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P1 and P3 Proteins of Influenza Virus Are Required for Complementary RNA Synthesis

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Members of two temperature-sensitive (ts) mutant groups of influenza A/WSN virus defective in complementary RNA synthesis were analyzed with respect to the identity of their defective genes. RNA analysis of recombinants having a ts^+ phenotype derived from the mutants and HK virus permitted the identification of RNA 1 and RNA 2 as the single defective gene in mutant groups I and III, respectively. Based on knowledge obtained by mapping the WSN virus genome, it then was possible to determine that biologically functional P3 protein (coded for by RNA 1) and P1 protein (RNA 2) are required for complementary RNA synthesis of influenza virus.

Studies in several laboratories have clearly established that the RNA segments contained in influenza virus particles must first be transcribed into complementary (c) RNA before translation on polysomes can occur (5-7, 12, 17, 25, 34). However, the enzymatic processes and the specific proteins required for the transcription into cRNA and replication of virion RNA have not been fully characterized. Chow and Simpson (4) were the first to describe polymerase activity in purified influenza virus particles. Subsequent biochemical analysis of the transcriptase-positive ribonucleoprotein complex of WS (2) and WSN viruses (30) and of a nucleocapsid fraction from infected cells (3) revealed the presence of P1 and possibly P2 proteins as well as a nucleoprotein, which suggested that any one or all of these three proteins might be required for cRNA synthesis. More recently, a third virus-coded P protein was identified in virus particles and in influenza virus-infected cells in our laboratory (18, 28, 29) and independently by other groups (10, 15), adding yet another virus-coded protein which might be required for transcriptase activity.

Up to now, attempts to demonstrate transcriptase activity with isolated components of the transcriptase complex have not been successful. As a result, it has not been possible to biochemically define which of the four proteins (P1, P2, P3, NP) are required for cRNA synthesis. In a previous communication (14) we reported that two of the seven temperature-sensitive (ts) mutant groups of influenza A/WSN virus described by Sugiura et al. (32, 33) had defects in cRNA synthesis. Analysis of cRNA from cells infected with mutant virus from different groups revealed that only members of mutant groups I and III failed to synthesize cRNA at the nonpermissive temperature or after shift from the permissive to the nonpermissive temperature.

These observations suggested that at least two of the gene products in the ribonucleoprotein core are required for cRNA synthesis. In the present communication, we describe how the analysis of RNAs of recombinants derived from influenza A/HK/8/68 and ts mutants in groups I and III permits us to establish that the specific defects of these groups are in P3 and P1 proteins, respectively, and to deduce that these two proteins are required for cRNA synthesis.

MATERIALS AND METHODS

Cells and viruses. MDBK (bovine kidney) and MDCK (canine kidney) cells were grown as previously described (13, 32, 33). Influenza A/HK/8/68 (H3N2) virus (HK virus) was propagated in the allantoic cavity of embryonated hen eggs; influenza A/WSN/33 (HON1) virus (WSN virus) and ts1 and ts6 mutants from complementation-recombination group I and ts15 and ts101 mutants from group III were grown in MDBK cells according to published procedures (24, 32, 33).

Preparation of recombinant viruses. Confluent MDCK cell monolayers on plastic dishes were mixedly infected with 1 to 10 PFU per cell of mutant virus and with approximately 1 PFU per cell of HK virus in the presence of 2 μg of TPCK trypsin (Worthington) per ml. Cells were washed and kept for 16 h at 33°C. The yield was assayed for plaques at 33

and 39.5°C in MDCK cells in the presence of 2 μg of TPCK trypsin per ml or in MDBK cells. Plaquing of the mixed yield in MDBK cells at 39.5°C provided a ready system to insure the identification of recombinants, because HK virus does not replicate in MDBK cells and, by definition, the ts mutants do not replicate at 39.5°C. Control dishes that were infected with either one of the ts mutants or HK virus alone were identically treated. Plaques obtained from the mixed virus yield in dishes kept at 39.5°C were picked. After preparation of seed virus, the RNA of these recombinants was labeled. (These recombinants are referred to as wild-type [ts$^+$] recombinants.)

