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Temperature-Sensitive Mutants of Chandipura Virus

I. Inter- and Intragroup Complementation

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Fifty temperature-sensitive (*ts*) mutants of Chandipura virus, a human rhabdovirus, have been classified into six complementation groups, designated ChI, ChII, ChIII, ChIV, ChV, and ChVI and containing 44, 2, 1, 1, 1, and 1 mutants, respectively. Weak complementation was observed within group ChI, allowing the division of the group into subgroups ChIA and ChIB. Intragroup complementation was most extensive within subgroup ChIB, and one mutant in this subgroup complemented all but one (*ts* Ch598) of the mutants in group ChI. If *ts* Ch598 had been omitted from the analysis the number of complementation groups would have been increased to seven. Consequently, in circumstances where intragenic and intergenic complementation cannot be clearly distinguished, the number of complementation groups identified in rhabdoviruses could be overestimated. The identification of six complementation groups in three different rhabdoviruses need not imply the existence of an as yet unidentified sixth virus-specified polypeptide. The extensive intragroup complementation observed in Chandipura virus suggests that the functional form of one at least of the virion proteins of Chandipura virus is a multimer.

Chandipura virus is a rhabdovirus which was first isolated in India from the sera of two patients with febrile illness (1). Chandipura virus is functionally and structurally similar to vesicular stomatitis virus (VSV), but is serologically distinct (4, 7). It possesses an RNA genome similar in electrophoretic mobility to the RNA of VSV, and the virion contains five proteins analogous to the L, G, NS, N, and M proteins of VSV (2).

Extensive genetic studies of temperature-sensitive (*ts*) mutants of the Indiana and New Jersey serotypes of VSV have identified six complementation groups in each serotype (19). However, only five virus-specified mRNA's and five viral polypeptides, all of which appear as structural proteins in the virion, have been identified unambiguously in infected cells.

The sixth virus-specified polypeptide suggested by the existence of six complementation groups remains to be identified.

This genetic study of Chandipura virus was undertaken to reinvestigate complementation analysis in rhabdoviruses with particular regard to the status of the sixth complementation group, and at the same time to make available a range of mutants of a human rhabdovirus.

In this paper we show that 50 temperature-sensitive mutants of Chandipura virus can be classified into six distinct complementation groups. Group ChI, to which 44 of the mutants belong, could be subdivided into groups ChIA and ChIB on the basis of weak complementation with the prototype mutant of the group. Efficient complementation was observed between certain combinations of mutants of subgroups ChIA and ChIB. It is concluded that the number of complementation groups identified could be increased fortuitously in a small sample of mutants. Consequently, in rhabdoviruses the number of complementation groups identified could exceed the number of gene products where intra- and intergroup complementation cannot be discriminated. Since most, if not all, of the five virion proteins of rhabdoviruses have multiple functions, intragenic complementation could be observed in any group where a sufficiently large sample of mutants were available.

The phenotypic properties of these mutants will be described separately. In vitro experiments suggest that complementation group ChV represents the M protein transcriptional unit of the Chandipura virus genome (10). Recently Dal Canto et al. (6) have reported that *ts* Ch472 of group ChIV induced an immunologically mediated primary demyelination in mice.

MATERIALS AND METHODS

Cells. The BS-C-1 line of African green monkey kidney cells was propagated in roller-bottle cultures in Eagle minimum essential medium (Glasgow modifi-

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cation) supplemented with 10% calf serum. BHK-21 clone 13 cells were supplied routinely by the Cytology Department of the Institute of Virology.

Virus. Chandipura virus was obtained from F. Murphy, Center for Disease Control, Atlanta, Ga., and cloned by three sequential isolations from single plaques in BHK-21 cell monolayers.

Isolation of ts mutants. BS-C-1 or BHK-21 cultures were infected with wild-type Chandipura virus, which had been grown in the presence of varying concentrations of 5-fluorouracil (Sigma Chemicals Limited, London). Well-separated plaques were transferred using Pasteur pipettes to monolayers in 30-ml bottles to obtain stocks of each clone. Temperature-sensitive mutants were detected by screening at a permissive temperature of 31°C and a restrictive temperature of 39°C.

