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Common Sequence at the 5′ Ends of the Segmented RNA Genomes of Influenza A and B Viruses

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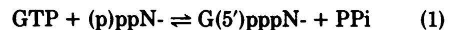
Guanylyl- and methyltransferases, isolated from purified vaccinia virus, were used to specifically label the 5′ ends of the genome RNAs of influenza A and B viruses. All eight segments were labeled with [α-^{32}P]guanosine 5′-triphosphate or *S*-adenosyl[*methyl*-^{3}H]methionine to form "cap" structures of the type m^{7}G(5′)pppN^m-, of which unmethylated (p)ppN- represents the original 5′ end. Further analyses indicated that m^{7}G(5′)pppA^m, m^{7}G(5′)pppA^{m}pGp, and m^{7}G(5′)pppA^{m}pGpUp were released from total and individual labeled RNA segments by digestion with nuclease P1, RNase T1, and RNase A, respectively. Consequently, the 5′-terminal sequences of most or all individual genome RNAs of influenza A and B viruses were deduced to be (p)ppApGpUp. The presence of identical sequences at the ends of RNA segments of both types of influenza viruses indicates that they have been specifically conserved during evolution.

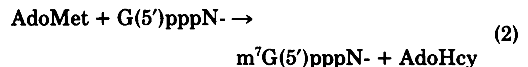
Influenza viruses possess segmented RNA genomes (8, 29) accounting for their high rates of genetic recombination (4, 13, 34). Recent studies indicate that type A strains have eight negative-strand RNA segments (2, 18, 28, 30, 33) in agreement with the seven to eight recombination groups of temperature-sensitive mutants of influenza A viruses (35). Moreover, from the analysis of recombinants between two strains of influenza A viruses, the protein corresponding to each of the eight genes has been identified (24, 26, 31, 32). Structural studies indicate that each RNA segment possesses a unique nucleotide sequence (6, 14, 18) but that all or most of the segments contain pppA at the 5′ end (39) and uridine at the 3′ end (17).

Considerably less is known about the RNA genomes of influenza B and C than about influenza A viruses. Influenza B viruses also undergo genetic recombination (2, 36), and eight RNA segments have been separated (30). Thus far, only four to six RNA segments have been resolved from influenza C virus (5, 7, 30). Recombination between group A, B, and C viruses has not been observed.

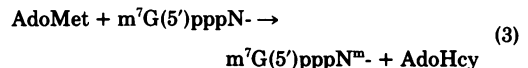
We considered that a recently described method of specifically labeling the 5′ end of RNA by using "capping" and methylating enzymes isolated from vaccinia virus (9, 19, 23) might prove useful for studies with influenza RNAs. These enzymes catalyze the transfer of the [α-^{32}P]guanosine 5′-monophosphate (GMP) moiety of guanosine 5′-triphosphate (GTP) and methyl groups from *S*-adenosyl[*methyl*-^{3}H]-methionine (Ado[*methyl*-^{3}H]Met) to RNA molecules containing a di- or triphosphate at the 5′ end. The reactions occur in the following order:



$$\text{GTP} + \text{(p)ppN-} \rightleftharpoons \text{G(5')pppN-} + \text{PPi} \quad (1)$$



$$\text{AdoMet} + \text{G(5')pppN-} \rightarrow \text{m}^7\text{G(5')pppN-} + \text{AdoHcy} \quad (2)$$



$$\text{AdoMet} + \text{m}^7\text{G(5')pppN-} \rightarrow \text{m}^7\text{G(5')pppN}^\text{m}\text{-} + \text{AdoHcy} \quad (3)$$

By labeling influenza virus RNAs in this manner and characterizing the oligonucleotides released by digestion with specific ribonucleases, a single trinucleotide sequence present at the 5′ ends of most or all RNA segments of influenza A and influenza B viruses was identified.

MATERIALS AND METHODS

Purification of influenza RNAs. Influenza A/PR/8/34 (H0N1), A/MS-SL/2996/76 (H3N2), and B/Lee/40 viruses were grown in 10-day-old embryonated eggs. All viruses were labeled in the presence of [^{32}P]phosphate in MDCK (canine kidney) cells and purified as described previously (30, 31). Methods used for isolation of the RNA and electrophoresis in 6 M urea-polyacrylamide gels were also reported previously (25, 26).

