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Isolation and Characterization of Sendai Virus Temperature-Sensitive Mutants

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Ten temperature-sensitive mutants of Sendai virus, a paramyxovirus, were isolated and partially characterized. The mutants replicated in chicken embryo lung cells at 30 C, but not at 38 C; wild-type virus grew equally well at both temperatures. Complementation tests divided the mutants into seven groups. Six groups synthesized neither infectious virus nor RNA when incubated at 38 C from the beginning of infection. Temperature shift-up experiments demonstrated that three of these complementation groups were blocked in early steps required for RNA synthesis, but these gene functions were not needed throughout the replicative cycle. In contrast, the other three RNA-negative complementation groups were defective throughout the replicative cycle in functions required for virus-specific RNA synthesis. Only one mutant, which complemented all of the above, synthesized RNA but not infectious virus when placed at 38 C; the hemagglutinin of this mutant functioned only at the permissive temperature.

Temperature-sensitive (ts) mutants of animal viruses have been useful in studying virus replication (6, 7). With respect to paramyxoviruses, a number of Newcastle disease virus (NDV) nonconditional-lethal mutants as well as ts mutants have been isolated, but these have been used mainly to study genetic interactions (4, 8, 9). We have isolated and characterized a number of Sendai virus ts mutants to learn more about biochemical events in paramyxovirus replication. Preliminary genetic and biochemical analyses of 10 of these mutants are the subject of this report.

MATERIALS AND METHODS

Virus and cells. A clone of wild-type Sendai virus was plaque purified from the Enders strain of Sendai virus and was free from incomplete virions (17). Stocks of virus were prepared by infecting 10-day-old embryonated hens' eggs with 0.01 hemagglutinating unit and incubating for 72 h at 37 C. Methods for preparing chicken embryo lung (CEL) cell cultures, for growing Sendai virus in these cells, and for plaque assay were described (5), but in the present work, Eagle minimal essential medium was used as growth medium and in the plaque overlay.

Virions were labeled with radioactive amino acids as described (18), except that labeled precursors were added 16 h after infection and virions were collected 32 h later. Culture fluids containing released virus were centrifuged for 10 min at 3,600 × *g* to remove cells and debris. Virus was pelleted (78,000 × *g*, 5 C, 30 min) and resuspended in 0.01 M sodium phosphate (pH 7.2) for fractionation experiments.

RNA extraction, rate-zonal centrifugation, and radioactivity determinations. These methods have all been fully described (13).

Selection of ts mutants. The methods for mutagenization of wild-type virus and isolation of ts mutants were similar to those described for Sindbis virus (2) and vesicular stomatitis virus (14). After mutagenization with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (2), the remaining infectious titer was 10^{-3} of the initial titer. Mutagenization by 5-fluorouracil (FU) (14) was done by treating CEL cell monolayers with 200 μg of the compound per ml for 1 h, by infecting with 10 plaque-forming units (PFU) of Sendai virus per cell, and by allowing the virus to grow for 48 h in the presence of FU. After treatment with mutagens, the virus stocks were diluted to produce a few plaques at 30 C. After incubation for 7 to 10 days, well-isolated plaques were picked, suspended in 1 ml of phosphate-buffered saline (PBS) containing 5% fetal calf serum, and replated at permissive (P) temperature (30 C) and nonpermissive (NP) temperature (38 C). Ten isolates were temperature-sensitive, whereas all others tested were not. Stocks of each ts isolate were grown in CEL cell monolayers at 39 C for further testing. Virus was harvested when the hemagglutinin titer was 27/ml or greater.

Complementation tests. Complementation tests were done in CEL cell monolayer cultures containing about 10^6 cells at the time of infection. Sendai virus ts mutants were diluted in PBS to give an input multiplicity of 5 PFU/cell in a single infection or 2.5 PFU/cell of each mutant in mixed infection. Virus was adsorbed for 30 min at 25 C, the monolayers were washed with PBS, and 5 ml of growth medium was added. After incubation at 38 C for 4 h, cultures were washed again, prewarmed growth medium was added, and monolayers were reincubated at 38 C for 48 to 72

h. The medium was then assayed for plaque formation on CEL cell monolayers at 30 C. Complementation levels were calculated according to Burge and Pfefferkorn (3).

