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Seven Complementation Groups of Respiratory Syncytial Virus Temperature-Sensitive Mutants

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Fifteen temperature-sensitive mutants of the RSN-2 strain of respiratory syncytial virus have been classified into six complementation groups, two of which appeared to be homologous with two of the three complementation groups of the A2 strain described by Wright et al. (P. F. Wright, M. A. Gharpure, D. S. Hodes, and R. M. Chanock, Arch. Gesamte Virusforsch. 41:238–247). Thus seven complementation groups of respiratory syncytial virus, designated A, B, C, D, E, F, and G, have been defined. The frequency and type of mutant isolated varied according to strain; group C was unique to the A2 strain, and groups D, E, F, and G were unique to the RSN-2 strain. The highest complementation indexes were obtained by preincubation for 7 h at permissive temperature, followed by incubation at restrictive temperature for 40 to 50 h in the case of A2 strain mutants or 80 to 90 h for RSN-2 strain mutants. Genetic recombination was not detected.

Temperature-sensitive (*ts*) mutants have been isolated from two strains of human respiratory syncytial (RS) virus. Seven mutants were isolated from the A2 strain by Gharpure et al. (5) and classified into three groups (designated A, B, and C) by genetic complementation tests (11). Five of these seven mutants belonged to complementation group A. Another 36 mutants were isolated from the RSN-2 strain of RS virus by Faulkner et al. (4), and a minimum of three complementation groups was recognized on the basis of genetic and physiological studies. We now report further genetic characterization of the *ts* mutants of the RSN-2 strain and identification of the homologies of the complementation groups of the A2 and RSN-2 strains. Optimal conditions for complementation were quite distinct in the two strains. Definition of these conditions was an essential preliminary to demonstration of interstrain complementation. We suggest that a similar approach may be appropriate with other viruses which do not grow to high titer and where complementation has been difficult to demonstrate.

Fifteen of the RSN-2 *ts* mutants have been classified into six complementation groups, two of which appeared to be homologous with complementation groups A and B of the A2 strain. Thus seven complementation groups are recognized now in RS virus: groups A, B, and C, represented by A2 strain mutants, and groups A, B, D, E, F, and G, by RSN-2 strain mutants.

Genetic recombination between complementing *ts* mutants was not detected in agreement with the previous findings of Wright et al. (11) and Faulkner et al. (4). It is likely, therefore, that the RS virus genome is an unsegmented molecule and that there may be at least seven primary gene products. The identification of between six and eight virus-specified polypeptides in partially purified RS virus and in extracts of RS virus-infected cells reinforces this conclusion (3, 7, 13). However, because five of the seven groups are represented by single mutants, the occurrence of intracistronic complementation cannot be excluded.

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MATERIALS AND METHODS

Cells. The BS-C-1 and HeLa cell lines were obtained from J. F. Williams, Institute of Virology, and propagated in roller bottle cultures in Eagle minimum essential medium with twice the normal concentration of amino acids (Glasgow modification) and supplemented with 5% fetal calf serum (virus and mycoplasma screened, Gibco-Biocult, Glasgow).

Virus. The A2 strain wild type and mutants *ts*1 (A2), *ts*2 (A2), and *ts*7 (A2) were provided by Linda Richardson, National Institutes of Health, Bethesda, Md. The origin and characteristics of the RSN-2 wild type and *ts* mutants have been described previously (4). In this paper, to avoid confusion, the strain of origin of individual mutants will be indicated in parentheses following the mutant number.

Complementation tests. Complementation tests were carried out by the method described by Faulkner et al. (4), with the additional modifications described in the text. The complementation index was calculated as the ratio: yield from the mixed infection (*tsx* + *tsy*) at 39°C/sum of the yields of the single infections (*tsx*) + (*tsy*) at 39°C, where the yields are assayed at 31°C,

and *tsx* and *tsy* are any pair of mutants. The multiplicity of each mutant was the same in mixed infection and self-infection; i.e., the total multiplicities were not adjusted. Previous experiments showed that this did not bias the results.