Complementation. Complementation tests were performed as described previously (18) and in the text. The complementation index (CI) was calculated as the ratio of the yield of infectious virus obtained from the mixed infection at 39°C to the sum of the yields of the single infections at 39°C, all of which were assayed at 31°C; i.e., $[(ts\ A + ts\ B)^{39°}]/[(ts\ A)^{39°} + (ts\ B)^{39°}]$.

Although a CI greater than 1 represents positive complementation, throughout these experiments a mean value of 3 was chosen arbitrarily as the base line for significant complementation. The weak CIs presented in Tables 4, 5, 6, 7, and 8 are the means of at least two, and frequently more, separate determinations. The results of duplicate determinations were tested for significance by the *t* test devised by R. A. Elton and described previously (21), and when the result was doubtful additional complementation tests were carried out.

RESULTS

Isolation of ts mutants. Two series of *ts* mutants of Chandipura virus have been isolated. Twenty *ts* mutants were obtained by Pringle and Wunner (21) from 5-fluorouracil-mutagenized virus grown entirely in BHK-21 cells. In this first series there was no association between the number of mutants isolated and the concentration of mutagen. Another 30 mutants have now been obtained by mutagenization, cloning, and screening entirely in BS-C-1 cells. In this second series the frequency of isolation of *ts* mutants increased with increasing concentration of mutagen up to 200 μg/ml. The plating efficiencies of the mutants used in these experiments are given in Table 1: *ts* Ch1, *ts* Ch4, and *ts* Ch5 are mutants from the BHK series, being the prototype mutants of complementation groups ChI and ChII (21), and the remaining 30 are all those isolated from BS-C-1 cells.

Classification of mutants into complementation groups. BS-C-1 monolayers in 1-ounce (30-ml) bottles were used for all complementation experiments. The bottles were totally immersed in a water bath at 39.5°C to obtain accurate control of temperature. The multiplicity of infection and length of incubation at the nonpermissive temperature for optimum complementation were similar to those determined previously for VSV Indiana *ts* mutants (18). The mixed-infected (10 PFU/cell) and singly infected (5 PFU/cell) cultures were incubated for 7 h at 39.5°C in duplicate, and the total virus yield from each culture bottle was estimated by titration on BS-C-1 monolayers.

TABLE 1. *Plaque-forming ability of the Chandipura virus ts mutants at the permissive (31°C) and nonpermissive (39°C) temperatures*

Mutant	Log_{10} PFU/ml		
	31°C	39°C	31°C − 39°C
ts Ch1	8.30	3.30	5.0
ts Ch4	8.40	3.00	5.4
ts Ch5	7.40	<3.00	>4.4
ts Ch83	7.70	<4.00	>3.7
ts Ch84	7.85	<4.00	>3.9
ts Ch90	6.54	<2.00	>4.5
ts Ch128	7.75	<2.00	>5.8
ts Ch144	7.78	<2.00	>5.8
ts Ch188	8.45	4.00	4.5
ts Ch203	8.30	4.00	4.3
ts Ch315	7.85	<2.00	>5.9
ts Ch319	7.00	4.18	2.8
ts Ch346	7.88	4.70	3.2
ts Ch363	7.88	4.18	3.7
ts Ch449	8.00	3.40	4.6
ts Ch465	7.30	3.00	4.3
ts Ch472	7.70	<3.00	>4.7
ts Ch482	6.70	<2.00	>4.7
ts Ch484	7.65	2.00	5.7
ts Ch540	8.88	3.70	5.7
ts Ch544	8.00	3.18	5.8
ts Ch595	8.00	4.00	4.0
ts Ch598	7.00	4.00	3.0
ts Ch615	7.00	<2.00	>5.0
ts Ch715	8.48	2.70	5.8
ts Ch743	7.00	4.00	3.0
ts Ch763	8.30	<2.00	>5.3
ts Ch808	7.95	<3.00	>5.0
ts Ch824	7.18	<2.00	>5.2
ts Ch851	7.78	3.48	4.3
ts Ch867	7.81	3.18	4.6
ts Ch895	7.65	3.00	4.7
ts Ch962	7.18	3.30	3.9
Wild type	8.70	8.30	0.4

