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Expression of Defective-Interfering Influenza Virus-Specific Transcripts and Polypeptides in Infected Cells

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We have previously shown that influenza virus defective-interfering particle (DI) RNAs can be transcribed into polyadenylated complementary RNAs in vitro (Chanda et al., J. Virol. 45:55–61, 1983). In this paper we report that influenza virus DI RNAs can be transcribed into mRNAs in infected cells as well. The DI-specific RNAs (both plus and minus strands) were found to be synthesized in molar excess compared with RNAs of standard virus segments. In addition, two DI preparations (DI_3 and DI_7) produced novel polypeptides not present in standard virus-infected cells. These novel polypeptides in DI-infected cells were of PB_2 origin, as were the major DI RNA species in both DI preparations. Furthermore, these polypeptides were shown to arise from the translation of functional mRNAs transcribed from DI_3 and DI_7 RNAs and not from either the degradation of PB_2 protein or the incomplete translation of PB_2 mRNA. Using mixed-infection tests with different DI preparations, we found that the ability of DI to produce detectable novel polypeptides does not necessarily confer any replicative or interfering advantage over other DI which do not produce detectable DI-specific polypeptides. The possible role of DI-specific polypeptides in DI-mediated interference is discussed.

Influenza virus when serially passaged at a high multiplicity of infection produces defective-interfering particles (DI). These particles are noninfectious and, therefore, need the helper function of the standard virus for replication. In addition, they interfere with the multiplication of standard virus (18, 19; D. P. Nayak, T. M. Chambers, and R. K. Akkina, Curr. Top. Microbiol. Immunol., in press). Studies from a number of laboratories (2, 11, 17, 21) have shown that influenza virus DI contain, in addition to standard virus gene segments, small RNA molecules of various sizes. These small RNAs (DI RNAs) have been shown to be responsible for interference with standard virus multiplication (11). Influenza virus DI RNAs are of the same polarity as standard virus RNAs and arise from standard virus gene segments during virus multiplication by aberrant replicational events (3, 4, 8, 12, 20). Sequence analyses of a number of influenza virus DI RNAs have indicated that they are of the 5′-3′ type, i.e., they retain the 5′- and 3′-terminal regions of the progenitor genes (12, 19, 20, 24). In contrast, the majority of DI RNAs of nonsegmented negative-strand RNA viruses such as Sendai virus and vesicular stomatitis virus are of the 5′ type, as they retain only the 5′ genomic terminus (15). Since the influenza virus DI RNAs retain both the 5′ and 3′ termini of the progenitor genes, they are likely to possess polymerase-replicase binding, transcription initiation, and transcription termination as well as polyadenylic acid [poly(A)] addition sites, and therefore, they are expected to serve as templates for transcription (20).

In vitro transcription studies have shown that DI RNAs are transcribed into complementary RNAs (cRNAs) of plus sense with poly(A) tails of various lengths, indicating that these DI RNAs probably can produce functional mRNAs (1a). Therefore, it is of interest to see whether DI-specific mRNAs and DI-specific polypeptides are also indeed produced in infected cells. If so, DI-specific transcriptional or translational products may have an important function in the mechanism of interference mediated by influenza virus DI. In this paper, we report that influenza virus DI RNAs are transcribed into polyadenylated [poly(A)$^+$] cRNAs in infected cells and that at least some of these cRNAs are translated into novel DI-specific polypeptides. We further discuss the possible role of transcriptional and translational products in virus multiplication and interference.

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MATERIALS AND METHODS

Viruses and cells. Influenza virus strain A/WSN/33 was grown in Madin-Darby bovine kidney (MDBK) cells as previously described (21). Individual plaques were isolated and grown separately to produce standard virus preparations. Each standard virus preparation was passaged serially at a high multiplicity of infection to generate and amplify DI [DI-ts^+(Tobita), DI_3, DI_7, etc.] as described previously (21). Subsequently, large amounts of individual DI virus stocks were made, and samples were frozen. The same batch of DI preparation was used throughout these experiments. Procedures for assaying DI units (DIU), PFU, and hemagglutinating units have been previously described (10).

Isolation and fractionation of viral RNA. MDBK cells were either infected with standard virus alone or coinfected with DI (3 DIU per cell) and standard viruses (3 PFU per cell), and the viral RNA was labeled with ^{32}P (200 μCi/ml) as previously described (21). Virus purification and viral RNA extraction were performed as previously described (22). The RNA was fractionated by polyacrylamide gel electrophoresis (PAGE) on 3% gels containing 6 M urea (22).

Isolation and analysis of poly(A)$^+$ and poly(A)$^-$ RNAs from infected cells. At various times postinfection (p.i.), cytoplasmic RNAs from infected cells were extracted (7) and separated into poly(A)$^+$ and non-polyadenylated [poly(A)$^-$] fractions by oligodeoxythymidylate cellulose chromatography (6). For analysis on gels, poly(A) tails were removed by annealing poly(A)$^+$ RNAs with polydeoxythymidylate and digesting them with RNase H (5). The removal of poly(A) tails of various lengths was necessary to avoid heterogeneous migration of poly(A)$^+$ mRNAs.

To determine whether the DI-specific poly(A)$^+$ RNAs were true complements of their corresponding DI RNAs found in the DI, the ^{32}P-labeled poly(A)$^+$ DI-specific transcripts and the corresponding unlabeled DI RNAs were eluted from gels and specifically hybridized to each other (1). The hybrids were then treated with RNases A (4 μg/ml) and T1 (20 U/ml), denatured, and electrophoresed on a 3% polyacrylamide–6 M urea denaturing gel.

Northern blot analysis of DI-specific RNAs. Virus RNA and cytoplasmic poly(A)$^+$ cRNA were prepared as described above. The RNAs were glyoxal denatured, electrophoresed on a 1.1% agarose (sodium phosphate, pH 6.5) gel, and blotted to Genescreen transfer paper (New England Nuclear Corp., Boston, Mass.), as described previously (23). The blots were baked, prehybridized, and then hybridized at 42°C with ca. 20 ng (specific activity, 5×10^4 cpm/ng) of denatured cDNA probes corresponding to the PB_1, PB_2, PA, or NS influenza virus gene segments per ml, labeled with ^{32}P by nick translation. Each hybridization mixture also included a cDNA probe for the M gene for use as an alignment marker. The hybridized blots were washed, dried, and autoradiographed (23).