Preparation of mutant stocks. RS virus does not grow to high titer, thereby limiting the multiplicity of infection which can be achieved. The following method of stock preparation was found to give the highest virus titers. Each mutant was inoculated into a 30-ml (1-ounce) bottle containing 10^6 BS-C-1 cells and incubated until cytopathic effect was extensive. The monolayer was scraped into suspension, and the whole culture was inoculated immediately into a 2-liter (80-ounce) roller bottle containing 10^8 BS-C-1 cells. The culture was incubated for 1 h at 31°C to allow absorption of the inoculum. Then 40 ml of incubation medium was added, and the culture was incubated at 31°C for 5 days. The incubation medium was discarded and replaced by 30 ml of fresh incubation medium. Incubation was continued at 31°C for a further 48 to 72 h until cytopathic effect was almost complete. The attached cells were rapidly scraped into the medium, which was immediately dispensed into vials and frozen in liquid nitrogen. Approximately 10^8 PFU were obtained from one roller bottle culture.

Infectivity assay. Infectivities were measured on BS-C-1 cell monolayers. Dilutions of virus-containing fluids were inoculated in 0.2-ml volumes and incubated for 30 min at 31°C for absorption. Then the monolayers were overlaid with 5 to 7 ml of Eagle medium containing 0.9% agar and 2% fetal calf serum. The infected monolayers were incubated for 5 to 7 days at 39°C (the restrictive temperature) or 7 to 10 days at 31°C (the permissive temperature) in CO_2-gassed and humidified incubators. The monolayers were fixed with Formol saline and stained with Giemsa. At both temperatures, RS virus plaques appeared as darkly staining heaps of cells, as described previously (4, 12). The nonsyncytial plaque morphology of mutant *ts*2 (A2), which according to Gharpure et al. (5) distinguishes this mutant from the other A2 strain mutants, was not observed in BS-C-1 cells because all strains of human and bovine RS virus so far examined produce the same uniform focal plaque on BS-C-1 monolayers under agar overlay (3).

Growth curve determination. Replicate cultures of 2×10^6 HeLa cells in 30-ml (1-ounce) screw-capped bottles were infected with approximately 1 PFU of the wild type of either the A2 or the RSN-2 strain per cell. The infected cultures were incubated at 39°C for 30 min to absorb the inoculum. The inoculum was then removed by two washes of cold medium, and finally 5 ml of warm incubation medium was added and the bottles were placed in a water bath at 39°C. Two bottles were removed at intervals up to 44 h after infection and frozen at −70°C. The infectivities were measured on BS-C-1 monolayers at 31°C in the normal manner.

RESULTS

Growth curve of wild-type virus at restrictive temperature. Figure 1 shows that the growth of wild-type virus of the A2 and RSN-2 strains in HeLa cells at 39°C was essentially similar. The yield of the RSN-2 strain virus in HeLa cells was lower, although the cultures had been infected at the same nominal multiplicity. The incremental yields, however, were comparable. This result is probably a reflection of the past history of the two strains and the fact that the inoculum virus titers were determined on BS-C-1 cell monolayers. The RSN-2 strain had been grown exclusively on BS-C-1 cells and exhibited some adaptation to these cells (4), whereas the A2 strain had been grown predominantly in HeLa cells.

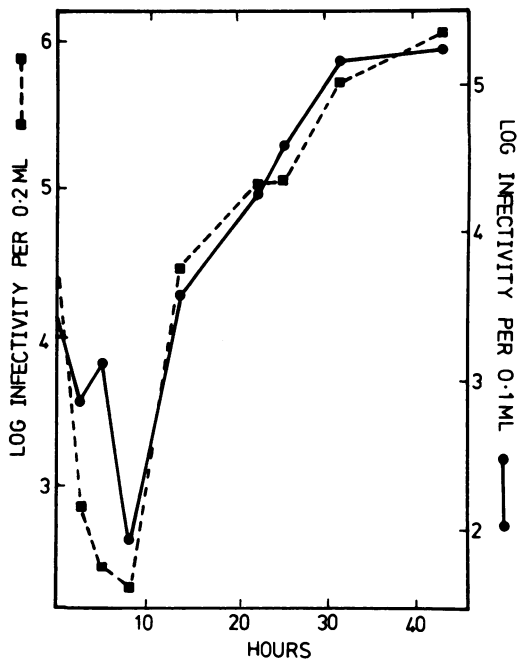

FIG. 1. *Growth of wild-type virus (ts^+) of the A2 and RSN-2 strains in HeLa cells at 39°C. Monolayers of HeLa cells in 1-ounce (ca. 30-ml) bottles were infected with 1 PFU of ts^+ (A2) or ts^+ (RSN-2) per cell as described in the text and incubated in a water bath at 39°C. Replicate cultures were harvested by freeze-thawing at various times after infection, and total infectivity was assayed on BS-C-1 cell monolayers at 31°C. (●——●) ts^+ (RSN-2); (■----■) ts^+ (A2).*

