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Extra RNAs of von Magnus Particles of Influenza Virus Cause Reduction of Particular Polymerase Genes

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Extra RNAs, or RNA species other than eight gene segments, in von Magnus particles of the influenza virus WSN strain were studied by polyacrylamide gel electrophoresis and oligonucleotide mapping. From the original virus stock, various cloned stocks were obtained, each giving rise to a characteristic set of extra RNAs. One cloned virus stock contained a large number of von Magnus particles. The RNA pattern was characterized by two prominent extra RNAs (X_1 and X_2) and a decrease in the content of two polymerase genes, P_1 and P_2. Segregation of the two extra RNAs was carried out by coinfection of cells with a von Magnus particle and infectious virions. The results showed that the presence of one of the extra RNAs (X_2) was associated with a reduction in the amount of the P_1 gene and that the presence of the other extra RNA (X_1) was associated with a reduction in the amount of the P_2 gene. Oligonucleotide mapping showed that both extra RNAs, X_1 and X_2, were derived from the P_1 gene. The results suggested that an extra RNA did not necessarily cause the reduction of the progenitor polymerase gene, but might cause the reduction of another polymerase gene.

Since the introduction of polyacrylamide gel electrophoresis in the presence of a high concentration of urea in the analysis of influenza virus RNA, it has often been observed that preparations of von Magnus particles contain RNA species of various size classes not necessarily present in the genome of the standard virus (4, 17, 18, 20). We termed these RNA species extra RNAs and presented evidence based on oligonucleotide mapping that the extra RNA of a von Magnus virus preparation was generated from a polymerase gene by deletion (17) in a manner similar to the generation of the abbreviated RNA in defective interfering particles of other animal viruses, as reviewed by Huang and Baltimore (9). Extra RNAs were found to be associated with a reduction in the amount of large gene segments (4, 10, 17, 18). The above preparation of von Magnus particles studied by us contained an extra RNA derived from the P_3 gene, and the P_3 gene itself was markedly diminished in intensity on the electropherogram. Therefore, we postulated that an extra RNA caused the loss of a gene from which it was derived (17). We found that a virus stock of the WSN strain reproducibly gave rise to progeny containing multiple extra RNAs. During an attempt to obtain the standard virus free from extra RNA by cloning the original virus stock, we collected various cloned virus stocks, each giving rise to a characteristic set of extra RNAs. Among them was a cloned stock characterized by two prominent extra RNAs and two reduced polymerase genes, P_1 and P_2, which appeared to offer a suitable material for examining the possible specific relationship between an extra RNA and the loss of a polymerase gene, as postulated. This report describes an attempt to segregate two extra RNAs for the purpose of determining whether a particular extra RNA is associated with the loss of a polymerase gene and, if so, whether the extra RNA is derived from the polymerase gene.

MATERIALS AND METHODS

Virus strains and cells. The WSN strain of influenza virus and the recombinant virus R107, obtained from the cross between the WSN and Hong Kong (Aichi/2/68) strains, were employed in this study. Electrophoretic analysis showed that R107 had received the P_1 and NP genes from the Hong Kong parent and six other genes from the WSN parent. Various clones obtained from WSN virus are described in the text. Viruses were propagated in MDBK cells, and infectivity was determined by plaque assay on MDBK cells (25).

Preparation of ^{32}P-radiolabeled viral RNA. Monolayer cultures of MDBK cells were infected at a multiplicity of about 10 PFU/cell, unless otherwise stated. After adsorption for 30 min at room temperature, the inoculum was removed and the monolayers were rinsed three times with phosphate-free Earle balanced salt solution, overlaid with phosphate-free maintenance medium containing $^{32}P_i$, and incubated at 37°C until more than 80% of the cells developed a cytopathic effect. Virions grown from cells maintained in 10 ml of medium containing 200 μCi of $^{32}P_i$ were

sufficient for one-dimensional electrophoretic analyses of viral RNA segments. For oligonucleotide mapping of individual RNA segments, however, virions collected from cultures maintained in 50 ml of medium in the presence of 3 mCi of $^{32}P_i$ were required. The purification of virions and the extraction of viral RNA were performed by the method described previously (20).

