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Biochemistry

Segment 8 of the influenza virus genome is unique in coding for two polypeptides

(hybrid-arrested translation/gene structure/genetic recombinants/peptide mapping/myxovirus proteins)

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ABSTRACT **In previous studies we showed that a ninth polypeptide with a molecular weight of ≈11,000 (NS_2) found in influenza virus-infected cells was unique, that it could be synthesized *in vitro*, and that its expression *in vivo* required early protein synthesis. On the basis of these results we suggested that one of the eight genome RNA segments of influenza virus codes for two polypeptides [Lamb, R. A., Etkind, P. R. & Choppin, P. W. (1978) *Virology* 91, 60–78]. We describe here differences in the electrophoretic mobility of the NS_2 polypeptides of different strains of influenza A virus. These results provided further evidence that NS_2 is virus coded and also made possible genetic studies using recombinants between two virus strains (HK and PR8) whose NS_2 polypeptides differ. These studies showed that the gene for NS_2 reassorts with that of the nonstructural polypeptide NS_1, which is coded by genome segment 8. A mRNA for NS_2 has been separated from that of NS_1 and the other viral polypeptides by centrifugation and has been translated *in vitro*. Hybridization of genome segment 8 to the total mRNAs from infected cells specifically prevented the synthesis of NS_2 and NS_1. These results indicate that influenza virus genome segment 8 is transcribed into two separate mRNAs that code for two polypeptides, NS_1 and NS_2. Possible mechanisms for the transcription of the two mRNAs from either contiguous or overlapping genes are discussed.**

The genome of influenza virus consists of eight single-stranded RNA segments (1–3), which have been shown 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) (4–8). Assignments of these eight polypeptides to the eight genome segments have been made by several methods (8–11). It had therefore been assumed that the virus possesses eight genes, each on a separate segment of RNA and coding for a single polypeptide. In addition to the eight established virus-specific polypeptides, we and others have observed a small polypeptide of ≈11,000 M_r variously designated "9," "NS_2," or "4" (5, 12–16). In this communication "NS_2" will be used for this polypeptide and "NS_1" for the original ≈23,000 M_r NS polypeptide.

In previous studies (14–16) we presented evidence that NS_2 was a ninth influenza virus polypeptide. These studies indicated that NS_2 is a late gene product whose synthesis is dependent on early virus protein synthesis, that it can be synthesized *in vitro* by using mRNA from infected cells, and that its tryptic peptides are unique in comparison to those of the other eight gene products (14, 15). On the basis of these studies we suggested that NS_2 was an influenza virus-coded polypeptide. Because only eight unique RNA segments have been found in the virus, we also suggested that one of the viral genes could code for two polypeptides (14), and that this could arise either from two contiguous genes on one of the genome RNA segments or from translation of a second mRNA in a different reading frame, with early viral protein synthesis required for translation of NS_2 in either case (14). At that time it was not known which genome segment was involved, and the possibility that it was one that coded for a P polypeptide was considered. In this paper we present evidence that, in addition to coding for NS_1, genome segment 8 of influenza virus codes for NS_2.

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MATERIALS AND METHODS

Viruses. The following strains of influenza A were used: WSN/33 (H_0N_1), PR/8/34 (H_0N_1), HK/8/68 (H_3N_2), RI/5+/57 (H_2N_2), FW/1/50 (H_1N_1), FM/1/47 (H_1N_1), Brazil/11/78 (H_1N_1), USSR/90/77 (H_1N_1). The GL/1760/54, MB/50, and Lee/40 strains of influenza B were used. All viruses were grown in embryonated eggs, except WSN, which was grown in MDBK cells (17). Eleven recombinants between PR8 and HK in which individual genes were exchanged were generously provided by J. Schulman, M. Lubeck, and P. Palese.

Cells. Primary chicken embryo fibroblasts (CEF) and the MDBK (bovine), HeLa (human), and HKCC (hamster) cell lines were grown as described (7, 17).

