

GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF TEMPERATURE-SENSITIVE MUTANTS OF BLUETONGUE VIRUS

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ABSTRACT

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Complementation studies were carried out, using temperature-sensitive (t-s) mutants of bluetongue virus (BTV). The results proved to be inconclusive as only low indices of complementation were obtained. No discrepancy was found between the previous classification of these mutants in 6 recombination classes and the complementation data recorded. In general, the t-s mutants require a latent growth period of 16-20 h at 28 °C and maximum titres can be demonstrated 40-48 h post-infection. One mutant, (F211), however, consistently had a growth lag phase of 32 h. Mutants of the 6 recombination groups were further classified into 2 groups by temperature-shift studies. One class of mutants expressed their t-s lesion prior to 24 h and the other class only after 24 h post-infection. Mutant F73 was found to be defective in its ability to synthesize ssRNA at a late stage in the replication cycle at the non-permissive temperature.

Résumé

CARACTÉRISATION GÉNÉTIQUE ET PHYSIOLOGIQUE DE MUTANTS THERMOSENSIBLES DU VIRUS DE LA FIÈVRE CATARRHALE DU MOUTON

On a effectué des études de complémentation avec des mutants thermosensibles (t-s) du virus de la fièvre catarrhale du mouton. Les résultats n'ont pas été concluants, du fait qu'on n'a pu obtenir que de faibles indices de complémentation. On n'a toutefois pas trouvé de désaccord entre la classification préalable de ces mutants en 6 classes de recombinaison et les données de complémentation qu'on a enregistrées. De façon générale, les mutants t-s ont besoin à 28 °C d'une période de croissance latente de 18 à 20 heures et les titres les plus élevés peuvent être mis en évidence de 40 à 48 heures après l'infection. Un des mutants néanmoins (F211) a eu de manière constante un retard à la croissance de 32 heures. Par des études de déplacement thermique on a subdivisé en 2 groupes les mutants des 6 groupes de recombinaison. Les mutants d'une classe ont manifesté leur lésion t-s dans un délai de 24 h après l'infection, ceux de l'autre classe ne l'ont fait qu'après 24 h. On a constaté que le mutant F73 était déficient du point de vue de sa capacité de synthèse de ssRNA à un stade tardif du cycle de reproduction à la température non-permissive.

INTRODUCTION

RNA isolated from bluetongue virus (BTV) can be fractionated by means of electrophoresis on polyacrylamide gels into 10 distinct size classes (Verwoerd, Louw & Oellermann, 1970). The conclusion that the genome of BTV is segmented makes this virus, together with reovirus and influenza virus, a unique genetic model. A high frequency of recombination was shown for both reovirus (Fields & Joklik, 1969) and for influenza virus t-s mutants (Tobita & Kilbourne, 1974). Similar results have recently been obtained for co-infecting BTV t-s mutants (Shipham & De la Rey, 1976). This high frequency of recombination suggests that an assortment of genome segments, rather than crossing over, is the probable mechanism of gene rearrangement in these viruses (Fields, 1971; Fields & Joklik, 1969; Mackenzie, 1970; Simpson & Hirst, 1968; Sugiura, Tobita & Kilbourne, 1972).

To date, 7 BTV specific capsid polypeptides have been identified and a correlation, based on molecular mass determinations between individual genome segments and polypeptides, has been suggested (Verwoerd, Els, De Villiers & Huismans, 1972). Studies, using t-s mutants of reovirus, have indicated that it is possible to correlate certain viral functions with t-s lesions in single genome segments (Ito & Joklik, 1972 a,b; Matsuhisa & Joklik, 1974; Morgan & Zweerink, 1974; Schuerch & Joklik, 1973; Spandidos & Graham, 1976). For a review see also Joklik (1974). Temperature-sensitive mutants of BTV were therefore used for similar studies of the functions of specific gene products.

In this study we report on complementation studies, using BTV t-s mutants, as well as on the physiological characterization of some of these mutants in terms of early or late functions affected and on their ability to synthesize RNA.

