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Temperature-Sensitive Mutants of Influenza WSN Virus Defective in Virus-Specific RNA Synthesis

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Influenza WSN virus temperature-sensitive (*ts*) mutants were examined for defects in viral complementary RNA (cRNA) synthesis. The synthesis of viral cRNA was determined by hybridizing RNA from infected cells to radiolabeled virion RNA of known specific activity. Mutants in complementation groups I and III synthesized little, or no, cRNA at the nonpermissive temperature (39.5 C). When cells infected by these mutants were incubated for 5 h at the permissive temperature (33 C) and were then shifted to 39.5 C, net synthesis of cRNA ceased. This strongly suggests that mutants in these two complementation groups possess a *ts* defect in the transcriptase complex. Mutants in group II and group V synthesize reduced amounts of cRNA at 39.5 C. In contrast to the group I and III mutants, cRNA synthesis in cells infected by a group II or a group V mutant continues after a shift-up. This indicates that these mutants do not possess a *ts* transcriptase complex and that these mutants are most probably defective in some step in the amplification of cRNA synthesis. As will be discussed, the most likely defect in these mutants is in the synthesis of virion-type RNA. These results suggest that there are two influenza viral gene functions required for transcription and most likely two additional gene functions required for RNA replication.

The steps in the transcription and replication of influenza viral RNA are poorly understood at present. Temperature-sensitive (*ts*) mutants, which have been isolated in several laboratories (11, 19, 25, 27, 30-32), would be expected to aid the elucidation of these steps. Most of the previous studies directed at determining whether *ts* mutants were defective in virus-specific RNA synthesis used actinomycin D to suppress host cell RNA synthesis (11, 31). The addition of this drug during the first 2 to 4 h of infection inhibits viral replication almost completely (1, 12), and even when added later, viral replication is still curtailed (13). When the drug is added 3 to 4 h after infection, some virus-specific RNA synthesis persists: 40 to 50% of virion RNA synthesis and 0 to 10% of viral complementary RNA (cRNA) (RNA complementary to virion RNA [vRNA]) synthesis (23, 26). Consequently, if a viral mutant was unable to induce the synthesis of actinomycin D-resistant RNA at the nonpermissive temperature, it has been presumed that this mutant was defective in some aspect of virus-specific RNA synthesis (11, 31).

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The present study was undertaken to analyze influenza viral *ts* mutants for RNA synthesis defects without the use of actinomycin D. For this study, we used the *ts* mutants of WSN virus originally isolated by Sugiura et al. (30, 31). These mutants fall into at least seven complementation-recombination groups. We have analyzed the synthesis of viral cRNA during infection by hybridizing RNA from infected cells to radiolabeled virion RNA of known specific activity (16). Mutants in four of the seven complementation groups were found to be defective in cRNA synthesis at the nonpermissive temperature. The results of temperature-shift experiments strongly suggest that the mutants in two of the complementation groups possess a *ts* transcriptase-template complex. In contrast, the mutants in the other two groups appear to be defective in some other aspect of virus-specific RNA synthesis, with the most likely possibility being that they are defective in virion RNA synthesis which would result in a reduction in the amplification of cRNA synthesis.

MATERIALS AND METHODS

Cells. MDBK (bovine kidney) cells were grown as described previously (15, 30).

Virus. Wild-type WSN virus was grown and purified as described previously (15). To radiolabel the viral RNA, [^{3}H]adenosine at a concentration of 10 μCi/ml was present throughout infection. Stocks of the *ts* mutants were prepared as described previously (20, 30, 31).

Infection of cells. Monolayer cultures of MDBK cells in 25 cm^2-plastic bottles were infected with wild-type or mutant virus at a multiplicity of 5 to 10 except where indicated. The virus was adsorbed for 1 h at 4 C or for 40 min at room temperature. After adsorption, prewarmed growth medium (reinforced Eagle medium containing 0.2% bovine serum albumin) was added, and the bottles were completely immersed in a water bath maintained at either 33 C (permissive temperature) or 39.5 C (nonpermissive temperature). Zero time corresponds to the time at which the cells were brought to 33 or 39.5 C.

Preparation of infected cell RNA. Before the collection of the cells, [^{14}C]uridine at 0.5 μCi/ml was added to label the infected cell RNA. The isotope was present for 30 min at 39.5 C or for 45 min at 33 C. The radioactivity in the infected cell RNA enabled us to monitor the steps during the preparation of this RNA for annealing. However, the amount of ^{14}C-labeled radioactivity was kept at a minimal level so as not to interfere with the measurements of ^{3}H-labeled virion RNA in the annealing experiments (see below).

