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Identification of the Defective Genes in Three Mutant Groups of Influenza Virus

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Seven complementation-recombination groups of temperature-sensitive (ts) influenza WSN virus mutants have been previously isolated. Recently two of these groups (IV and VI) were shown to possess defects in the neuraminidase and the hemagglutinin gene, respectively, and two groups (I and III) were reported to have defects in the P3 and P1 proteins which are required for complementary RNA synthesis. In this communication we report on the defects in the remaining three mutant groups. Wild-type (ts^+) recombinants derived from ts mutants and different non-ts influenza viruses were analyzed on RNA polyacrylamide gels. This technique permitted the identification of the P2 protein, the nucleoprotein, and the M protein as the defective gene products in mutant groups II, V, and VII, respectively. Based on the physiological behavior of mutants in groups II and V, it appears that P2 protein and nucleoprotein are required for virion RNA synthesis during influenza virus replication.

Temperature-sensitive (ts) mutants of influenza A/WSN/33 virus were classified into seven complementation-recombination groups by Sugiura and co-workers (31, 32). Examination of the physiological properties of these mutants led to the conclusion that members of group IV were defective in neuraminidase (19) and that members of group VI were defective in hemagglutinin (34). Recently we also succeeded in establishing the entire map of the RNA genome of different influenza viruses (13-15, 17, 18, 23, 24). It is therefore possible to conclude that group VI mutants have a defect in their RNA 4 segment (hemagglutinin gene) and that group IV mutants have a defective RNA 6 segment (neuraminidase gene). The identification of the viral polypeptide for which each RNA segment codes was, in part, made possible by the discovery that the RNA segments of different influenza A viruses have strain-specific mobilities on polyacrylamide gels. RNA analysis of recombinants with a ts^+ phenotype, which was derived from ts mutants and non-ts marker viruses, now enables us to determine which RNA segment, and consequently which viral protein, is defective in each of the mutant groups. Using this technique, we describe in the present report the identification of the defective proteins in mutant groups II, V, and VII. A similar approach was used to identify the P3 and P1 proteins as the defective proteins in groups I and II (14), and hemagglutinin and neuraminidase mutants were previously described (19, 34). The present analysis completes the identification of the defective genes in all complementation-recombination groups of WSN virus mutants.

MATERIALS AND METHODS

Cells and viruses. The MDBK (bovine kidney) line of cells, which has been used in previous studies of ts mutants, was also employed here (31, 32). MDCK (canine kidney) cells, grown on Medium 199 (Microbiological Associates, Inc.) plus 10% calf serum, were used for recombination experiments, plaque assays, and labeling of viral RNAs (12-14, 17, 18, 21, 23). The viruses employed in this study included ts53, ts4, ts56, ts12, and ts51 of the A/WSN/33 ts mutant collection isolated previously (31, 32), A/HK/8/68 (H3N2) virus (HK virus), A/FM/1/47 (H1N1) virus (FM1 virus), and A/NED/84/68 (H2N2) virus (NED virus). Seed virus for A/WSN/33 virus (WSN virus) was prepared in MDBK cells; all other seed viruses were grown in the allantoic cavity of embryonated hen eggs. The liquid and agar overlay media for growing virus have been described (8, 19, 31, 32). Two micrograms of TPCK trypsin (Worthington) per ml was routinely added to the overlay medium of MDCK cells and to MDBK cells that were mixedly infected during recombination experiments.

Rescue experiments. MDCK or MDBK cells were co-infected with a ts mutant (multiplicity of infection, 10 to 100) and another influenza A virus (multiplicity of infection, approximately 1) under liquid overlay. Dishes were also singly infected with each of the viruses at the same multiplicity of infection used in the double infection. Incubation was at 35 or 39.5°C for a total of 10 to 16 h. At 3 h postinfection,

the infected dishes were washed to remove any remaining unadsorbed virus. At the end of the incubation period, the supernatant fluids were harvested and titrated by plaque assay at 39.5°C in MDBK cells. When the yield from doubly infected cells was greater than that from singly infected cells, plaques were isolated and seed virus was prepared in MDBK cells at 39.5°C. These recombinants showed ts^+ or wild-type phenotype.