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MATERIALS AND METHODS

Cells and media

NCTC clone 929 L-cells and BHK21 (C-13) cells obtained from the American Type Culture Collection were used throughout. The growth and maintenance of cells were carried out as described by Verwoerd, Oellermann, Broekman & Weiss (1967).

Eagle's medium was prepared according to the prescription of MacPherson & Stoker (1962), as modified by Verwoerd, Oellermann, Broekman & Weiss (1967).

Virus

The isolation, titration, propagation and mutant nomenclature of BTV t-s mutants have been previously described (Shipham & De la Rey, 1976).

Complementation

L-cell monolayers prepared in 60 mm petri dishes were either singly or doubly infected at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (PFU)/cell for each virus. Virus was allowed to adsorb for 1 h at 38 °C, after which the cell cultures were rinsed and then incubated at 38 °C for 48 h. Infected cells were harvested by scraping them off the glass and were then titrated for virus at 28 °C and 38 °C. The complementation index (CI) for any 2 t-s mutants, A and B, was calculated according to the formula given by Fields & Joklik (1969),

$$CI = \frac{(AB)_{28} - (A)_{28} - (B)_{28}}{(A)_{28} + (B)_{28}}$$

where $(AB)_{28}$ and $(AB)_{38}$ are the virus titres of the doubly-infected cultures determined at 28 °C and 38 °C respectively, and $(A)_{28}$ and $(B)_{28}$ the virus titres of the singly-infected cultures as determined at 28 °C.

Growth cycle determinations

Growth cycle studies of BTV t-s mutants were carried out in BHK cell monolayers in Roux flasks. Virus was inoculated at a m.o.i. of 5–10 PFU/cell and allowed to adsorb for 2 h at 20 °C prior to incubation at the required temperature. The infected Roux flask cultures were collected at 4-hour intervals, the cells harvested as described above and the virus titres determined at the permissive temperature.

Determination of RNA synthesis

Monolayers of L-cells were inoculated with virus at a m.o.i. of 20–30 PFU/cell. After virus was allowed to adsorb for 2 h at 20 °C, the cells were rinsed and incubated at 28 °C in fresh Eagle's medium containing 0,1 µg/ml actinomycin D.* Infected cells were trypsinized 30 min. prior to pulse-labelling, suspended at a cell concentration of 5×10^6 /ml, and the actinomycin D concentration increased to 0,5 µg/ml. ³H-Uridine† was added to give a final concentration of 3 µCi/ml and the cultures maintained in suspension with continuous stirring for a further 2 hours. The reaction was stopped by rapid chilling and the cells pelleted by centrifugation at 1 200 g. The cell pellet was taken up in a 10 mM Tris-HCl buffer, pH 8,0, containing 0,5% Nonidet P40‡ and kept at 4 °C for 15 minutes. The suspension was homogenized with 3–4 strokes in a Dounce homogenizer and the nuclei pelleted by centrifugation at 1 200 g for 10 min. The supernatant was treated with a sodium dodecyl sulphate (SDS)-urea-sodium acetate solution (final concentrations 1%, 0,5% and 100 mM respectively), prior to layering over preformed sucrose-SDS gradients (4,40% sucrose in 100 mM NaCl, 5 µM Tris, 0,5% SDS, pH 7,2). Centrifugation was at 185 000 × g for 4 h at 20 °C using a SW 50 Beckman rotor. Each fraction was treated with excess 10% TCA, kept for at least 1 h at 4 °C, and the acid insoluble precipitate collected on Millipore filters (porosity 0,8 µ). The filters were dried at 100 °C for 1 h and radio-activity (cpm) determined in toluene scintillation fluid.

RESULTS

Complementation

Complementation studies were carried out to ascertain the relationship between recombination and complementation classes of the same set of t-s mutants. Previous studies allowed the classification of 19 BTV t-s mutants into 6 recombination classes (Shipham & De la Rey, 1976). Further 4 t-s mutants were isolated but no new recombination classes could be demonstrated (Shipham, 1977).