Isolation of virion glycoproteins. Sendai virions were fractionated by treating them (1 mg of protein per ml) with 2% Trition X-100 and 1 M KCl for 20 min at 25 C (15, 16). The mixture was centrifuged at 100,000 × *g* for 30 min at 5 C, and the glycoproteins in the 100,000 × *g* supernatant were separated from the smallest virion polypeptide by dialysis and centrifugation (15, 16).

Acrylamide gel electrophoresis. This method was previously described (18).

Neuraminidase assay. Fetuin was used as a substrate. Free sialic acid was measured by the thiobarbituric acid procedure (19, 20). Duplicate samples of virions or the glycoprotein fraction from virions (15, 16) were diluted with 0.2 M sodium phosphate buffer (pH 5.9) to 0.05 ml, and 0.05 ml of fetuin (6.25 mg/ml) in the same buffer was added. The incubation temperature was 30 or 38 C.

RESULTS

Isolation of ts mutants. With nitrosoguanidine and FU mutagenesis, about 1.5 and 1.0%, respectively, of the plaque isolates contained useful ts mutants. When mutant stocks were tested at 38 and 30 C, ratios of plaques produced ranged from less than 1.6×10^{-5} (ts 271) to 2.5×10^{-8} (ts 935) (Table 1). Only a few plaques from the 38 C plates were not temperature-sensitive when picked and tested again at 38 and 30 C, indicating that the mutants had low back mutation frequencies and were slightly "leaky." The frequency of wild-type revertants in mutant stocks ranged from 3.8×10^{-4} to 4.5×10^{-6}.

Growth of ts mutants at P and NP temperatures. To examine the growth of ts mutants, cultures were infected with an input multiplicity of about 5 PFU/cell and incubated at P and NP temperatures. Growth curves are shown for wild-type virus (Fig. 1A) and mutant ts 271 (Fig. 1B). Wild-type virus grew equally well at 30 and 38 C. Most ts mutants were like ts 271, producing virus at approximately the same rate at 30 C as wild-type virus, but little or no virus at 38 C. Typical amounts of virus released 48 h after infection by each mutant are shown in Table 2. All mutants synthesized substantial amounts of virus at 30 C, and some (ts 74, 105, 245, and 271) grew better than wild-type virus at 30 C. In contrast to growth under permissive conditions, mutant yields at

TABLE 1. *Efficiency of plating of wild-type virus and ts mutants*

Virus	38 C/30 C ratio (PFU/ml)
Wild-type	1.0
ts 74	2.4×10^{-7}
ts 105	1.2×10^{-7}
ts 245	1.2×10^{-7}
ts 271	1.6×10^{-5}
ts 348	$<2.7 \times 10^{-5}$
ts 557	6.0×10^{-7}
ts 595	$<1.0 \times 10^{-7}$
ts 642	1.0×10^{-5}
ts 840	$<8.0 \times 10^{-7}$
ts 935	2.5×10^{-8}

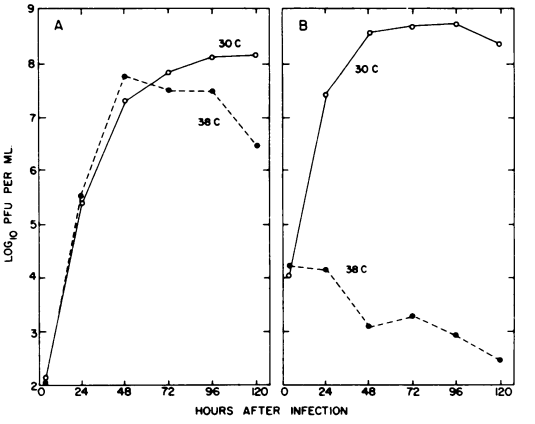

FIG. 1. *Growth of wild-type Sendai virus and mutant ts 271 at 30 and 38 C. CEL cell monolayers were infected with an input multiplicity of 5 PFU/cell. After adsorption (30 min at 24 C), monolayers were washed and incubated at 30 or 38 C. At intervals after adsorption, culture fluids were removed from the monolayers and assayed for infectious virus at 30 C by the plaque method. A, Wild-type virus; B, mutant ts 271. ●, 38 C; ○, 30 C.*