To map the genome of WSN virus, recombinants of WSN wild-type virus and HK virus were obtained by co-infection of the allantoic sac of embryonated hen eggs and by selection in the presence of antiserum using established techniques (22). Recombinants obtained by these techniques were also used for RNA labeling. Serological confirmation of the hemagglutinin and neuraminidase of each influenza virus was done according to published procedures (1, 20).

Labeling of RNA and analysis on polyacrylamide gels. Monolayers of MDBK or MDCK cells were infected with different virus strains at a multiplicity of 1 to 10 PFU/cell in phosphate-free medium containing 5 to 15 mCi of ^{32}P per dish. After 16 h the virus was purified, and RNA was extracted as previously reported (18, 19, 21-23, 27-29). ts mutants and ts$^+$ recombinants were labeled in MDBK cells, whereas HK virus was labeled in MDCK cells. (RNA patterns of the mutants and of the ts$^+$ recombinants grown in the two cell systems were identical, but the yield was greater in MDBK cells.) RNAs were then analyzed after electrophoresis on urea-polyacrylamide gels according to procedures originally described by Floyd et al. (8) and modified by us (18, 19, 21-23, 27-29).

RESULTS

RNA analysis of ts mutants. In a previous communication we identified two ts mutant groups of influenza WSN virus which had defects in genes required for the synthesis of cRNA in vivo (14). Mutants in group I later were shown to possess a thermolabile transcriptase in vitro (16). Preliminary experiments also revealed that mutants in these groups (I and III) direct little or no virus-specific protein synthesis when grown at the nonpermissive temperature (K. Tobita, M. Ueda, R. M. Krug, and P. Palese, unpublished observations). In addition, it was found that virus-specific proteins made by these mutants at permissive temperature did not differ from those made by the wild-type virus when analyzed on gradient polyacrylamide gels.

However, the analysis of the RNAs of ts mutants and the wild-type virus revealed differences in some instances. Figure 1 demonstrates that the slowest moving RNA (RNA 1) of ts1 (group I) moves faster than the corresponding RNA of the wild-type virus (arrow). A second mutant in group I (ts6) and ts101 (group III) had RNA patterns that were indistinguishable from that of the wild-type virus (results not shown). But another mutant (ts15, group III) also contained one RNA segment (RNA 2) that differed in its migration rate from the equivalent segment of wild-type virus (Fig. 2, arrow). ts1 and ts15 were obtained after chemical mutagenesis of the wild-type virus with 5-fluorouracil (32), and differences in the migration rate of individual RNAs in the two mutants undoubtedly reflect mutations in these genes. However, there was no proof that mutations detectable by polyacrylamide gel electrophoresis corresponded to the lesions responsible for the ts defects, and independent evidence had to be provided to verify that mutants in groups I and III have ts lesions in RNA 1 and RNA 2, respectively.

Selection of wild-type recombinants. Figure 3 schematically shows the approach that was taken to identify the ts genes of mutant viruses. Cells were simultaneously infected with mutants, and HK virus and recombinants were selected at nonpermissive temperature. Infection with ts mutants was done at a high multiplicity, and infection with HK virus was done at a low multiplicity to permit the selection of viable recombinants, in which most of the genes were derived from WSN virus. Similar procedures to obtain recombinants, which derive most of the genes from one or the other parent have been described previously (31). RNA analysis of the ts$^+$ recombinants allowed the identification of genes that were derived from HK virus and thus replaced the ts genes of the mutant viruses.

Mapping of WSN virus genome. To identify the defective gene products of ts mutants of WSN virus, it was first necessary to establish a genetic map of the wild-type virus. This was accomplished by selection of recombinants derived from wild-type A/WSN virus and from the influenza A/HK/8/68 virus we had previously mapped (18, 22, 28, 29). For example, recombinants containing only the neuraminidase from WSN virus were shown to contain only RNA 6 from WSN virus, whereas all other RNAs were derived from HK virus (gel not shown); hence RNA 6 codes for neuraminidase. Similar analysis permitted the identification of the gene products of RNAs 1 through 8 (Fig. 1 and 2). It can be seen that the gene order of WSN virus is identical to that of influenza A/PR/8/34 virus, another virus with the HON1 serotype (18, 22, 28, 29).

