products. Experiments in which the *Hae* III A fragment (0.34–1.0 units) was used as a probe produced a band of ≈615 nucleotides, corresponding to the reduced large mRNA, and another band of ≈340 nucleotides, corresponding to the intact small mRNA (lane b in both panels). On the other hand, similar experiments using the *Hae* III B probe (0–0.34 units) produced a single band of ≈300 nucleotides corresponding to the 5′ end of the NS_1 mRNA, and no other discrete DNA segment was observed. To locate the map positions of the NS_2 mRNA precisely, mRNA/DNA hybridization was carried out using the *Hae* III A/*Alu* I fragment (0.34–0.77 units). A DNA band of ≈410 nucleotides and another band of ≈160 nucleotides were detected (Fig. 2, lane d). The large DNA segment was derived from protection by the NS_1 mRNA, and the small segment was derived from protection by the NS_2 mRNA. From these results it can be deduced that the map position of the NS_1 mRNA is located between 0.05 and 0.95

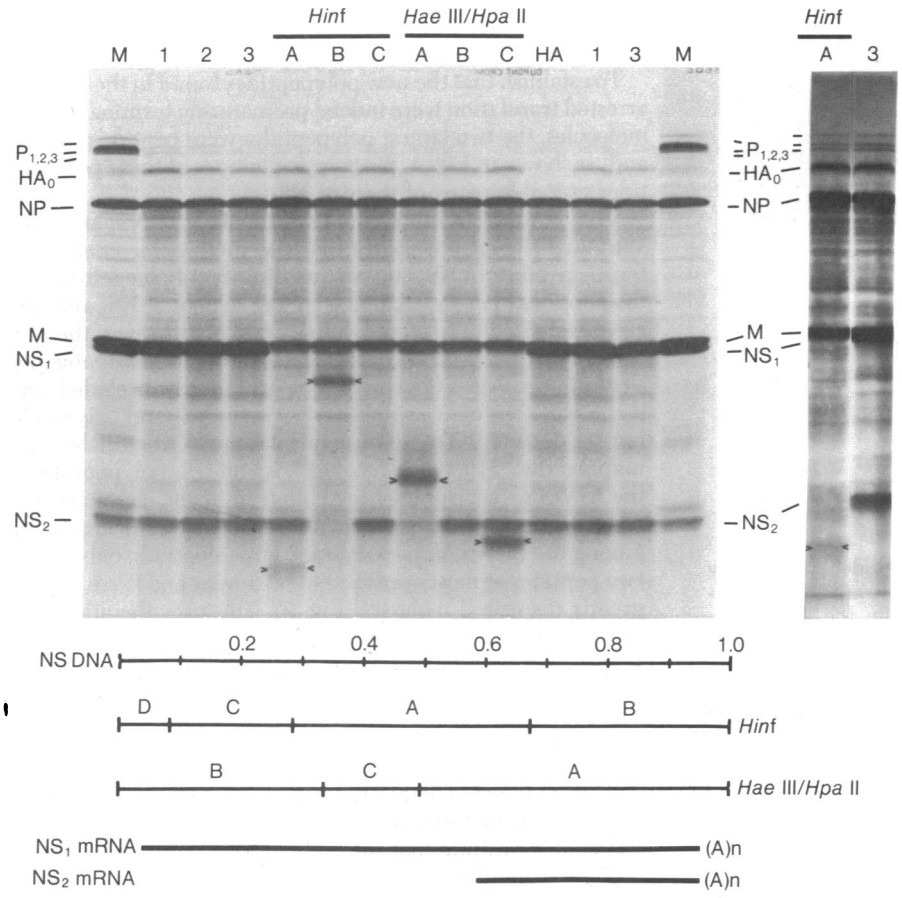

FIG. 3. Hybrid-arrested translation of the NS_1 and NS_2 mRNAs with NS DNA restriction endonuclease fragments. Poly(A)-containing mRNAs from influenza virus-infected HeLa cells were hybridized with fragments of NS DNA obtained with different restriction endonucleases. Both mRNAs and DNA were first melted at 100°C for 1 min in 5 μl of H_2O, quenched at −60°C, and hybridized in 80% formamide and buffer at 44°C for 2 hr. The hybrid molecules were freed of formamide by ethanol precipitation and translated in wheat germ extracts. Samples were analyzed by electrophoresis on a 17.5% polyacrylamide/4 M urea gel (14), and the gel was subjected to autoradiography. (*Left*) Lanes M: Influenza virus marker polypeptides synthesized in influenza virus-infected HeLa cells mixed with a wheat germ extract that had been incubated with no exogenous RNA. The dark band comigrating with the three P polypeptides is glycosylated HA synthesized *in vivo* which migrates more slowly than HA_0 synthesized *in vitro*. Lanes 1: mRNA in H_2O frozen at −20°C for 2 hr and then treated for translation as described in *Materials and Methods*. Lane 2: mRNA incubated at 44°C for 2 hr in buffer with H_2O instead of formamide. Lanes 3: mRNA incubated at 44°C for 2 hr in buffer and 80% formamide. Lanes *Hinf* A, B, and C: hybridization under conditions given for 3 but with *Hinf*I A, B, or C fragments added. Lanes *Hae* III/*Hpa* II A, B, and C: hybridizations as above but with *Hae* III/*Hpa* II A, B, or C fragments added. Lane HA: hybridization as above but with cloned DNA of the hemagglutinin (HA) gene (PFV 88/HA) added. (*Right*) Lane *Hinf* A: mRNA and *Hinf*I A fragment hybridized at 44°C for 5 hr in buffer and 80% formamide. Lane 3: mRNA incubated at 44°C for 5 hr in buffer and 80% formamide without DNA. A schematic representation of the fragments used is shown below.