TABLE 2. *Growth of Sendai virus mutants at 30 and 38 C*[a]

Virus	Yield (PFU/ml)		38 C/30 C ratio (PFU/ml)
	30 C	38 C	
Wild-type	2.0×10^{7}	6.0×10^{7}	3
ts 74	3.4×10^{8}	1.8×10^{2}	5.3×10^{-7}
ts 105	2.0×10^{8}	$<10^{1}$	$<5.0 \times 10^{-8}$
ts 245	6.3×10^{7}	$<10^{1}$	$<1.6 \times 10^{-7}$
ts 271	3.6×10^{8}	1.2×10^{3}	3.3×10^{-6}
ts 348	6.3×10^{5}	$<10^{1}$	1.6×10^{-5}
ts 557	5.0×10^{4}	$<10^{1}$	$<2.0 \times 10^{-4}$
ts 595	1.6×10^{7}	$<10^{1}$	$<6.3 \times 10^{-7}$
ts 642	2.5×10^{7}	5.0×10^{4}	2.0×10^{-3}
ts 840	4.0×10^{5}	$<10^{1}$	$<2.5 \times 10^{-5}$
ts 935	2.0×10^{6}	$<10^{1}$	$<5.0 \times 10^{-6}$

[a] CEL cell monolayers were infected with an input multiplicity of 5 to 10 PFU/cell. After 30 min at 24 C, cultures were washed and then were incubated at 30 or 38 C for 48 h. Culture fluids were cleared of debris by low-speed centrifugation and then were assayed for infectivity by the plaque method at 30 C.

the restrictive temperature were less than 0.2%. Most of the virus produced at NP temperature was of the mutant genotype, unable to produce virus when tested again at 38 C, indicating slight "leakiness." Additionally, these results confirmed the previous observation that the mutants have a low reversion frequency to wild type.

Complementation. The results of complementation experiments are shown in Table 3. On the basis of these data, we have assigned the mutants to seven complementation groups (A to G). Five complementation groups (A and D to G) are represented by single mutants (245, 105, 348, 74, 271). Group B is represented by two mutants (557, 642), and group C by three mutants (595, 840, 935). Within a group, each mutant complemented all other mutants, but not mutants in the same group. Thus, the groups are nonoverlapping.

Virus-specific RNA synthesis by ts mutants. The ability of Sendai virus ts mutants to synthesize the various species of virus-specific RNA (1, 13) under restrictive conditions was examined. After adsorption of virus at 24 C for 30 min, infected monolayers were incubated at 38 C for 48 h. At this time, when viral RNA synthesis is maximal for wild-type virus (13), cultures were treated with actinomycin D and labeled with [^{3}H]uridine in the presence of the drug. The RNAs were extracted and sedimented in sucrose gradients. Results shown in Fig. 2 are representative of each type of physiological behavior observed, although a member of every complementation group was examined.

Similar amounts of viral RNA were synthesized by ts mutant 271 (group G) at P and NP temperatures (excluding the adsorption period) (Fig. 2A). However, this was threefold less than wild-type virus under similar conditions. The distribution of radioactivity was the same as that found when wild-type virus was incubated under the same conditions (13), i.e., virus-specific 18*S* messenger RNA and 50*S* genomes were made. Thus, we have designated complementation group G RNA$^+$. Mutants belonging to complementation groups A to F synthesized little or no RNA when incubated at the restrictive temperature from the beginning of infection, and we have designated them RNA$^-$ (Fig. 2B, C, and D).

To distinguish viral gene functions required for RNA synthesis throughout replication from those required only early in the cycle, temperature shift (30 to 38 C) experiments were done. Infected cultures were incubated at the P temperature for 48 h and then were shifted to the NP temperature for 24 h. In all cases, virus production ceased within 24 h. After the 24 h shift-up, the cultures were labeled with [^{3}H]uridine for 1 h in the presence of actinomycin D, and the RNAs were extracted and analyzed as described above. As expected, ts mutant 271 (group G, RNA$^+$) synthesized normal amounts of viral RNA after shift-up (Fig. 2A). Both the mutant and wild-type virus synthesized more RNA after being shifted from the P to NP temperature.