Separation of viral mRNAs by sucrose velocity gradient centrifugation. The cytoplasmic mRNAs, isolated from infected cells at 5 h p.i., were separated according to their size by sucrose velocity gradient centrifugation as described by Lamb and Choppin (14), except that sodium dodecyl sulfate (SDS) was omitted. Briefly, viral mRNA was dissolved in 0.5 ml of water, boiled for 2 min, and immediately quenched in a dry ice-ethanol bath. The RNA solution was adjusted to 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA before being layered onto a linear gradient of 5 to 30% sucrose (wt/vol) and centrifuged at 27,000 rpm in a Spinco SW28 rotor for 18 h at 22°C. Fractions were collected from the bottom. To each fraction 10 μg of *Escherichia coli* tRNA was added as carrier, and the RNA was precipitated with ethanol. The RNA precipitate was dissolved in 10 μl of distilled water and used for in vitro translation.

Analysis of viral polypeptides in infected cells. MDBK cells were either infected with standard virus alone or coinfected with DI and standard virus (21) and labeled for 1 h with 50 μCi of [^{35}S]methionine per ml in methionine-free medium. The labeled viral polypeptides were analyzed by SDS-PAGE on 13% gels containing 4 M urea and detected by autoradiography (13).

In vitro translation of viral mRNAs. The viral mRNAs were isolated from infected cells at 5 h p.i. as described above and used for in vitro translation with wheat germ lysates (6). Poly(A)-selected and total cytoplasmic RNAs gave identical results. The polypeptide products of the in vitro translation reactions were analyzed by SDS-PAGE.

Hybrid selection of DI mRNAs for translation. Selection of DI-specific mRNA was achieved by the method of Miller et al. (16) with some modifications. Briefly, plasmids (pBR322) containing influenza virus A/WSN/33 polymerase cDNA inserts were used for hybrid selection. Approximately 10 to 20 μg of each linearized plasmid in 10 μl of distilled water was boiled for 60 s and immediately quenched on ice. The DNA was spotted onto nitrocellulose filters (1 cm^2) and dried at room temperature. The filters were washed for 30 min in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried overnight at room temperature, and baked for 2 h at 70°C in a vacuum oven. Then the filters were cut into squares (3 by 3 mm) and transferred to microcentrifuge tubes. Prehybridization was carried out in 300 μl of hybridization buffer (0.4 M sodium acetate [pH 6.0], 0.001 M EDTA) with 50 μg of *E. coli* tRNA at 60°C for 1 h. The filters were washed 10 times with 1× SSC containing 0.5% SDS and 10 times with 10 mM Tris-hydrochloride, pH 7.8. The actual hybridization was carried out with total cytoplasmic mRNA from DI-infected cells in 300 μl of hybridization buffer for 4 h at 60°C. After being washed, the hybridized mRNA was eluted by boiling the filter for 60 s in 300 μl of 1 mM EDTA, pH 8.0. The eluate was chilled quickly in ice, and 10 μg of *E. coli* tRNA was added. The mRNA was precipitated with ethanol and dissolved in 10 μl of water. Hybrid-selected mRNAs were translated in vitro as described above, and the polypeptide products were analyzed by SDS-PAGE.

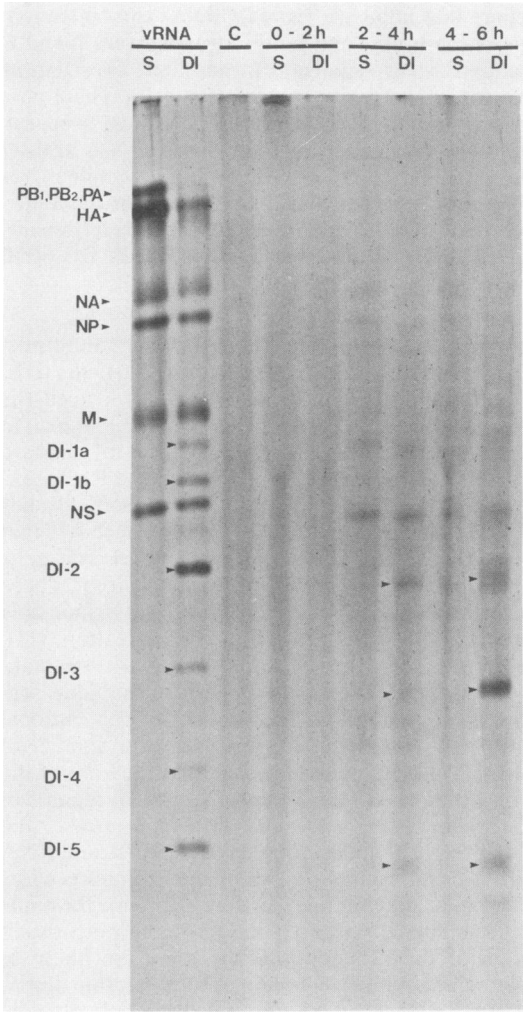

FIG. 1. Cytoplasmic poly(A)$^+$ cRNA from cells infected with standard virus and DI. S, RNA from MDBK cells infected with standard WSN virus. DI, RNA from cells coinfected with standard virus and DI-*ts*$^+$(Tobita). C, RNA from mock-infected cells. vRNA, Marker RNA extracted from standard virus and DI. The indicated times are the ^{32}P-labeling periods p.i. At the end of the labeling period, the cells were lysed, and cytoplasmic RNA was extracted. The poly(A)$^+$ and poly(A)$^-$ RNA fractions were separated by oligodeoxythymidylate cellulose chromatography. Subsequently, poly(A) tails were removed from the poly(A)$^+$ fraction by hybridization to polydeoxythymidylate and treatment with RNase H (5). RNAs were electrophoresed on a 3% polyacrylamide–6 M urea gel. Arrows indicate positions of DI-specific poly(A)$^+$ cRNAs corresponding to DI RNAs.