In the present study 2 t-s mutants were chosen from each recombination class except in the case of mutants in groups IV and VI, where only 1 mutant of each class was isolated. Cultures were inoculated in triplicate and the complementation index calculated, as described under Materials and Methods. Indices represent the average of 3 determinations. The results are shown in Table 1.

A complementation index of 1 or less than 1 implies that no complementation has occurred. Since low complementation indices were found for most of the crosses between the t-s mutants, complementation

only occurs to a minimal extent, if at all. No complementation was found between mutants within the same recombination group.

TABLE 1 Complementation indices from pair-wise crosses of BTV t-s mutants

Group and mutant	I F211	II F207	III G1	IV G345	V F210	VI F73
I F211.....	—	1,0	3,5	1,9	15,3	3,6
G339.....	0,3	1,5	3,5	5,0	1,5	2,0
II F207.....	1,0	—	5,1	3,7	2,7	5,0
P9.....	0,9	0,9	2,5	3,9	3,1	3,0
III G1.....	3,5	5,1	—	3,6	9,5	29,7
G320.....	8,8	14,0	0,9	2,1	2,7	16,0
IV G345.....	1,9	3,7	3,6	—	3,4	6,0
V F210.....	15,3	2,7	9,5	3,4	—	1,6
G262.....	2,2	3,0	19,0	3,2	0,5	4,4
VI F73.....	3,6	5,0	29,7	6,0	1,6	—

Growth kinetics of BTV t-s mutants

Since no previous growth studies at 28 °C have been reported for BTV, it was thought necessary to determine the growth characteristics of wild type (WT) BTV and the BTV t-s mutants at this temperature. The t-s mutants of BTV have been classified into 6 recombination groups (Shipham & De la Rey, 1976) and for practical purposes only representatives of each of these groups were chosen for these studies. Where possible 2 mutants of each group were selected.

Cultures were inoculated, sampled and titrated, as described under Materials and Methods. The resulting growth curves are shown in Fig. 1.

Although the rate of viral replication of WT virus was considerably retarded at 28 °C, compared with the rate at 38 °C, there was no difference between the maximum viral titres obtained at the 2 temperatures. In general, the t-s mutants had a replication pattern similar to that of the WT at 28 °C, represented by the patterns of 2 mutants, G361 and F207 shown in Fig 1. The only exception to this general growth pattern was found for the mutant F211 of Group I which consistently had an extended lag phase of 32 h. The mutant G361 belongs to the same recombination group as F211, but does not exhibit this extended lag phase.

Temperature-shift studies

The isolation and study of BTV t-s mutants was primarily aimed at the identification of a specific t-s lesion with a particular gene function. It was therefore important to determine when these t-s lesions are expressed, a determination which can most effectively be done by temperature-shift experiments.

BHK cell monolayers were inoculated with virus at a m.o.i. of 5–10 PFU/cell and after 30 min at 4 °C were rinsed with prewarmed medium and incubated at the required temperature for 24 h prior to shifting, either up to the non-permissive temperature, or down to the permissive temperature. Immediately prior to shifting, all cultures were rinsed with prewarmed medium. After a 48 h total incubation period, infected cultures were harvested as described above and virus titres determined. The results expressed as PFU/cell are given in Table 2.

* Merck, Sharp & Dohme, Rahway, N. J., U.S.A.

