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The smallest genome RNA segment of influenza virus contains two genes that may overlap

(fowl plague virus/cell-free translation/peptide mapping/RNA·RNA hybridization)

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ABSTRACT **The genome of influenza virus consists of eight segments of single-stranded RNA, each of which encodes a different polypeptide. In addition to the eight recognized gene products, the virus specifies a distinct smaller nonstructural polypeptide (NS_2), which is translated from a separate species of virus-specific mRNA. The location on the virus genome of the gene encoding this polypeptide was investigated by hybridization of the NS_2 mRNA with isolated subgenomic RNA species, and by correlation of the inheritance of a strain-specific NS_2 with inheritance of particular genome RNA segments during recombination between two different virus strains. The genetic information for NS_2 was found to reside in the smallest genome RNA segment of the virion, which also encodes the NS_1 polypeptide. Considering the sizes of the molecules involved, it is likely that the coding sequences for the two polypeptides overlap.**

The genetic material of influenza A virus consists of single-stranded RNA, of opposite sense to mRNA, which exists as eight separate molecular species (1–3). A separate polypeptide-coding function has been ascribed to each RNA segment (4–7), suggesting that the subgenomic fragments represent single genes; the eight recognized gene products include seven virion structural polypeptides [M (matrix), NA (neuraminidase), NP (nucleoprotein), HA (hemagglutinin), and P_1, P_2, and P_3 (polymerase-associated proteins)] which have molecular weights between 25,000 and 95,000, and a nonstructural polypeptide (NS_1) of molecular weight about 23,000 (8, 9). However, it is clear that the synthesis of an additional polypeptide, of molecular weight 11,000, is induced in influenza virus-infected cells (10–13). This polypeptide has a tryptic peptide map that is unrelated to maps of the other virus polypeptides (10, 14) and is translated from a separate species of mRNA in the infected cell (14, 15). These observations suggested that the polypeptide, which has been described as a possible second nonstructural polypeptide (NS_2), is a separate virus gene product. Our recent studies using the avian influenza fowl plague virus (FPV) have confirmed these results and have shown directly that the information for the NS_2 polypeptide is provided by the virus genome (ref. 16; unpublished results). We therefore concluded that NS_2 is encoded by a virion RNA (vRNA) segment that also specifies another polypeptide.

We show here that the smallest vRNA (segment 8) fulfils this role. Because the size of this RNA appears to be insufficient to code for both polypeptides independently, it seems likely that the NS_1 and NS_2 genes overlap. Furthermore, if, as the peptide maps suggest, the NS_2 and NS_1 polypeptides are completely unrelated, the genetic code of the shared nucleotide sequences would have to be read in two different frames.

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METHODS

Cells and Virus. Primary cultures of chicken embryo fibroblasts (CEF) and stocks of FPV were prepared as described (17). Procedures for infection of cells and purification of virus are detailed elsewhere (8).

Extraction and Fractionation of RNA. Cytoplasmic RNA was prepared from FPV-infected CEF (6) and fractionated by oligo(dT)-cellulose chromatography as described (18). RNA was separated according to size on linear gradients containing 5–20% sucrose in LETS buffer (100 mM LiCl/10 mM Tris·HCl, pH 7.6/4 mM EDTA/0.1% sodium dodecyl sulfate). Gradients were centrifuged at 130,000 × *g* in an IEC SB283 rotor at 4°C and fractionated on an ISCO automatic fractionator. RNA was recovered from gradient fractions by ethanol precipitation in the presence of 20 μg of carrier tRNA. Unlabeled vRNA and individual genome segments were prepared as before (6). The procedure for isolation of separate ^{125}I-labeled vRNA segments was as described (19).

Cell-Free Protein Synthesis. The procedures for preparation of wheat germ extracts, the conditions for cell-free translation, and the preparation of samples for polyacrylamide gel electrophoresis were as described (6).

RNA·RNA Hybridization. Pre-annealing of mRNA with unlabeled vRNA prior to cell-free translation was carried out in 63% dimethyl sulfoxide as described (6). Hybridization of mRNA with ^{125}I-labeled vRNA segments was performed in 2 times concentrated standard saline citrate for 18 hr at 68°C. The extent of double-strand formation was analyzed by treatment with RNases A and T1 (20).