Growth, Isotopic Labeling, and Purification of Virus. These procedures were done as described (7). ^{32}P-Labeled virus was grown in MDBK cells in Eagle's medium deficient in phosphate and containing [^{32}P]orthophosphate (100 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) and trypsin (1 μg/ml).

Isotopic Labeling of Polypeptides in Infected Cells and Polyacrylamide Gel Electrophoresis. These were done as described (14), using 17.5% acrylamide gels containing 4 M urea.

Peptide Maps. Peptide mapping was as described (14).

Virion RNA and mRNA Extraction and Protein Synthesis *In Vitro*. Purified virions in a buffer containing 100 mM NaCl, 10 mM Tris·HCl (pH 7.4), and 1 mM EDTA were made 1% in sodium dodecyl sulfate, extracted three times with phenol (2), and precipitated with ethanol. mRNA was extracted from postnuclear supernatants of infected cells with phenol/chloroform/isoamyl alcohol, and those molecules containing poly(A) were selected by oligo(dT)-cellulose chromatography as described by Etkind and Krug (18). *In vitro* protein synthesis in nonpreincubated wheat germ extracts was done as described previously (14) except that 100-μl assays were used.

Hybrid-Arrested Translation. The RNA segments in 250 μg of ^{32}P-labeled RNA from purified virions were separated at 30°C on a 17-cm 2.6% acrylamide/6 M urea gel (1) buffered with Tris/borate (19). After location by autoradiography, individual RNA segments were extracted from the gel by elution and precipitated with perchloric acid (19, 20). Influenza virus mRNA and individual virion RNA segments were hybridized as described by Ito and Joklik (21) in 1.5-ml Eppendorf microcentrifuge tubes. After 16 hr at 37°C, samples were precipitated with 2.5 vol of ethanol, and RNA was collected by centrifugation (15,000 × *g* for 15 min) and resuspended in 30

μl of H_2O. For translation in wheat germ extracts, 5–10 μl of RNA was used.

RESULTS

Strain-Specific Differences in NS_2. The synthesis of NS_2 has been observed in seven different cell types infected with WSN influenza virus (14, 15). To extend this observation and to provide further evidence that NS_2 is virus-coded, HeLa cells were infected with different strains of influenza A virus, including several recent isolates. As shown in Fig. 1 *left*, a polypeptide of $\approx$11,000 M_r was synthesized in each case. Small but distinct differences in the mobility of NS_2 could be observed among the strains in the same host cell type, providing further evidence that NS_2 is virus coded. Cells infected with three strains of influenza B viruses were also examined for the synthesis of a small polypeptide. As shown in Fig. 1 *right*, a polypeptide of $\approx$10,000 M_r was detected in these cells.

To demonstrate that the NS_2 polypeptides of different strains were analogous, the NS_2 polypeptides from cells infected with the WSN and PR8 strains were compared by tryptic peptide mapping (Fig. 2). The peptides were coincident except for one peptide, indicating their similarity.

Evidence for a Separate mRNA for NS_2. A previous analysis of infected cell mRNAs had indicated that a mRNA coding for an $\approx$11,000 M_r polypeptide could be found (22), and, using total mRNA from infected cells, we were able to synthesize *in vitro* a polypeptide with a peptide composition similar to that of NS_2 synthesized *in vivo* (14). To establish that a specific mRNA for NS_2 existed, the cytoplasm of cells infected with the WSN strain was fractionated on sucrose gradients, and the mRNAs in each fraction were extracted and translated in a wheat germ system. Near the top of the gradient a fraction (no. 6) was found that yielded on translation a polypeptide of $\approx$11,000 M_r (Fig. 3). The size of the mRNA in this fraction would be equivalent to 350–450 nucleotides, as estimated by its sedimentation in comparison with 4S tRNA and 18 and 28S ribosomal RNAs. The other influenza virus mRNAs were translated from the gradient fractions that corresponded to their sizes (23, 24). To ensure that