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Labeling of RNAs with capping enzymes. Routinely, an enzyme mixture containing guanylyltransferase, guanine-7-methyltransferase, and nucleoside-2′-methyltransferase extracted from vaccinia virus cores and freed of nucleic acids by passage through a DEAE-cellulose column (9) was used. In some experiments, a highly purified guanylyltransferase-guanine-7-methyltransferase complex (19) was substituted. Neither preparation nicked radioactively labeled rRNA, as measured by sucrose gradient sedimentation. For methyl labeling the 5′ ends of influenza virus RNAs, a 0.5-ml reaction mixture containing 0.05 M Tris-hydrochloride (pH 7.5), 1 mM dithiothreitol, 2 mM GTP, 2 mM $MgCl_2$, 2 μM Ado[*methyl*-^{3}H]Met (7 Ci/mmol), 25 μg of RNA, and 25 μl of enzyme was incubated at 37°C for 15 to 30 min. For ^{32}P labeling, a 0.1-ml reaction mixture containing 0.05 M Tris-hydrochloride (pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, 50 μM *S*-adenosylmethionine (AdoMet), 2 μM [α-^{32}P]GTP (135 to 250 Ci/mmol), 5 μg of RNA, and 5 μl of enzyme was incubated at 37°C for 15 to 30 min. "Capping" is incomplete under the latter conditions because of the low concentration of high-specific-activity GTP used.

Analysis of reaction products. Approximately 50 μg of carrier yeast RNA was added to the reaction mixture, which was then extracted with sodium dodecyl sulfate-phenol-chloroform (27), precipitated with ethanol, passed through a G-75 Sephadex column (1 by 25 cm) equilibrated with 0.05 M ammonium acetate or neutral triethylamine carbonate, and lyophilized.

RNase digestions, electrophoresis, and homochromatography of oligonucleotides were carried out essentially as described by Brownlee (3). Methods for analysis of cap structures and methylated nucleosides were previously reported (22). Fluorographs were made by using Kodak XR-2 film and DuPont Cronex intensifiers at −70°C. Comigration of ^{3}H- and ^{32}P-labeled oligonucleotides was demonstrated by outlining the spot revealed by ^{32}P fluorography and then by scintillation counting of material eluted from the spot and from the four quadrants of concentric circles drawn around the spot.

Materials. Marker nucleosides, nucleotides, and dinucleoside triphosphates were purchased from Sigma or P-L Biochemicals. AdoMet was from Boehringer Mannheim Corp. [α-^{32}P]GTP was obtained from either Amersham/Searle or New England Nuclear, and Ado[*methyl*-^{3}H]Met was obtained from the latter source. Commercial enzymes were purchased from Worthington Biochemicals Corp., except for nuclease P1, which came from Yamasa Shoyu Co. (Chosi, Japan). Cellogel strips, DEAE-81 paper, and cellulose-DEAE (9:1) thin-layer plates (20 by 40 cm) were purchased from Kalex Scientific, Whatman, and Analtech, respectively.

RESULTS

5′-Terminal sequence of influenza A virus RNAs. Initial experiments indicated that RNA extracted from purified influenza A/PR/8/34, A/MS-SL/2996/76, and B/Lee/40 viruses served as acceptors for the guanylyltransferase isolated from vaccinia virus. All eight RNA segments of influenza A/PR/8/34 and B/Lee/40 viruses propagated in embryonated eggs were enzymatically labeled with [α-^{32}P]GTP, as indicated by radioautographs of polyacrylamide gels (Fig. 1). For comparison, influenza RNAs uniformly labeled with ^{32}Pi in vivo in MDCK cells were analyzed in parallel gels. Enzymatic labeling appeared to produce little degradation of the RNA species. Identical results were obtained by using either the mixture of enzymes obtained after passage of soluble extracts of vaccinia virus cores through a DEAE-cellulose column or highly purified guanylyltransferase-guanine-7-methyltransferase complex. The nonmolar amounts of the largest RNA species in both uniformly and 5′-labeled preparations appear to be due to the presence of defective particles.