Polyacrylamide gel electrophoresis. Viral RNA segments were separated by electrophoresis on a 2.8% polyacrylamide slab gel (14 by 28 by 0.15 cm) containing 6 M urea at 120 V for 14 to 44 h at room temperature (26). Gels were dried and autoradiographed.

Oligonucleotide mapping. An RNA segment was located on a gel slab, excised, eluted, and precipitated by the method described previously (16), except for the following modification. Before elution of RNA, excised gel pieces were soaked in water for 1 h at 37°C to remove water-soluble polyacrylamide, instead of precipitating RNA with perchloric acid after elution. The elimination of polyacrylamide was not complete by this method, but the nonspecific loss of RNA was less. A small amount of polyacrylamide remaining in RNA preparations as a result did not interfere with subsequent procedures. The digestion of RNA with RNase T_1 and the separation of oligonucleotides by two-dimensional electrophoresis were performed by the methods described previously (6, 16).

RESULTS

Diverse pattern of extra RNAs among cloned virus stocks of the WSN strain. By electrophoretic analysis of viral RNA, the stock of the WSN strain that we were using was found to contain, in addition to eight gene segments, multiple extra RNA segments which migrated faster than the NS gene segment. Furthermore, the intensities of all three polymerase gene segments were reduced (Fig. 1, lane O). This finding indicated that the particular virus stock, although it had been grown from a single plaque, contained von Magnus particles. We then attempted to prepare virus stocks free from von Magnus particles or, more specifically, without extra RNAs, by cloning. Well-isolated plaques on MDBK cells were picked and grown individually to virus stocks. Radiolabeled virions were prepared from these cloned virus stocks by high-multiplicity infection, and viral RNA was examined by electrophoresis. Figure 1 shows the RNA pattern of the original WSN and cloned virus stocks on the first undiluted passage. Of 10 clones examined, only 1 (clone 10) was free from extra RNA bands. All of the other clones gave rise to extra RNAs in various amounts. Some clones, i.e., clones 1, 4, 6, 7, and 8, contained one or two extra bands with mobilities that were the same as those present in the parent virus. Most extra bands produced by cloned virus stocks, however, migrated differently from those of the parent and of the other cloned stocks. Some clones (clones 4, 7, and 9) contained extra RNAs larger than the NS gene. This result indicated that extra RNAs of various size classes were generated randomly and with a fairly high fre-