RNA. All WSN viruses and recombinants were labeled by inoculating MDBK cells at a multiplicity of infection of 1 to 10 and incubating in phosphate-free medium in the presence of 5 to 15 mCi of ^{32}P (as orthophosphate, ICN). The other influenza A viruses were labeled in MDCK cells. After 18 h the virus was harvested and purified, and its RNA was extracted as previously described (13, 15-18, 21-23). The RNA was analyzed on urea-polyacrylamide (2.6%) gels using established procedures (2, 13, 15-18, 21-23). The temperature and time of electrophoresis were adjusted to allow maximum separation of the corresponding bands of the viruses employed. It should be noted that virus grown under high multiplicity does not always contain equimolar amounts of each RNA segment.

RESULTS

The ts mutants of WSN virus were recombined with other influenza A viruses with the hope of obtaining viruses that derived one or a few genes from the rescuing virus and which were no longer temperature sensitive. It was expected that RNA analysis of these recombinants would then enable us to identify the ts gene of the mutant virus. MDBK and MDCK cells at both permissive and nonpermissive temperatures were employed for the mixed infection as described in Materials and Methods. Recombination was assumed to have taken place if the yield from mixedly infected cells assayed in MDBK cells at 39.5°C exceeded that from cells infected with the mutant alone. It should be noted that all influenza A viruses used as rescuing viruses in this study do not plaque in MDBK cells. In general, the yield of recombinants from a particular mixture appeared to depend more on the mutant and the rescuing virus than on the cell culture system or the temperature at which the recombination experiment was done. Increases in titer of the mixed yield over the yield of mutant virus alone at 39.5°C ranged from 2- to 10,000-fold. Clones of virus were not usually isolated and examined unless at least a 10-fold increase in virus titer was observed in the yield from the mixed infections compared to infections of mutants alone. The reason for this was that too many revertants of the WSN mutant virus were isolated unless a high level of recombination was initially observed. After purification, clones of virus from suitable recombination mixtures were isolated and purified at 39.5°C to be sure that they were no longer temperature sensitive. Subsequently, the RNA of each clone was labeled and examined on urea-polyacrylamide gels.

Mutants from group VII. The temperature sensitivity of WSN ts51 virus was eliminated by rescue with HK virus. A representative recombinant of ts51 and HK virus is shown in Fig. 1. The genes of the parental HK and WSN viruses (lanes 1 and 3) have previously been identified (13, 14, 17, 23, 24). Each and every corresponding band of these two viruses is distinguishable from each other, and thus it can be determined that the recombinant virus (lane 2) contains all RNA segments from ts51 except RNA7, which is derived from HK virus. Because this recombinant is no longer temperature sensitive and contains only one RNA from HK virus, we conclude that segment 7, the membrane protein gene, was defective in the ts51 parent. Two other recombinants, similar to the one just described, were also isolated (Table 1), confirming our conclusion. However, we experienced difficulties in always separating RNA 7 of HK virus from that of the WSN viruses, since both segments have similar migration rates in polyacrylamide gels. To obtain independent evidence that RNA 7 is indeed defective in group VII mutants, a recombinant between ts51 and NED virus was isolated. The derivation of its RNA segments is shown in Fig. 2. Lanes 2 and 3 show the parental viruses and demonstrate that their corresponding RNA segments are clearly distinguishable from each other. Lane 4 just shows a longer exposure of the sample in lane 3. The recombinant in lane 1 can be seen to derive all of its segments from WSN virus except for RNAs 2 and 7, which are derived from NED virus (arrows). Since segment 2 was shown to be defective in group III mutants and since this segment was not exchanged in recombinants derived from HK virus, segment 7 must be the defective gene in group VII mutants.

Mutants from group V. The ts defect of ts56 was lost after recombination with FM1 virus or NED virus. Figure 3 shows the RNA patterns of ts56, FM1 virus, and two non-ts recombinants derived from them. It can be seen that all of the recombinants derive most of their genes from the ts56 parent; the exceptions are gene 5, the nucleoprotein gene, and RNA 8 in one recombinant, which are clearly derived from the FM1 parent. We thus conclude that group V mutants are defective in their nucleoprotein. Two additional recombinant clones were isolated, con-