Mutants belonging to groups A, B, and C (RNA$^-$) synthesized substantial amounts of 18 and 50*S* RNA after being shifted to the NP temperature for 24 h (Fig. 2B). When the interval at the NP temperature was extended to 48 h before labeling (data not shown), the

TABLE 3. *Complementation levels*[a]

Mutant	Mutant									
	935	840	642	595	557	348	271	245	105	74
74	31	5	48	8	120	8	2,050	1,390	83	
105	7	5	2	38	6	3	40	22		
245	65	1,220	15	460	140	660	30,000			
271	14	6,750	10	3,300	6,600	1,175				
348	50	20,000	2	13	48					
557	7	90	0.8	16						
595	0.07	0.3	8							
642	20	14								
840	0.3									
935										

[a] Complementation tests were done as described in Materials and Methods. Complementation levels were calculated according to Burge and Pfefferkorn (3). All values given are the average of three experiments. These levels were significantly greater than 1 by the Student's *t* test at $P < 0.05$. Complementation levels divided the mutants into seven complementation groups: group A, mutant 245; group B, 557 and 642; group C, 595, 840, and 935; group D, 105; group E, 348; group F, 74; and group G, 271.

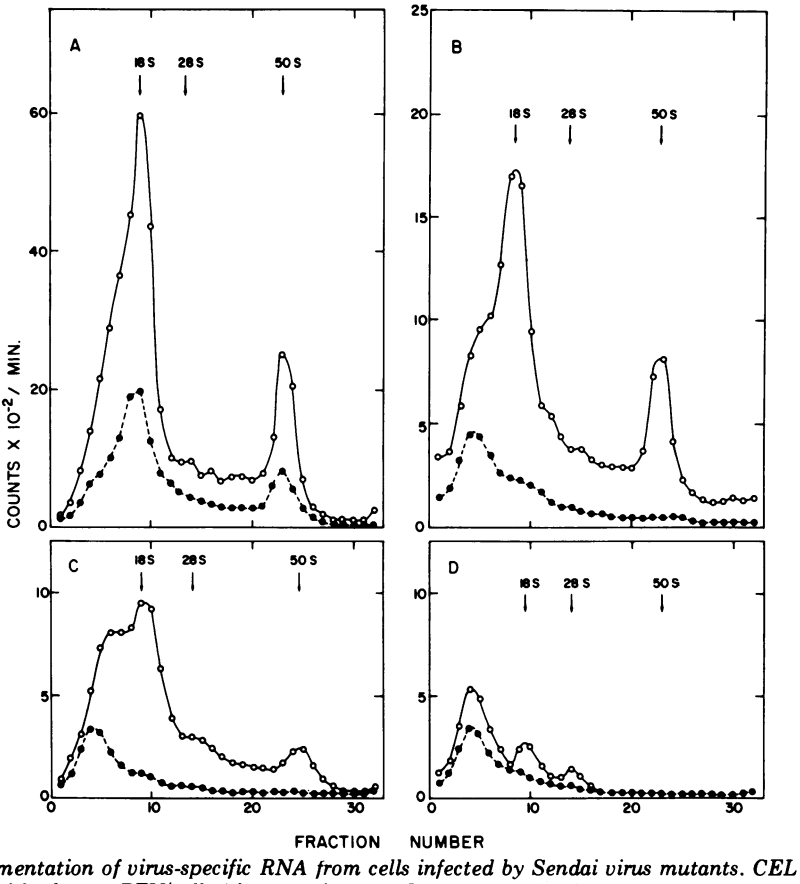

FIG. 2. *Sedimentation of virus-specific RNA from cells infected by Sendai virus mutants. CEL cell cultures were infected with about 5 PFU/cell. After 30 min at 24 C one group of cultures (●) was incubated for 48 h at 38 C. Another group (○) was incubated at 30 C for 48 h and then was shifted to 38 C for 24 h. Each group then was treated for 1 h with 50 μg of actinomycin D per ml and labeled for 1 h with 50 μCi of [³H]uridine per ml in the presence of the drug, all at 38 C. RNA was extracted with sodium dodecyl sulfate-phenol (13) and centrifuged at 20,000 rpm at 20 C for 16 h in 34-ml linear 15 to 30% sucrose gradients (13). A, Infection with mutant ts 271 (complementation group G); B, mutant ts 557 (complementation group B); C, mutant ts 74 (complementation group F); and D, mutant ts 105 (complementation group D).*

amount of virus-specific RNA made was the same as after the 24 h shift. Thus, these mutants are defective in early functions essential for viral RNA synthesis, but these functions are not required later in the replicative cycle.

Mutants ts 348 and 74 (groups E and F, RNA⁻) synthesized low levels of virus-specific RNA after shift-up for 24 h (Fig. 2C). Viral RNA synthesis decreased even further (in contrast to groups A to C) as the interval at the NP temperature was extended to 48 h (data not shown).