Enzyme-catalyzed incorporation of Ado[*methyl*-^{3}H]Met into influenza A virus RNA was dependent upon the presence of GTP (not shown) since guanylylation (cap formation) precedes both methylation steps. The resistance of cap structures to the action of nuclease P1 and bacterial alkaline phosphatase was used to demonstrate the specific location of the methyl groups. After such a digestion, the methyl-labeled material from influenza A virus RNA cochromatographed with m^{7}G(5′)pppA^m and incompletely methylated m^{7}G(5′)pppA (Fig. 2A). Upon further digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase, 7-methylguanosine and 2′-*O*-methyladenosine were identified (Fig. 2B). From these results we deduced that the original 5′ ends of influenza A virus RNAs were (p)ppA.

To identify the nucleotides adjacent to the 5′ end, samples of the methyl-labeled influenza A virus RNAs were digested with either RNase T1 (specific for cleavage after guanosine) or RNase A (specific for cleavage after pyrimidines) and then chromatographed on DEAE-cellulose columns under conditions in which oligonucleotides are separated by chain length (37). A single RNase T1 product eluted with a net negative charge of approximately −5, consistent with the structure m^{7}G(5′)pppA^{m}pGp (not shown). The succeeding nucleoside was identified as a pyrimidine since the RNase A product eluted with almost one additional net negative charge. To determine whether the pyrimidine was C or U, the m^{7}G residue was removed from intact methyl-labeled influenza A RNA by periodate oxidation and β-elimination (22). After this procedure, the terminal triphosphate was removed with bacterial alkaline phosphatase, and the resulting RNA ending in A^{m}pGpPyrpN- was digested with RNase T1 or RNase A. When the methyl-labeled oligonucle-

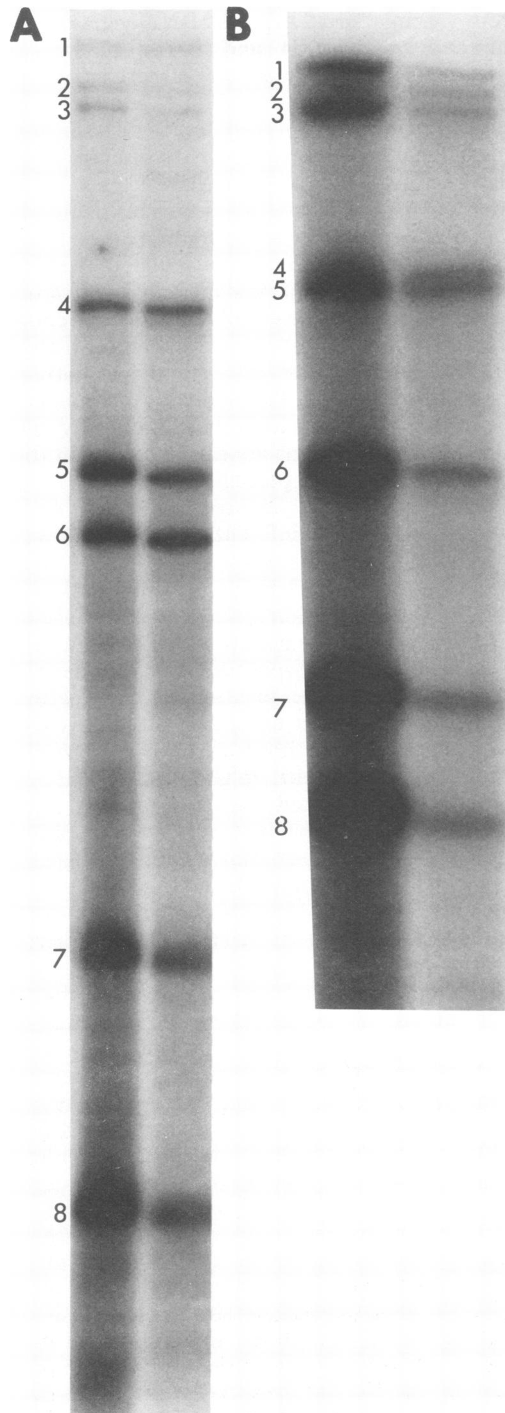

FIG. 1. *Radioautography of influenza virus RNA segments separated by polyacrylamide gel electrophoresis. (A) Influenza A/PR/8/34 virus. Left: RNA of virus grown in embryonated eggs and then labeled with "capping" enzymes in vitro. Right: RNA of virus uniformly* ^{32}*P-labeled in vivo in MDCK cells. (B) Influenza B/Lee/40 virus. Left: RNA of virus grown in embryonated eggs and then labeled with "capping" enzymes in vitro. Right: RNA of virus uniformly* ^{32}*P-labeled in vivo in MDCK cells.*