RESULTS

DI-specific transcripts in infected cells. To determine whether DI RNAs were transcribed into poly(A)$^+$ cRNAs, cells infected with standard virus alone or coinfected with DI and standard viruses were labeled with ^{32}P, and cytoplasmic poly(A)$^+$ RNAs were isolated. Subsequently, poly(A) tails were removed, and labeled mRNAs were analyzed by PAGE. Novel poly(A)$^+$ RNAs, not found in cells infected with standard virus alone, were present in cells coinfected with DI and standard virus (Fig. 1). As we expected, these RNAs were slightly shorter than the corresponding DI RNAs, as determined by relative electrophoretic mobility. DI-specific transcripts were found with different DI preparations, and their sizes varied in accordance with the sizes of the DI RNAs in specific DI preparations. Pulse labeling at different times p.i. indicated that these DI-specific transcripts were detectable at 2 to 4 h p.i. and became more prominent at 4 to 6 h p.i. (Fig. 1). Our results also indicate that DI-specific transcripts are synthesized in molar excess over the standard virus gene segment transcripts between 3 and 6 h p.i. Furthermore, some DI RNA transcripts are made in greater abundance than others (1). To determine whether the DI RNA transcripts arose because of incomplete transcription of standard RNA segments or whether they were true complements of specific DI RNAs, labeled DI-specific transcripts (plus strand) and corresponding unlabeled DI RNAs (minus strand), isolated from virus particles, were eluted from gels and specifically hybridized to each other. These hybrids were resistant to RNase digestion. Furthermore, the labeled cRNAs in these RNase-treated hybrids were of nearly the same size as the DI RNAs in denaturing gels (Fig. 2). These results show that DI-specific transcripts were indeed faithful copies of DI RNAs and could not be incomplete transcripts of progenitor RNAs. Poly(A)$^+$ DI-specific transcripts have been found in all DI preparations we examined, indicating that most, if not all, influenza virus DI RNAs are capable of undergoing in vivo transcription. The cytoplasmic poly(A)$^-$ RNA, presumably consisting of plus-strand replicative template as well as progeny viral RNA (minus strand), was also analyzed by PAGE. DI-specific poly(A)$^-$ RNAs were first detectable at 2 to 4 h p.i. and became more prominent at 4 to 6 h p.i. (Fig. 3). DI-specific poly(A)$^-$ RNAs were also synthesized in excess over standard RNAs.

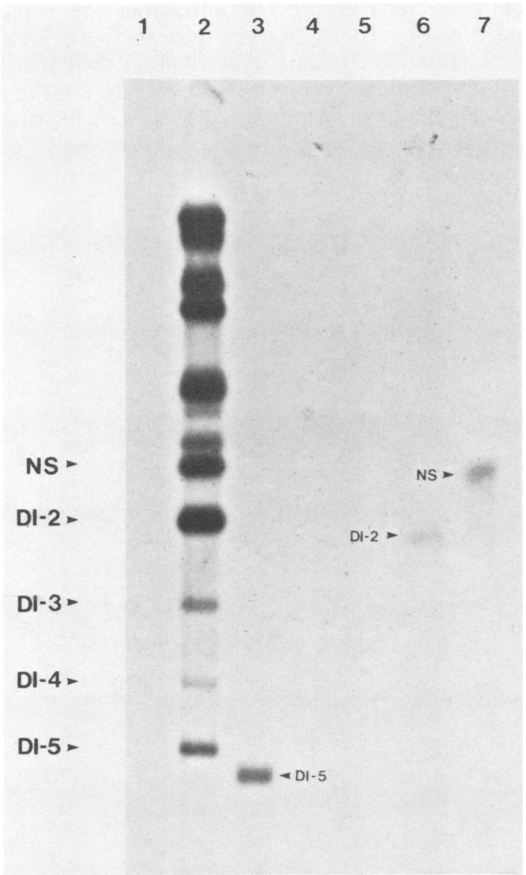

FIG. 2. RNase resistance of individual DI-specific cytoplasmic poly(A)$^+$ cRNAs after hybridization to the corresponding DI RNA segments. Individual ^{32}P-labeled cRNA bands and their corresponding unlabeled viral RNA bands were eluted from gels (see Fig. 1) and specifically hybridized to each other. The hybrids were treated with RNases A and T1 and then phenol-chloroform extracted, denatured, and electrophoresed on a 3% polyacrylamide–6 M urea gel. Lane 2, Marker viral RNA extracted from DI-*ts*$^+$(Tobita). Lanes 3 to 7, Specific hybrids of cRNA and viral RNA of DI_5, DI_4, DI_3, DI_2, and NS segments, respectively, with arrows indicating the positions of visible protected cRNAs. Lane 1 contains DI (DI-5, DI-4, DI-3, and DI-2, respectively), cRNA equivalent to the cRNA in lane 6 but hybridized with itself and then treated as described above. Counts in DI_3 (lane 3) and DI_4 (lane 4) hybrids were too low to show as distinct bands although some faint bands were visible after long exposure.

DI-specific polypeptides in infected cells. To determine whether DI-specific transcripts were translated into specific polypeptides, cells either infected with standard virus alone or coinfected with DI and standard viruses were labeled with [^{35}S]methionine and analyzed for viral polypeptides on SDS-PAGE gels. Novel DI-specific polypeptides were detected with two different DI preparations, DI_3 and DI_7 (Fig. 4). Both DI_3 and DI_7 preparations contained DI RNAs which were transcribed into poly(A)$^+$ cRNAs in vivo as well as in vitro (data not shown). These novel polypeptides were not present in standard virus-infected cells. They have approximate molecular weights of 22,000 (DI_3) and 8,000 (DI_7) and do not correspond to any known influenza virus polypeptides. Both of these polypeptides were first observed at ca. 3 h p.i., reached maximum synthesis at 5 to 7 h p.i., and continued to be synthesized up to 9 to 10 h p.i. (Fig. 5).