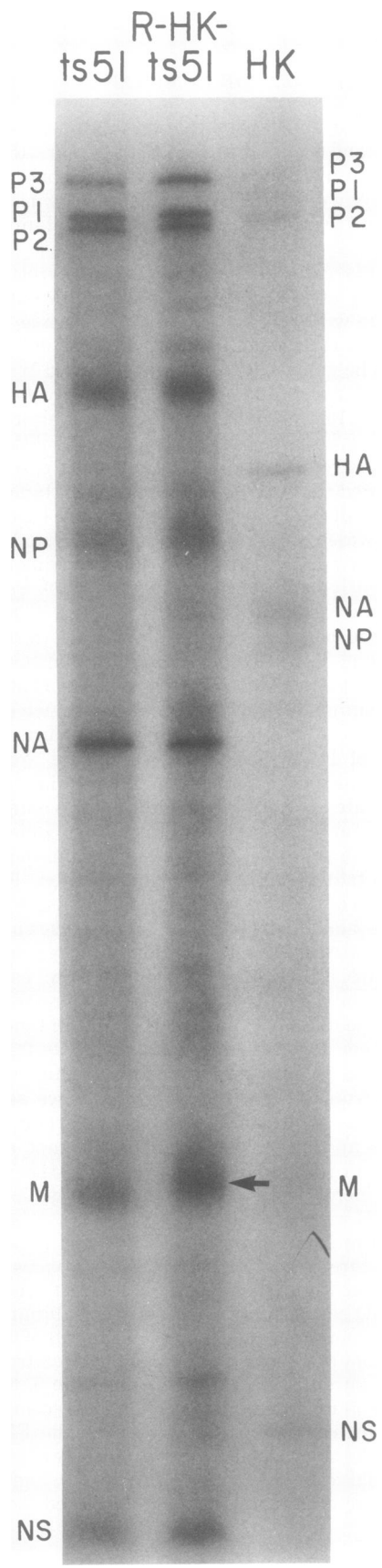

R-HK-
ts51 ts51 HK
P3
P1
P2
HA
NP
NA
M
NS
P3
P1
P2
HA
NA
NP
M
NS

FIG. 1.

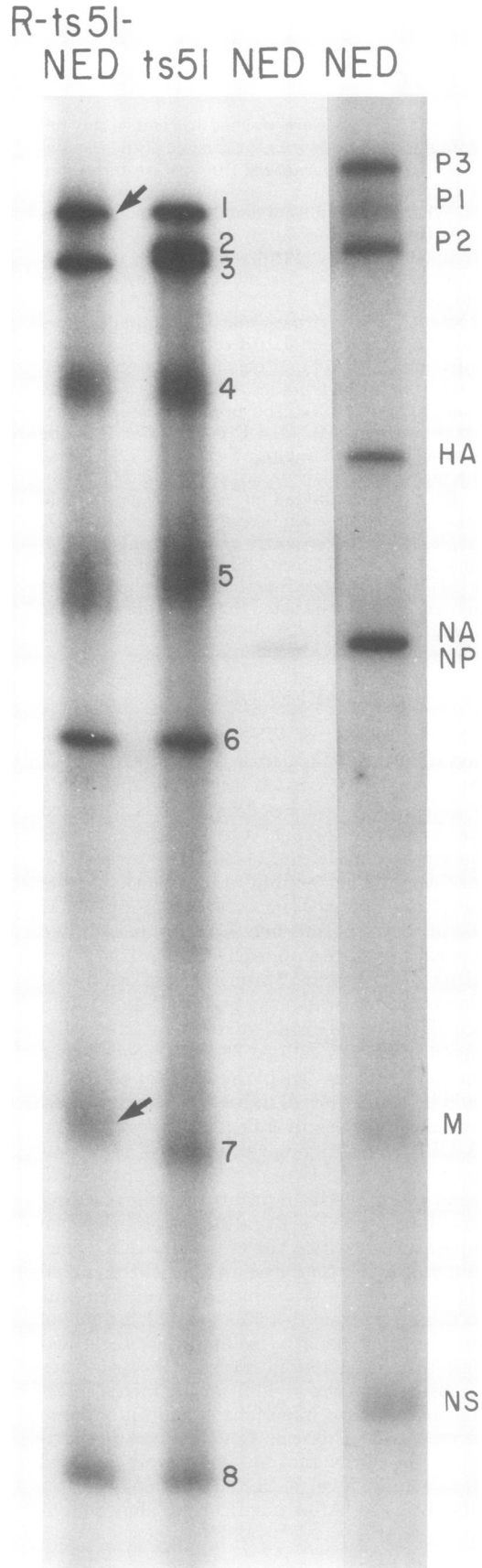

R-ts51-
NED ts51 NED NED
1
2
3
4
5
6
7
8
P3
P1
P2
HA
NA
NP
M
NS

FIG. 2.