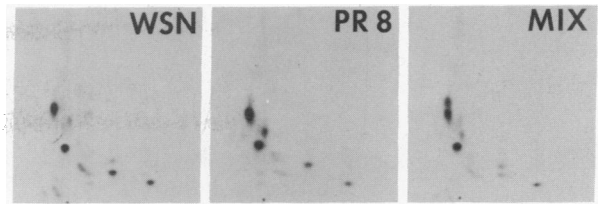

FIG. 2. Tryptic peptide maps of NS_2 obtained from HeLa cells infected with the WSN or PR8 strains of influenza A virus and labeled with [^{35}S]methionine (250 μCi/ml) for 2 hr. MIX, tryptic peptides from the two NS_2s were mixed before mapping.

the $\approx$11,000 M_r polypeptide translated from fraction 6 was authentic NS_2, its tryptic peptides were mapped and compared with those of NS_2 isolated from infected cells (Fig. 4, panels 3 and 4). The peptides of major intensity (A–D) were the same, as were nearly all the minor-intensity peptides as determined by mixing experiments. Fig. 4 also shows the tryptic peptide maps of NS_1 made *in vivo* (panel 1) and *in vitro* (panel 2). These indicate that NS_1 and NS_2 are unique polypeptides that do not share any common peptides, as shown by the mixing experiments (panels 5 and 6).

Genetic Evidence That the Gene for NS_2 Reassorts with That for NS_1. When the RNA segments of two strains of influenza virus were compared on gels, it was found that they had different electrophoretic mobilities, and this made it possible to determine the gene constellations of recombinant viruses obtained from two parental types (8, 25). This, together with the finding that the polypeptides of various strains had different electrophoretic mobilities, permitted a determination of which gene coded for the viral polypeptides (8, 26, 27). The difference we observed in the mobility of the NS_2 polypeptides of the PR8 and HK strains (Fig. 1 *left*) made it possible to determine which viral RNA segment coded for NS_2 by an analysis of the polypeptides synthesized in cells infected with 11 recombinants between PR8 and HK that were selected to contain different genes. The constellations of these recombinants had been determined previously by Schulman and Palese and their co-