otides were subjected to paper electrophoresis at pH 3.5, the mobilities of the RNase A and T1 products (Table 1) indicated that the pyrimidine was U since A^{m}pGpUp, but not A^{m}pGpCp, would be expected to migrate ahead of A^{m}pGp (3). Similarly, the much slower migration of the RNase A product compared with that of the RNase T1 product upon DEAE-paper electrophoresis in 7% formic acid was consistent with the presence of a U residue (Table 1).

Since the proposed (p)ppApGpUp sequence of influenza A/PR/8 virus RNA is identical to that described for satellite tobacco necrosis virus (STNV) RNA (15, 38), the latter was used as a marker to confirm our results. By using the guanylyl- and methyltransferases of vaccinia virus, the 5′ ends of STNV RNA were labeled with Ado[*methyl*-^{3}H]Met, and the 5′ ends of influenza A/PR/8 virus RNAs were labeled with [α-^{32}P]GTP. Separate RNase A and T1 digests were prepared, and the products were found to co-elute from DEAE-cellulose columns (Fig. 3A and B), confirming the proposed (p)ppApGpPyrp sequence. Moreover, the capped oligonucleo-

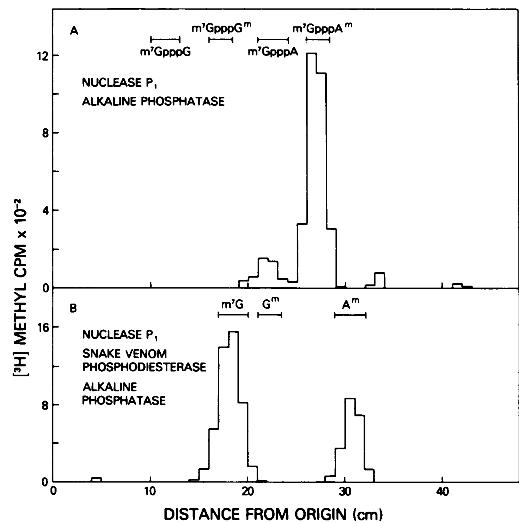

FIG. 2. *Paper chromatography of the digestion products of methyl-labeled influenza A/PR/8/34 virus. (A) RNA was digested with nuclease P1 and then bacterial alkaline phosphatase. Descending paper chromatography was in isobutyric acid–0.5 M* NH_4OH *(10:6). (B) RNA was digested with nuclease P1 and then with a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase. Descending paper chromatography was in isopropanol–water–concentrated* NH_4OH *(7:2:1).*

TABLE 1. *Electrophoretic mobilities of oligonucleotides obtained from the 5′ ends of influenza A/PR/8 virus RNAs*[a]

Oligonucleotide	Paper electrophoresis		DEAE-paper electrophoresis	
	cm	R_{Up}	cm	R_{Up}
A^mpGp	15.0	0.73	32.5	0.68
A^mpGpPyrp	20.5	1.0	12.5	0.25

[a] Paper electrophoresis was at pH 3.5 and DEAE-paper electrophoresis was in 7% formic acid. A^mpGp and A^mpGpPyrp were obtained by RNase T1 and RNase A digestions, respectively, of β-eliminated and phosphatase-treated methyl-labeled influenza A/PR/8 virus RNA. R_{Up} is the mobility relative to that of Up.

tides of STNV RNA and influenza A virus RNA migrated similarly upon Cellogel electrophoresis at pH 3.5 followed by transfer to DEAE-cellulose paper and electrophoresis in 7% formic acid (Fig. 4). Comigration was demonstrated by eluting the ^{3}H-labeled m^{7}G(5′)pppA^{m}pGpU oligonucleotide from STNV RNA at the precise position of the ^{32}P-labeled oligonucleotide from influenza virus RNA. Similar results were obtained when the doubly labeled RNAs were digested with RNase A and the products were analyzed by Cellogel electrophoresis in the first dimension and homochromatography in the second (Fig. 4). From these results we conclude that the 5′-terminal trinucleotide of influenza A virus RNA is identical to the (p)ppApGpUp end of STNV RNA.