TABLE 1. *ts⁺ recombinants derived from ts mutants and different rescuing viruses*

Wild-type (ts⁺) recombinant[a]	Parent viruses		Derivation of proteins[b]							
	Mutant (A/WSN virus)	Rescuing virus	P1	P2	P3	HA	NP	NA	M	NS
RII-1	ts53 (group II)	A/HK/8/68	H	H	H	W	W	W	?	W
RII-2	ts53 (group II)	A/FM/1/47	F	F	F	W	W	W	W	W
RII-3	ts4 (group II)	A/FM/1/47	F	F	F	W	F	W	F	W
RV-1	ts56 (group V)	A/FM/1/47	W	W	W	W	F	W	W	F
RV-2	ts56 (group V)	A/FM/1/47	W	W	W	W	F	W	W	W
RV-3	ts56 (group V)	A/FM/1/47	W	W	W	W	F	W	W	W
RV-4	ts56 (group V)	A/FM/1/47	W	W	W	W	F	W	W	W
RV-5	ts56 (group V)	A/NED/84/68	N	N	N	W	N	W	W	N
RV-6	ts56 (group V)	A/NED/84/68	N	N	N	W	N	W	W	N
RV-7	ts12 (group V)	A/FM/1/47	F	F	F	W	F	W	F	W
RV-8	ts12 (group V)	A/FM/1/47	F	F	F	W	F	W	F	W
RV-9	ts12 (group V)	A/FM/1/47	F	F	F	W	F	W	F	W
RV-10	ts12 (group V)	A/FM/1/47	F	F	F	W	F	W	F	F
RVII-1	ts51 (group VII)	A/HK8/68	W	W	W	W	W	W	H	W
RVII-2	ts51 (group VII)	A/HK/8/68	W	W	W	W	W	W	H	W
RVII-3	ts51 (group VII)	A/HK/8/68	W	W	W	W	W	W	H	W
RVII-4	ts51 (group VII)	A/NED/84/68	N	W	W	W	W	W	N	W

[a] Phenotypically wild-type recombinants were derived from group II, group V, and group VII mutants and from the rescuing viruses A/HK/8/68, A/FM/1/47, and A/NED/84/68.

[b] The derivation of proteins in all recombinants was established by RNA analysis. Proteins derived from the WSN mutants and from A/HK/8/68, A/FM/1/47, and A/NED/84/68 viruses are identified by the letters W, H, F, and N, respectively.

firming these results (Table 1). Recombinants selected from mixed infection of ts56 and NED virus derived most of the genes, including the nucleoprotein gene, from NED virus (Table 1). Likewise, recombinants between ts12, another group V mutant, and FM1 virus or NED virus, which were selected for their ability to grow at nonpermissive temperature, derived most of their genes, including the nucleoprotein gene, from either FM1 or NED virus (Table 1). Although these latter recombinants do not allow us to pinpoint the segment that is defective in group V mutants, they are consistent with the defect being localized in the nucleoprotein gene. It is interesting to note that recombinant RV-10 has only two genes from WSN virus but forms plaques in MDBK cells. This indicates that not more than the hemagglutinin and neuraminidase genes are necessary for the ability to plaque in MDBK cells.

Mutants from group II. The temperature sensitivity of ts53 was lost when it was recombined with HK virus or with FM1 virus. The RNA patterns of one recombinant and its parents, ts53 and FM1 virus are shown in Fig. 4. It can be seen that this recombinant derives the three slowest moving RNA segments from FM1 virus and the rest from the ts53 parent. Because the P3 protein (coded for by RNA segment 1) was shown to be defective in group I mutants and the P1 protein (RNA segment 2) was determined to be defective in group III mutants (14), we conclude that the P2 protein (RNA segment 3) is defective in group II mutants. Recombinants derived from ts53 and HK virus and from ts4 (another group II mutant) and FM1 virus, which were also selected for the loss of the ts characteristic, have RNA patterns that are consistent with this interpretation (Table 1).