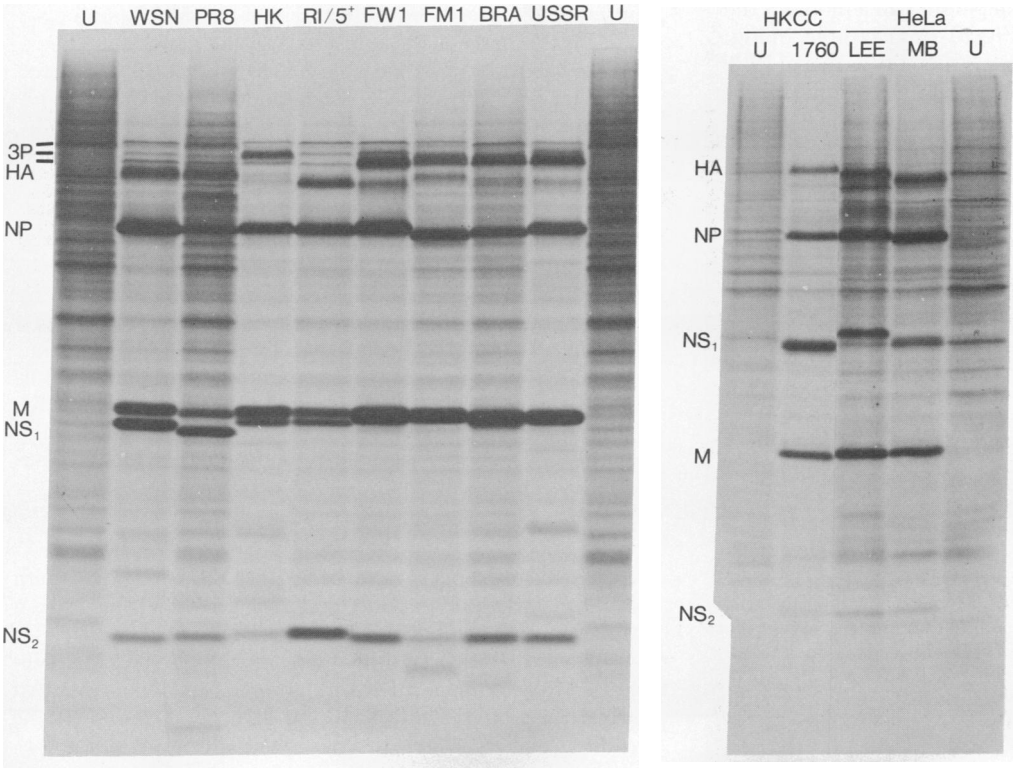

FIG. 1. (*Left*) Synthesis of NS_2 in HeLa cells infected with various strains of influenza A virus. At 5 hr after infection cells were labeled with [^{35}S]-methionine (20 μCi/ml) for 30 min and subjected to electrophoresis on a 17.5% acrylamide/4 M urea gel. U, uninfected cells. The strains used were WSN, PR8, HK, RI/5$^+$, FW1, FM1, Brazil (BRA), and USSR, as described in *Materials and Methods*. (*Right*) Synthesis of NS_2 in cells infected with three strains of influenza B virus. At 19 hr after infection with B/1760, HKCC cells were labeled with [^{35}S]methionine (20 μCi/ml) for 30 min, and at 6 hr after infection with B/MB or B/Lee, HeLa cells were labeled similarly Cell lysates were subjected to electrophoresis as described for *Left*.

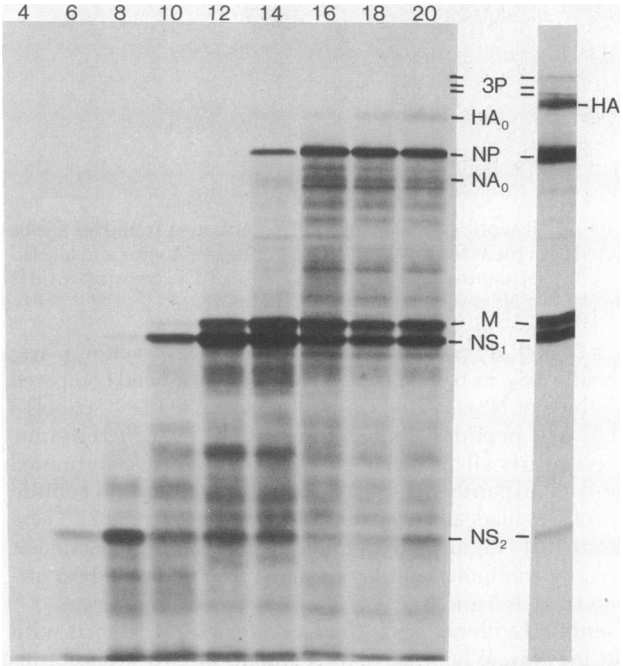

FIG. 3. Translation *in vitro* of influenza virus mRNAs separated on a sucrose gradient. At 3 hr after infection WSN-infected CEF cells were harvested and lysed in RSB buffer (10 mM NaCl/10 mM Tris·HCl, pH 7.4/1.5 mM $MgCl_2$) by Dounce homogenization, and a postnuclear supernatant was prepared. This was made 2% in sodium dodecyl sulfate, layered onto 15–30% (wt/wt) sucrose/100 mM NaCl/10 mM Tris·HCl (pH 7.4)/0.1 mM EDTA gradients containing 0.5% sodium dodecyl sulfate, and centrifuged in a Spinco SW 27 rotor for 20 hr at 24,000 rpm at 22°C. Fractions were collected from the top of the gradient and each fraction was precipitated with 2.5 vol of ethanol. RNA was extracted from each fraction and poly(A)-containing RNA was translated in wheat germ extracts. Fraction numbers are indicated at the top. The right lane contains a sample of WSN-infected CEF cell lysate to show viral polypeptides as markers.

workers (8, 25, 28, 29), and are indicated in Table 1. By examination of the mobility of NS_2 from each recombinant and the two parents, a parental type was assigned for the NS_2 of each recombinant by determining which parent had a mobility the same as that of the recombinant (Fig. 5). For example, in Fig. 5 the NS_2 of recombinant 1 has the same mobility as that of PR8; recombinant 2, that of PR8; 3, that of HK, etc. From this experiment, the parental types of NS_2 of recombinants 1–11 were found to be: P, P, H, P, P, H, P, P, H, P, and H, respectively, in which P = PR8 and H = HK. This correlation fits only with RNA segment 8, which codes for NS_1 (Table 1). This provides strong genetic evidence that RNA segment 8 codes for two unique polypeptides, NS_1 and NS_2.