The finding of a single spot on both two-dimensional systems suggested that all eight segments of influenza A/PR/8/34 virus RNA have the same 5′-terminal trinucleotide. Additional evidence to support this was obtained by analyzing RNA segments that had been labeled with [α-^{32}P]GTP and then separated by gel electrophoresis as in Fig. 1. It was necessary to elute the first three RNA segments together from the polyacrylamide gel, but the next five were eluted individually. The electrophoretic mobilities (DEAE-paper, 7% formic acid) of the oligonucleotides obtained from the individual RNA segments coding for hemagglutinin, nucleoprotein, neuraminidase, M, and NS protein, and from the mixture of the RNAs coding for the three P proteins were the same after digestion with nuclease P1, with RNase T1, and with RNase A (not shown).

5′-Terminal sequence of RNAs from other influenza A and B viruses. A similar approach was used to identify the 5′-terminal trinucleotides of the RNAs of influenza A/MS-SL/2996/76 and B/Lee/40 viruses (Fig. 1). From Fig. 5 it is apparent that the electrophoretic migration on DEAE-paper of the capped oligonucleotides obtained upon digestion with nuclease P1, RNase T1 and alkaline phosphatase, and with RNase A and alkaline phosphatase were the same as those obtained upon digestion of influenza A/PR/8/34 virus and STNV RNAs.

DISCUSSION

Capping and methylating enzymes (guanylyl- and methyltransferases), isolated from vaccinia virus, have been used to label the 5′ ends of the negative-strand genome RNA segments of influenza A and B viruses. Formation of terminal m^{7}G(5′)pppA^m- structures confirmed previous evidence (39) that influenza A virion RNA has pppA- termini. Why the negative-strand RNAs have free triphosphate ends whereas the complementary mRNA's are capped (16) is unknown, although a similar situation exists for reovirus (11) and vesicular stomatitis virus (12, 23). The present studies clearly show that the negative-strand RNAs of influenza virus can be capped, at least by the vaccinia virus guanylyltransferase, as can the negative-strand virion RNA of vesicular stomatitis virus (R. A. Lazzarini, personal communication). Possibly, the capping enzymes used by RNA viruses exhibit se-

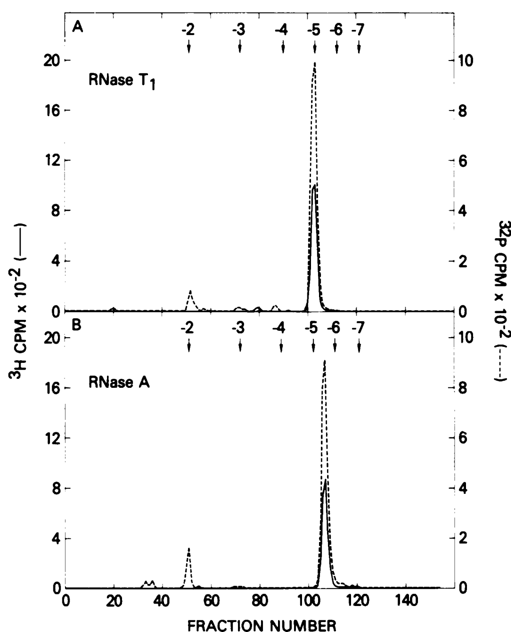

FIG. 3. *DEAE-cellulose column chromatography of the digestion products of influenza A/PR/8/34 virus RNA. Influenza virus RNA labeled with [α-^{32}P]GTP (---) and satellite tobacco necrosis virus RNA (——) labeled with Ado[methyl-^{3}H]Met were digested with RNase T1 or RNase A and chromatographed on DEAE-cellulose columns in 7 M urea at pH 7.6 as described (22).*