Effect of the period of incubation at 31°C on complementation yields. Factors affecting the efficiency of the standard complementation test were examined to improve consistency and reduce variability between experiments. It was confirmed that complementation indexes were higher and more reproducible when HeLa cells were used as host for the mixed infection rather than BS-C-1 cells (4). Another important factor was the length of the period of incubation at permissive temperature (31°C) before removal of the inoculum and incubation at restrictive

temperature (39°C). Table 1 illustrates that the complementation index in a mixed infection of two A2 strain mutants was increased from 100 to 813 by increasing the incubation period at 31°C from 1 to 7 h. Other experiments showed that increasing the 31°C incubation period beyond 7 h did not further enhance the complementation index.

Effect of the period of incubation at restrictive temperature on complementation. The effect of increasing periods of incubation at 39°C on the complementation index is illustrated in Table 2. HeLa cells were infected with two complementing mutants from the RSN-2 strain, using two different periods of preliminary incubation at 31°C, namely, 1.5 h and the optimal 7 h. The complementation indexes increased up to 88 h after absorption and subsequently declined. At all times the longer incubation period at 31°C gave higher complementation indexes with these RSN-2 strain mutants, as observed previously with A2 strain mutants (Table 1).

Optimum incubation periods at 39°C. Initially, the optimum period of incubation at 39°C had been determined for mutants of the A2 strain and found to be between 40 and 50 h. This is illustrated in Fig. 2a for mixed infection by *ts*1 (A2) and *ts*7 (A2). However, several experiments showed that complementation between mutants of the RSN-2 strain was poor under these conditions. The experiment with mutants *ts*17 (RSN-2) and *ts*19 (RSN-2) illustrated in Fig. 2b shows that much longer incubation was required to obtain satisfactory complementation with RSN-2 strain mutants. The optimal period of incubation at 39°C was between 80 and 90 h, i.e., twice as long as in the case of the A2 strain mutants. Under these conditions of infection the observed complementation indexes were higher than previously reported for any pair of RS virus *ts* mutants. The data illustrated in Fig. 2a, b, and c were obtained in a single experiment, and therefore the indexes are directly comparable. Figure 2c illustrates the complementation indexes obtained in a mixed infection with one mutant from each strain, *ts*1 (A2) and *ts*19 (RSN-2). It is apparent that interstrain complementation experiments between the A2 strain and the RSN-2 strain require careful design and evaluation.

The data in Table 3 show that it was necessary to measure complementation indexes in interstrain experiments over a range of incubation times spanning the optima of the two strains. In this experiment, two *ts* mutants of the A2 strain, representing complementation groups A and B, were crossed with four mutually complementing