FIG. 1. *RNA analysis of ts⁺ (wild-type) recombinant derived from mutant ts51 (group VII) and HK virus. Lane 1, RNA of WSN mutant ts51; lane 2, RNA of a recombinant with ts⁺ phenotype derived from HK virus and ts51; lane 3, RNA of HK virus. Letters identify the genes of ts51 (identical to WSN wild-type virus) and HK virus, which have been previously mapped (12, 14, 17, 23). Arrow indicates that only one gene in the recombinant was derived from HK virus. Conditions for polyacrylamide gel electrophoresis are described in the text.*

FIG. 2. *RNA analysis of ts⁺ (wild-type) recombinant derived from mutant ts51 (group VII) and NED virus. Lane 1, RNA of recombinant with ts⁺ phenotype derived from ts51 and NED virus; lane 2, RNA of ts51; lane 3, RNA of NED virus; lane 4, longer exposure of lane 3 (to permit exact identification of all eight RNA segments). Letters identify the gene products coded for by individual RNA segments. RNA 2 and RNA 7 of the recombinant in lane 1 are derived from NED virus (arrows).*

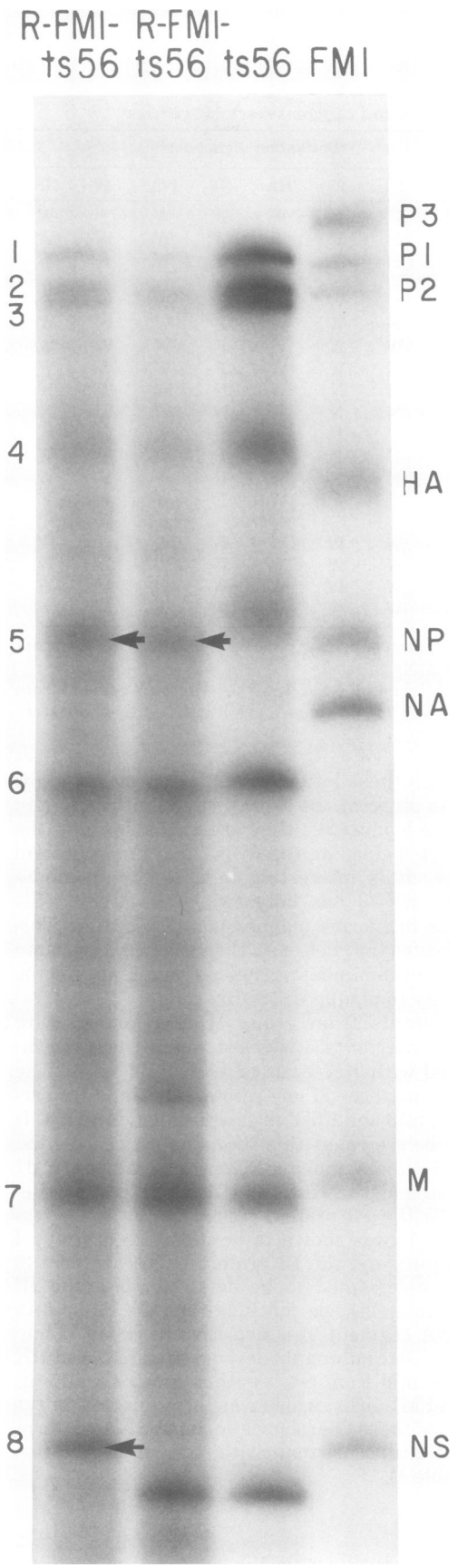


FIG. 3. *RNA analysis of ts⁺ (wild-type) recombinants derived from ts56 (group V) and FM1 virus. Lane 1, RNA of recombinant with ts⁺ phenotype derived from ts56 and FM1 virus (RV-1); lane 2, RNA of recombinant with ts⁺ phenotype derived from ts56 and FM1 virus (RV-2); lane 3, RNA of ts56; lane 4, RNA of parent virus FM1. Letters identify the gene products for which the individual RNA segments code. RNAs 5 and 8 of the recombinant in lane 1 and only RNA 5 of the recombinant in lane 2 are derived from the FM1 parent (arrows).*

TABLE 2. *ts defects in mutant groups of influenza WSN virus*

Group	Defective protein	Function
I	P3	Complementary RNA synthesis
II	P2	Viral RNA synthesis (?)
III	P1	Complementary RNA synthesis
IV	NA	Removal of neuraminic acid, prevention of autoaggregation
V	NP	Viral RNA synthesis (?)
VI	HA	Attachment to cells
VII	M	Assembly (?)