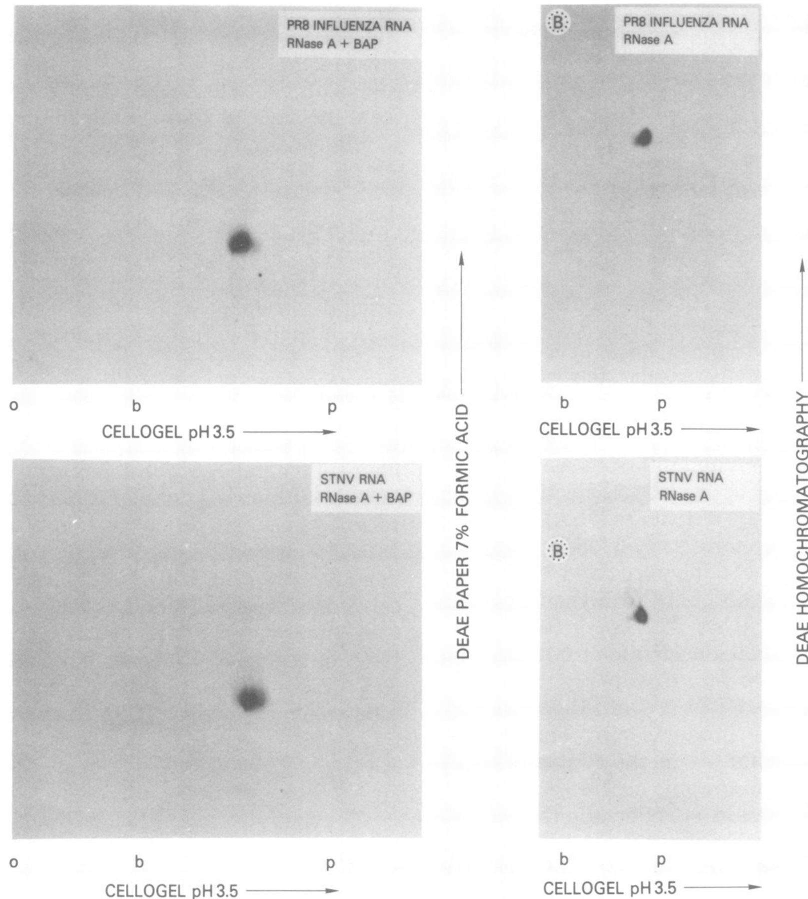

FIG. 4. *Two-dimensional analysis of the 5'-terminal oligonucleotide of influenza A/PR/8/34 virus RNA. Influenza virus RNA and STNV RNA were "capped" with [α-^{32}P]GTP. In addition, STNV RNA was labeled with Ado[methyl-^{3}H]Met and mixed with both ^{32}P-labeled preparations. After digestion with RNase A and bacterial alkaline phosphatase or RNase A alone, electrophoresis was carried out on Cellogel at pH 3.5 in the first dimension and either on DEAE-paper in 7% formic acid or by DEAE-thin-layer homochromatography for the second dimension. Fluorographs are shown. The position of blue xylene cyanol dye is indicated by the letter B.*

quence specificity or the negative strands are made in a way or location that does not permit capping to occur. Analogous explanations are also needed to account for the lack of poly(A) at the 3' end of negative-strand RNAs.

From a characterization of the products obtained by digestion of total and individual labeled RNA segments with RNase T1 and RNase A, evidence was obtained that the terminal pppA is followed by guanosine and then uridine. Coincidently, the same (p)ppApGpUp sequence is present in satellite tobacco necrosis virus RNA (15, 38); this served as a marker to confirm our results. We do not yet know the position at which the sequences of the individual influenza RNA segments diverge. Previously, Horst et al. (14) reported that a 29-unit oligonucleotide appeared to be present in all three general size classes of RNA from the WSN strains of influenza A virus. Since the oligonucleotide was obtained by RNase T1 digestion, it could not include the 5'-terminal nucleotides but might lie immediately adjacent to them.