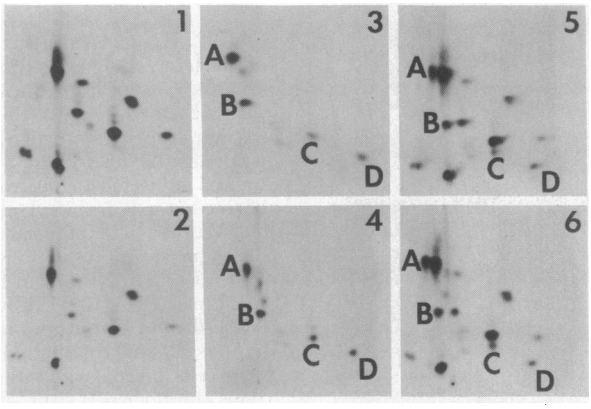

FIG. 4. Tryptic peptide maps of NS_1 and NS_2 synthesized *in vivo* in HeLa cells, and *in vitro* in wheat germ extracts. NS_1 was obtained by translation *in vitro* of fractions 12 and 14, and NS_2 by translation of fractions 6 and 8 from a gradient similar to that in Fig. 3, except that the mRNA was obtained from infected HeLa cells. Panel 1, NS_1 synthesized *in vivo*; 2, NS_1 synthesized *in vitro*; 3, NS_2 *in vivo*; 4, NS_2 *in vitro*; 5, a mixture of NS_1 and NS_2 synthesized *in vivo*; 6, a mixture of NS_1 and NS_2 synthesized *in vitro*. To each plate, 40,000 cpm of NS_1 and 20,000 cpm of NS_2 were applied.

Table 1. Recombinants between influenza A virus strains PR8 and HK

Recombinant	Genes derived from PR8 or HK*							
	P_1	P_2	P_3	HA	NP	NA	M	NS_1
1	P	P	P	H	P	H	P	P
2	H	H	H	H	P	H	P	P
3	H	H	H	H	H	H	P	H
4	H	P	P	H	P	H	P	P
5	P	P	P	H	P	P	H	P
6	P	P	P	H	P	P	P	H
7	H	H	P	P	H	H	P	P
8	H	P	H	P	P	H	P	P
9	H	H	H	P	H	P	H	H
10	H	H	H	P	H	P	H	P
11	H	H	H	H	P	H	P	H

*P, PR8; H, HK.

Prevention of Translation of NS_2 by Hybridization of Its mRNA to Genome Segment 8. To confirm the above observation by a biochemical approach, we used the RNA·RNA hybrid-arrest translation scheme that had been used to assign gene functions to the segments of an avian influenza virus, fowl plague (10). In this system the addition of a viral RNA segment to the total mRNAs from infected cells specifically prevents the translation of the polypeptide for which it codes by hybridizing to its mRNA. Individual segments of influenza virus RNA (Fig. 6 *left*) were hybridized to influenza virus-specific mRNA, and after hybridization the total RNA was translated in wheat germ extracts. As shown in Fig. 6 *right*, segments 1–3, which were not separated, prevented the translation of P_1, P_2, and P_3, and segment 4 prevented that of the unglycosylated hemagglutinin (HA_0). Segment 5 greatly reduced the translation of NP; segment 6 prevented the translation of NA_0, and segment 7, that of polypeptide M. Of particular significance was the finding that segment 8 prevented the translation of both NS_1 and NS_2. Thus, on the basis of these results and the genetic evidence presented above (Fig. 5), it can be concluded that RNA segment 8 codes for both NS_1 and NS_2 polypeptides. These findings and the above evidence that a separate mRNA codes for NS_2 indicate that RNA segment 8 is transcribed into two mRNAs, which are translated into two unique polypeptides, NS_1 and NS_2.