It was possible to classify the 50 *ts* mutants into six complementation groups, and Table 2 shows CIs obtained with representative mutants which define the six complementation groups. The distribution of the mutants between the six groups is shown in Table 3. Of the 50 mutants, 44 were initially classified in the complementation group designated ChI by their failure to complement *ts* Ch1, the prototype mutant of group ChI. Both *ts* mutants belonging to group ChII were isolated in the first series from BHK

TABLE 2. *CIs for the prototype mutants of the six complementation groups*

Mutant	CI for group (mutant):					
	ChI (*ts* Ch1)	ChII (*ts* Ch4)	ChIII (*ts* Ch465)	ChIV (*ts* Ch472)	ChV (*ts* Ch851)	ChVI (*ts* Ch319)
ts Ch1		142	21	196	345	48
ts Ch4			80	147	91	348
ts Ch465				30	150	23
ts Ch472					178	32
ts Ch851						69

cells. Groups ChIII, ChIV, ChV, and ChVI are represented by single mutants, and these four mutants were isolated in the second series from BS-C-1 cells.

Complementation within group ChI. The 44 mutants in group ChI had been allocated in a semiquantitative manner, and additional experiments using the series of mutants isolated from BS-C-1 cells were carried out to examine the homogeneity of group ChI.

Table 4 shows that twenty-five group ChI mutants isolated from BS-C-1 cells complemented efficiently mutants representing groups ChII, ChIII, ChIV, ChV, and ChVI.

The 17 mutants in the first section of Table 4 failed to complement *ts* Ch1 (CI < 3). The eight mutants in the second section exhibited low levels of complementation (CI = 3.0 to 8.8) with mutant *ts* Ch1. Consequently, complementation group ChI has been subdivided into two subgroups, ChIA and ChIB. The initial choice of mutant *ts* Ch1 as the prototype of group ChI in the intergroup test shown in Table 2 was arbitrary. It remains to be determined whether the other group ChI mutants isolated in BHK cells resemble *ts* Ch1 in this respect.

Complementation within subgroup ChIA. Of the 17 *ts* mutants of subgroup ChIA, 11 were chosen for further studies. Complementation tests were carried out with all possible combinations of these mutants (Table 5). The majority of the crosses failed to show any significant complementation. Only 8 (14.6%) of the 55 possible combinations of these 11 subgroup ChIA mutants yielded CIs greater than 3. Three mutants, *ts* Ch90, *ts* Ch449, and *ts* Ch615, did not complement any mutant within the subgroup ChIA. Mutant *ts* Ch540, on the other hand, showed the greatest number of significant CIs, complementing five mutants within subgroup ChIA. The highest CI (75) was obtained with the combination of *ts* Ch363 and *ts* Ch540.

Complementation within subgroup ChIB. All eight mutants in subgroup ChIB were compared in all combinations. Significant complementation was obtained with 17 (60.7%) of the 28 possible combinations of the eight mutants of subgroup ChIB (Table 6). A gradation in the degree of complementation was observed; mutant *ts* Ch808 complemented only one of the mutants (*ts* Ch315) in subgroup ChIB, whereas *ts* Ch315 complemented all the mutants of subgroup ChIB with the exception of *ts* Ch598. The remaining mutants were between these two extremes. The mutants in this group, therefore, can be arranged according to their ability to complement increasing numbers of *ts* mutants within subgroup ChIB. Based on increasing numbers of positive CIs (CI > 3), their ranking is *ts* Ch808, *ts* Ch128, *ts* Ch203, *ts* Ch346, *ts* Ch598, *ts* Ch144, *ts* Ch188, *ts* Ch315.

TABLE 3. *Distribution of Chandipura virus ts mutants in six complementation groups*

Complementation group	No. and origin of mutants		Total
	BS-C-1 cells	BHK-21 cells[a]	
ChI (A + B)	26	18	44
ChII	0	2	2
ChIII	1	0	1
ChIV	1	0	1
ChV	1	0	1
ChVI	1	0	1

[a] Data from Pringle and Wunner (21).