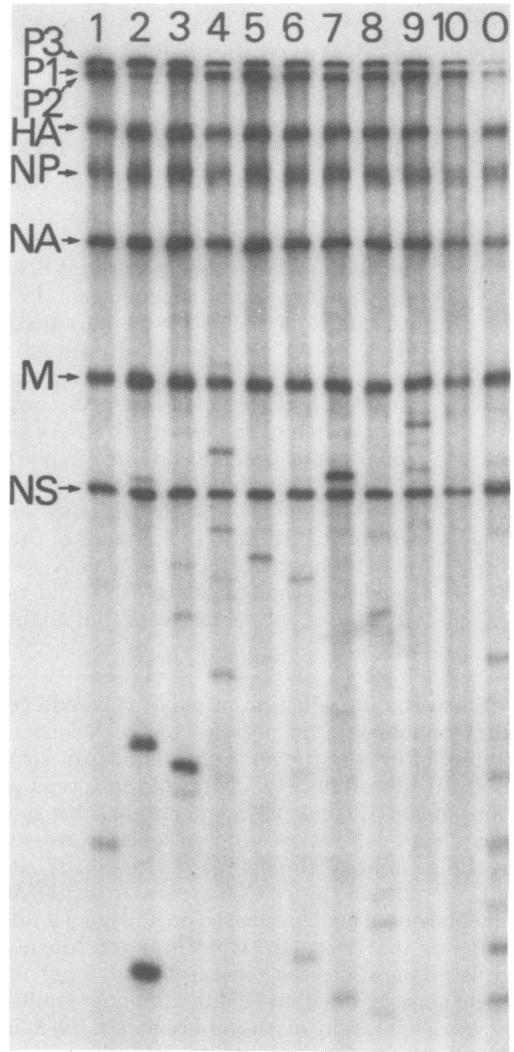

FIG. 1. *Polyacrylamide gel electrophoresis of RNAs of the original stock of WSN virus and cloned virus stocks derived from it. Each virus stock was inoculated into MDBK cells at a multiplicity of 10 PFU/cell. Cultures were maintained in phosphate-free medium containing 20 μCi of $^{32}P_i$ per ml. After overnight incubation, virions were purified from infected culture fluids. RNA was extracted and electrophoresed for 18 h as described in the text. The assignment of eight individual RNA segments to specific viral proteins was based on the previously established maps of the WSN strain of influenza A virus (19, 21, 23). Lane O, the original WSN virus stock; lanes 1 to 10, cloned virus stocks derived from the original virus stock.*

quency during virus replication, resulting in a quite diverse pattern of extra RNAs by the first undiluted passage. The loss of polymerase genes was not apparent except in clones 2 and 3. Both cloned stocks were characterized by pronounced extra RNAs. Only the P_2 band was diminished in intensity in clone 3 which contained one extra RNA band, whereas the amount of both the P_1 and P_2 genes was reduced in clone 2 which contained two extra RNAs. Thus, preparations of von Magnus particles were diverse not only in the size class of extra RNAs which appeared but also in the pattern in which polymerase genes were lost. Table 1 is a comparison of the hemagglutinin titer and infectivity of the original WSN virus and some cloned virus stocks derived from it. As predicted from the RNA pattern, clone 10 had the highest infectivity (PFU)-to-hemagglutinin ratio. The PFU/hemagglutinin ratio was 20- to 40-fold lower for the original WSN and clone 2 viruses and 3-fold lower for clone 3 virus. When MDBK cells in suspension were coinfected with 0.1 PFU of clone 10 virus per cell and 3 PFU of clone 2 virus per cell and plated as infective centers on fresh monolayers of MDBK cells, the resulting plaques were markedly smaller than those arising from infective centers initiated by clone 10 alone, although the plaque numbers were unchanged. This finding showed the presence of interfering particles in the clone 2 virus preparation. Therefore, in subsequent experiments, clone 2 virus was used as the virus preparation largely consisting of von Magnus particles, and clone 10 virus was used as the standard virus.

Segregation of extra RNAs of clone 2 virus. The previous study with a preparation containing predominantly von Magnus particles suggested that the extra RNA derived from the P_3 gene caused the reduction of the P_3 gene (17). Clone 2 virus, with two prominent extra RNA bands, designated as X_1 and X_2, and two reduced polymerase bands, appeared to be a suitable material for examining the possible specific relationship between an extra RNA and the loss of a polymerase gene. We then attempted to segregate two extra RNAs. Because von Magnus particles required standard virions as a helper for replicating themselves, cells were mixedly infected with standard virus (clone 10) and clone 2 virus at multiplicities of 1 and 0.00125 to 0.01 PFU/cell, respectively. At this multiplicity of clone 2 virus, cells were unlikely to be infected with more than one von Magnus particle. Cells were plated as infective centers on fresh monolayers of MDBK cells, which were then overlaid with agar overlay medium. Small plaques were among those formed after a 3-day incubation because such plaques were likely to be initiated by infective centers which were coinfected with von Magnus particles, as described above. Virus stocks were grown individually from 17 plaques. Radiolabeled virion RNA was prepared in the first undiluted passage and examined by electrophoresis. Two virus stocks produced an RNA pattern identical to that of clone 2 virus, namely, the presence of two extra RNA bands comigrating with X_1 and X_2 and the diminished intensity of the P_1 and P_2 bands (Fig. 2). The RNA pattern of two other virus stocks showed only the faster-migrating extra RNA, X_2. The P_1 band was reduced (clone B in Fig. 2). Another virus stock was characterized by the presence of slow-migrating extra RNA, X_1, and the reduction of the P_2 gene (clone A in Fig. 2). Twelve remaining

TABLE 1. *Infectivity and hemagglutinin (HA) titer of the original WSN and cloned virus stocks*

Virus stock	Infectivity (PFU/ml)[a]	HA titer	PFU/HA ratio ($\times 10^3$)
WSN original	1.3×10^8	2,048	64
Clone 2	3.8×10^7	1,024	37
Clone 3	2.6×10^8	512	508
Clone 10	7.5×10^8	512	1,465

[a] Plaque titration was done in MDBK cells.