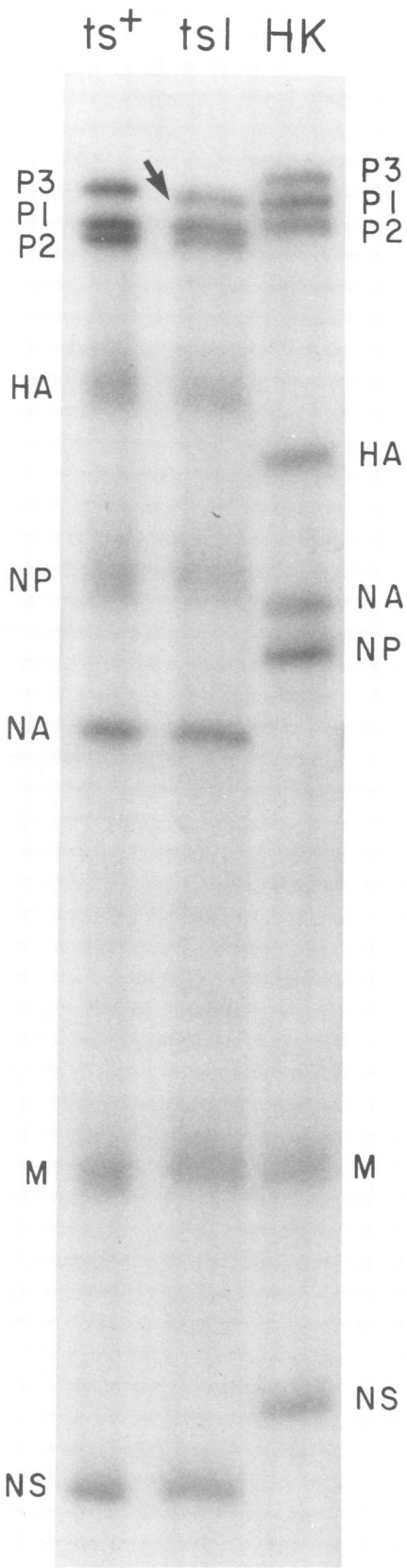

FIG. 1. *Analysis of influenza virus RNAs on a 6 M urea-polyacrylamide gel. Lane 1, RNA of WSN wild-type (ts^+) virus; lane 2, RNA of mutant tsl (group I); lane 3, RNA of HK virus. Letters next to the RNA segments of ts^+ and HK viruses identify the gene products coded for by these genes. The arrow next to RNA 1 of tsl virus indicates its unusual migration rate compared to the corresponding RNA segment of ts^+ virus.*