At the indicated times, the infected cells were collected into reticulocyte standard buffer (0.01 M KCl; 0.0015 M $MgCl_2$; 0.01 M Tris-hydrochloride, pH 7.4), and were fractionated into cytoplasm and nucleus as described previously (15, 16). As 95% of viral cRNA has been shown to accumulate in the cytoplasm (16), only the cytoplasmic fraction of infected cell RNA was analyzed for viral cRNA. The cytoplasmic extract was made 2% in sodium dodecyl sulfate and 0.01 M in EDTA, and the RNA was extracted three times with water-saturated phenol. Phenol was removed by three ether extractions and the ether was removed by evacuation under low pressure. The RNA was desalted by chromatography through Sephadex G-50 in water. The RNA in the excluded volume (monitored by its ^{14}C radioactivity) was pooled and lyophilized to dryness. To verify the absence of viral cRNA in the nucleus, in several experiments the nuclear RNA was extracted (15, 16) from wild-type or mutant (ts-6, ts-7, ts-15) virus-infected cells and was analyzed in annealing experiments as described below for cytoplasmic RNA; little, or no, viral cRNA was detected in the nucleus.

Annealing experiments. The lyophilized cytoplasmic RNA was taken up in a small volume (0.10 to 0.15 ml) of a buffer containing 0.025 M KCl, 0.002 M EDTA, and 0.01 M Tris-hydrochloride, pH 7.4 (buffer L). The concentration of RNA was determined by the absorbancy at 260 nm, and within each annealing experiment the concentration of RNA in each sample was adjusted to be the same. The RNA of [^{3}H]adenosine-labeled virus was extracted with phenol, and was desalted by Sephadex G-50 chromatography in water. The vRNA was lyophilized to dryness and was taken up in a small volume of buffer L. The specific activity of the vRNA was 8×10^3 to 12×10^3 counts/min per μg.

Annealing was carried out in a final volume of 0.1 to 0.2 ml as described previously (16). To 3 μg of ^{3}H-labeled vRNA was added either approximately 20 μg of infected cell cytoplasmic RNA or an equivalent volume of buffer L. In these low salt conditions, the mixture was heated at 98 C for 5 min to release the viral cRNA from any double-stranded RNA forms. Under these conditions, virus-specific double-stranded RNA forms are denatured (Krug and Etkind, unpublished data). After boiling, the mixture was immersed in a 68 C water bath, the salt concentration was increased to 2.4× KKC (KKC = 0.15 M KCl, 0.0015 M potassium citrate), and incubation at 68 C was continued for 4 h. The annealed mixtures were slowly cooled over an 18-h period, and were then divided in half. One-half was left untreated, and the second half, in 2.4× KKC, was treated with 10 μg of pancreatic RNase per ml and 50 units of T1 RNase per ml for 30 min at 37 C. The amount of acid-insoluble ^{3}H-labeled virion RNA in the untreated and RNase-treated samples was determined. After annealing in the absence of infected-cell cytoplasmic RNA, 1 to 1.4% of the ^{3}H-labeled virion RNA was RNase resistant (control value). This control value, determined in duplicate in each annealing experiment, was subtracted from the amount of ^{3}H-labeled virion RNA rendered RNase resistant after annealing to infected-cell cytoplasmic RNA. This calculation yields the amount of viral cRNA in the sample of cytoplasmic RNA. Maximal amounts of ^{3}H-labeled virion RNA were rendered RNase resistant when either 3 or 6 μg was added to the annealing mixture, indicating that the cRNA in 20 μg of cytoplasmic RNA was saturated by 3 μg of the ^{3}H-labeled virion RNA.

Materials [2,8-^{3}H]adenosine and [2-^{14}C]uridine were purchased from New England Nuclear Corporation, Boston, Mass.