Polyacrylamide Gel Electrophoresis and Tryptic Peptide Mapping. [^{35}S]Methionine-labeled polypeptides were separated by discontinuous polyacrylamide gel electrophoresis and detected by autoradiography (8). Two-dimensional tryptic peptide maps of ^{35}S-labeled polypeptides were prepared by elution of polypeptides directly from gels in the presence of trypsin (8).

RESULTS

Both the NS_1 and NS_2 mRNAs specifically hybridize to vRNA segment 8

We previously developed a method for identification of the protein-coding functions of individual segments of the genome by cell-free translation (6). mRNA, prepared from infected cells, is annealed in the presence or absence of a purified subgenomic RNA species. The annealed RNAs are precipitated from the reaction and added directly to the cell-free system.

Abbreviations: M, matrix protein; NP, nucleoprotein; HA, hemagglutinin; P_{1-3}, polymerase-associated proteins; vRNA, virion RNA; NS, nonstructural; FPV, fowl plague virus; CEF, chicken embryo fibroblasts.

mRNAs that are complementary to the genome segment used are converted into a double-stranded form and so are no longer active; synthesis of the polypeptide encoded by that segment is therefore specifically decreased.

Fig. 1 shows the results of such an experiment. Poly(A)-containing RNA was extracted from infected cells and annealed with increasing amounts of purified genome segment 7 or segment 8. Annealed RNAs were subsequently added to the cell-free system, and the products were analyzed by gel electrophoresis. Virus-specific polypeptides were identified among the cell-free products by tryptic peptide mapping (ref. 6; unpublished results). Pre-annealing with genome segment 8 (lanes 2–5) affected the cell-free translation products in two ways. First, increased amounts of vRNA in the annealing mixture resulted in an overall, nonspecific reduction in protein synthesis.