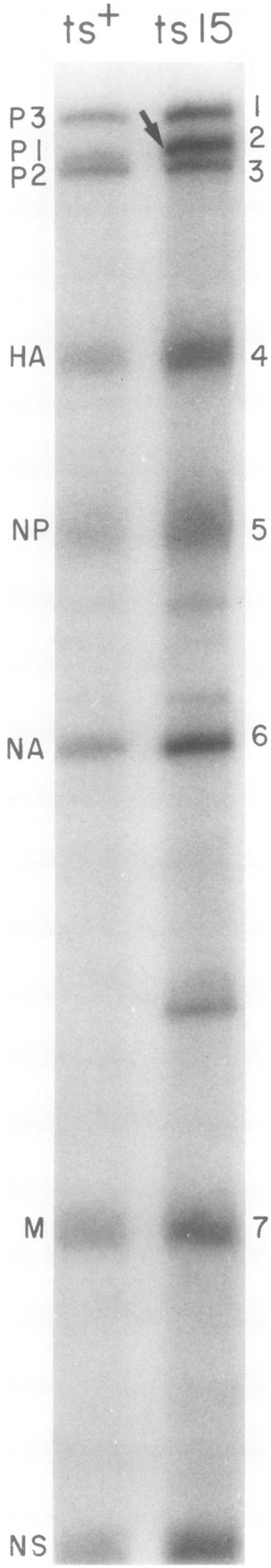

FIG. 2. *Comparison of the RNA pattern of WSN wild-type (ts^+) virus and that of a ts mutant (ts15). Lane 1, RNA of WSN wild-type (ts^+) virus; lane 2, RNA of mutant ts15 (group III). The letters next to the RNA segments of ts^+ virus identify the gene products for which those segments code. The arrow in lane 2 marks the slower moving RNA 2 of the mutant. Additional bands between RNAs 5 and 7 of ts15 were seen on occasion and possibly represent breakdown products of larger RNAs.*

Identification of the defective proteins in mutants of groups I and III. After identifying the genes of influenza WSN wild-type virus, we attempted to obtain recombinants derived from ts mutants and HK virus by mixedly infecting eggs at 37°C and selecting for ts$^+$ recombinants in MDCK cells at the nonpermissive temperature. Viruses that were isolated by this procedure were mostly revertants instead of recombinants. Hence, all subsequent recombination experiments were performed in tissue culture. Recombinational mixtures of ts1 and HK viruses yielded six different isolates that were phenotypically ts$^+$ and could be identified as recombinants by RNA analysis (Table 1). Figure 4 demonstrates the RNA pattern of one isolate (RI-1) that clearly derives RNA 1 (P3 protein) from HK virus. Similar analysis of all six recombinants (Table 1) reveals that RNA 1 (gene for P3 protein) is the only RNA in common that is derived from the "rescuing" HK virus, suggesting that only this gene is defective in mutants of group I.

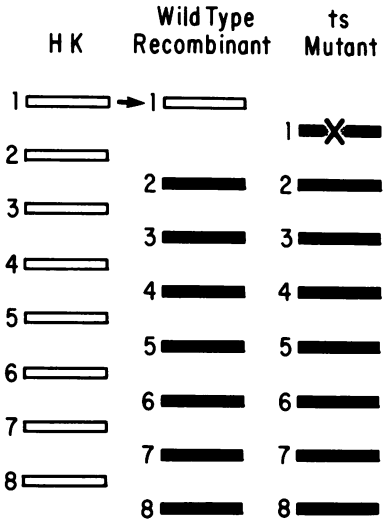

FIG. 3. *Schematic diagram of the strategy employed to identify the defective gene in ts mutants. Bars in this figure represent the RNA segments of the parent viruses (HK virus and ts mutant) and the recombinant. Cells are co-infected with mutant virus (in 10-fold excess) and a rescuing virus (HK virus). Viable recombinants are selected at a temperature at which the mutant does not grow, and in a cell system in which HK virus does not replicate. Such recombinants with wild-type (ts$^+$) phenotype uniformly derive one particular RNA segment from the nondefective HK virus parent. Subsequent RNA analysis of the recombinant permits identification of the ts gene in the mutant. In this figure, only RNA 1 of the recombinant is shown to be derived from the rescuing virus (open bar).*

It should be noted that we encountered difficulties in distinguishing RNA 7 of HK virus from RNA 7 of WSN virus. However, on occasion we were able to demonstrate that RNA 7 of HK virus migrates slightly slower than RNA 7 of wild-type WSN virus and also slower than RNA 7 of ts$^+$ recombinants derived from mutants in groups I and III. In addition, we were able to demonstrate that mutants in another complementation-recombination group (group VII) have a defect in RNA 7 (26). Therefore, the possibility that mutants in groups I and III have a ts defect in RNA 7 (M protein) can be excluded. All attempts to select ts$^+$ recombinants of ts6, another group I mutant, and HK virus failed. The reasons for this are not clear, but it appears that HK virus interferes with the replication of ts6 (unpublished observation).