RESULTS

Characteristics of the production of viral cRNA. As described previously (16), the net production of viral cRNA during infection can be measured by annealing infected-cell RNA to an excess of virion RNA of known specific activity. The amount of virion RNA rendered RNase resistant represents the amount of viral cRNA in the sample of infected-cell RNA. It was shown previously that in MDCK (canine kidney) cells infected with wild-type virus at 37 C, net synthesis of cRNA continued for the first 4 h of infection and subsequently the amount of cRNA remained constant or decreased slightly (16). In initial experiments, we determined the kinetics of accumulation of cRNA in MDBK (bovine kidney) cells infected by wild-type virus at 39.5 C and at 33 C. One representative experiment is shown in Fig. 1. At 39.5 C, the kinetics of cRNA production were similar to that in MDCK cells at 37 C. Net production of cRNA continued for the first 4 to 6 h. Subsequently, the amount of cRNA remained constant or decreased slightly (not

shown in Fig. 1). At 33 C, the kinetics of cRNA production were slower. The time at which net cRNA synthesis ceased at 33 C has been somewhat variable, and occurred between 6 to 9 h after infection. The maximum production of cRNA by wild-type virus at 39.5 C was 1.2 to 3.0 times that at 33 C.

Using virus radiolabeled in its RNA as inoculum, Bean and Simpson were able to detect primary transcription in influenza virus-infected chicken embryo fibroblasts (2). Their data, however, indicated that the amount of transcription due to primary transcription represents an extremely small fraction of the transcription occurring after amplification. The assay for viral cRNA used in the present study does not detect the small amount of cRNA synthesized as a result of primary transcription. Thus, when 80 μg of cycloheximide per ml was added at the start of wild-type virus infection, little or no cRNA production was detected (Fig. 1). Less than 0.002 μg of cRNA was produced at 39.5 C in the presence of cycloheximide as compared to 0.235 μg in the absence of cycloheximide. This result indicates that this assay will detect mutants with two different types of defects: (i) those which do not produce cRNA at 39.5 C because of a *ts* defect in the enzyme-template complex which synthesizes cRNA; and (ii) those which do not produce cRNA at 39.5 C because of a *ts* defect in the steps involved in the amplification of cRNA synthesis beyond that due to primary transcription.

Screening mutants for ts defects in viral cRNA production. Wild-type virus and a group IV mutant (ts 11), which possesses a *ts* neuraminidase (20), synthesize more cRNA at 39.5 C than at 33 C (Table 1). A group VI mutant (ts 61S), which possesses a *ts* hemagglutinin (M. Ueda, unpublished data), also exhibits this behavior. In contrast, a group I (ts 6) and a group III (ts 15) mutant synthesize little, or no, cRNA at 39.5 C. The inability to synthesize any cRNA at 39.5 C is also possessed by at least one other mutant in each of these two complementation groups, ts-1 in group I and ts-101 in group III. This indicates that a ts defect in cRNA synthesis is a characteristic of these two complementation groups. Other mutants in these two groups (e.g., ts 8 in group I and ts-5 in group III) exhibit a partial, rather than a complete, reduction in cRNA production at the nonpermissive temperature, and were not studied further.

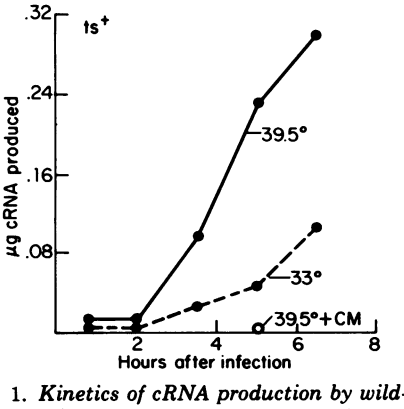

FIG. 1. *Kinetics of cRNA production by wild-type (ts+) virus in MDBK cells at 39.5 and at 33 C. Experimental procedures were as described in Materials and Methods. Virus adsorption was for 1 h at 4 C. For treatment with cycloheximide (CM), this drug at a final concentration of 80 μg/ml was added immediately after adsorption.*

Mutants in two other complementation groups (II and V) also are defective in the synthesis of cRNA at 39.5 C (Table 2). The amount of cRNA synthesized at 39.5 C by ts 53 (group II) and by ts 56 (group V) was 24 and 17 to 26%, respectively, of that synthesized at 33 C. In contrast, wild-type virus produced 1.2- to 1.5-fold more cRNA at 39.5 C than at 33 C. More cRNA was synthesized at 39.5 C by ts 53 and ts 56, the group II and V mutants, than by

TABLE 1. *Synthesis of cRNA in MDBK cells infected with influenza WSN virus ts mutants*

Virus	Complementation group	cRNA produced (μg)[a]	
		33 C	39.5 C
ts+	Wild type	0.196	0.316
ts11	IV	0.208	0.312
ts6	I	0.120	0.003
ts15	III	0.136	0.004

[a] Infected cells were collected at 9 h at 33 C and at 7.5 h at 39.5 C.