DISCUSSION

Identification of the defective gene in different WSN influenza virus mutants has been a primary goal for many years. Biochemical techniques have been decisive in these efforts, which first led to the characterization of one mutant group that was defective in the neuraminidase gene (group IV) (19). Subsequently, WSN virus mutant groups with defects in virus-specific RNA synthesis (groups I, II, III, and V) (8, 32) and a defect in the hemagglutinin molecule (group VI) (34) were identified.

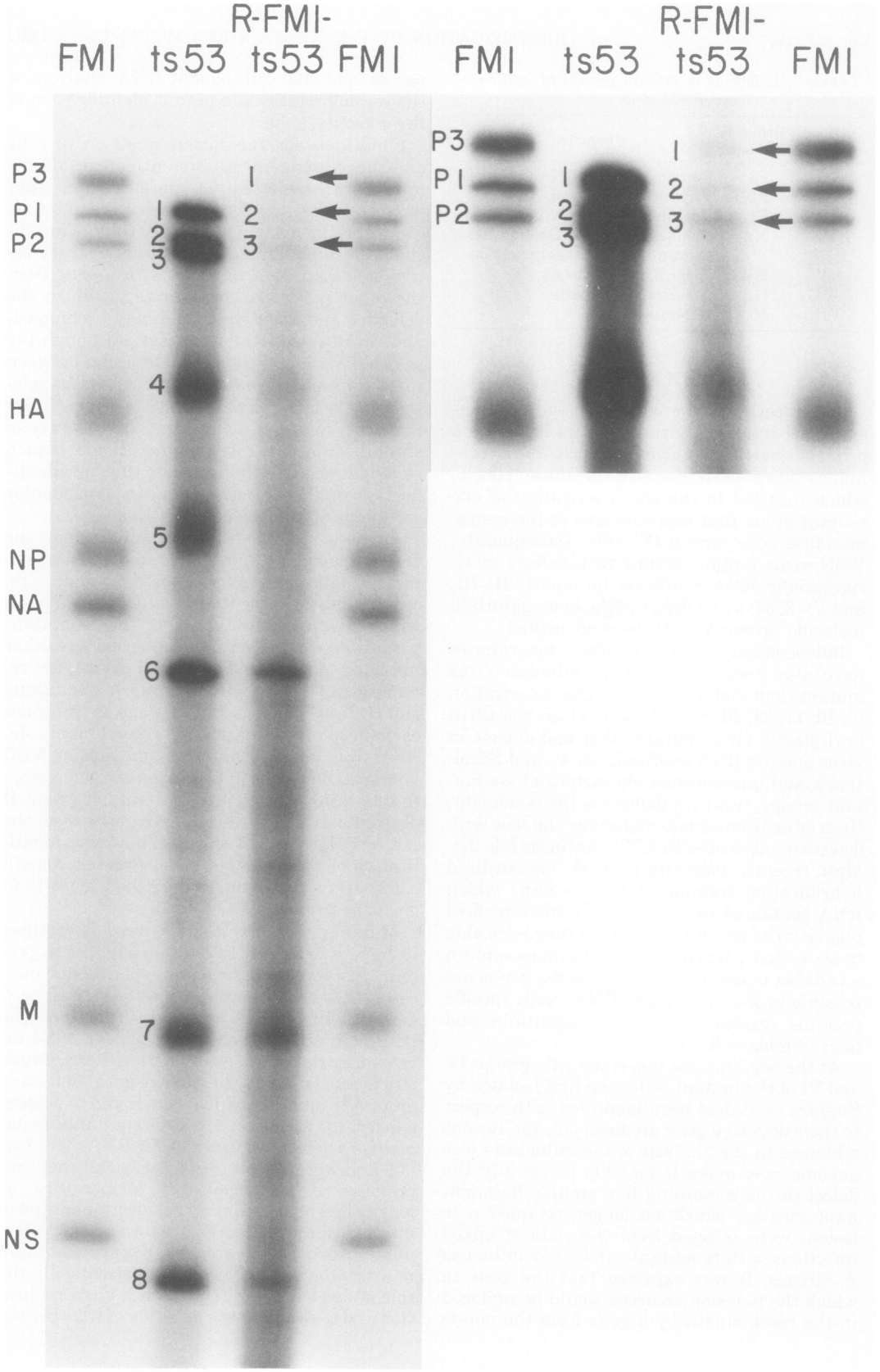
Independently, several other laboratories have also been interested in influenza virus mutants and their biochemical characterization (5, 10, 11, 29, 30, 33). Ghendon et al. identified fowl plague virus mutants that had defects in virus-specific RNA synthesis (3, 4), and Scholtissek and collaborators characterized six mutant groups, two with defects in the hemagglutinin or neuraminidase molecules and four with defects in virus-specific RNA synthesis (25, 27). Most recently Scholtissek et al. (26) utilized hybridization techniques to determine which RNA contained the ts defect in different fowl plague virus mutants. Although they were able to show that particular mutant groups contain a ts defect in specific RNA segments, it was not possible to associate these RNAs with specific proteins (except for the hemagglutinin and neuraminidase RNAs).