The results of the experiments shown in Fig. 6 have also provided direct *in vitro* translation evidence confirming the gene assignments for the WSN strain made previously (26). In addition they provide evidence for the synthesis *in vitro* of the unglycosylated neuraminidase polypeptide. RNA segment 6 of the WSN strain has been shown by genetic and immunological analysis to code for the neuraminidase (26); thus it follows that the polypeptide designated NA_0 in Fig. 6, which is of the appropriate size and whose translation is specifically inhibited by genome segment 6, is the unglycosylated neuraminidase polypeptide.

DISCUSSION

We demonstrated in previous studies that NS_2 is a unique, ninth influenza virus polypeptide, and that a polypeptide with a similar peptide composition could be synthesized *in vitro* (14, 15). It was further found in studies of the polypeptides synthesized from primary mRNA transcripts of the input RNA that early viral protein synthesis was apparently required for the synthesis of NS_2, which is synthesized late in infection (14).

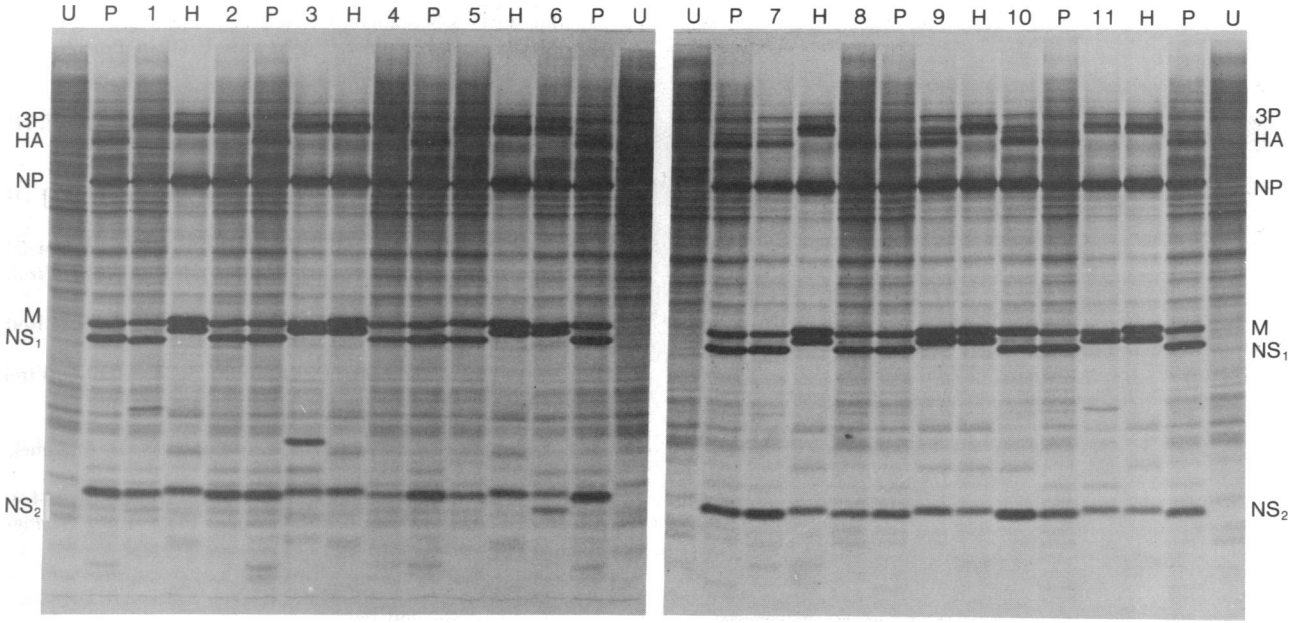

FIG. 5. An analysis of NS_2 synthesized in HeLa cells infected with different recombinants between the PR8 and HK strains. HeLa cells were infected with PR8, HK, and 11 recombinants. At 5 hr after infection, cells were labeled with [^{35}S]methionine (20 μCi/ml) and subjected to electrophoresis. U, uninfected; P, PR8 parent; H, HK parent; numbers indicate the recombinants shown in Table 1.