In vitro translation of DI-specific polypeptides. Next, we wanted to determine whether the novel polypeptides observed in DI-infected cells were primary translation products of mRNAs or degraded products of standard virus polypeptides. Accordingly, mRNAs were isolated from DI- and standard virus-infected cells and translated in vitro by the wheat germ translation system (6). Again, the two novel polypeptides which correspond to the DI-specific polypeptides in infected cells were observed in the in vitro translational products of mRNAs obtained from cells coinfected with DI and standard virus but not in products obtained from cells infected with standard virus alone (Fig. 6). Poly(A)-selected and total cytoplasmic RNAs from infected cells produced identical results in the in vitro translation reactions.

Size-analysis of DI mRNAs in sucrose velocity gradients. To determine whether the mRNAs giving rise to novel polypeptides belong to a size class different from that of other viral messages, the mRNAs from DI-infected cells were analyzed in 5 to 30% sucrose velocity gradients. RNAs from different gradient fractions were collected and translated in vitro for polypeptide synthesis. As shown in Fig. 7, the mRNAs giving rise to DI-specific polypeptides were smaller than the

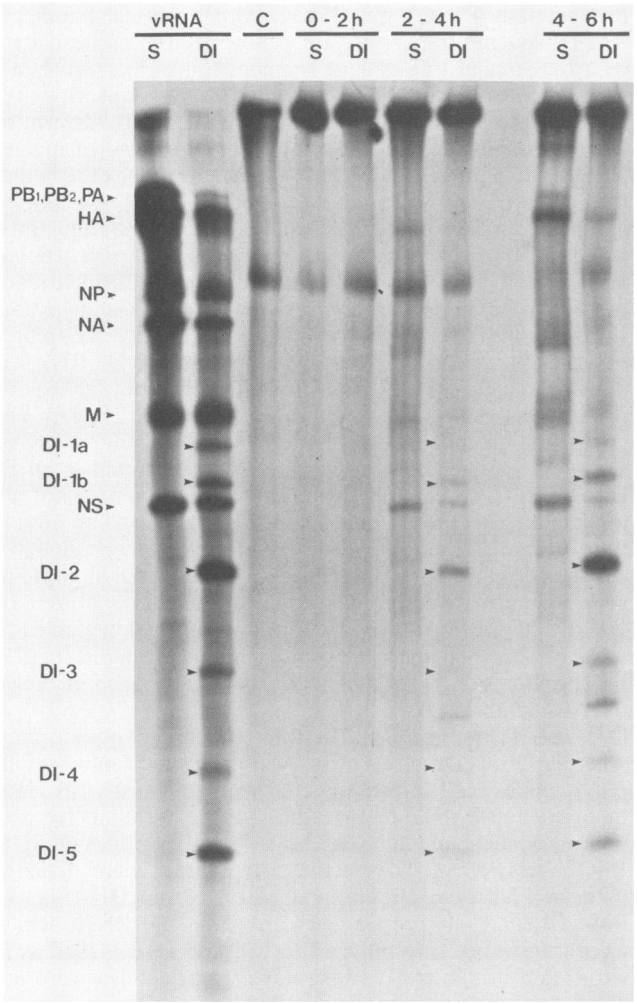

FIG. 3. Cytoplasmic poly(A)$^-$ RNA from cells coinfected with standard virus and DI. Labels and experimental methods are as described in the legend to Fig. 1. Arrows indicate the positions of DI-specific poly(A)$^-$ RNAs corresponding to DI RNAs.

standard virus mRNAs, including the M_1 and NS_1 mRNAs. This was expected because DI polypeptides are smaller than known standard virus polypeptides, with the exception of M_2 and NS_2. These results, therefore, demonstrate that DI-specific polypeptides cannot be the products of incomplete translation of standard virus mRNAs.

Origin of the DI RNAs, DI transcripts, and DI polypeptides in DI_3 and DI_7 preparations. The next question was whether the transcripts of the prominent DI RNA species present in DI_3 and DI_7 preparations were responsible for giving rise to these novel polypeptides. We therefore first determined the origin of these DI_3 and DI_7 RNA segments. The prominent DI RNAs and DI-specific transcripts in the DI_3 and DI_7 preparations were analyzed by Northern blot hybridization (23) with nick-translated cDNA copies of WSN polymerase and NS genes and were found to arise from the PB_2 gene (Fig. 8). Finally, to determine whether these polypeptides were encoded by mRNAs of PB_2 origin, mRNAs were isolated from cells coinfected with DI and standard viruses and hybrid selected with different cloned cDNA copies of standard influenza virus RNA segments. The hybrid-selected mRNAs were translated in vitro, and the polypeptide products were analyzed by SDS-PAGE. We found that both DI_3 and DI_7 mRNAs were hybrid selected with cloned PB_2 DNA and that they produced corresponding DI-specific polypeptides after translation in vitro (Fig. 9).

Interfering and replicative efficiencies of different DI preparations. Since prominent DI-specific polypeptides were detected in only a few DI preparations, we wanted to see whether DI which produce prominent DI-specific polypeptides in infected cells possessed any replicative and interfering advantages over other DI, such as DI-*ts*$^+$(Tobita), which do not produce detectable novel polypeptides in infected cells. First, we quantitated the interfering property of these DI preparations by assaying the DIU per milliliter and the PFU/HAU ratios in DI_3, DI_7, and DI-*ts*$^+$(Tobita) preparations. By both methods, the observed hierarchy of increasing interfering ability was DI_3, DI_7, DI-*ts*$^+$(Tobita) (Table 1). This hierarchy suggests that neither DI_3 nor DI_7, each of which produces prominent DI-specific polypeptides, possessed any greater interfering ability than did DI-*ts*$^+$(Tobita), which does not produce any detectable DI-specific polypeptides. Furthermore, to determine the replicative advantage of one DI RNA over the other, we performed mixed-infection experiments with different combinations of these DI preparations. When the labeled RNA of the resultant virus populations was analyzed by PAGE, it was found