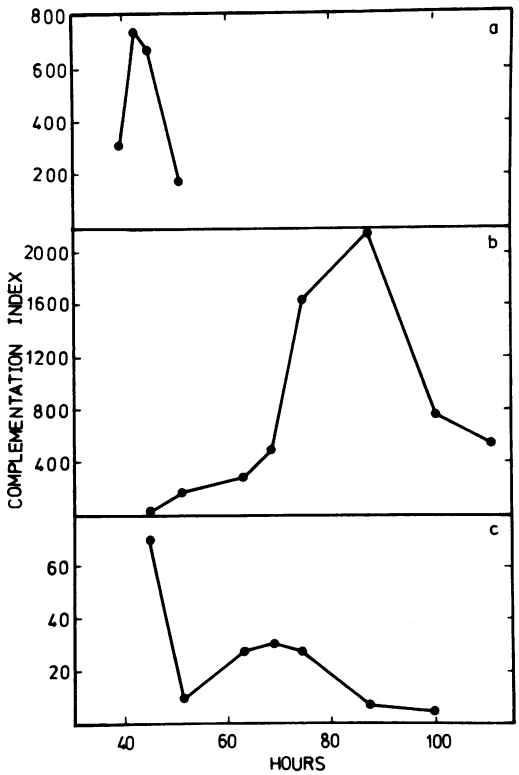

FIG. 2. *Complementation index after various periods of incubation at restrictive temperature. (a) Complementation indexes in a mixed infection with two A2 strain mutants, ts1 (A2) and ts7 (A2). (b) Complementation indexes in a mixed infection with two RSN-2 strain mutants, ts17 (RSN-2) and ts19 (RSN-2). (c) Complementation indexes in an interstrain mixed infection, ts1 (A2) and ts19 (RSN-2).*

TABLE 1. *Increase in complementation index (CI) with time of absorption at 31°C*

Mutants	Host cell	Absorption period at 31°C (h)	Incubation time at 39°C (h)	CI
*ts*1 (A2) + *ts*7 (A2)	HeLa	1	47	100
		2.5	45.5	160
		4	44	240
		7	41	813

TABLE 2. *Complementation indexes in mixed infection of HeLa cells with mutants ts17 (RSN-2) and ts19 (RSN-2)*

Absorption time at 31°C (h)	Complementation index at time of incubation (h) at 39°C:					
	63	72	88	96	112	135
1.5	19	75	78	76	6	10
7	64	90	210	145	13	38

TABLE 3. *Interstrain complementation; effect of varying the period of incubation at 39°C*

Mutant	Complementation indexes in mixed infection at time of incubation (h) at 39°C[a]:									
	37		43		50		61		75	
	*ts*1 (A2)	*ts*2 (A2)	*ts*1 (A2)	*ts*2 (A2)	*ts*1 (A2)	*ts*2 (A2)	*ts*1 (A2)	*ts*2 (A2)	*ts*1 (A2)	*ts*2 (A2)
*ts*1 (RSN-2)	0.8	<1	*96*	<1	4	*50*	2.7	1	5.5	<1
*ts*17(RSN-2)	<1	0.4	*73*	<1	2	1.3	<1	1.1	1.1	<1
*ts*19(RSN-2)	5.4	0.2	*535*	<1	7	*78*	ND	<1	ND	<1
*ts*20(RSN-2)	<1	<1	*67*	<1	2.6	*86*	0.5	<1	3.7	<1

[a] Optimum values are indicated in italics. ND, Not done.

mutants [*ts*1 (RSN-2), *ts*17 (RSN-2), *ts*19 (RSN-2), and *ts*20 (RSN-2)] of the RSN-2 strain. Mutant *ts*1 (A2) complemented all four RSN-2 *ts* mutants optimally at 43 h, whereas mutant *ts*2 (A2) complemented mutants *ts*1 (RSN-2), *ts*19 (RSN-2), and *ts*20 (RSN-2) at 50 h only. Mutant *ts*2 (A2) did not complement mutant *ts*17 (RSN-2) at any time, and both mutants have been assigned to the same complementation group (group B) on the basis of these and additional data. Mutants *ts*1 (RSN-2), *ts*19 (RSN-2), and *ts*20 (RSN-2) complemented both *ts*1 (A2) and *ts*2 (A2) at either 43 or 50 h, and therefore did not belong to complementation group A or B. Since these three RSN-2 *ts* mutants were also complemented by *ts*7 (A2), representing group C, they are assigned to new complementation groups, which have been designated D, E, and F, respectively (see Table 4).

The seven complementation groups. Of the remaining 32 RSN-2 strain mutants, only one [mutant *ts*6 (RSN-2)] complemented each of the six mutants representing groups A, B, C, D, E, and F. Mutant *ts*6 (RSN-2), therefore, represented the seventh complementation group (Table 5). Table 5 also shows that mutant *ts*26 recombinants was very low, they could have (A2), *ts*1 (RSN-2), *ts*17 (RSN-2), *ts*19 (RSN-2), *ts*20 (RSN-2), and *ts*6 (RSN-2), but not *ts*1 (A2). Mutant *ts*26 (RSN-2), therefore, was considered to belong to complementation group A. Another nine RSN-2 *ts* mutants complemented the mutants representing groups A, C, D, E, F, and G, but not *ts*17 (RSN-2) (Table 6) or each other (data not shown). Therefore, these mutants have been placed in complementation group B. The remaining 21 RSN-2 strain *ts* mutants failed to complement, or complemented sporadically, and could not be classified into any complementation group.