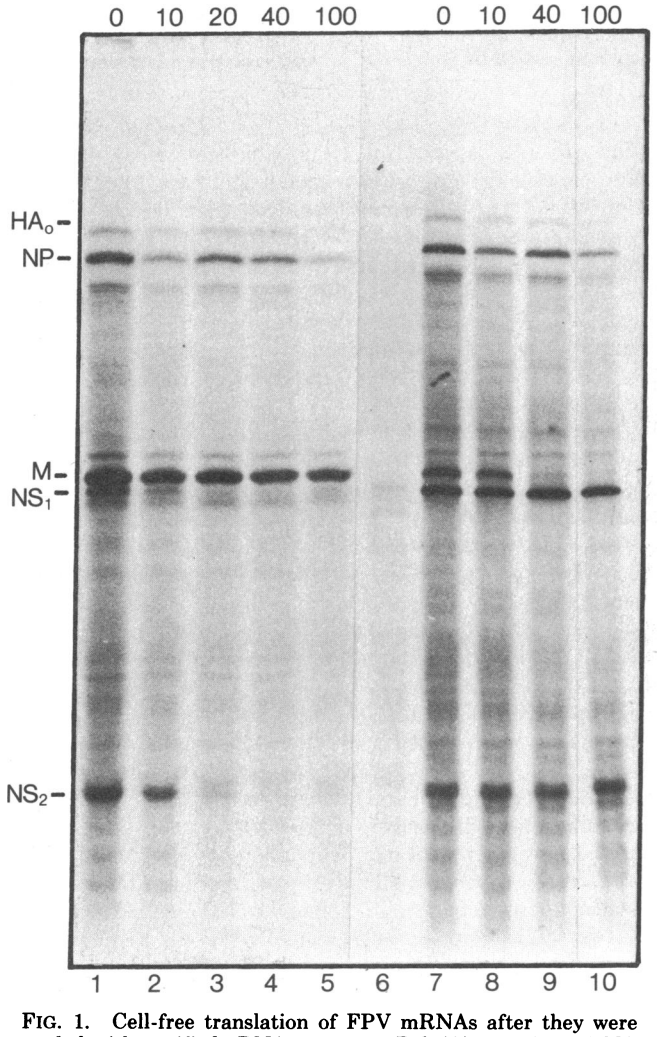

FIG. 1. Cell-free translation of FPV mRNAs after they were annealed with purified vRNA segments. Poly(A)-containing RNA was prepared from the cytoplasm of FPV-infected CEF at 4.5 hr postinfection, and 1-μg samples were hybridized with or without vRNA for 24 hr at 37°C in 63% dimethyl sulfoxide (6). RNAs were precipitated, dissolved in 10 μl of H_2O, and added to the wheat germ cell-free protein synthesizing system at a 1:5 dilution. Translation products were labeled with [^{35}S]methionine, separated on a 17.5% polyacrylamide gel, and detected by autoradiography. Lanes 1–5: mRNA was hybridized in the presence or absence of purified segment 8 RNA; the vRNA was present in the amounts (in nanograms) indicated above each lane. Lanes 7–10: a different mRNA preparation was hybridized with and without purified segment 7 RNA; the amount of vRNA (in nanograms) used is shown above each lane. Lane 6: cell-free products in the absence of added RNA.

This was also observed by using genome segment 7 (lanes 7–10) although to a lesser degree. The most likely explanation for this general inhibition is that the purified vRNA preparations contained residual impurities from the gel extraction procedure, which interfered with translation. The second effect of pre-annealing with segment 8 was a specific decrease in synthesis of NS_1 and also of NS_2 relative to the other virus polypeptides. The inhibition of NS_2 synthesis was not noted before (6), because the identity of the polypeptide was not then recognized. The decrease did not occur when segment 7 was used. Instead, synthesis of the M polypeptide was specifically inhibited (see ref. 6). Pre-annealing of mRNA with the other vRNA segments also had no specific effect on synthesis of NS_2 (data not shown). This experiment strongly suggests that the mRNA encoding NS_2 is complementary to genome segment 8.

We tested this possibility further by hybridization studies with ^{125}I-labeled genome segments. We fractionated polyadenylylated RNA from FPV-infected CEF according to size by sucrose gradient sedimentation, using conditions that allowed clear separation of the NS_1 and NS_2 mRNAs (unpublished results). RNA was recovered from each fraction of the relevant part of the gradient and dissolved in 50 μl of water, and a sample was added to the cell-free system (Fig. 2 *upper*). It is clear that the peak of mRNA activity for the NS_1 and NS_2 polypeptides occurs in fractions 15 and 23, respectively. Additional samples of the gradient fractions were analyzed by hybridization. Initially, a constant amount of ^{125}I-labeled segment 8 RNA was hybridized with increasing amounts of RNA from fraction 15 or fraction 23 (Fig. 2 *lower*, left panel). Approximately 75–80% of the radioactivity could be converted into a nuclease-resistant form by hybridization with RNA from fraction 23 or by hybridization with a mixture of RNA from fractions 23 and 15. This figure was considered rather low, because it appears that the NS_1 mRNA is complementary to almost the whole length of segment 8 (21, 22). However, 10% of the labeled preparation would not hybridize with unfractionated RNA from infected cells (which should contain sequences complementary to the entire genome), and therefore is probably not virus-specific. The remaining discrepancy may be due to the presence in the radioactive probe of contaminating sequences derived from other vRNAs. When the radioactive preparation was hybridized with RNA from fraction 15, only 50–55% protection was achieved. This suggests that fraction 15 contains sequences complementary to a smaller proportion of segment 8 than does fraction 23.

We next analyzed the distribution, across the gradient, of RNA complementary to segment 8 by hybridization of a constant amount of the radioactive segment with equivalent proportions of each gradient fraction (Fig. 2 *lower*, middle panel). A proportion was chosen so that no fraction could protect more than 65% of the added radioactivity from nuclease digestion. Although this experimental approach does not necessarily provide accurate measurements of the relative concentrations of complementary sequences in each fraction, it should give a broad indication of the sequence distribution throughout the gradient. As expected, sequences complementary to segment 8 were most abundant around fraction 23, the fraction containing the greatest proportion of NS_1 mRNA. However, an additional peak of hybridization was observed (fractions 14–17), which coincided exactly with the area of the gradient containing the NS_2 mRNA (see Fig. 2 *upper*). This secondary peak, although small and poorly resolved from the main peak, was nonetheless reproducible in different experiments. When the same experiment was performed by using ^{125}I-labeled segment 7 RNA, the second peak was not evident (Fig. 2 *lower*, right panel). These results provide further evidence that the NS_2