At the beginning of this study only groups IV and VI of the mutant collection first isolated by Sugiura et al. had been identified with respect to their defective gene products (19, 33). Recent advances in the analysis of the influenza virus genome now make it possible to identify the defect in the remaining five groups. Recombinant viruses, which no longer retained a ts lesion, were selected from the yield of mixed infections with ts mutants and other influenza A viruses. It was expected that the gene in which the ts lesion occurred would be replaced in the recombinant by a gene from the non-ts parent and that subsequent RNA analysis of the recombinant would permit identification of the defective gene.

Conditions for the mixed infection of cells and the subsequent isolation at nonpermissive temperature selected for those viruses that were no longer temperature sensitive and that were able to plaque in MDBK cells. This included recombinants in which only the defective gene had been replaced with a gene from the other parent, recombinants in which the defective and other genes had been exchanged, and revertants of the ts parent. Although the possibility of observing recombination between revertants and the other parent exists, the relative yields from mixed infection versus infection with mutants alone, plus the recovery of several different recombinant clones which gave identical results, indicate that misidentification of the defective gene in a particular group due to this event is not likely.

As discussed above, the identification of the defective gene in each of the complementation-recombination groups was based on finding recombinants no longer temperature sensitive, in which one or a few genes of the ts mutant parent were replaced by genes from the other influenza A parent. The ts^+ (or wild type) recombinants derived from group VII mutants and HK or FM1 virus had only one or two genes exchanged. In contrast, ts^+ recombinants derived from group V mutants and FM1 or NED virus received one, five, or even six genes from the rescuing parent, and mutants of group II derived between three and five genes from the HK or FM1 virus. In all cases, however, identification of the defective genes was not complicated by recombinants, which had more than one gene exchanged (Table 1).

Mutants in groups II and V were determined to have a defect in P2 protein and nucleoprotein, respectively. Previous experiments indicated that these mutants most likely have a defect in virion RNA synthesis. Therefore, the P2 protein and nucleoprotein appear to be involved in the synthesis of virion RNA. Much less is known about the physiological defects of group VII mutants which were found to possess a defect in the M protein. These mutants do not possess a defect in virus-specific RNA synthesis (32) and were shown to synthesize all the viral proteins at nonpermissive temperature as judged by polyacrylamide gel electrophoresis of infected cell proteins (K. Tobita and P. Palese, unpublished observations). Experiments are now in progress to define more specifically the role of the M protein in influenza virus replication and to examine the question of whether the


R-FMI-
FMI
ts53
ts53
FMI
FMI
ts53
ts53
FMI
P3
P1
P2
HA
NP
NA
M
NS
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P3
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synthesis of M protein is indeed a rate-limiting step in virus production (6, 9). It should be noted that there are eight influenza virus genes (1, 12, 17, 20, 22) and that ts mutants have been identified for all of the influenza A virus genes except gene 8, which codes for the nonstructural protein. We are presently attempting to identify mutants with defects in this protein.

In summary, the work described here and in the companion paper (14), along with previous investigations, have permitted us to identify the exact defect in seven mutant groups of influenza WSN virus and to correlate biological activities with particular gene products (Table 2).

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FIG. 4. *RNA analysis of ts⁺ (wild-type recombinants) derived from ts53 (group II) and FM1 virus. Lanes 1 and 4, RNA of FM1 virus; lane 2, RNA of ts53; lane 3, RNA of recombinant with ts⁺ phenotype derived from FM1 virus and ts53. Letters identify the gene products for which the individual RNA segments of FM1 code. RNAs 1, 2, and 3 of the recombinant are derived from the FM1 parent (arrows). The insert (right) shows a longer exposure of the upper part of the gel in order to permit a better identification of the RNA segments of the recombinant virus.*

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