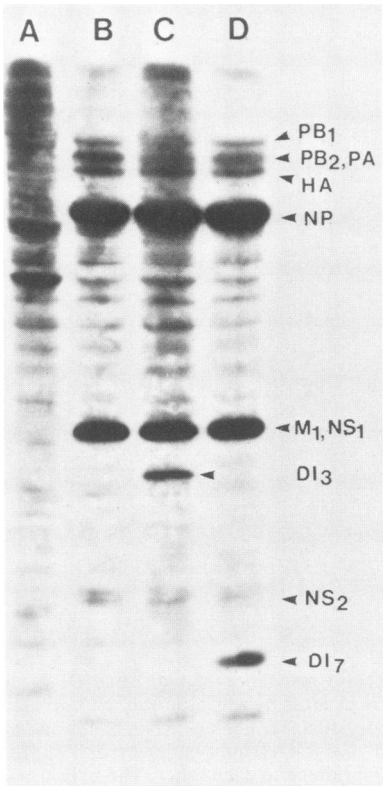

FIG. 4. DI_3 and DI_7 viral polypeptides in infected cells. Cells were infected with standard virus alone or coinfected with either DI_3 and standard virus or DI_7 and standard virus. Viral polypeptides were labeled for 1 h with [^{35}S]methionine (50 μCi/ml) at 4 h p.i. Cell lysates were analyzed by SDS-PAGE on 13% gels containing 4 M urea. Lane A, Uninfected cells; lane B, standard virus-infected cells; lane C, DI_3 and standard virus coinfected cells; lane D, DI_7 and standard virus coinfected cells. The positions of the standard virus and DI polypeptides are shown at the right.

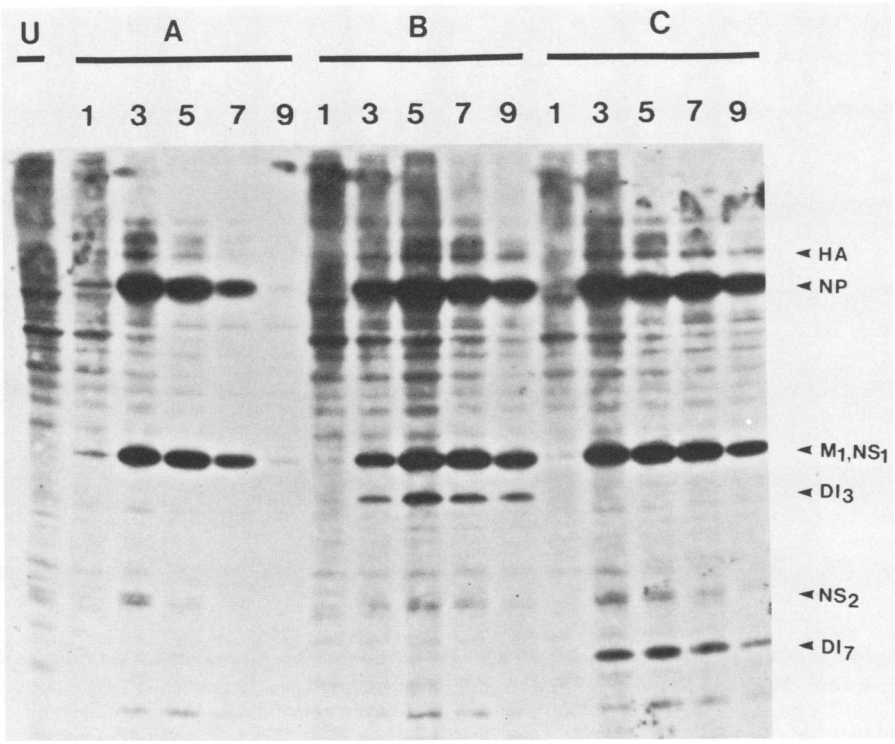

FIG. 5. Kinetics of synthesis of DI viral polypeptides in infected cells. Cells were either infected with standard virus alone or coinfected with DI and standard virus. Cells were labeled at various times with [^{35}S]methionine (50 μCi/ml) for 1 h at different times p.i. Cell lysates were analyzed by SDS-PAGE on 13% gels containing 4 M urea. Panel U, Uninfected cells; panel A, standard virus-infected cells; panel B, DI_3 and standard virus coinfected cells; panel C, DI_7 and standard virus coinfected cells. The number at the top of each lane represents the time (in hours) p.i. when the cells were labeled.

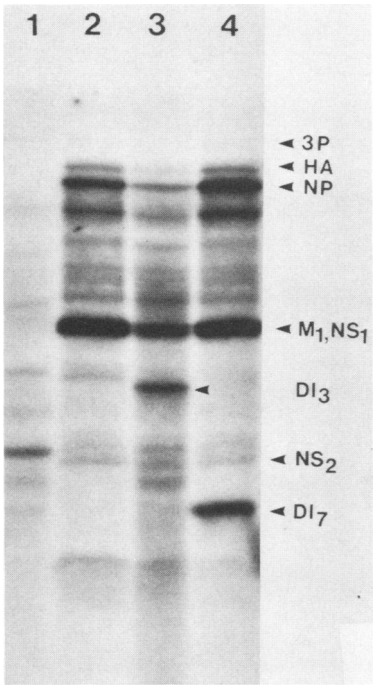

FIG. 6. In vitro translation of viral mRNAs in the wheat germ cell-free translation system. mRNAs were purified from DI- or standard virus-infected cells at 5 h p.i. and translated in vitro with wheat germ extracts. Translational products were analyzed by SDS-PAGE on 13% gels containing 4 M urea. Lane 1, Translation product of uninfected-cell mRNA; lane 2, translation product of mRNA from standard virus-infected cells; lane 3, translation product of mRNA from DI_3 and standard virus coinfected cells; lane 4, translation product of mRNA from DI_7 and standard virus coinfected cells.

that DI-ts^+(Tobita)-specific RNA was replicated more efficiently than was RNA of DI_3 or DI_7 (Fig. 10).