These studies lead to the hypothesis that one of the known viral genome segments coded for two polypeptides (14, 16), and in this paper we have presented evidence that the genome segment is segment 8, which also codes for NS_1.

The finding of mobility differences among the NS_2 polypeptides of different strains synthesized in the same host cell (Fig. 1) provided a means for determining which genome segment codes for NS_2. By examining recombinants with selected genotypes, it was shown (Fig. 5) that the gene coding for NS_2 reassorts with segment 8, which codes for NS_1.

We have also shown in the present studies that a separate mRNA with an estimated size of 350–450 nucleotides codes for NS_2, and that hybridization of genome segment 8 to mRNA from infected cells specifically prevents the synthesis of NS_2 as well as NS_1. Thus, on the basis of both genetic and biochemical evidence, genome segment 8 codes for NS_1 and NS_2. Inglis *et al.* (30), using an avian influenza virus (fowl plague), have also found that an NS_2 polypeptide analogous to that in WSN-infected cells is coded for by RNA segment 8.

The question remains as to how the mRNAs that code for NS_1 and NS_2 are generated. We previously discussed the possibilities, including nonoverlapping genes or overlapping genes with translation in a second reading frame (14, 16), using a segment coding for a P polypeptide as a model because of the possibility that the coding capacity exceeded the known polypeptide. It is obvious that the same reasoning could be applied to another genome segment, and the possible mechanisms are discussed again in light of recent findings.

The mRNA for NS_1 is nearly a full length copy of RNA segment 8 (23, 24) and thus must contain some of the same nucleotide sequences that are present in the NS_2 mRNA. The initiation of transcription of the mRNA for NS_2 could occur near the 3′ end of RNA segment 8 or internally. Alternatively, the mRNA for NS_2 could be spliced from the mRNA for NS_1. If splicing does occur, it would seem more likely to occur either immediately after the AUG for initiation of translation for NS_1 or in a noncoding region of NS_1, because splicing within the translated region would probably yield some common tryptic peptides in NS_1 and NS_2, which we have not found. If the mRNA is transcribed as a direct copy of the 3′ end of segment 8, or of any region that codes for NS_1, then a second reading frame for translation of NS_2 would have to be used to accommodate the above facts. Such second reading frames have been found for phage ϕX174 (31) and simian virus 40 (32).

On the other hand, internal initiation of transcription of the NS_2 mRNA could occur ≈350 nucleotides from the 5′ end of segment 8 without utilizing overlapping nucleotides in translation, if toward the 3′ end of the NS_1 mRNA there was a region of ≈350 nucleotides not translated during the synthesis of NS_1. The initiation site for synthesis of the NS_2 mRNA could be inaccessible until a modification of transcription late in infection. This could explain the observed requirement for protein synthesis. Examples of internal initiation of RNA synthesis have been found with α viruses (33, 34) and untranslated regions of mRNA that are easily translated from separate small mRNAs have been found with plant viruses (35, 36). However, this possibility depends on there being sufficient nucleotides in segment 8 to code for the two polypeptides. Recent estimates of the size of RNA segment 8 from cDNA copies suggest that it contains 890–970 nucleotides (37, 38). NS_1 has been estimated to have a molecular weight of ≈23,000, and NS_2 ≈11,000 (7, 14). Thus a combined coding capacity of 910 nucleotides would be needed, excluding any noncoding sequences. However, these size estimates of the polypeptides are based on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and thus the true values could be sufficiently different to permit coding of the two polypeptides by separate RNA sequences, particularly because NS_1 is a phosphoprotein (39), and phosphorylation may affect migration in gels. In summary, the available evidence does not permit one 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.