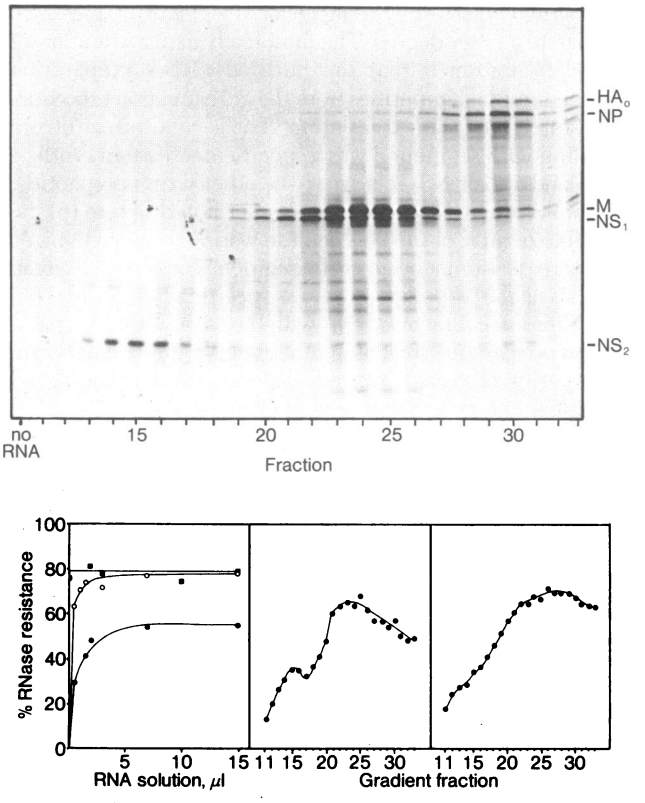

FIG. 2. Analysis of gradient-fractionated FPV-infected cell RNA by cell-free translation and hybridization with ^{125}I-labeled vRNA. (*Upper*) Poly(A)-containing RNA (50 μg) from infected CEF, was sedimented through a 12 ml sucrose density gradient. Fractions (0.25 ml) were collected, and the RNA from each was recovered by ethanol precipitation. RNAs were dissolved in 50 μl of H_2O, and samples were added to the wheat germ cell-free translation system at a 1:10 dilution. Cell-free products were labeled with [^{35}S]methionine, separated on a 17.5% gel, and detected by autoradiography. Only the translation products of RNAs from the area of the gradient containing virus mRNAs are shown. The fraction from which each RNA was obtained is indicated under each track. (*Lower*) RNA from the gradient fractions prepared in *Upper* was annealed in 100-μl vols with isolated ^{125}I-labeled vRNA segments (specific activity 5×10^6 cpm μg^{-1}), and the formation of labeled double-stranded material was monitored by treatment with RNase. Left panel: saturation hybridization with equal amounts (approx. 10,000 cpm) of segment 8 RNA. Annealing reactions contained increasing amounts of RNA from fraction 15 (●), increasing amounts of RNA from fraction 23 (○), or 7 μl of RNA from fraction 23 plus increasing amounts of RNA from fraction 15 (■). Middle panel: hybridization of 2-μl samples from each gradient fraction shown in *Upper* with equal amounts (approx. 10,000 cpm) of purified segment 8 RNA. Right panel: exactly as for middle panel except with purified segment 7 RNA.

mRNA as well as the NS_1 mRNA is complementary to genome segment 8.

NS_2 polypeptides of different strains of FPV segregate with genome segment 8 during recombination

Previous work in this laboratory led to the isolation and genetic characterization of a series of viruses produced by recombination between the Rostock and Dobson strains of FPV; during a mixed infection, reassortment of the genome RNAs of the parent viruses promotes formation of progeny bearing different combinations of vRNA segments. The parental origin of the genome RNAs of a number of these recombinants has been established (refs. 23 and 24; Table 1). We have recently shown that the Rostock and Dobson strains of FPV specify slightly different NS_2 polypeptides, which may be distinguished by tryptic peptide mapping (ref. 16; unpublished results). We were therefore able to determine for each recombinant the parental origin of its NS_2. We reasoned that if NS_2 were indeed encoded by genome segment 8, then a recombinant that contained the Dobson-specific RNA segment 8 would automatically specify the Dobson-specific NS_2 polypeptide, and of course the reverse would also be true. Peptide maps were therefore prepared from the NS_2 polypeptide synthesized in CEF after infection with the parent viruses and with a number of different recombinants.