Absence of genetic recombination. No wild-type recombinants have been found previously in the progeny of mixed infections of *ts* mutants of either the A2 strain (11) or the RSN-2 strain (4). Nevertheless, if the frequency of (RSN-2) complemented mutants *ts*2 (A2), *ts*7

TABLE 4. *Interstrain complementation, ts7 (A2) versus ts1 (RSN-2), ts17 (RSN-2), ts19 (RSN-2), and ts20 (RSN-2)*

A2 strain mutant	Incubation time at 39°C (h)	Complementation indexes in mixed infection[a] with RSN-2 mutants:			
		*ts*1 (RSN-2)	*ts*17 (RSN-2)	*ts*19 (RSN-2)	*ts*20 (RSN-2)
*ts*7 (A2)	43	*76*	*26*	*17*	3.3
	49	ND	25	13	*5.8*
	60	17	5.3	8.3	2.3
	67	4	4	2.3	1.4

[a] Mean of three experiments. The optimum values are indicated in italics. ND, Not done.

escaped detection in the earlier work. Therefore, the yield from the complementation experiment illustrated in Fig. 2b was examined to determine whether the generation of wild-type virus by recombination had contributed to the high yields obtained in this experiment. A series of clones was isolated from the complementation yields at 69, 75, 88, and 100 h, and these clones were screened for ability to form plaques at 39°C. All 317 clones examined were temperature sensitive like the parental virus; therefore, the wild-type recombinant frequency, even under conditions favoring the recovery of wild-type virus, could not have exceeded 0.03%.

DISCUSSION

Table 7 summarizes the classification of RS virus *ts* mutants into complementation groups. There is a marked difference in the distribution of mutants by groups and strain of origin. For instance, five of the 7 A2 strain mutants fall into complementation group A, whereas only 1 of the 15 RSN-2 strain mutants appears in this group. Similarly, 10 of the 15 RSN-2 strain mutants were classified in group B, but only 1 of the 7 A2 strain mutants. Group C is unique to the A2 strain, and groups D, E, F, and G are unique to the RSN-2 strain. These differences may indicate inherent differences in the mutability of the two strains. On the other hand, the A2 mutants

TABLE 5. *The seven complementation groups defined by inter- and intrastrain mixed infections*

Group	Mutant	Complementation indexes[a] in group: A *ts26* (RSN-2)	B *ts17* (RSN-2)	D *ts1* (RSN-2)	E *ts19* (RSN-2)	F *ts20* (RSN-2)	G *ts6* (RSN-2)
A	*ts1* (A2)	1.5	72	96	555	67	3
B	*ts2* (A2)	14	1.6	50	78	86	5
C	*ts7* (A2)	11	26	76	21	6	28
A	*ts26* (RSN-2)	-	11	6	10	4	23
B	*ts17* (RSN-2)	-	-	550	536	100	7
D	*ts1* (RSN-2)	-	-	-	150	9	23
E	*ts19* (RSN-2)	-	-	-	-	68	114
F	*ts20* (RSN-2)	-	-	-	-	-	60

[a] Highest value in three to five experiments.

TABLE 6. *Assignment of RSN-2 mutants to group B*

Mutant	Complementation index[a] *ts17* (RSN-2)	*ts19* (RSN-2)	*ts3* (RSN-2)	*ts4* (RSN-2)	*ts5* (RSN-2)	*ts12* (RSN-2)	*ts16* (RSN-2)	*ts18* (RSN-2)	*ts23* (RSN-2)	*ts25* (RSN-2)	*ts37* (RSN-2)
ts17 (RSN-2)	(2)	650 (3)	0.4 (160)	0.2 (2)	0.7 (120)	1 (0)	1.5 (1)	0.5 (0)	1.2 (2,000)	0.6 (300)	0.3 (89)

[a] Result of single experiment; optimum time of incubation for RNS-2 mutants. Figures in parentheses are number of PFU in self-infection.