units and the NS_2 mRNA is between 0.59 and 0.95 units, and that the two mRNAs have the same 3′ terminus to which poly(A) is added.

Hybrid-Arrested Translation of NS_1 and NS_2 mRNAs with Restriction Fragments of NS DNA. To confirm the results of the mapping of the NS_1 and NS_2 mRNAs obtained by the nuclease S1 technique and to obtain information concerning the extent of translation of the NS_1 mRNA, we used the hybrid-arrested translation technique of Paterson and coworkers (24). The cloned NS gene DNA was digested with restriction enzymes and the resulting fragments were purified by gel electrophoresis. Total poly(A)-containing mRNA from infected cells was hybridized with a 10- to 20-fold molar excess of the NS DNA fragments in 80% formamide at 44°C for 2 hr. After the hybrid molecules were free of formamide, the mRNAs were translated *in vitro* by using wheat germ extracts. Hybridization of a restriction fragment to a mRNA would be expected to stop translation proceeding downstream from the 5′ terminus of the mRNA at the region of the boundary of the hybrid. The *Hinf* A fragment (0.28–0.67 units) prevented the synthesis of full-length NS_1 but yielded a shortened NS_1 polypeptide (M_r, ≈8900), and NS_2 was synthesized normally (Fig. 3). This indicates that when the DNA fragment hybridized to the NS_1 the mRNA translation was stopped prematurely. Fragment *Hinf* B (0.67–1.0 units) inhibited the synthesis of NS_2 and yielded a short NS_1 (M_r, ≈23,400). Fragment *Hinf* C stopped the synthesis of NS_1, and NS_2 was synthesized normally. The *Hae* III/*Hpa* II A fragment (0.49–1.0 units) almost completely prevented the synthesis of NS_2 and yielded a short NS_1 (M_r, ≈15,700). Fragment *Hae* III/*Hpa* II B (0–0.34 units) prevented the synthesis of NS_1, and NS_2 synthesis occurred normally. Fragment *Hae* III/*Hpa* II C (0.34–0.49 units) allowed the normal synthesis of NS_2 and yielded a short NS_1 (M_r, ≈10,200). The previous results from nuclease S1 mapping indicated that the NS_2 mRNA extended ≈80 nucleotides into the *Hinf* A fragment (0.28–0.67 units), yet under the conditions used this fragment did not inhibit the synthesis of NS_2.