The group D mutant (ts 105, RNA⁻) was clearly separated physiologically from groups E and F. After shift-up for 24 h, ts 105 synthesized little or no virus-specific RNA (Fig. 2D). (The peak of RNA found at about 5S was also found in uninfected cells.) When the shift intervals were decreased to 2 or 4 h (data not shown), ts 105 still showed a marked decline in viral RNA synthesis, indicating that this mutant ceases RNA synthesis rapidly under restrictive conditions. (When examined in the same way, mutants belonging to groups E and F synthesized normal amounts of viral RNA.) From these data it is apparent that mutants belonging to complementation groups D, E, and F are defective in functions required for virus-specific RNA synthesis throughout the replicative cycle, but the defect in group D may be more intimately related to the synthetic machinery for viral RNA.

Mutant ts 271 has a hemagglutinin that does not function at NP temperature. Mutant ts 271 synthesized RNA, but not infectious virus, at the NP temperature. When the ability

of this mutant to agglutinate erythrocytes was examined, agglutination occurred at P, but not at NP temperature. Wild-type virus and the other mutants agglutinated chicken erythrocytes at both temperatures (Table 4). When the mixture of virus and erythrocytes was shifted from NP to P temperature, the erythrocytes became fully agglutinated. When the agglutinated mixture then was shifted back from P to NP temperature, the hemagglutinin pattern was again lost. These results indicated that the hemagglutinin of mutant ts 271 did not function at NP temperature, but that function was restored at P temperature. However, the inability to hemagglutinate chicken erythrocytes does not exclude the possibility that the hemagglutinin was still capable of binding to receptors.

Sendai virions contain at least six polypeptides (12). By analogy with other paramyxovirions, the largest glycopolypeptide with an apparent molecular weight of 70,000 probably represents the hemagglutinin (15, 16). The inability of mutant ts 271 to agglutinate chicken erythrocytes at NP temperature might be due to an alteration in some other virion protein which makes the hemagglutinin nonfunctional. Therefore, we tested isolated glycopolypeptides (15, 16) of ts 271 for the ts phenotype. The preparations contained all of the hemagglutinating activity, and mainly virion glycopolypeptides were seen in polyacrylamide gels (Fig. 3, peaks 2 and 5). When this material was tested for ts hemagglutinin activity, the results were similar to those obtained with intact virions. Wild-type

TABLE 4. *Hemagglutination by wild-type Sendai virus and mutants*[a]

Virus	Hemagglutination (per ml)		
	30 C	38 C	38 C/30 C
Wild-type	330	150	0.5
ts 557	41	81	2.0
ts 348	120	160	1.3
ts 935	120	160	1.3
ts 642	200	200	1.0
ts 105	68	54	0.8
ts 595	54	41	0.8
ts 840	160	120	0.8
ts 74	160	68	0.4
ts 245	240	68	0.3
ts 271	200	<2	<0.01

[a] Chicken erythrocytes (0.5% in PBS, pH 7.2) and viruses were separately warmed in a water bath at 30 or 38 C, mixed, and maintained at the respective temperatures. The test was performed in tubes, and titers represent the reciprocal of the highest dilution showing hemagglutination at 30 min after mixing.