Table 1. Parental origin of NS_2 polypeptides specified by recombinants between the Rostock and Dobson strains of FPV

Recombinant	Parental origin of vRNA segment encoding*								Parental origin of NS_2
	P_1	P_2	P_3	HA	NP	NA	M	NS_1	
Di47-2	R	R	R	R	R	R	R	D	D
Di47c-1	R	R	R	R	R	R	R	D	D
Di34-3	R	R	R	R	D	R	R	R	R
Di44-2	R	D	R	R	D	R	R	D	D
Di44-4	R	D	R	D	D	R	D	R	R
Di44-7	R	D	R	R	D	D	D	D	D
DimN5c-4	D	D	R	D	D	R	?	D	D
Dd45-g	D	R	D	D	R	?	?	R	R

* P polypeptide nomenclature is based on the order of gel migration of the polypeptides in the Rostock (R) strain (P_1 is the largest, etc.). It should be noted that P_2 and P_3 of Rostock are functionally equivalent to P_3 and P_2, respectively, of Dobson (D) (8). Therefore, where Dobson P_3 replaces Rostock P_2 in a recombinant, D is included under the column heading P_2. Likewise, a D is shown in the column headed by P_3 when Rostock P_3 is replaced by Dobson P_2.

The results of this experiment are shown in Fig. 3 and Table 1. The tryptic peptide map of the Rostock-specific NS_2 (Fig. 3*a*) is very similar to that of the Dobson NS_2 (Fig. 3*b*), but contains two extra peptides (arrowed). This difference was observed in several different experiments and was confirmed by analysis of mixed samples (data not shown). To ensure that these extra spots were genuinely associated with NS_2, and were not derived from fragments of other virus polypeptides, we prepared a peptide map from the NS_2 polypeptide synthesized by the wheat germ cell-free system in response to purified NS_2 mRNA from cells infected with the Rostock strain (Fig. 3*c*). The NS_2 mRNA was isolated by gradient sedimentation as before (Fig. 2 *upper*). The relative amounts of the two Rostock-specific peptides were more or less unchanged in this map; the possibility that the peptides were not derived from NS_2 is therefore remote. Several of the minor peptides observed in the NS_2 maps prepared by *in vivo* labeling were greatly diminished in their relative intensity in the map prepared from the *in vitro* product, and therefore may not be associated with NS_2. Fig. 3*d* shows a map of the NS_2 polypeptide specified by a recombinant that derives all its vRNA segments, with the exception of segment 8, from the Rostock parent virus (see Table 1). The map does not contain the Rostock-specific peptides. The NS_2 map of a second recombinant whose vRNA segments are mostly derived from the Dobson parent, but which retains the Rostock-specific segment 8, is shown in Fig. 3*e*; the Rostock-specific peptides are clearly evident. The Rostock peptides are also visible in Fig. 3*f*, which shows the map of NS_2 specified by another recombinant with the Rostock-specific segment 8. Five further recombinants were analyzed in this way, and the results are summarized in Table 1. The presence of the Rostock- or Dobson-specific NS_2 polypeptide correlated perfectly with the presence of the corresponding parental segment 8. This correlation was not apparent for any other genome segment. These