Table 1. Comparison of sizes of prematurely terminated polypeptides with sizes of restriction fragments suggests that the carboxyl terminus of NS_1 (M_r, ≈25,000) is at map unit ≈0.76

Restriction sites, map units*	Estimated Bases from 1st AUG† of mRNA	Polypeptide size	
		Predicted‡	Actual§
0.28 (*Hinf*I C–A)	220	8,200	8,900
0.34 (*Hae* III B–C)	270	10,100	10,200
0.49 (*Hpa* II C–A)	420	15,700	14,100
0.67 (*Hinf*I A–B)	590	22,000	23,400

* The cloned NS gene is 950 base pairs, including ≈20–30 dG/dC residues added at each end. Map units were determined from sizes of restriction enzyme fragments.

† Calculated from the restriction enzyme sites, with subtraction of 20 bases for terminal linker residues and 30 bases to the first AUG in mRNA (26).

‡ Calculated from the number of bases in column 2, and on the basis that three bases code for 112 daltons of protein.

§ Calculated from the products of hybrid-arrested translation experiments using polypeptide markers of known size.

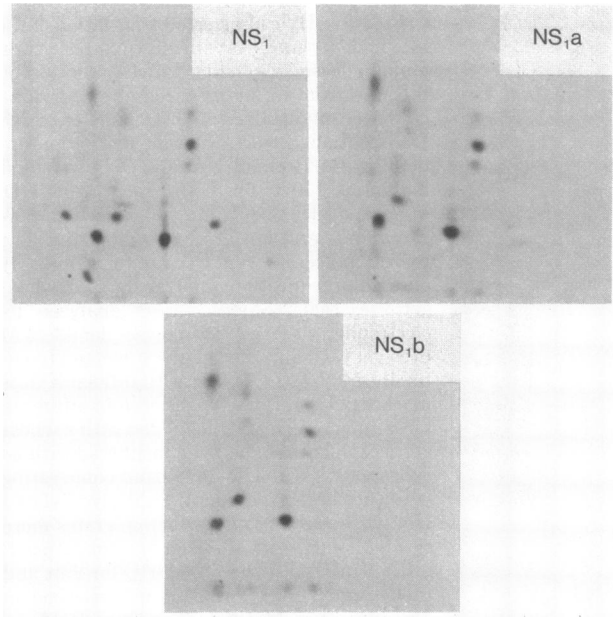

FIG. 4. Peptide mapping of the NS_1 polypeptides prematurely terminated by hybridization of DNA fragments to mRNA. The following polypeptides were digested with trypsin: the intact NS_1 polypeptide synthesized *in vitro* in wheat germ extracts; the M_r, ≈23,000 polypeptide synthesized *in vitro* in wheat germ extracts after hybridization of mRNA with the *Hin*fI B fragment (designated NS_1a); and the M_r, ≈14,000 polypeptide synthesized *in vitro* after hybridization with the *Hae* III/*Hpa* II A fragment (designated NS_1b). Peptide maps were obtained as described (14) using 15,000 cpm of [^{35}S]methionine-labeled tryptic peptides.

When the hybridization conditions were made more stringent by incubation at 44°C for 5 hr, the *Hin*f A fragment inhibited the synthesis of NS_2 and still yielded the short NS_1 (M_r, ≈8200), as shown in Fig. 4 *Right*. A control used in these experiments was the cloned DNA of the hemagglutinin (HA) gene (PFV 88/HA) (18), which inhibited the synthesis of HA_0. Thus, these hybrid-arrested translation results confirm the nuclease S1 data indicating that the mRNA for the NS_2 polypeptide maps between 0.59 and 0.95 units. In addition, the size estimates of the short NS_1 polypeptides and the location of the restriction enzyme cleavage sites suggest that the location of the carboxyl terminus of NS_1 is at ≈0.76 map unit (Table 1). These calculations indicate that the translated regions of the NS_1 and NS_2 mRNAs overlap by ≈45% of the NS_2 polypeptide and ≈25% of the NS_1 polypeptide—i.e., an overlap of translated nucleotides equivalent to 48–53 amino acids (M_r, 5500–6000).