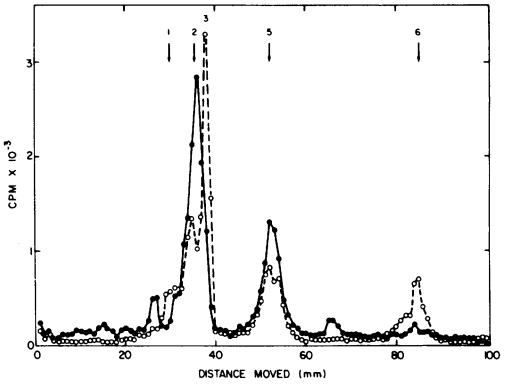

FIG. 3. *Polyacrylamide gel electrophoresis of Sendai mutant ts 271 [^3H]glycopolypeptides prepared as described in Materials and Methods. [^{14}C]polypeptides from whole wild-type Sendai virions were added before electrophoresis. The numbering system is that of Mountcastle et. al. (12). Migration is from left to right.* ●, ^3H; ○, ^{14}C.

virion glycoproteins isolated the same way agglutinated chicken erythrocytes at 30 and 38 C, whereas ts 271 glycoproteins were unable to agglutinate erythrocytes at 38 C (data were identical to those shown in Table 4). We attempted to identify which of the two glycopolypeptides from wild-type or mutant ts 271 was the hemagglutinin, but we were unable to separate the glycopolypeptides by centrifugation in sucrose gradients (15, 16). However, it is clear that the ts hemagglutinin resides in one of the solubilized viral glycopolypeptides, and not in another viron component which might affect hemagglutinating activity in intact virions.

If the same glycopolypeptide has neuraminidase and hemagglutinin activities (15, 16), then mutant ts 271 might have a ts neuraminidase. To test this possibility, equivalent amounts of glycopolypeptides were isolated from wild-type virus and ts 271, and neuraminidase activity was measured at the P and NP temperatures (Fig. 4). The rate of sialic acid release by ts 271 enzyme was about the same at 30 and 38 C, indicating that the neuraminidase was not temperature-sensitive. Increasing the temperature to 46 C did not change the results. However, compared with wild-type, the mutant neuraminidase functioned much less efficiently (Fig. 4), even at the P temperature. Additionally, when ts 271 and wild-type virions were tested for heat stability at 54 C, it was found that the mutant hemagglutinin and neuraminidase activities were more heat labile than wild-type virus. After 90 s at 54 C, wild-type virions had 35% of their original hemagglutinin and 30% of their original neuraminidase activities, but mutant

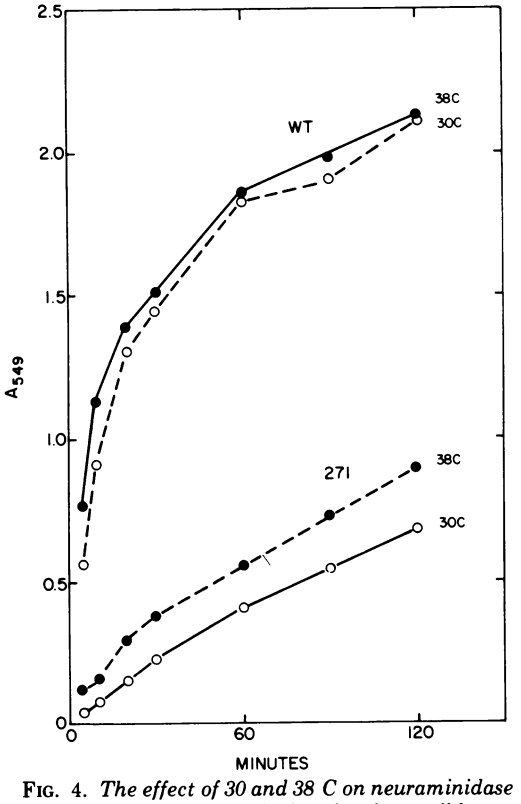

FIG. 4. *The effect of 30 and 38 C on neuraminidase activities of the glycoprotein fraction from wild-type Sendai virus and ts 271. Replicate tubes containing 50 hemagglutinating units were placed at 30 or 38 C. The wild-type virus preparation contained 5 μg of protein per ml, and ts 271 contained 10 μg of protein per ml. At intervals, one tube of each was removed, rapidly chilled, and assayed for released sialic acid (19, 20).*