TABLE 2. *Synthesis of cRNA in MDBK cells infected with influenza WSN virus ts mutants*

Virus	Complementation group	cRNA produced (μg)			
		Exp 1[a]		Exp 2[b]	
		33 C	39.5 C	33 C	39.5 C
ts+	Wild type	0.228	0.345	0.200	0.240
ts53	II	0.162	0.039	0.190	0.046
ts56	V	0.200	0.053	0.220	0.038

[a] Infected cells were collected at 7 h at 33 C and at 6 h at 39.5 C.

[b] Infected cells were collected at 9 h at 33 C and at 7 h at 39.5 C.

two of the group I (ts 6, ts 1) and two of the group III (ts 15, ts 101) mutants. The amount of cRNA produced at 39.5 C by ts 53 and ts 56 (group II and V) appears to be related to the number of vRNA genomes per cell, as increasing the multiplicity of infection from 5 to 20 increases the ratio of cRNA production at 39.5 C to that at 33 C by two- to threefold. This behavior is not exhibited by wild-type virus, and as will be discussed later, is consistent with the nature of the putative defects in group II and group V mutants. Mutant ts 66, which is a double mutant (group V and VI) is similar to ts 56 (group V) in that this mutant also induces only a small amount of cRNA at 39.5 C. That this is a property of the mutation in the group V cistron of ts 66 is indicated by the fact that the group VI mutant, ts 61S, produces wild-type levels of cRNA at 39.5 C. Ts 4 and ts 7 (group II) and ts 12 (group V) were found to produce variable amounts of cRNA at 39.5 C, ranging from 20 to 100% of that produced by wild-type virus. As will be discussed later, a tendency for leakiness in cRNA synthesis is not surprising for mutants with the putative defect(s) of group II and group V mutants.

Temperature-shift experiments. To distinguish viral gene functions required for cRNA synthesis throughout replication from those required only early during infection, we carried out temperature-shift (33 to 39.5 C) experiments. Cells infected by a given *ts* mutant were incubated at the permissive temperature (33 C) for 4 or 5 h to allow the synthesis of virus-specific RNA, both vRNA and cRNA, and the synthesis of virus-specific proteins. The infected cells were then shifted to the nonpermissive temperature, and the amount of cRNA synthesized after the shift-up was determined. For these experiments, we used one "prototype" mutant from each of the four complementation groups defective in cRNA synthesis: ts 6 (group I), ts 53 (group II), ts 15 (group III), and ts 56 (group V).

After shifting up cells infected by either ts 6 (group I) or ts 15 (group III), little, or no, cRNA was produced (Fig. 2). This indicates that the group I and group III functions are required for cRNA synthesis throughout infection. Mutants in group I specify a *ts* protein distinct from that specified by group III mutants, as indicated by the fact that mutants in group I complement those in group III: in cells doubly infected at 39.5 C with a group I and a group III mutant, cRNA is made (Table 3) and virus is produced (30).

In contrast to the group I and group III

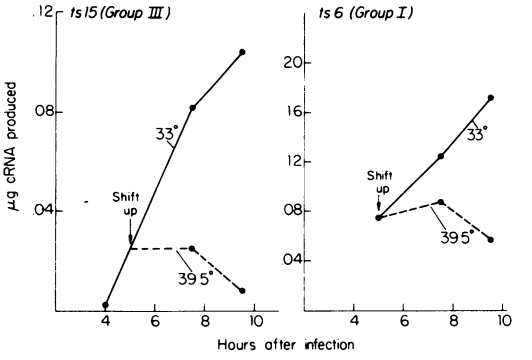

FIG. 2. *Temperature-shift experiments with a group I (ts 6) and a group III (ts 15) mutant. Experimental procedures were as described in Materials and Methods.*

mutants, cells infected by ts 53 (group II) and ts 56 (group V) continue to synthesize cRNA after a shift-up (Fig. 3). With ts 53, the rate of cRNA synthesis immediately after the shift-up was similar to, or slightly greater than, the rate in the cells maintained at 33 C. After 1.5 h at 39.5 C, there was no further increase in the amount of cRNA, whereas in the cells maintained at 33 C cRNA production continued for at least a further 1.5 h. With ts 56, cRNA synthesis after the shift-up proceeded at a rate much greater than in cells maintained at 33 C and continued for at least 1.75 h. In this particular experiment net cRNA synthesis in the cells maintained at 33 C ceased a little early, at 5.75 to 6 h. These results indicate that the group II and group V gene functions are required for cRNA synthesis primarily only during early times of infection.