As noted previously, the fact that RNA 2 of

TABLE 1. *ts$^+$ recombinants derived from ts mutants and HK virus*

Wild-type (ts$^+$) recombinant[a]	Parent viruses		Derivation of proteins[b]							
	Mutant (A/WSN virus)	Rescuing virus	P1	P2	P3	HA	NP	NA	M[c]	NS
RI-1	ts1 (group I)	A/HK/8/68	W	W	H	W	W	W	W	W
RI-2	ts1 (group I)	A/HK/8/68	W	W	H	W	W	W	W	W
RI-3	ts1 (group I)	A/HK/8/68	W	W	H	W	W	W	W	W
RI-4	ts1 (group I)	A/HK/8/68	H	W	H	W	W	W	W	W
RI-5	ts1 (group I)	A/HK/8/68	H	W	H	W	W	W	W	W
RI-6	ts1 (group I)	A/HK/8/68	H	W	H	W	W	W	W	W
RIII-1	ts101 (group III)	A/HK/8/68	H	W	W	W	W	W	W	W
RIII-2	ts101 (group III)	A/HK/8/68	H	W	W	W	W	W	W	W
RIII-3	ts101 (group III)	A/HK/8/68	H	W	W	W	W	W	W	W
RIII-4	ts101 (group III)	A/HK/8/68	H	W	W	W	W	W	W	W

[a] Phenotypically wild-type (ts$^+$) recombinants (RI or RIII) were obtained by recombination of HK virus and ts mutants in group I or III.

[b] Derivation of proteins was determined by RNA analysis after establishing a genetic map. The letter W or H describes the derivation of the protein from WSN virus or HK virus, respectively.

[c] Identification of the derivation of the M protein by RNA analysis alone was difficult (see text).

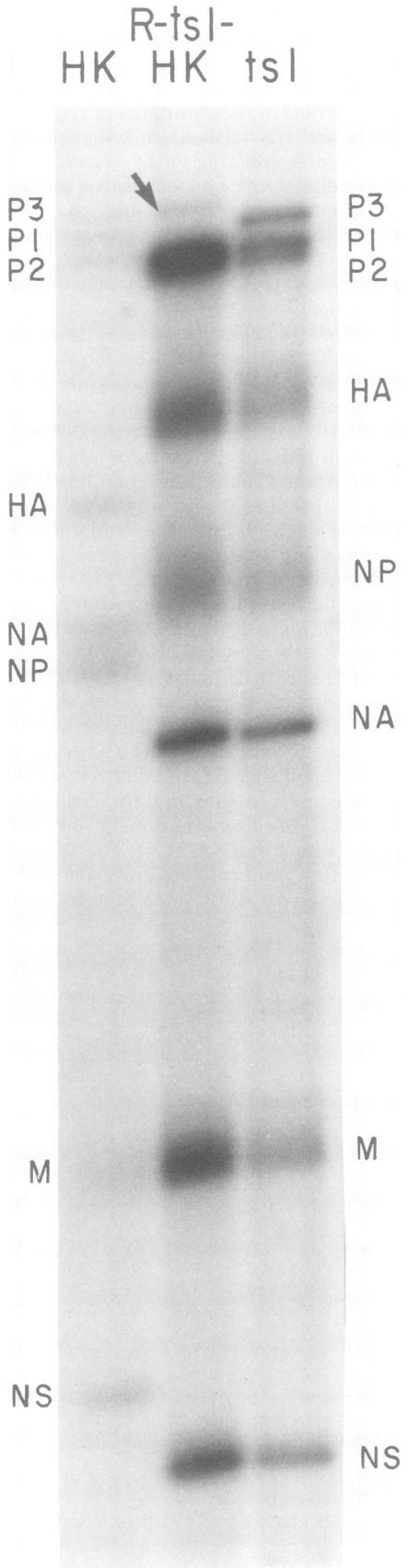

FIG. 4. *RNA analysis of HK virus, tsl, and a wild-type (ts^+) recombinant derived from them. Lane 1, RNA of HK virus; lane 2, RNA of a recombinant derived from tsl and HK virus (RI-1 in Table 1); lane 3, RNA of mutant tsl. Letters identify the gene products of each of the RNA segments in the HK and tsl virus genomes. Arrow indicates that RNA 1 in the recombinant is derived from the HK parent virus. It should be noted that the RNA segments of the different viruses are not present in equimolar amounts.*