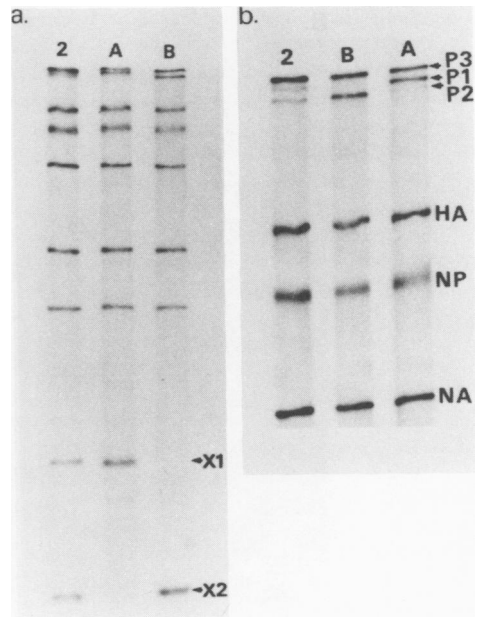

FIG. 2. *Polyacrylamide gel electrophoresis of RNAs of clone 2 virus and two representative clones derived from it. (2) Clone 2 virus; (A and B) clones obtained from infective centers mixedly infected with clone 2 virus and standard virus as described in the text. Electrophoresis was for 14 h (a) and for 44 h (b).*

clones gave rise to extra RNAs with electrophoretic mobilities different from those of either X_1 or X_2, in addition to X_2 in three clones, X_1 and X_2 in three clones, and neither X_1 nor X_2 in six clones (data not shown). New extra RNAs were probably generated independently from the two preexisting extra RNAs, confirming the randomness and high frequency with which extra RNAs were generated, as described above. The above result demonstrated that the presence of X_1 was associated with the reduction of the P_2 gene and that the presence of X_2 was associated with the reduction of the P_1 gene.

Origin of extra RNAs. We attempted to determine whether extra RNAs of clone 2 virus, like the extra RNA described previously (17), were also derived from a polymerase gene(s) and, if so, from which one. The X_1 and X_2 bands were cut out from the gel after electrophoresis of labeled clone 2 virus RNA. To label clone 10 virions, cells were infected at a low multiplicity (about 0.1 PFU/cell) to minimize a possible generation of von Magnus particles and a decrease in polymerase gene content as a consequence. The close proximity of the P_1 and P_2 genes of WSN virus on the electropherogram made the separation of these two genes difficult, and they were excised as combined $P_1 + P_2$. RNase T_1-resistant oligonucleotide maps of X_1, X_2, P_3, and $P_1 + P_2$ are shown in Fig. 3 and 4. The following conclusions were drawn from the comparison of oligonucleotide patterns. First, all unique spots of both X_1 (Fig. 3a) and X_2 (Fig. 3b) matched some of the spots present in $P_1 + P_2$ (Fig. 4b), but they were distinct from those of P_3 (Fig. 4a) and did not overlap those of the HA, NP, NA, M, and NS gene segments (data not shown). Second, X_1 and X_2 did not share any spots, indicating that unique spots of the two extra RNAs were derived either from different gene segments or from different parts of the same gene segment. Separation of the P_1 and P_2 genes was then attempted. For this purpose, a recombinant obtained from the cross between the WSN and Hong Kong strains, R107, was chosen. The gene constellation of R107 was described above. With this recombinant, the P_2 gene derived from WSN virus was electrophoretically separable from the P_1 gene of the Hong Kong virus, because of the slower migration of the latter compared with that of the corresponding gene of WSN virus (Fig. 4c, inset). RNA of R107 virus was labeled, again during a low-multiplicity infection as described above. The P_2 gene segment was excised from the gel and subjected to oligonucleotide mapping (Fig. 4c). Spots of rel-