DISCUSSION

The single-stranded RNA genome (vRNA) of influenza virus is segmented (4, 18, 28). The *ts* mutants of influenza WSN virus grown in MDBK cells fall into at least seven complementation-recombination groups (30, 31), and the working hypothesis is that there is a one-to-one correspondence between a complementation-recombination group and a vRNA segment. Mutants in two complementation groups, IV and VI, possess a *ts* defect in the neuraminidase (20) and in the hemagglutinin (Ueda, M., unpublished data), respectively. Our results indicate that mutants in four other complementation groups (I, II, III, and V) have a *ts* defect in virus-specific RNA synthesis. Consequently, at least four of the seven or more vRNA segments appear to code for proteins involved in virus-specific RNA synthesis.

TABLE 3. *Complementation between group I and group III mutants for cRNA synthesis*

Inoculum virus	cRNA produced at 39.5 C (μg)[a]
ts6 (group I)	0.007
ts15 (group III)	0.007
ts6 + ts15	0.118
ts$^+$	0.168

[a] Infected cells were collected at 7.5 h.

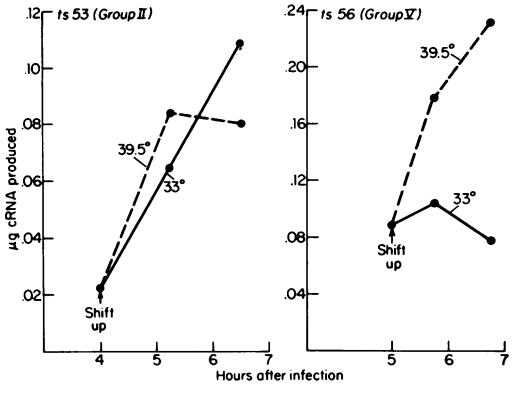

FIG. 3. *Temperature-shift experiments with a group II (ts 53) and a group V (ts 56) mutant. Experimental procedures were as described in Materials and Methods.*

Mutants in two of the complementation groups, groups I and III, apparently specify a transcriptase-ribonucleoprotein complex which is temperature sensitive. The strongest evidence for this conclusion comes from the results of the temperature-shift experiments. When cells infected by ts-6 (group I) or by ts-15 (group III) were incubated for 5 h at 33 C and were then shifted to 39.5 C, little or no cRNA production occurred after the shift-up. The most likely interpretation of this result is that the transcriptase formed at the permissive temperature by these mutants does not function at 39.5 C. It is unlikely that the cessation in cRNA synthesis is due to a *ts* block in vRNA synthesis or in the synthesis of new transcriptase molecules. Experiments using cycloheximide indicate that cRNA synthesis does not require continuing synthesis of vRNA or of transcriptase molecules (26; Krug and Etkind, unpublished data). Further proof of the existence of a *ts* transcriptase complex in these group I and group III mutants would derive from the demonstration that the virion-associated transcriptase is *ts* in vitro. However, this is very difficult to demonstrate with WSN virus because the transcriptase of the wild-type virus is thermolabile and has only very minimal activity in vitro at 39.5 C (3).

Whether primary transcription occurs at 39.5 C in cells infected by the two group I mutants (ts 6, ts 1) or by the two group III mutants (ts 15, ts 101) cannot be ascertained definitively, as the assay we have used does not detect primary transcription. Nonetheless, overall transcription at 39.5 C with these two group I and two group III mutants is barely detectable. Since viral cRNA is most probably the viral mRNA (10, 14, 22), these mutants at 39.5 C induce the synthesis of only minimal amounts of virus-specific proteins, as evidenced by analysis of infected cells by immunological and enzymatic assays (30, 31) and by polyacrylamide gel electrophoresis (Palese, P., Tobita, K., Ueda, M. and Krug, R., unpublished data).