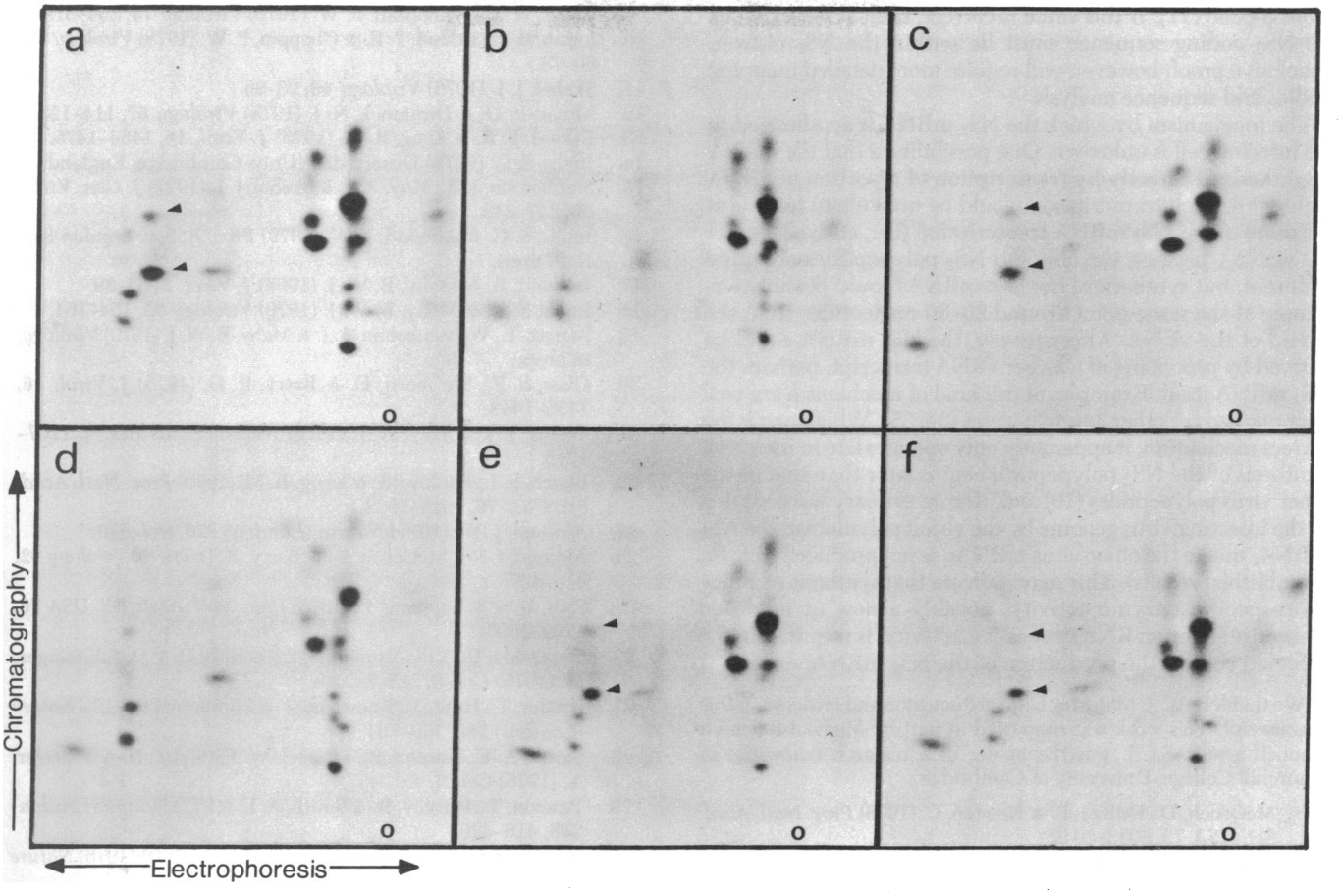

FIG. 3. Two-dimensional tryptic peptide maps of the NS_2 polypeptide synthesized in FPV-infected CEF (*a, b, d, e, f*) and in the wheat germ cell-free system (*c*). Cells were infected with either the Rostock (*a*) or Dobson (*b*) parent strains, or recombinants Di47c-1 (*d*), Dd45-g (*e*), and D34-3 (*f*), and polypeptides were separated by gel electrophoresis after being labeled with [^{35}S]methionine (100 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) from 2–6 hr postinfection. The cell-free incubation contained, in a final volume of 200 μl, 10 μl of a mixture of gradient fractions 14 and 15 (see Fig. 2 *Upper*); translation products were labeled with [^{35}S]methionine (1 mCi/ml) and separated by gel electrophoresis. Maps were prepared from polypeptides excised directly from gels (9). Peptides were separated on thin-layer plates by electrophoresis at pH 6.5 followed by ascending chromatography, and were detected by autoradiography. Peptides were applied at the spot marked **o**.

data provide confirmation that the information for the NS_2 polypeptide resides in genome segment 8.