DISCUSSION

The data presented in this paper show that the majority, if not all, of the influenza virus DI RNAs are transcribed into poly(A)$^+$ cRNAs in cells coinfected with DI. We have previously shown (1a) that influenza virus DI RNAs are transcribed into poly(A)$^+$ cRNAs in in vitro transcription reactions and that the reaction conditions, including primer requirements, are essentially the same as those required for standard influenza viruses. Similarly, the transcripts made both in vitro and in vivo were slightly shorter than the template, as we expected. The transcripts contained poly(A) tails at the 3′ end and most likely possessed host-derived 5′ cap structures, as has been shown by primer-dependent transcription in vitro (1a), but they were otherwise faithful copies of the DI RNAs. These DI transcripts, therefore, possess the essential characteristics of standard virus mRNAs and are likely to act as functional mRNAs in infected cells. We have not shown that DI transcripts are associated with polyribosomes in infected cells, but we expect to find that this is so. The data in this paper confirm the prediction of sequence analyses (12, 19, 20; Nayak et al., in press) that influenza virus DI RNAs are capable of functioning as templates for transcription. Furthermore, the data also show that transcriptase- and replicase-binding sites

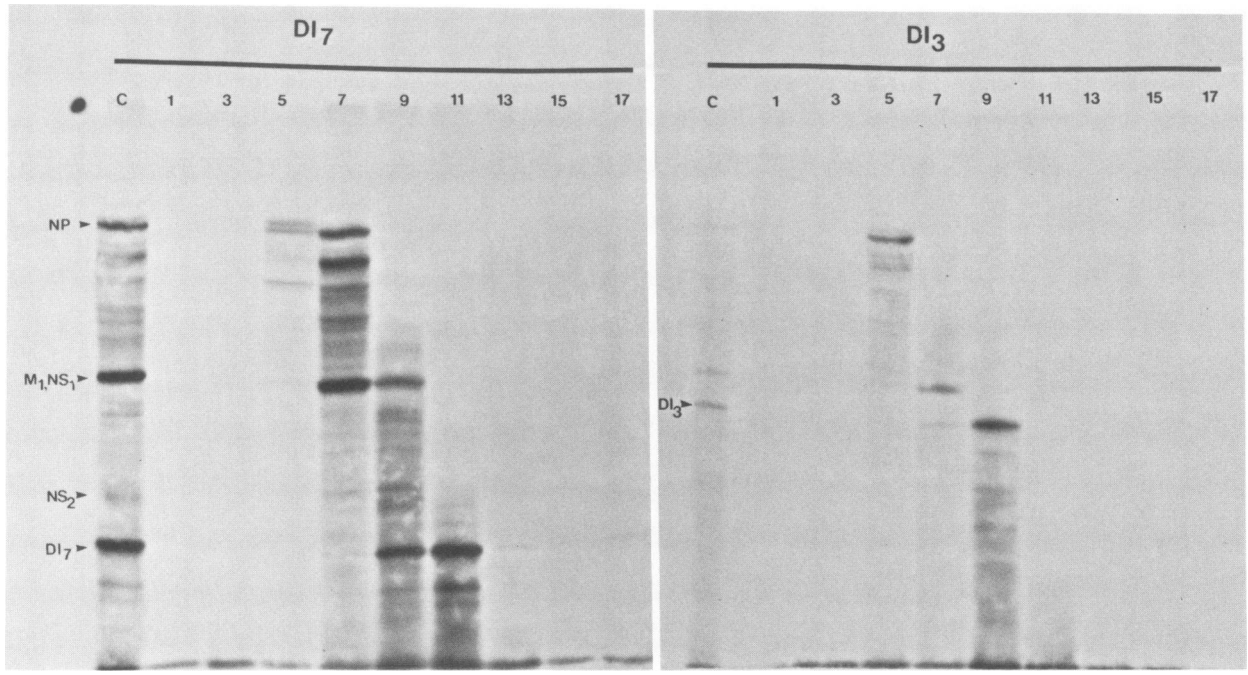

FIG. 7. Translation in vitro of DI_3 and DI_7 viral mRNAs separated on sucrose velocity gradients. DI_3 or DI_7 viral mRNA was extracted from virus-infected cells at 5 h p.i. The mRNA was dissolved in 500 μl of water, boiled for 2 min, immediately quenched in a dry ice-ethanol bath, and later adjusted to 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA. The mRNA sample was layered onto a 5 to 30% (wt/vol) linear sucrose gradient in 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA and centrifuged in a Spinco SW28 rotor at 22°C for 18 h at 27,000 rpm. Fractions (1 ml) were collected from the bottom. RNA was ethanol precipitated and translated in vitro by using the wheat germ cell-free system. Panel DI_7, Translation products of mRNA from DI_7-infected cells; panel DI_3, translation products of mRNA from DI_3-infected cells. Lanes C, Translation products of unfractionated mRNAs. The number at the top of each lane represents the density gradient fraction number.