The fact that two different complementation-recombination groups appear to specify a *ts* transcriptase strongly suggests that the transcriptase-ribonucleoprotein complex is comprised of at least two proteins required for activity. Three proteins have been found to be associated with the transcriptase complex: the nucleocapsid protein and the largest virion proteins, P1 and P2 (5, 6, 29). With another RNA virus, vesicular stomatitis virus, the nucleocapsid protein and the largest virion protein have been shown to be required for the activity of the virion transcriptase (8, 9).

It has been reported that group I and III mutants do not induce the synthesis of actinomycin D-resistant RNA after a shift-up (31). As more vRNA than cRNA synthesis persists in the presence of actinomycin D during wild-type infection (23, 26), these results suggest that group I and III mutants may not synthesize vRNA after a shift-up. It is quite possible that vRNA synthesis is dependent on cRNA synthesis, as has already been postulated for VSV replication (7, 17, 21, 24). It is, however, necessary, as verification, to use an assay which specifically and directly measures vRNA and to determine with this assay whether, in the absence of actinomycin D, vRNA is synthesized after a shift-up. It has also been reported that a *ts* mutant of fowl plaque virus (FPV) synthesizes neither cRNA nor vRNA after a shift-up, but it is not clear whether the particular mutant studied was actually a double mutant, as recombination or complementation studies were not carried out (25).

The mutants in the other two complementation groups which are defective in virus-specific RNA synthesis, groups II and V, do not appear to possess a defect in the transcriptase. After allowing amplification of transcription at the permissive temperature, cells infected by ts 53 (group II) or by ts 56 (group V) continue to

synthesize cRNA after a shift-up. Thus, the transcriptase specified by these mutants functions well at 39.5 C. Mutants in these two groups are most probably defective in the synthesis of a virus-specific product required for the amplification of cRNA synthesis, with the most likely defect being in the synthesis of vRNA. Consistent with an inability to synthesize vRNA, group II and group V mutants reportedly do not induce the synthesis of actinomycin D-resistant RNA after a shift-up (31). Further, a defect in vRNA synthesis is consistent with the behavior of these mutants at 39.5 C. If a mutant were defective in vRNA synthesis, cRNA synthesis at 39.5 C would result from primary transcription (parental transcriptase transcribing the parental genome) and from the transcription catalyzed by newly synthesized transcriptase molecues. Any vRNA synthesized at 39.5 C should be susceptible to transcription. With ts 53, ts 56 and ts 66, a low amount of cRNA is made at 39.5 C, presumably reflecting minimal synthesis of vRNA at 39.5 C. Increasing the number of parental genomes per cell by increasing the multiplicity of infection would be expected to increase the amount of cRNA synthesized, as was observed. Any leakiness in vRNA synthesis should be reflected in an augmented leakiness in cRNA synthesis. Presumably, this is the situation with ts 4, ts 7, and ts 12. Clearly, to substantiate a vRNA defect in group II and V mutants, it is necessary to measure vRNA synthesis directly.

The results so far obtained with the WSN virus *ts* mutants allow a tentative classification of the defects in these mutants as shown in Table 4. With respect to defects in RNA synthesis, it is possible to make an initial comparison with the FPV *ts* mutants described by Ghendon *et al.* (11). These authors examined one mutant in each of five complementation groups, and found that four of the five mutants possessed a defect in some aspect of virus-specific RNA synthesis. One mutant, in complementation group D, possessed a *ts* virion transcriptase and synthesized little, or no, virus-specific proteins. Consequently, complementation group D of FPV most probably corresponds to complementation group I or III of WSN virus. A second complementation group of FPV defective in transcription has not yet been described. Three other FPV mutants did not induce the synthesis of actinomycin D-resistant RNA at the nonpermissive temperature. This was interpreted as indicating that these mutants were defective in vRNA synthesis. Two of these three FPV complementation groups (groups A, B, and C) probably correspond to complementation groups II and V of WSN virus. A complete comparison of the FPV and WSN virus mutants and the determination of the total number of influenza viral complementation groups defective in RNA transcription and/or replication must await further experimentation.

TABLE 4. *Tentative classification of the defects in influenza WSN virus ts mutants*[a]

Group	Defect	Defective protein
I	cRNA synthesis	NP or P
II	vRNA synthesis	?
III	cRNA synthesis	NP or P
IV	Neuraminidase	Neuraminidase
V	vRNA synthesis	?
VI	Hemagglutinin	Hemagglutinin
VII	?	?

[a] Compiled from present study, Palese et al. (20), Suguira et al. (30, 31), and M. Ueda (unpublished data).

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