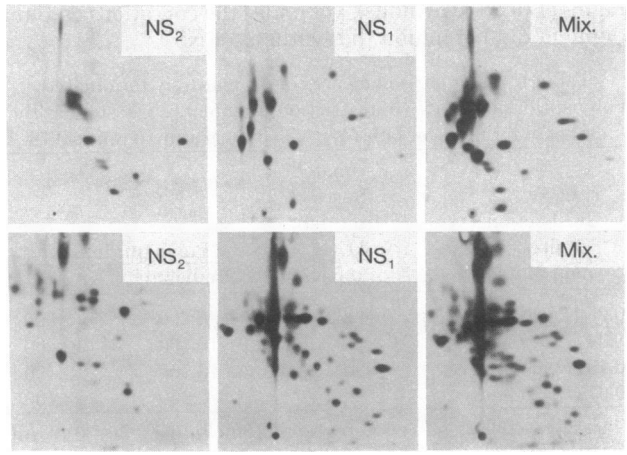

FIG. 5. Peptide maps of [^{3}H]leucine *in vivo*-labeled NS_1 and NS_2 polypeptides labeled with [^{3}H]leucine *in vivo* and digested with trypsin and chymotrypsin. Infected HeLa cells were labeled with [^{3}H]leucine and peptide maps were obtained as described (14). Top row: Tryptic peptide maps of NS_2, NS_1, and a mixture of the two (100,000 cpm of NS_1 and 50,000 cpm of NS_2 were used). Bottom row: Chymotryptic peptide maps of NS_2, NS_1, and a mixture of the two. The same amounts of NS_1 and NS_2 were used as above.

To establish that the new polypeptides found in the hybrid-arrested translation were indeed prematurely terminated NS_1 molecules, the two largest polypeptides were peptide mapped and, as shown in Fig. 4, the tryptic peptides of these polypeptides resemble many of those of NS_1.

Tryptic and Chymotryptic Peptide Maps of NS_1 and NS_2. We have shown previously that the methionine-containing tryptic peptides of NS_1 and NS_2 are distinct (14), suggesting that the peptides in the overlapping coding regions of NS_1 and NS_2 are translated in different reading frames. However, the possibility that NS_1 and NS_2 contained neither a methionine nor a trypsin cleavage site in this region could not be excluded, and if this were the case a similarity between NS_1 and NS_2 would not be detected. To attempt to investigate this further, we mapped the [^{3}H]leucine-labeled NS_1 and NS_2 polypeptides of the A/WSN/33 virus by using both trypsin and chymotrypsin. The tryptic peptides seemed to be completely distinct and, among the more complex chymotryptic peptides, only one or two peptides at most appeared to be similar and these were in greatly decreased molarity (Fig. 5). This provides additional suggestive evidence that NS_1 and NS_2 do not share a common sequence of 48–53 amino acids, and thus are synthesized from different reading frames.

DISCUSSION

The data presented in this paper, obtained by using the nuclease S1 mapping method and cloned NS gene DNA (≈950 base pairs), demonstrate that the body of the NS_1 mRNA contains ≈860 nucleotides and the body of the NS_2 mRNA contains ≈340 nucleotides. At the hybridization temperature used, the double-stranded DNA probe was kept dissociated and only the body of mRNA was protected. [Heterogeneous cellular sequences of ≈10–15 nucleotides added at the 5′ end of the mRNA and poly(A) sequences at the 3′ end would not be expected to hybridize with the cloned DNA probe.] The NS_1 mRNA has been found (27–29) to be an incomplete transcript of the genome segment 8 in that transcription of the mRNA terminates ≈30 nucleotides before the 5′ end of the virion DNA. The data obtained here are consistent with these observations and indicate that the NS_1 mRNA maps 0.05–0.95 units of the cloned NS gene. The location of the NS_2 mRNA was obtained by using restriction endonuclease-cleaved fragments of the NS gene with the nuclease S1 mapping technique and was found to lie between 0.59 and 0.95 units on the cloned NS gene. This suggests that the NS_1 and NS_2 mRNAs are 3′ coterminal, sharing the same poly(A) addition site. This would be analogous to the common poly(A) addition sites for the multiple mRNAs in adenovirus late mRNAs and the poly(A) addition sites for early and late simian virus 40 mRNAs (21, 22, 30–32). Because the hybridizations were done in molar excess of DNA, the relative amounts of the NS_1 and NS_2 mRNAs are represented on the gels, and it can be seen that the NS_1 mRNA is much more abundant than the NS_2 mRNA (Fig. 2).