† Radio Chemical Centre, Amersham, England

‡ Shell Chemicals Ltd, Johannesburg

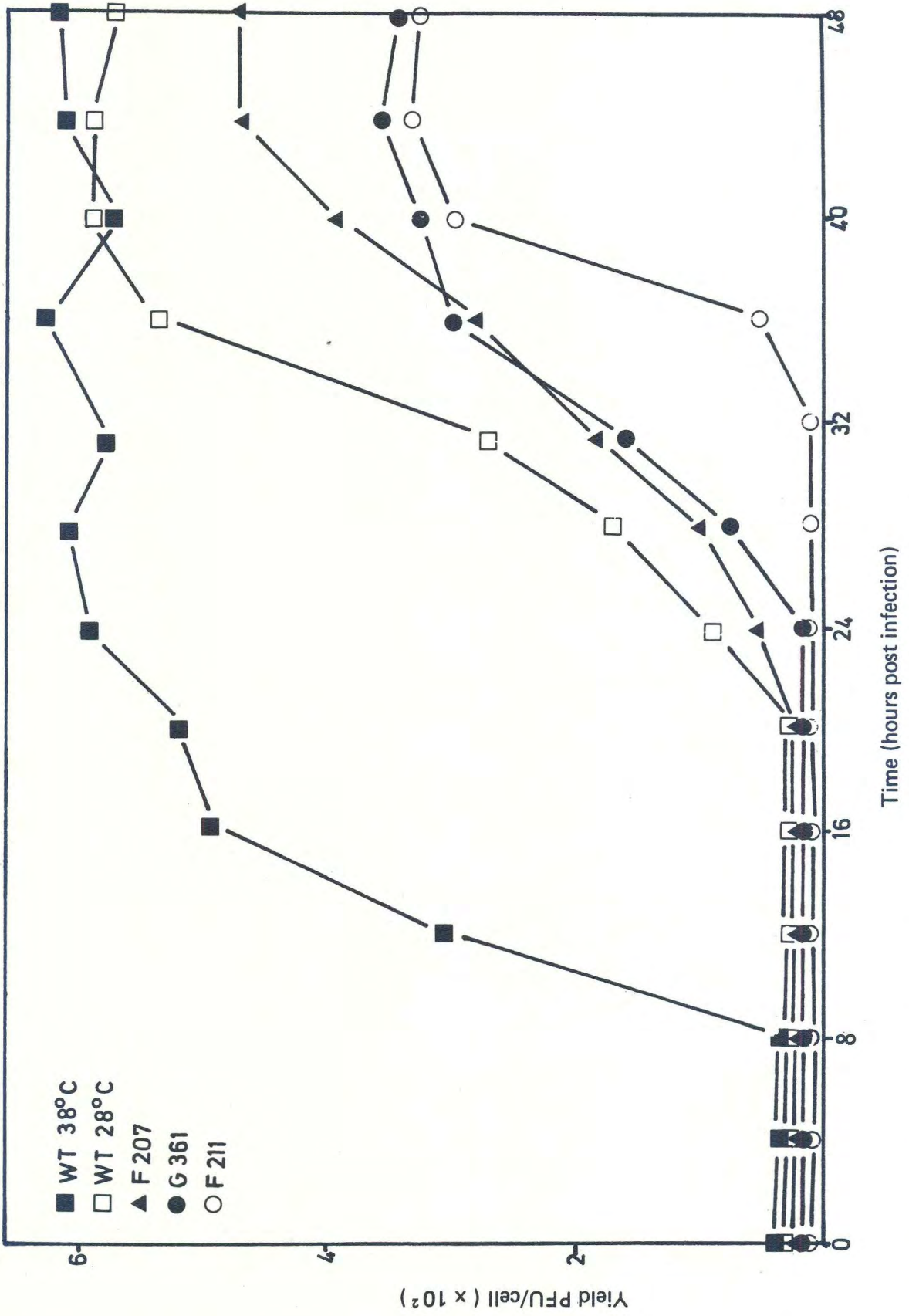


FIG. 1 Growth patterns of bluetongue virus wild type (WT) and temperature-sensitive (t-s) mutants