Complementation between subgroups ChIA and ChIB. The 11 mutants from subgroup ChIA and the 8 mutants from subgroup ChIB were tested against each other, and 39 (46.4%) of the possible 84 combinations showed significant complementation (Table 7). Certain combinations of mutants, e.g. *ts* Ch449 × *ts* Ch598, *ts* Ch715 × *ts* Ch598, *ts* Ch895 × *ts* Ch598, yielded CIs that were much higher than those observed in intra-subgroup combinations (Tables 5 and 6). The ranking of the mutants according to their ability to complement in intra-subgroup and inter-subgroup combinations was not the same, e.g., *ts* Ch144 (subgroup ChIB) complemented only one mutant (*ts* Ch540) from subgroup ChIA, whereas it complemented six mutants of subgroup ChIB. Conversely, *ts* Ch598 (ChIB) complemented 9 out of 11 mutants of subgroup ChIA and only 4 out of 7 mutants of subgroup ChIB.

Effect of incubation period on intragroup

TABLE 4. *Complementation between mutants of group ChI and mutants of the other five groups*

Mutant	CI of Complementation group (mutant):					
	ChI (*ts* Ch1)	ChII (*ts* Ch4)	ChIII (*ts* Ch465)	ChIV (*ts* Ch472)	ChV (*ts* Ch851)	ChVI (*ts* Ch319)
ts Ch83	1.0	10	90	100	500	ND[a]
ts Ch84	1.5	20	143	33	60	ND
ts Ch544	1.2	50	57	230	300	ND
ts Ch595	1.5	10	67	176	129	ND
ts Ch763	2.0	113	66	129	666	ND
ts Ch824	1.3	60	80	133	180	ND
ts Ch90	1.3	89	88	65	33	17
ts Ch449	1.9	88	400	1,000	222	483
ts Ch615	0.7	161	92	34	50	8
ts Ch363	0.8	65	80	50	150	50
ts Ch482	0.5	55	46	26	150	6
ts Ch867	1.3	50	66	100	500	67
ts Ch895	1.3	47	143	75	1,000	6
ts Ch962	1.1	250	73	666	666	6
ts Ch484	1.0	84	23	40	20	37
ts Ch715	1.6	58	111	33	140	29
ts Ch540	2.2	1,000	143	40	445	375
ts Ch808	7.0	40	150	53	140	4
ts Ch128	5.0	177	60	44	167	41
ts Ch203	3.7	125	77	210	461	12
ts Ch346	5.0	50	8	15	50	8
ts Ch598	3.0	10	16	43	50	12
ts Ch144	3.5	75	89	57	145	6
ts Ch188	3.3	50	100	15	500	10
ts Ch315	8.8	67	18	32	35	25

[a] ND, Not determined.

TABLE 5. *Complementation within subgroup ChIA*[a]

Mutant	*ts* Ch90	*ts* Ch449	*ts* Ch615	*ts* Ch363	*ts* Ch482	*ts* Ch867	*ts* Ch895	*ts* Ch962	*ts* Ch484	*ts* Ch715	*ts* Ch540
ts Ch90		1.3	0.6	0.6	0.9	1.0	0.6	1.0	0.7	0.5	0.3
ts Ch449			2.7	1.3	1.2	0.4	0.3	0.5	0.7	2.5	2.9
ts Ch615				1.0	1.2	0.4	0.8	2.4	0.8	1.4	0.4
ts Ch363					1.8	0.8	1.4	1.3	2.8	1.0	*75.0*
ts Ch482						1.7	1.1	1.2	*4.3*	0.6	0.4
ts Ch867							1.4	0.8	0.7	*3.1*	0.3
ts Ch895								0.7	0.9	0.2	*3.5*
ts Ch962									0.3	2.2	*5.0*
ts Ch484										*8.2*	*5.0*
ts Ch715											*3.8*

[a] Significant CIs (>3.0) are indicated by italics.

TABLE 6. *Complementation within subgroup ChIB*[a]

Mutant	*ts* Ch808	*ts* Ch128	*ts* Ch203	*ts* Ch346	*ts* Ch598	*ts* Ch144	*ts* Ch188	*ts* Ch315
ts Ch808		0.5	2.3	1.5	2.1	1.2	0.8	*20.0*
ts Ch128			1.2	1.0	0.6	*3.1*	*3.6*	*13.0*
ts Ch203				1.3	*12.3*	*7.0*	*24.0*	*6.6*
ts Ch346					*12.2*	*3.6*	*6.9*	*6.5*
ts Ch598						*5.0*	*7.1*	1.3
ts Ch144							*7.0*	*36.0*
ts Ch188								*10.3*

[a] Significant CIs (>3.0) are indicated by italics.