virions retained only 3% of their hemagglutinin and less than 1% of their neuraminidase activities. These data are consistent with the idea that a single glycopolypeptide has neuraminidase and hemagglutinin activities, but other explanations are not ruled out.

DISCUSSION

The Sendai virus genome has a molecular weight of 6×10^6 (10). The virion polypeptides have molecular weights from 40,000 to 80,000 (12). Thus, the genome may contain enough information for eight to ten polypeptides. Complementation experiments (Table 3) indicated that our Sendai virus ts mutants were defective in seven different cistrons; thus, this small series of mutants may already represent most of the viral gene functions. However, the number of complementation groups may be high, because low complementation values could reflect intracistronic complementation or complementation between mutants with multiple temperature-sensitive lesions.

The complementation groupings were confirmed in part by the physiological properties of the mutants. We were able to classify the mutants into four categories according to their ability to synthesize virus-specific RNA under restrictive conditions. Complementation group G was RNA^+. Complementation groups A to F synthesized neither viral RNA nor infectious virus when incubated at the NP temperature from the beginning of infection. However, after shift-up late in infection, groups A, B, and C synthesized substantial amounts of virus-specific RNA, demonstrating that three gene functions were required transiently in the replicative cycle for RNA synthesis to be initiated. Groups D, E, and F appeared to be defective in functions required continuously for viral RNA synthesis. The reason for the slow decline in RNA synthesis with groups E and F after shift-up is not apparent. One possibility is that at NP temperature the temperature-sensitive defect prevents the formation of a protein required for RNA synthesis, but once the protein is formed it remains functional after shift from 30 to 38 C. Such mutants have been described for Semliki forest virus (11). Mutant ts 105 (group D) showed a rapid decline in viral RNA synthesis after shift-up, suggesting that its gene product turns over rapidly or is unable to maintain a functional configuration after shift to NP conditions.

It may seem surprising that a virus with such a small amount of genetic information should possess six functions related to RNA synthesis. But the three early functions represented by complementation groups A, B, and C are probably involved in preparing the viral transcriptive machinery for productive interaction with the cell (entry, "uncoating," transport, etc.), because they are not required later in infection. It should be emphasized that these functions are only "early" with respect to RNA synthesis: with respect to virus production they are also "late," according to the results of shift-up experiments. This suggests that they represent virion structural components.

One structural element of virions whose function may not be required for viral RNA synthesis is the hemagglutinin, as exemplified by the RNA^+ phenotype of mutant ts 271. But it is possible that other mutants of this polypeptide will be discovered which will affect RNA synthesis. Recently, we showed that virion envelope glycoproteins (12) and the smallest virion polypeptide, also thought to be an envelope

component (10), inhibit virion transcriptase in vitro (P. A. Marx, A. Portner, and D. W. Kingsbury, manuscript submitted for publication). Thus, these polypeptides are all candidates for a role in regulating viral RNA synthesis.

RNA synthesis functions continually required during infection, for which complementation groups D, E, and F are candidates, would include transcription and genome replication. Transcriptive complexes isolated from virions and infected cells contain two polypeptides, the nucleocapsid structure unit (molecular weight 60,000) and the largest virion polypeptide (molecular weight 75,000) (18; P. A. Marx, A. Portner, and D. W. Kingsbury, manuscript submitted for publication). Both of these may be needed for transcriptase activity. Polypeptides involved in genome replication have not been identified.

It is clear that we cannot exclude any virus-specified polypeptide from an involvement in paramyxovirus RNA synthesis at this stage in our understanding. Hopefully, additional work on the biochemistry of Sendai virus ts mutants will contribute useful answers to these questions.

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