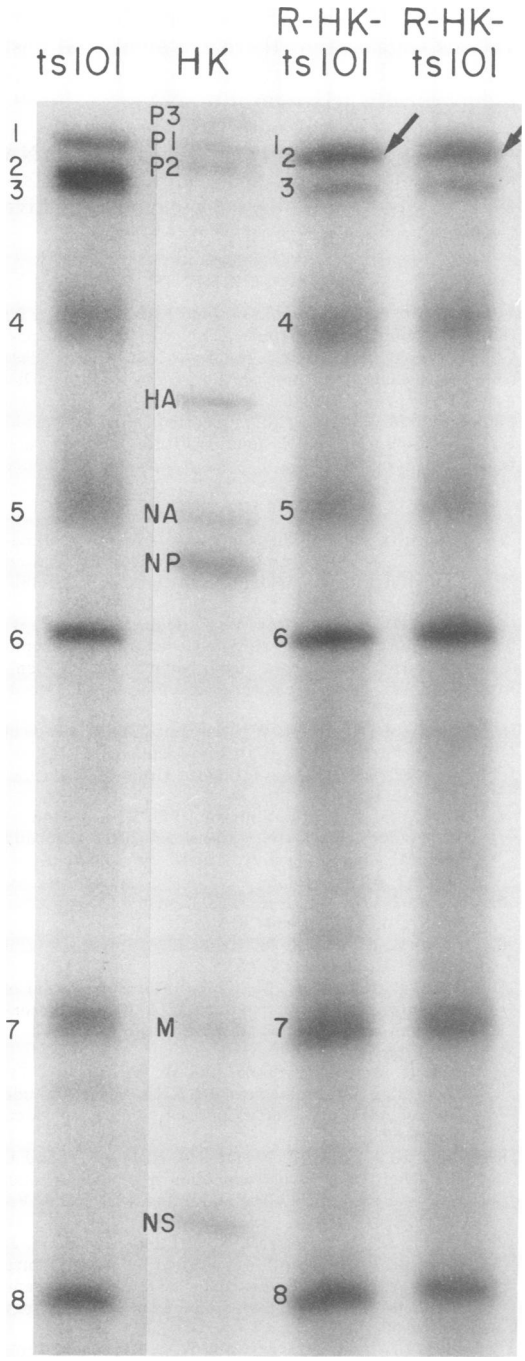

FIG. 5. *Analysis of the RNAs of two recombinants derived from HK virus and the mutant virus ts101. Lane 1, RNA of ts101; lane 2, RNA of HK virus; lane 3, RNA of recombinant (RIII-1) derived from HK virus and ts101; lane 4, RNA of recombinant (RIII-2) derived from HK virus and ts101. Arrows in lanes 3 and 4 identify the double band consisting of RNAs 1 and 2. RNA 2 in the recombinants is derived from HK virus. It should be noted that the RNAs of the parent virus ts101 were separated on the same gel but not right next to the other three samples.*

ts15 (group III) has an unusual migration rate suggested that the ts defect of group III mutants might be in RNA 2. This was confirmed by RNA analysis of four different ts$^+$ recombinants derived from ts101, another group III mutant, and HK virus (Table 1). ts101 rather than ts15 was used, since it was easier to isolate recombinants of this virus than of ts15. For purposes of illustration, the RNA patterns of two ts$^+$ recombinants and those of ts101 and HK virus are shown in Fig. 5. It can be seen that RNAs 2 and 3 of ts101 appear as very closely spaced double bands (lane 1). In contrast, RNA 2 of the two recombinants migrates more slowly (lanes 3 and 4) in a position similar to that of RNA 2 of HK virus (lane 2). As a result, RNAs 1 and 2 of the two ts$^+$ recombinants appear as double bands (arrows) compared to RNAs 2 and 3 in ts101. Previously, RNA 2 was shown to code for P1 protein. Hence, mutants in group III have a ts defect in P1 protein.