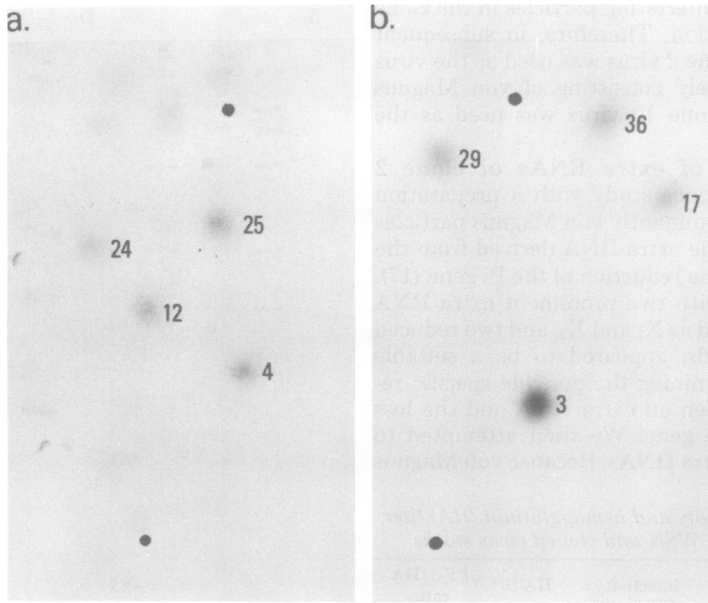

FIG. 3. *Oligonucleotide maps of extra RNAs. Segments of extra RNAs, X_1 and X_2, were obtained by polyacrylamide gel electrophoresis of ^{32}P-labeled clone 2 virus RNA, as described in the text. Nuclease T_1 digestion and two-dimensional gel electrophoresis were performed by previously described methods (6, 16). First dimension, Left to right; second dimension, bottom to top. (a) X_1; (b) X_2. Dots at the top and the bottom indicate the positions of bromophenol blue and xylene cyanol dye markers, respectively. Oligonucleotide spots were identified and numbered on the background of the $P_1 + P_2$ (Fig. 4b and 5a).*

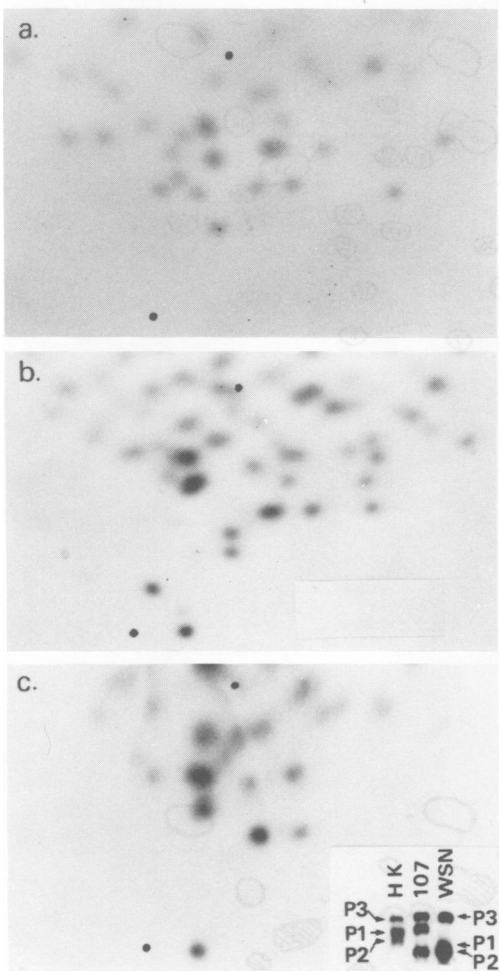

FIG. 4. *Oligonucleotide maps of the P_3, P_1 + P_2, and P_2 gene segments of WSN virus. The direction of migration was as in Fig. 3 (a) P_3 gene segment; (b) mixture of P_1 and P_2 gene segments; (c) P_2 gene segment. The P_2 gene segment was obtained by polyacrylamide gel electrophoresis of the R107 virus RNA shown in the inset.*

atively large oligonucleotides of P_1 + P_2 were numbered from 1 to 37 (Fig. 5a). Spots present in the P_2 gene were identified on the background of the P_1 + P_2 map. The oligonucleotide map of the P_1 gene was constructed by subtracting the P_2 spots from the background and is shown as shaded spots in Fig. 5b. By a comparison of oligonucleotide maps, it was evident that all major spots of X_1 RNA were a part of the P_1 oligonucleotide spots (Fig. 3a and 5b). On the other hand, three of four major spots (spots 3, 17, and 36) present in X_2 RNA matched those of the P_1 gene (Fig. 3b and 5b). One remaining spot (spot 29) was found in the map of the P_2 gene, but since the spots of the P_1 gene were identified indirectly, it is possible that the P_1 gene also contained the oligonucleotide with a migration pattern similar to spot 29. We concluded that both X_1 and, most likely, X_2 originated in the P_1 gene.