TABLE 7. *Complementation between mutants of subgroups ChIA and ChIB*[a]

Subgroup ChIA mutant	CI with subgroup ChIB mutants:							
	ts Ch808	*ts* Ch128	*ts* Ch203	*ts* Ch346	*ts* Ch598	*ts* Ch144	*ts* Ch188	*ts* Ch315
ts Ch90	0.3	1.4	*4.0*	*7.2*	*5.2*	0.9	0.1	*6.0*
ts Ch449	0.5	0.9	0.1	1.1	*200*	2.6	0.7	*4.3*
ts Ch615	ND[b]	0.3	0.8	1.2	1.2	0.6	1.5	*17.0*
ts Ch363	0.9	*9.5*	*19.0*	*6.7*	*14.5*	1.4	*5.0*	*80.0*
ts Ch482	ND	1.4	*3.2*	0.9	2.5	2.2	*12.5*	*5.0*
ts Ch867	0.9	0.3	0.2	2.8	*12.0*	1.7	*7.5*	*14.3*
ts Ch895	ND	2.3	1.5	*17.0*	*170.0*	0.6	0.8	*8.7*
ts Ch962	*3.0*	2.3	1.5	*13.0*	*27.0*	0.7	0.2	*13.3*
ts Ch484	*5.0*	1.4	2.5	0.7	*5.0*	1.5	2.9	*10.0*
ts Ch715	ND	*5.0*	*8.0*	*14.0*	*109.0*	0.4	2.2	*9.4*
ts Ch540	0.8	2.1	0.3	*23.0*	*62.0*	*14.3*	*25.0*	*5.0*

[a] Significant CIs (≥3.0) are indicated in italics.
[b] ND, Not determined.

complementation. The optimum conditions for complementation assays had been established for mutants representing two different groups. It was necessary, therefore, to study the effect of incubation period on intragroup complementation. For this purpose mutants *ts* Ch315 (ChIB), *ts* Ch540 (ChIA), and *ts* Ch962 (ChIA) were chosen to represent complementation group ChI, and *ts* Ch319 of group ChVI was included as a control for intergroup complementation. Singly and mixed-infected cultures were harvested after various periods of incubation at 39.5°C. The results, given as CIs, are shown in Table 8.

For intergroup complementation, all three combinations showed the highest CIs after 7 h of incubation. In the intragroup combinations, the highest CIs were obtained at 7 h with *ts* Ch315 and *ts* Ch962, at 9 h with *ts* Ch315 and *ts* Ch540, and at both 7 and 9 h with *ts* Ch540 and *ts* Ch962. These results indicate that harvesting after 7 h of incubation is unlikely to have produced a systematic bias in the intragroup CI values.

TABLE 8. *Effect of incubation period on intragroup and intergroup complementation*[a]

Incubation period (h)	Mutant	CI for mutant (group):			
		ts Ch315 (ChIB)	*ts* Ch540 (ChIA)	*ts* Ch962 (ChIA)	*ts* Ch319 (ChVI)
5.5	*ts* Ch315		1.7	1.7	8
	ts Ch540			1.0	16.3
	ts Ch962				2.0
7	*ts* Ch315		8	*41*	*20*
	ts Ch540			7.5	*108*
	ts Ch962				*6*
9	*ts* Ch315		*25*	36	7
	ts Ch540			*8*	26
	ts Ch962				4
11.5	*ts* Ch315		6	10	8.6
	ts Ch540			3.8	15.6
	ts Ch962				3.3

[a] The highest CI for each pair of mutants is indicated in italics.