The hybrid-arrested translation experiments using fragments of the cloned NS DNA to inhibit the *in vitro* synthesis of NS_1 or NS_2 polypeptides provided confirmation of the results obtained by nuclease S1 mapping and are consistent with the NS_2 mRNA extending from 0.59 to 0.95 map units. When a restriction endonuclease fragment is hybridized to a mRNA downstream from the site of initiation of protein synthesis, translation of a mRNA stops in the region of the junction of the hybrid. With restriction fragments of the NS gene and hy-

bridization to NS_1 mRNA, premature termination products of NS_1 were produced (see Fig. 3). By using the size of the premature termination products, calculated from gels, and the location of the restriction endonuclease sites, it can be estimated that termination of translation of NS_1 (M_r, ≈25,000) is at ≈0.76 map unit. If the M_r of NS_2 (≈11,000 as calculated from gels) is not aberrant, and because its mRNA contains ≈340 nucleotides, nearly all the NS_2 mRNA must be used for translation. Therefore, the maximal overlap that can be predicted is 25% of the codons used to translate NS_1 and 45% of the codons used to translate NS_2—i.e., polypeptide of M_r 5500–6000. Because the methionine- and leucine-labeled tryptic peptides of NS_1 and NS_2 do not contain any similar peptides and the leucine-labeled chymotryptic peptides of NS_1 and NS_2 contain at most one or two similar peptides, which may be fortuitous, it would appear that these polypeptides are completely distinct. However, because we do not know the size of the individual peptides, the distribution of methionine and leucine, or the location of the trypsin and chymotrypsin sites, it cannot be totally excluded that NS_1 and NS_2 do share any peptide sequences. If they do not, as the data strongly indicate, then the translation of the NS_2 mRNA must be in a reading frame different from that used for NS_1.

Further investigation will be needed to demonstrate how the mRNA for NS_2 arises. Its synthesis is late in infection, requiring early protein synthesis (14), and this could occur by RNA processing of the NS_1 mRNA to produce NS_2 mRNA or, alternatively, a second site for the transcription of NS_2 might be revealed on the virion RNA segment 8 by an alteration of the RNA by protein arising from early protein synthesis—e.g., removal of secondary structure. It would also be interesting to know the nucleotide structure at the 5′ end of the NS_2 mRNA and to compare it with that of the mRNAs made early in infection. Both *in vivo* and *in vitro*, the cap structure and several nucleotides are transferred from cellular mRNAs to the eight virion mRNAs made early in infection, and this mediates transcription from the 3′ end of the virion RNA (33, 34). Nucleotide sequencing of the 5′ ends of cloned DNAs which contain the 5′ ends of the *in vivo* mRNAs has shown that different cellular sequences are used (35). In addition, it would be interesting to investigate further whether any part of the 5′ end of the NS_2 mRNA is the same as that of the NS_1 mRNA. With the simian virus 40 early and late mRNAs the 5′-terminal leader sequences of the mRNAs are quite large (18, 21), but the adenovirus leader sequences of the late mRNAs are small (36). If a region of the NS_2 mRNA were the same as that of the NS_1 mRNA, it would presumably be less than 50 nucleotides, because a band larger than this would have been observed in the nuclease S1 analysis of the mRNAs (Fig. 3, lane C). In addition, in the hybrid-arrested translation experiments, the *Hae* III B fragments (0–0.34 units) did not inhibit the synthesis of NS_2 under stringent conditions in which a hybrid of ≈80 nucleotides using the *Hin*f A fragment (0.28–0.67 units) inhibited the translation of NS_2. These results suggest that the mRNAs do not share 5′ regions, but a small common region has not been excluded.

Determination of the nucleotide sequence of the NS gene should aid in elucidating the encoded polypeptide structure. However, the functional role of the NS_1 and NS_2 polypeptides in viral replication is unknown, apart from the observations that NS_1 accumulates in the nucleus (7, 37) and is also associated with polyribosomes (38) whereas NS_2 is found predominantly in the cytoplasm (16, 39). Furthermore, the viral components of enzyme activities that may be involved in influenza virus RNA replication have yet to be described in detail, and NS_1 or NS_2 or both could be components of this system.

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