DISCUSSION

Diverse patterns of extra RNAs produced by cloned virus stocks indicated that a given virus strain or a given line of the virus strain was not predisposed to generate a particular set of extra RNAs, but that the generation of extra RNAs in influenza virus was a random and unpredictable event as in vesicular stomatitis virus (8) and Sendai virus (13). A similar observation has been made with a temperature-sensitive mutant of the WSN strain by Janda et al. (10). Another feature of influenza virus extra RNAs revealed in this study is their apparently high rate of generation. Even on the first undiluted passage of a virus stock grown from a single virus particle, most virus stocks gave rise to extra RNA bands of varying, but clearly recognizable, intensity. The replication of WSN virus in MDBK cells was characterized as a lesser production of incomplete virus than that in other influenza virus-cell systems (2). Bunyamwera virus also produced a small amount of defective interfering virus in MDBK cells compared with BHK and Vero cells (12). The rate at which extra RNAs are generated in MDBK cells shows that the presence of von Magnus particles must always be considered for any virus stocks of influenza virus.

The loss of large RNA species from von Magnus particles has been observed consistently (1, 3, 7, 18, 22), but exactly what gene segment(s) was lost has not been determined unambiguously. The finding in our previous study (17) and this study and the study made by Janda et al. (10) showed that the loss of polymerase genes varied from one von Magnus preparation to another, just as the species of extra RNAs contained in them were variable. In the von Magnus preparation used in our previous study, the appearance of the extra RNA was associated with a reduced amount of the P_3 gene, whereas the present study showed that the presence of the extra RNAs X_1 and X_2 was associated with a reduction in the amount of the P_2 and P_1 genes, respectively. Therefore, it appears that it is the species of extra RNAs that determines the species of polymerase genes lost from von Magnus particles.

With von Magnus preparations used in our previous study (17) and in the study of Janda et al. (10), infective centers mixedly infected with