Since the description of NS_1 (4), little has been learned about its role in infected cells, and nothing is known about the role of NS_2. After synthesis, NS_1 accumulates in the nucleus (4) and is probably associated with the nucleolus (40, 41), and NS_2 is found in the cytoplasm (16). Examination of a temperature-sensitive mutant in the NS_1 gene (27) suggested that, although viral RNA synthesis was reduced at nonpermissive temperatures, NS_1 was unlikely to be involved in the replicase enzyme, but might be involved in the switch from early to late protein synthesis (42). The synthesis of virus mRNAs and their trans-

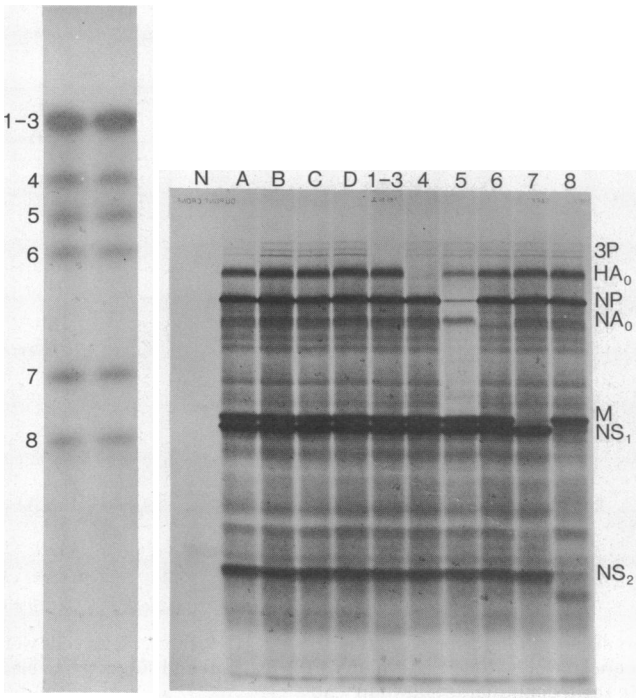

FIG. 6. (*Left*) Analysis of ^{32}P-labeled WSN RNA on a 2.6% polyacrylamide/6 M urea gel before elution of the individual segments to be used in hybrid-arrested translation. (*Right*) Translation *in vitro* of influenza virus mRNA after hybridization of mRNA from WSN-infected cells to individual genome RNA segments. N, no RNA added to wheat germ. A, mRNA frozen at −20°C in 1 mM EDTA for 16 hr. B, mRNA frozen at −20°C in 1 mM EDTA and 90% (vol/vol) dimethyl sulfoxide (Me_2SO) for 16 hr. C, mRNA incubated in 1 mM EDTA and 90% Me_2SO at 45°C for 30 min, then at 37°C for 16 hr. D, mRNA incubated in 1 mM EDTA and 90% Me_2SO at 45°C for 30 min, then the salt was adjusted to 30 mM NaCl/10 mM Tris·HCl, pH 7.4/2 mM EDTA and the Me_2SO concentration was reduced to 63% before incubation at 37°C for 16 hr. Lanes 1–3, 4, 5, 6, 7, and 8 show the polypeptides synthesized after hybridization of the respective virion segments to mRNA. Hybridization was carried out under conditions listed for D above.

lation is tightly controlled (6, 7, 15, 23, 43) and because NS_2 is made late in infection it is possible that its biological role is to regulate mRNA synthesis, promote the synthesis of full-length template cRNA, or function in the replication of vRNA strands.

Although such functions for NS_2 are attractive, we cannot totally exclude the possibility that NS_2 is synthesized as a consequence of a virus–host cell interaction leading to defective virus replication. We have observed variations in the amount of NS_2 synthesized with the same strain in different cell types (14) and among different strains in the same cell type (ref. 16 and see Fig. 1), but we have not been able to correlate this with defective virus formation. Further, analysis of 30 plaque-purified clones of WSN yielded similar amounts of NS_2 synthesis in the same host cell (unpublished observations). Nevertheless, the variation in the amount of NS_2-related RNA synthesized in different cell types merits attention, as well as the mechanism of transcription of its mRNA and its function.

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