Origin of Small RNA in von Magnus Particles of Influenza Virus

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A clone of recombinant virus obtained from the cross between WSN and Hong Kong strains of influenza virus gave rise to progeny containing predominantly von Magnus particles. In the electropherogram of virus RNA, the P3 gene was markedly diminished, and a new species of RNA (extra RNA) was present in addition to eight gene segments. The origin of the extra RNA was studied by two-dimensional gel electrophoresis of T1 RNase-generated oligonucleotides. Four out of five large oligonucleotide spots present in the extra RNA matched to those contained by the P3 gene. It was concluded that the extra RNA was derived from the P3 gene probably by deletion. The possible origin of the spot which was present in the extra RNA but not in eight gene segments including P3 was discussed.

Serial undiluted passage of many animal viruses results in the generation of defective virus particles which interfere with the production of standard infectious viruses. Such defective interfering (DI) particles of several viruses have been shown to contain only a part of the genome of the standard virus as reviewed by Huang and Baltimore (5). von Magnus particles of influenza virus (22) have many properties common to DI particles in other viruses. Earlier electrophoretic analyses of virus RNA indicated that von Magnus virus was characterized by the loss of the largest RNA segment(s) and the appearance of small, heterogeneous RNA (1, 3, 16). Recently, Nayak and co-workers found that the small RNA was resolved into several distinct species and that at least a major species of them was hybridizable with DNA complementary to influenza virus RNA, thus establishing its viral origin (12). They suggested that it was these small RNAs that were responsible for the interference by von Magnus virus. RNA species which are found in von Magnus particles but not necessarily in standard virus preparations (extra RNA) might represent a part of the genome as in DI particles in other viruses. Because of the segmented nature of the influenza virus genome, it seemed pertinent to look for the origin of the extra RNA. In the study described below, we tried to answer the following questions: whether an extra RNA segment was derived from a particular gene segment and, if so, from which segment.

MATERIALS AND METHODS

Virus and cells. The recombinant viruses (H3N1) were prepared from A/Aichi/2/68(H3N2) and WSN(H3N1) in MDCK and MDBK cells. The media employed for preparation and maintenance of cells have been described previously (19, 20).

Isolation of 32P-labeled viral RNA. MDBK cells were infected with about 1 PFU of viruses per cell. After adsorption for 30 min at room temperature, inoculum was removed and phosphate-free maintenance medium containing 20 to 40 µCi of sodium 32P, per ml was added. When complete cytopathic effect was observed, culture fluid was collected. Viral RNA was extracted by the method described by Palese and Schullman (14).

Polyacrylamide gel electrophoresis. Electrophoresis of RNA was done in either 2.6 or 2.8% polyacrylamide slab gel at 120 V at room temperature (21).

Extraction of RNA segments. After electrophoresis, the gel slab was covered with Saran Wrap and X-ray film. The gel slab was placed on the developed film, and pieces of gel corresponding to RNA segments were cut out. The excised gel was transferred to a tube containing 1 ml of elution buffer (0.1 M NH4Cl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 50 µg of yeast RNA per ml) and incubated for 20 to 48 h at 37°C. The fluid was filtered through glass wool. RNA was precipitated with 2.5 volumes of ethanol. Water-soluble polyacrylamide coprecipitated with RNA. RNA was separated from polyacrylamide by precipitation with perchloric acid (6). Precipitated RNA was dissolved in 0.3 ml of 100 mM Tris-hydrochloride (pH 8.0) and 20 mM KCl and again precipitated with ethanol. Of the RNA originally present in the gel, 30 to 40% was recovered in the final precipitate.

Oligonucleotide map of RNA. Nuclease digestion of RNA and separation of oligonucleotides by two-dimensional gel electrophoresis were done by the method described previously (2, 11).

RESULTS

Extra RNA of von Magnus virus. During the course of a study for another purpose, we
collected many clones of the recombinant virus which had received five genes, P₁, P₃, P₅, HA, and NP, from the Hong Kong parent and three, NA, M, and NS, from the WSN parent. One of them, clone 50A, had a low PFU-to-hemagglutinin ratio (hemagglutinin titer, 1:2,048; 2.7 × 10⁷ PFU/ml) compared with other clones. Furthermore, it interfered with the formation of plaques by other clones of the same genotype. The electropherogram of the RNA showed that 50A contained a prominent extra RNA which migrated slightly ahead of the NS gene and that the P₃ gene was markedly reduced (Fig. 1). The same electrophoretic pattern was obtained invariably in different occasions as long as labeled virus was prepared from the same virus stock of this clone. Although virus stocks of all clones were grown from a single plaque in a similar way, virus stock 50A apparently contained von Magnus particles in a higher proportion than did other clones. A similar difference in the content of DI particles had also been described for clones of Sendai virus (9). Because of simplicity of analysis, we chose this clone, giving rise to a single extra RNA species for study. Another clone, 50B, which contained no extra RNA, with full complement of eight gene segments in normal amounts and a high PFU/hemagglutinin ratio (hemagglutinin titer, 1:1.024; 2 × 10⁸ PFU/ml), was used as the standard virus.