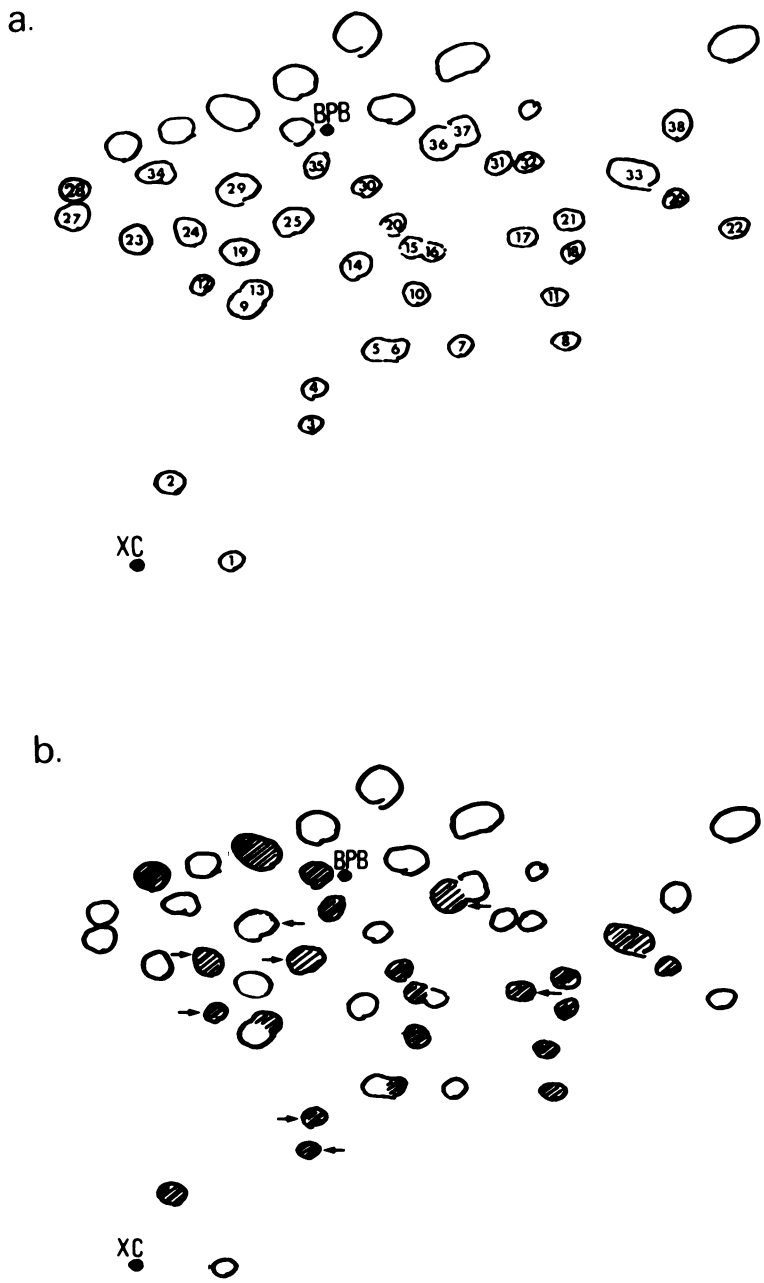

FIG. 5. *Diagrams of the oligonucleotide maps of the P_1 + P_2 genes and the P_1 gene. (a) P_1 + P_2 genes (diagram of the map shown in Fig. 4b). Spots of the large olignucleotides were numbered 1 through 37. (b) Spots contained in the P_1 gene were identified by subtracting the P_2 spots (Fig. 4c; shown here as empty circles) from the background of P_1 + P_2 (Fig. 4b) and are shown as shaded spots. Arrows pointing to the right indicate the spots present in X_1, and arrows pointing to the left indicate those present in X_2. BPB, Bromophenol blue; XC, xylene cyanol.*

a von Magnus particle and standard virions failed to form plaques. The latter authors used the infective-center reduction for quantitating von Magnus particles. Clone 2 virus, the von Magnus preparation in the present study, on the other hand, markedly reduced the size of plaques formed by standard virus, but never suppressed plaque formation entirely, providing a means for

segregating two extra RNAs. The different behavior of von Magnus preparations probably reflects the different interfering capacity of von Magnus particles or extra RNAs contained in them, an additional feature of diversity observed in von Magnus particles. The interference of von Magnus particles with the replication of influenza virus is most likely mediated by a reduced synthesis of polymerase genes. However, a detailed mechanism of interference is still obscure. Our previous postulate that the extra RNA causes a reduction in the amount of the gene segment from which it originated may not be valid in view of the finding that the X_1 RNA that originated from the P_1 gene was associated with a reduction in the amount of the P_2 gene. It might be speculated that the X_1 RNA has lost the sequence of the P_1 gene that could be recognized as P_1 and, instead, the new sequence that resembled that of the P_2 gene has been generated as a result of deletion. We need to know more about the sequence of polymerase genes that are interfered with and extra RNAs that do interfere, before the precise mechanism of interference is clear.

The X_1 and X_2 extra RNAs which were derived from the common progenitor RNA, i.e., the P_1 gene, shared none of the large oligonucleotide spots. Since large oligonucleotide spots represented less than 10% of the entire sequence (16), it is possible that two extra RNAs nevertheless had an overlapping sequence that could not be detected by the present method of mapping. However, the present study showed, at least, that the smaller X_2 RNA contained the sequence not present in the larger X_1 RNA. Therefore, the deletion in influenza virus extra RNA may be different from the progressive deletion observed in defective interfering RNA of some other viruses, in which the sequence present in smaller defective interfering RNA is always included in the sequence present in larger defective interfering RNA (11, 14, 15, 24). A nonoverlapping deletion was also observed by Davis and Nayak in influenza defective interfering RNA (5). The different portion deleted from the P_1 gene is probably reflected in the difference of the two extra RNAs in causing the reduction of polymerase genes.

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