DISCUSSION

The induced *ts* mutants of Chandipura virus have been classified into six complementation groups, as had previously the spontaneous and induced *ts* mutants of VSV Indiana and the induced *ts* mutants of VSV New Jersey. Most of the Chandipura mutants belonged to a single group, and in this respect Chandipura virus resembles VSV Indiana more than VSV New Jersey. Chandipura virus differs, however, in the extent of the weak complementation observed between combinations of mutants within the majority group. Indeed it was possible to arbitrarily subdivide this group on the basis of the occurrence or absence of weak complementation in mixed infection with the prototype mutant (*ts* Ch1) of group ChI. Although isolated instances of weak complementation have been reported within group I of VSV Indiana (9, 25), the phenomenon described here is quantitatively different.

Sixty-one percent of the combinations of mutants within group ChIB showed significant (CI > 3.0) complementation, whereas only 15 gave positive complementation within group ChIA. The mutants of subgroup ChIB could be ranked by ability to complement other mutants within the subgroup, with *ts* Ch315 complementing all but one (*ts* Ch598). Indeed the inclusion of *ts* Ch315 in group I was based on this single negative combination, since *ts* Ch315 complemented all mutants in subgroup ChIA. Had mutant *ts* Ch598 not been available, mutant *ts* Ch315 would have been assigned to a separate (sev-

enth) complementation group.

Conversely, mutant *ts* Ch319, which has been classified as group ChVI, might have been included in group ChI if a larger sample of mutants had been available, since *ts* Ch319 complemented poorly with some group ChI mutants. Mutant *ts* Ch319 has an RNA-negative phenotype as do other mutants classified in group ChI (10). Thus in a small sample of mutants the number of complementation groups can be ambiguous unless inter- and intragroup complementation can be discriminated clearly.

The common feature of the three rhabdoviruses that have been investigated in detail is that mutants of two of the six complementation groups have RNA-positive phenotypes and presumably represent the genes determining the two polypeptides of the virion envelope (10, 19). The available evidence is in agreement with these assignments, since the RNA-positive group III of VSV Indiana and group ChV of Chandipura virus probably represent the M gene (10, 14, 15), and the RNA-positive group V of VSV Indiana represents the G gene (14). The existence of four RNA-negative complementation groups does not necessarily imply the existence of four virus-coded polypeptides, since at least two (the NS and L proteins) of the three core proteins are known to be multifunctional proteins (24). Both the L and NS polypeptides are required for transcriptase activity in vitro (8), and there is evidence for involvement of the M protein (3, 17) and host factors (20, 23) in both in vitro and in vivo transcription. The polymerase complex has also additional functions which include replication, capping, methylation, and polyadenylation. Consequently two mutants of any polypeptide in the polymerase complex could exhibit intragroup complementation if they were located in different functional domains of the molecule.

Alternatively, intragroup complementation could indicate that the functional form of the gene product was a dimer or multimer, and that complementation occurred by a protein-protein interaction where the conformation of the mutated region of one monomer was corrected by the unmutated region of the other monomer, and vice versa (5). A large number of proteins exist which function in dimeric or multimeric forms (13, 26), and an instance of intragenic complementation, involving deoxypyrimidine kinase mutants of herpes simplex virus type 1, has been reported in animal viruses (12).

The gene assignment for Chandipura virus complementation group ChI has not been determined, but by analogy with VSV Indiana group ChI can be equated to group I of VSV Indiana on the basis of mutant frequency and RNA-negative phenotype. This suggests that the L protein of these viruses may be either a dimer or multimer in its functional form, since in VSV Indiana the L protein has been assigned to group I (19). Formation of a heterodimer in mixed infection, where the subunits are contributed by different mutants, could result in an active enzyme and account for the efficient complementation observed with certain pairs of mutants (e.g., *ts* Ch363 and *ts* Ch315). This could be tested experimentally by dissociation and reconstitution of enzyme activity using soluble and template fractions prepared from complementing mutants with in vitro thermolabile enzyme activity. However, none of the Chandipura mutants examined so far has the requisite thermolabile enzyme activity (Gadkari and Pringle, unpublished data).

Finally, the different spectra of mutants isolated in the two series suggest that the host cell environment influences the type of mutant recovered. A similar phenomenon has been observed with respiratory syncytial virus (11). This was less obvious in the isolation of VSV mutants, except that group V mutants of VSV Indiana have not been recovered from mammalian cells without some selective procedure (16, 22).

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