Conservation of identical trinucleotide or longer sequences at the 5' ends of the segmented genomes of influenza A and B viruses suggests an important selection factor during evolution. One possibility is that the RNA polymerase exhibits template specificity, as has been shown for bacteriophage QB (10). Another possibility is that a specific RNA sequence is needed for packaging the genome RNAs. The situation with double-stranded RNA viruses is similar in that the segments of cytoplasmic polyhedrosis virus

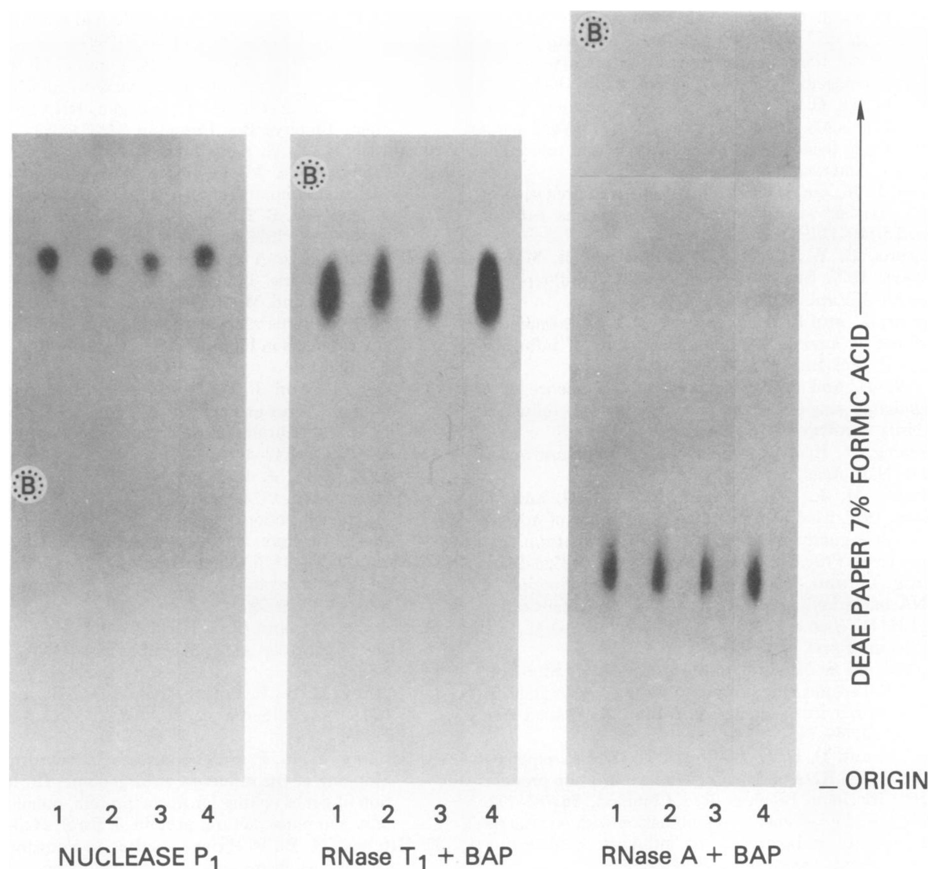

FIG. 5. *DEAE-paper electrophoresis of RNA digestion products from different strains of influenza virus. RNAs isolated from the influenza A virus strains A/PR/8/34 (1) and A/MS-SL/2996/76 (2), type B/Lee/40 (3), and satellite tobacco necrosis virus (4) were "capped" with [α-^{32}P]GTP and digested with nuclease P1 (left panel), with RNase T1 and bacterial alkaline phosphatase (middle panel) and with RNase A and bacterial alkaline phosphatase (right panel) and then analyzed by one-dimensional electrophoresis on DEAE-paper in 7% formic acid. Fluorographs are shown. The position of blue xylene cyanol dye is indicated by the letter B.*

(20) and reovirus (21) have identical ends, although the sequences are different for the two viruses. It may be useful to extend this type of investigation to include additional strains of influenza virus with different host ranges (e.g., influenza C virus), other myxoviruses, and other groups of segmented RNA viruses such as bunyaviruses.

It is interesting to note that reassortment between influenza A and B virus strains has not been observed despite the sharing of many characteristics, including identical 5′ ends, the same number of gene segments, and similar functions of the gene products. Lack of reassortment may be explained upon further sequence analysis of the genome RNAs.

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ADDENDUM IN PROOF

The 5′-terminal labeling procedure used in this report, because of its specificity for di- or triphosphate ends of RNA, should be useful in conjunction with recently described sequencing methods [H. Donis-Keller, A. M. Maxam, and W. Gilbert, Nucleic Acids Res. 4:2527–2538, 1977; A. Simoncsits, G. G. Brownlee, R. S. Brown, J. R. Rubin, and H. Guilley, Nature (London) 269:833–836, 1977].

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