DISCUSSION

Our experiments indicate that the NS_1 and NS_2 polypeptides of the avian influenza FPV are translated from two separate mRNAs, both of which are complementary to the smallest genome RNA of the virion, segment 8. It is clear from the work of others that the NS_1 mRNA is virtually a complete transcript of genome segment 8, lacking only 20–30 nucleotides from the 5′ end (21, 22). Therefore the nucleotide sequences of the NS_2 mRNA must also be present in the NS_1 mRNA. Because the methionine-containing tryptic peptide maps of the NS_1 and NS_2 polypeptides show no similarities (10, 14), then at least part, and probably all, of the NS_2 coding sequences remain unexpressed during translation of the NS_1 mRNA. The reason for this is not known, but two possibilities are evident, both of which have precedents in other virus systems. First, the NS_2 and NS_1 cistrons could occupy separate parts of the mRNA. In this case, the NS_2 coding sequences would presumably lie towards the 3′ end of the molecule, but might fail to be translated through having a "closed" initiation site for protein synthesis. This initiation site would be available only on a separate species of mRNA that lacked the NS_1 coding sequences. A number of different plant and animal virus mRNAs are known to contain untranslated cistrons; the information in these is expressed only on separate, shorter species of mRNA (25–29). An alternative possibility is that the NS_2 coding sequences could be contained partly or wholly within the NS_1 cistron. For the NS_2 coding sequences to remain unexpressed in this form requires that the shared nucleotide sequences be translated in two different codon-reading frames. In the NS_1 mRNA, the reading frame would be such that only the NS_1-coding triplets were translated; the functional NS_2 mRNA would be a shorter molecule in which the reading frame was altered to allow expression of the NS_2 codons. The discovery of overlapping genes in the DNA of the bacteriophages ϕX174 and G4 (30, 31) and of the animal virus simian virus 40 (32) suggests that nucleotide sequences that code in two and even three different reading frames may not be uncommon. In fact, considering the sizes of the NS_1 and NS_2 polypeptides and of genome segment 8, an overlap between the two "genes" appears quite probable. The vRNA segment contains about 820 nucleotides (1), which if read in a single frame could encode a polypeptide with a maximum molecular weight of about 30,000. The actual protein-coding potential is likely to be somewhat less, because about 20–30 nucleotides from the 5′ terminus of segment 8 are not transcribed into mRNA (21) and because the first potential initiation sequence for translation (the anticodon 3′-UAC-5′) occurs 27 nucleotides from the 3′ end of segment 8 (21, 33). The combined molecular weight of the NS_1 and NS_2 polypeptides, estimated from their mobility in sodium dodecyl sulfate/polyacrylamide gels, is

about 34,000 (11). If this value is correct, then at least part of the NS_2 coding sequence must lie within the NS_1 cistron. Conclusive proof, however, will require more detailed mapping studies and sequence analysis.

The mechanism by which the NS_2 mRNA is synthesized in the infected cell is unknown. One possibility is that the mRNA is synthesized directly by transcription of a portion of vRNA segment 8. Such transcription would be unlikely to initiate at the same site as NS_1 mRNA transcription (i.e., at the 3′ end of the vRNA), because the NS_1 and NS_2 polypeptides are quite different, but synthesis of the two mRNAs could possibly terminate at the same point around 20–30 nucleotides from the 5′ end of the vRNA. Alternatively, the NS_2 mRNA could be derived by processing of a larger vRNA transcript, perhaps the NS_1 mRNA itself. Examples of this kind of mechanism are well documented for other animal viruses (34, 35). Whichever is the correct mechanism, it apparently only operates late in infection. Synthesis of the NS_2 polypeptide begins later than that of the other virus polypeptides (10) and, during primary transcription of the infecting virus genome by the virion polymerase, the NS_2 mRNA, unlike the other virus mRNAs, is not produced (ref. 10; unpublished results). This may indicate that synthesis of a new virus-specific enzyme activity, possibly a new or modified transcriptase or an RNA processing activity, is required in the infected cell for the production of the NS_2 mRNA.

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