TABLE 7. *Distribution of mutants into complementation groups according to strain*

Complementation group	Prototype mutant	No. isolated: A2 strain	RSN-2 strain	Total
A	*ts1* (A2) *ts26* (RSN-2)	5	1	6
B	*ts2* (A2) *ts17* (RSN-2)	1	10	11
C	*ts7* (A2)	1	0	1
D	*ts1* (RSN-2)	0	1	1
E	*ts19* (RNS-2)	0	1	1
F	*ts20* (RSN-2)	0	1	1
G	*ts6* (RSN-2)	0	1	1
Total		7	15	22
Unclassified		0	21	21

were isolated from HeLa cells and the RSN-2 mutants from BS-C-1 cells, and it is possible that the host cell may influence the type and frequency of mutants. Similar differences have been observed with *ts* mutants of the human rhabdovirus Chandipura virus after 5-fluorouracil mutagenesis in different host cells (Gadkari and Pringle, unpublished data). However, the different frequencies may merely reflect the different procedures employed in each laboratory. A high proportion (21/36) of the RSN-2 strain *ts* mutants could not be classified into complementation groups, suggesting either complex mutational events or a lesion affecting a nondiffusible gene product.

Two of the three unnamed complementation groups of the RSN-2 strain described by Faulkner et al. are equivalent to group B (mutant *ts23*) and group E (mutant *ts19*). The identity of the group represented by mutant *ts15* is obscure because the mutant *ts15* used by Faulkner et al. (4) could not be recovered from storage.

The absence of recombination and the appreciable number of complementation groups (7 from 22 mutants) are characteristic features of other paramyxoviruses. For example, *ts* mutants of Sendai virus have been classified into seven groups (8), five and a possible sixth complementation group have been described for measles virus (2), and five have been claimed for Newcastle disease virus (10). Therefore the size and structure of the RS virus genome may be similar to that of other paramyxoviruses.

The detection of complementation between *ts* mutants of RS virus depended on several factors, which included the host cell type, multiplicity of

infection, duration of preliminary incubation at permissive temperature, and period of incubation at restrictive temperature. Attempts to improve complementation by creation of viral aggregates using poly-L-ornithine, nucleohistone, or coprecipitation by PEG-6000 were ineffective. It was confirmed that HeLa cells were a better host than BS-C-1 cells, and that this was a consequence of the greater temperature sensitivity (lower leak) of mutants in these cells (data not shown). The multiplicity of infection used was the highest that could be achieved (about 1 PFU/cell at the best), but the low infectivity titers obtained with RS virus were probably responsible for the problems encountered in complementation analysis. A period of absorption at 31°C of 7 h increased the complementation index without increasing the self-infection yields. These factors applied to the mutants of both strains equally. However, the optimal time of incubation at 39°C differed (Fig. 2). The reason for this difference is not clear since the growth cycles of the wild types of the two strains in HeLa cells at 39°C did not show obvious differences (Fig. 1). Possibly amplification of gene products was required in the case of the RSN-2 mutants, which had lower effective multiplicities of infection in HeLa cells then the A2 strain mutants. Althouh 22 *ts* mutants have been grouped satisfactorily into seven groups, it is possible that these conditions were not appropriate for complementation between mutants with lesions in other gene products. The example of RS virus may be instructive in the genetic analysis of other animal viruses where difficulties have been encountered in demonstrating complementation.

Some phenotypic properties of the A2 strain mutants in groups A, B, and C have been described (1, 6, 9), the most striking being the nonsyncytial cytopathology associated with mutant *ts*2 (group B). It may be significant that none of the RSN-2 mutants presently classified in group B induced the nonsyncytial cytopathic effect in HEp-2 cells reported for *ts*2 (A2). Therefore the homology of these mutants may be spurious, and absence of complementation in this case must be treated with caution. The same reservation may apply to group A, since *ts*1 (A2) is thermosensitive and *ts*26 (RSN-2) is thermostable (unpublished data). Therefore seven is the minimum number of complementation groups resolved in the present study, if intracistronic complementation is discounted.

ACKNOWLEDGEMENTS

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