DISCUSSION

The biochemical identification of the active components of the influenza virus transcriptase complex has not been previously described. Studies of ts mutants of WSN virus revealed that at least two complementation-recombination groups (groups I and III) had ts defects associated with virus-specific cRNA synthesis in vivo (14). In this communication we provide evidence that members of these two groups have defects in P3 and P1 proteins, respectively.

RNA analysis of mutants in groups I and III demonstrated that one mutant in each group had an unusual RNA pattern with respect to the migration of one RNA segment. Although suggestive, this observation by itself does not establish that the same RNA segment is responsible for the ts defect. Subsequent confirmation was obtained by RNA analysis of ts$^+$ recombinants derived from HK virus and ts mutants in the same mutant groups, suggesting that the mutations identified by RNA analysis of the mutants were most likely in the same genes responsible for the ts lesion. However, it should be noted that most mutants have RNA migration patterns indistinguishable from that of wild-type WSN virus, and it is possible that mutants in which RNA segments other than those responsible for the ts defect migrate differently on polyacrylamide gels may be found. The nature of the mutations in ts6

and ts15 cannot be determined by these methods, as RNA analysis on polyacrylamide gels does not distinguish between point mutations and deletions.

Six recombinants with ts^+ phenotype derived from ts1 (group I) and HK virus had only one HK-derived RNA (RNA 1) in common. It should be noted that most genes of the recombinant viruses were derived from the mutant virus, as this virus was in excess in the recombinational mixture (31). Similarly, analysis of wild-type recombinants derived from ts101 (group III) and HK virus permitted the identification of RNA 2 as the gene carrying the ts defect. Our analysis of recombinant viruses was complicated by the fact that RNA 7 of both WSN virus and HK virus has a similar migration pattern. However, repeated analysis of the RNAs of the recombinants suggested that RNA 7 of all recombinants was derived from the mutant virus. In addition, mutants belonging to a different group (group VII) were shown to have a defect in RNA 7 (26). Members of groups I and III readily complement and recombine with mutants in group VII and members of all other groups (32, 33). Therefore, it is highly unlikely that mutants in groups I and III have an additional ts defect other than those in RNA 1 or 2. Using information obtained from maps of the WSN and HK virus genomes, we conclude that group I mutants have ts defects in P3 protein and group III mutants are defective in P1 protein. Because the ts defects in members of both of these groups result in the absence of RNA synthesis at the nonpermissive temperature, we further conclude that functional P1 and P3 proteins are required for cRNA synthesis.

Based on the identification of the specific ts defects in other mutant groups (24, 26, 35) and characterization of their biological properties (14, 32, 33), it is unlikely that either of the surface proteins or M, NP, and P2 proteins directly influence or regulate transcriptase activity in vivo. It is obvious that the nonstructural protein (NS) is not involved in the in vitro transcriptase activity of purified virus. However, unless mutants defective in nonstructural protein are identified, the possibility that nonstructural protein has a regulatory function during transcription in vivo will remain open.

Finally, several other questions remain unanswered at this time. Although actinomycin D has no effect on in vitro transcriptase activity (4), cRNA synthesis in vivo is inhibited by this drug. Furthermore, no viral protein synthesis takes place in enucleated cells (9, 11), and cRNA made in vitro is not translated in a cell-free wheat germ system (M. B. Ritchey and P. Palese, unpublished observation). These observations suggest that there are additional host factors which may be required for the synthesis of biologically active viral mRNA's.

In conclusion, our data indicate that P1 and P3 protein have an essential role during cRNA synthesis of influenza virus, but additional factors may be required for a biologically active transcriptase complex in vivo.

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