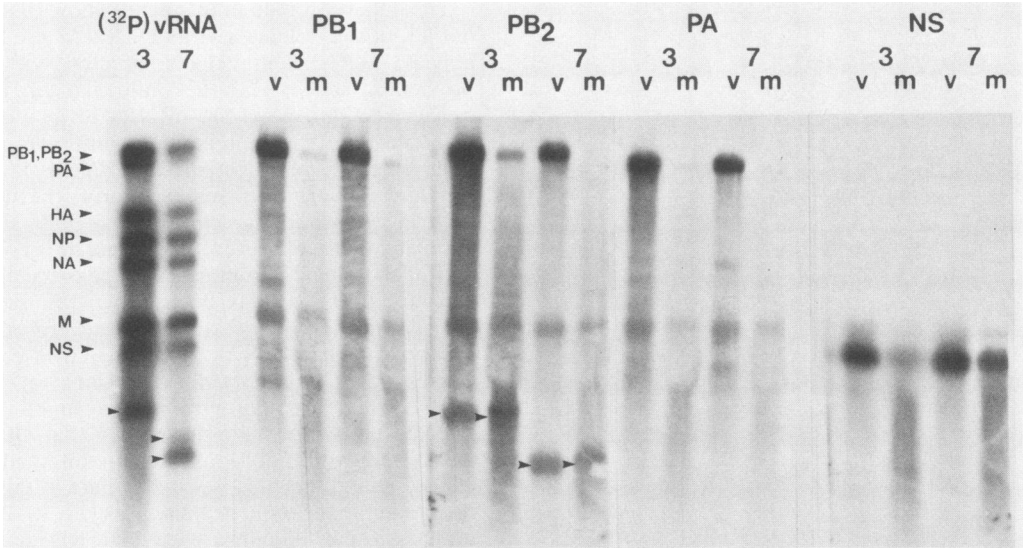

FIG. 8. Northern blot analysis of DI-specific RNAs. DI viral RNA (v) and infected-cell poly(A)$^+$ cRNA (m) were prepared with DI_3 (lanes 3) and DI_7 (lanes 7). The RNAs were electrophoresed, blotted, and hybridized as described in the text. The principal [^{32}P]cDNA probe used for each strip is given at the top (PB_1, PB_2, PA, NS). Also, each strip was hybridized with M segment [^{32}P]cDNA as an alignment marker. At the left are marker ^{32}P-labeled viral RNAs [(^{32}P)vRNA] of DI_3 and DI_7, which were electrophoresed and blotted together with the other unlabeled RNAs. The DI-specific RNAs of DI_3 and DI_7 are indicated with arrows. For both DI, the major DI-specific vRNA and mRNA hybridized with the PB_2 probe.

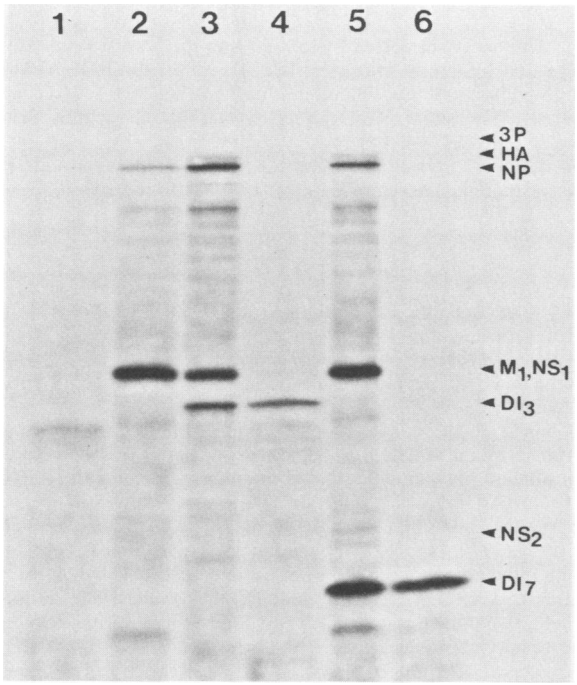

FIG. 9. Hybrid selection and translation of DI_3 and DI_7 viral RNAs. DI_3 and DI_7 viral mRNAs were hybrid selected with cDNA of the PB_2 gene segment as described in the text. The hybrid-selected mRNAs were translated in vitro by using the wheat germ cell-free system. Lane 1, Uninfected-cell mRNA; lane 2, standard virus mRNA; lane 3, DI_3 viral mRNA; lane 4, DI_3 viral mRNA hybrid selected with PB_2 cDNA; lane 5, DI_7 viral mRNA; lane 6, DI_7 viral mRNA hybrid selected with PB_2 cDNA.

are present at the 3′ termini of the DI RNA and that internal deletions of various lengths in influenza virus RNA segments do not affect the structural features required for the RNA to serve as a template for transcription or replication (Nayak et al., in press).

DI-specific transcripts were detectable by 4 h p.i. and reached maximum synthesis between 4 and 6 h p.i. Synthesis of transcripts continued beyond 6 h p.i. at a high level (data not shown), suggesting that the transcription of DI RNAs becomes amplified during the late phase of infection. Hay et al. (9) also reported that in fowl plague virus-infected chicken embryo fibroblast cells, the increased transcription of subgenomic RNA segments 9 and 10 also occurred relatively late in infection and that the transcription of segment 9, in particular, continued at its maximum rate toward the end of the infection cycle, whereas the transcription of polymerase genes reached a peak early and remained rather low and unamplified throughout the infection cycle. If this observation that the transcription of polymerase genes reaches a peak early in the infection cycle is also true in MDBK cells infected with WSN virus, it would appear that the transcription of DI RNAs and polymerase genes is not regulated by the same mechanism, suggesting that the deleted sequences may contain some structural features important in regulation of transcription. Deletion of these sequences would provide the DI RNA with a competitive edge in transcription and replication and, therefore, would be important in the mechanism of interference (Nayak et al., in press). We are now determining the kinetics of transcription of DI RNAs and their progenitor genes to elucidate the process of regulation.

The data presented in this paper indicate, for the following reasons, that the DI_3 and DI_7 polypeptides are translational products of the corresponding DI-specific mRNAs. (i) The polypeptides were found only in cells coinfected with DI_3 or DI_7 particles and not in cells infected with standard virus alone. (ii) The polypeptides were not found in cells infected with other DI preparations. Of the six DI preparations that

TABLE 1. Interfering ability of different DI preparations[a]

Virus	Interfering ability (per milliliter)			PFU/HAU ratio
	DIU	PFU	HAU	
DI-ts^+(Tobita)	4.1×10^7	8.5×10^5	1,024	8.3×10^2
DI_7	2.8×10^7	2.2×10^6	768	2.1×10^3
DI_3	1.3×10^7	7.7×10^6	1,024	1.0×10^4
Standard		6.2×10^7	2,048	3.0×10^4

[a] MDBK cells were infected with 3 DIU of each DI preparation per cell and 3 PFU of standard virus per cell or with standard virus only. Progeny viruses were harvested for the above assays at 24 h p.i. DIU were determined by the method of Janda et al. (10).