Oligonucleotide maps of RNA. Eight gene segments were isolated from 50B and the extra RNA segment from 50A as described above. RNA of the unfractionated entire genome of 50B and isolated segments were treated with RNase T₁ and T₂-digested oligonucleotides were separated by two-dimensional gel electrophoresis and autoradiographed. On the autoradiogram of the entire genome of 50B, 47 spots of relatively large oligonucleotides were chosen as unique spots and numbered in decreasing order of molecular size (Fig. 2). The oligonucleotide patterns of the P₃ gene (Fig. 3A and B) and the extra RNA (Fig. 3C and D) are shown. By comparing the autoradiogram of an individual gene segment with that of the entire genome, the oligonucleotide segments were identified on the map (Table 1). The oligonucleotide map of the P₃ gene (Fig. 3A) contained several faint spots derived from the P₁ gene (spots 11, 12, 13, 14, 21, etc.) in the background. These spots resulted from a small amount of the P₁ gene contained in the excised gel corresponding the P₃ gene because of the close proximity of the two RNA segments. The oligonucleotide pattern of the extra RNA shows only a very few spots, five spots of relatively large oligonucleotides and three of smaller oligonucleotides. All these spots other than spot a were present also in the map of the entire genome of 50B. Because spots of smaller oligonucleotides were often shared by more than one gene segment, they were not included in Table 1. The extra RNA did not contain any unique spot present in the P₁, P₃, HA, NA, M, and NS genes. On the other hand, four out of five spots in the extra RNA segment matched to those of the P₅ gene. One spot (spot 9) was contained by the NP as well as by the P₃ gene. Because spot 9 was more intense than other spots in the map of the entire genome but not in the map of either the NP or the P₅ gene, it was likely that NP and P₅ contained a stretch of oligonucleotide of a similar, if not identical, base composition. Therefore, it was concluded that the extra RNA had arisen from the P₅ gene by deletion. Spot a present in the extra RNA but not in P₅, then, might represent the sequence which was altered by the deletion. In the map of the entire genome of 50A, which is not presented here, spot a was also seen and the intensity of unique spots contained by the P₅ gene was markedly diminished, the pattern as expected from

![Fig. 1. Polyacrylamide gel electrophoresis of virus RNA. RNA was extracted from ³²P-labeled virus and subjected to electrophoresis as described in the text. The assignment of eight individual RNA segments to specific viral proteins was based on the previously established maps of WSN and Hong Kong strains of influenza A virus (13, 15, 17). The arrow indicates the extra RNA segment.](http://jvi.asm.org/)
Fig. 2. Oligonucleotide map of the RNA of 50B. RNA was extracted from 32P-labeled virus. T1 nuclease digestion and two-dimensional gel electrophoresis were described previously (2, 11). Large oligonucleotides were numbered from 1 to 47 (unique spots). Spot a is the oligonucleotide found in the extra RNA in Fig. 3. (×) indicates the position of dye markers, xylene cyanol and bromophenol blue.
FIG. 3. Oligonucleotide maps of the P3 gene segment isolated from 50B (A and B) and the extra RNA segment isolated from 50A (C and D). The diagram (B and D) shows the unique oligonucleotide spots in black on the background of the pattern of the entire genome taken from Fig. 2B.
the electropherogram shown in Fig. 1. Otherwise it was identical to the map of the entire genome of 50B.

**DISCUSSION**

The results presented above have shown that the extra RNA segment of recombinant virus 50A which contained von Magnus particles in high proportion was derived from the P₃ gene. We considered it unlikely that the extra RNA was a product formed by a random breakage of the P₃ gene for the following reasons. First, the complexity of the extra RNA estimated from the number of oligonucleotide spots was about one-fifth of that of the P₃ gene. Second, different preparations of 50A always contained the extra RNA of the same size whenever the same stock virus was inoculated at the same concentration into MDBGK cells. The generation with such a high reproducibility did not seem compatible with a random mechanism. The genesis of the extra RNA was, therefore, most easily explained by the deletion of a certain nucleotide sequence as in DI RNA in other viruses (7-10). If such was the case, the deleted portion might well have been contiguous to the newly generated oligonucleotide sequence detected as spot a. The oligonucleotide map of DI RNA of Semliki Forest virus was found to contain a novel oligonucleotide spot (spot X) absent in the map of standard virus RNA (18). It is also possible that the extra RNA was a snap-back molecule and the spot a contained the extragenomic sequence as envisioned by Huang (4). However, the finding of Nayak and co-workers that DI RNA, analogous to the extra RNA in this study, did not self-anneal argues against the presence of a snap-back RNA molecule in von Magnus particles of influenza virus (12). Our results suggested that the appearance of the extra RNA derived from the P₃ gene resulted in the reduced production and/or incorporation of P₃ gene into the virion. Whether such a specific inhibition by the extra RNA is a general phenomenon in the production of von Magnus virus is currently under investigation.

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**LITERATURE CITED**