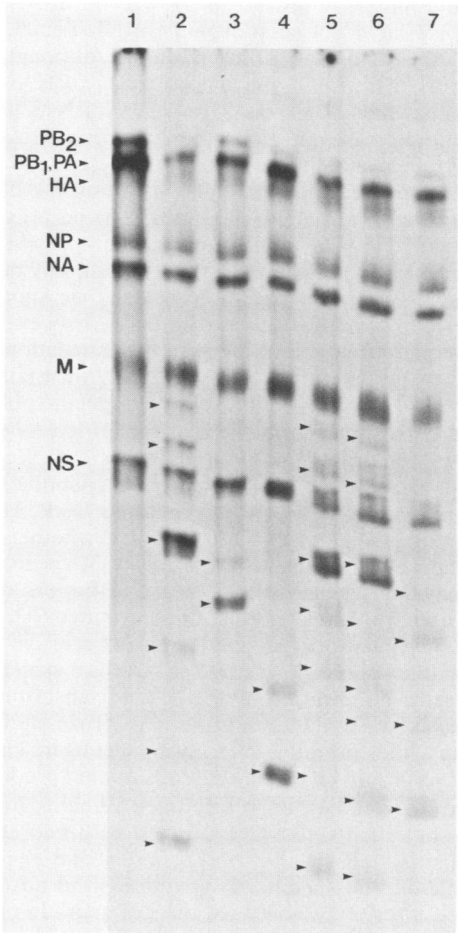

FIG. 10. Analysis of virion RNAs isolated from particles produced after mixed infections with polypeptide- and nonpolypeptide-producing DI preparations. Flasks were coinfected with 3 PFU of standard virus per cell and 3 DIU of each of the following per cell: lane 1, nothing; lane 2, DI-ts^+(Tobita); lane 3, DI_3; lane 4, DI_7; lane 5, DI-ts^+(Tobita) and DI_3; lane 6, DI-ts^+(Tobita) and DI_7; lane 7, DI_3 and DI_7. Infected cells were labeled with 0.2 mCi of ^{32}P per ml. Progeny viruses were harvested at 24 h p.i. and purified by sucrose density gradients (22). Viral RNAs were isolated from purified virus particles and separated by electrophoresis on a 3% polyacrylamide–6 M urea gel. Arrows indicate positions of DI RNAs.

we examined, only two synthesized DI-specific polypeptides. (iii) The polypeptides were produced by in vitro translation with poly(A)$^+$ mRNAs of DI-coinfected cells and not standard virus-infected cells, showing that neither of these polypeptides was a degradation product of standard PB_2 polypeptides. (iv) The polypeptides were translated from small mRNAs and, therefore, neither of these polypeptides resulted from incomplete translation of the standard PB_2 transcript. (v) Predominant DI-specific mRNAs of PB_2 origin were present in these DI-infected cells. (vi) The small mRNAs giving rise to DI-specific polypeptides could be hybrid selected from total viral mRNAs with cloned PB_2 cDNA, from which the major DI_3 and DI_7 RNAs arose. (vii) The complexity analysis showed that the DI_3 polypeptide (22 kilodaltons; ca. 170 amino acids) can be encoded by the DI_3 RNA (ca. 700 nucleotides). Similarly, the DI_7 polypeptide (8 kilodaltons, ca. 60 amino acids) can be encoded by the major DI_7 RNA (ca. 500 nucleotides). Preliminary data presented at a recent meeting by Penn and Mahy (22a) also support our conclusion by demonstrating the presence of subgenomic transcripts and polypeptides.

However, we have not seen prominent DI-specific polypeptides with the majority of DI preparations, although all of them transcribe distinct DI-specific poly(A)$^+$ cRNAs. One possibility is that these DI-specific poly(A)$^+$ cRNAs are not used as functional messages in protein synthesis. However, we think that this is an unlikely possibility because the major structural features of mRNAs, such as ribosome-binding sites and translation initiation sites, ought to be present in these DI-specific poly(A)$^+$ cRNAs. Another possibility is that these aberrant proteins are unstable and rapidly degraded. By using an in vitro translation system, we should be able to determine whether this is the case. However, the most likely explanation is that because the translation reading frame after the deletion is not selectively maintained in influenza virus DI RNAs (12, 20), these DI-specific polypeptides will not correspond to the size of the DI RNAs and in most cases will be small (20 to 60 amino acids). We have often seen minor, low-molecular-weight polypeptide bands in some DI preparations (R. K. Akkina and D. P. Nayak, unpublished data). We are now determining whether these low-molecular-weight polypeptides are also DI specific.

The mechanism by which influenza virus DI interfere with standard virus replication remains undetermined. The data presented in this and other studies (19; Nayak et al., in press) clearly show that influenza virus DI RNAs are structurally and functionally different from the 5′ nontranscribing DI RNAs of vesicular stomatitis and Sendai viruses (15) and, therefore, may interfere by a different mechanism. The role of either transcriptional products or translational products of influenza virus DI RNAs in interference remains unclear. Experiments in which cells were infected with two DI (one producing distinct DI-specific polypeptides and the other without detectable DI-specific polypeptides) did not reveal any increased interfering ability of the polypeptide-producing DI. However, without careful analysis for DI-specific, low-molecular-weight polypeptides in DI-infected cells, the role of DI-specific polypeptides cannot be excluded. If DI-specific polypeptides are involved in interference, the NH_2-terminal region of the polypeptide, which will correspond to the polypeptide of the progenitor gene and also will be common among the different DI RNAs arising from that gene, may be the site of interaction. By using synthetic peptides of various lengths, one can determine the effect of these polypeptides on transcription in vitro as well as on plus- and minus-strand RNA synthesis in infected cells.

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