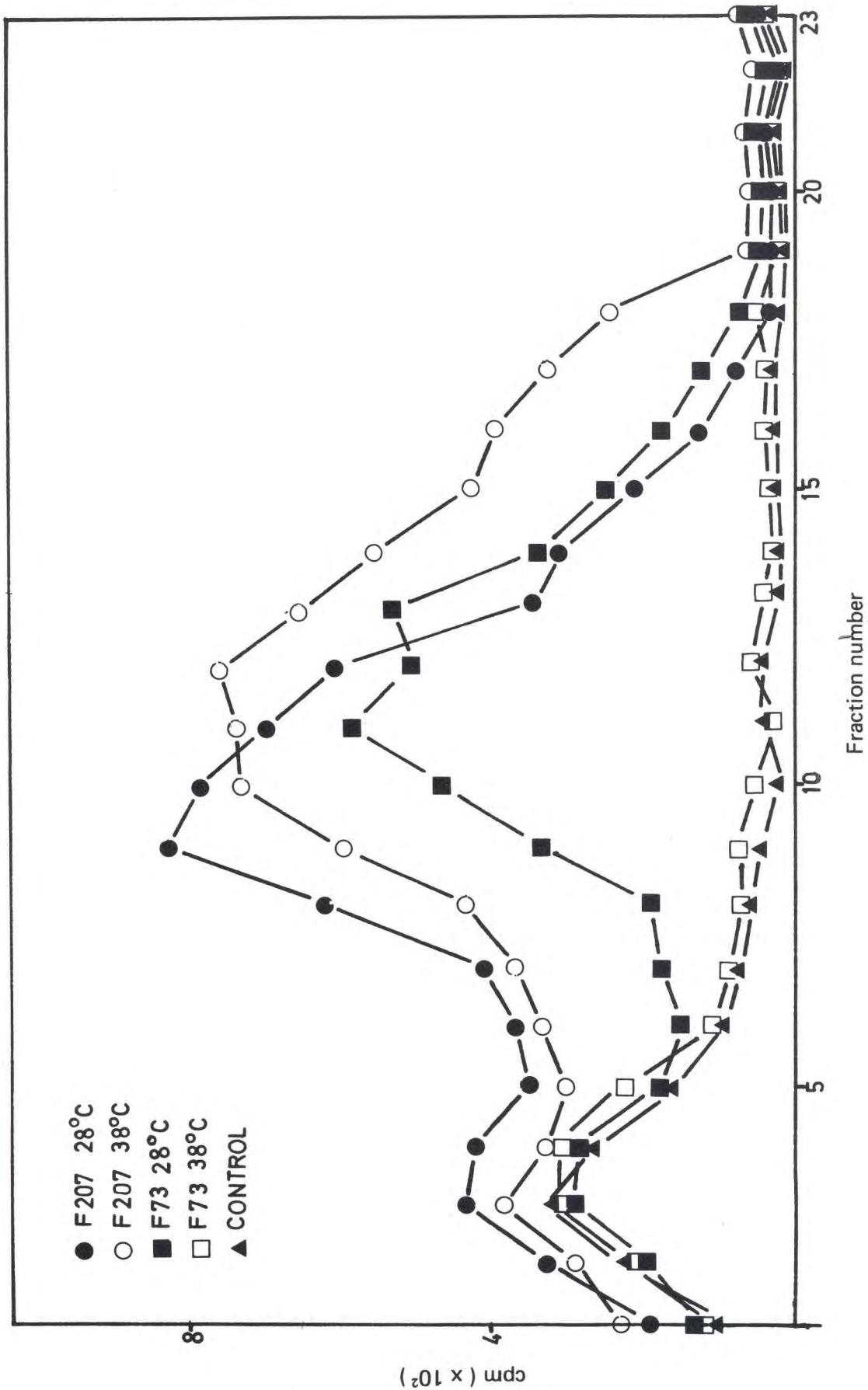


FIG. 2 Sucrose gradient sedimentation analysis of RNA synthesized by bluetongue virus t-s mutants. Sedimentation is from left to right

TABLE 2 Virus yields (PFU/cell) after temperature-shift. Cultures were incubated for 24 h prior to and after shift

Group and mutant	T0	T24 (28 °C)	T48 (38 °C)	T48 (28 °C)	T24 Shift-up	T24 Shift-down
I F211.....	8,2	21,0	13,0	514	145	26,0
G361.....	0,5	2,5	5,1	460	300	32,0
II F207.....	2,2	18,2	1,7	695	55	610
G231.....	2,1	21,9	6,2	216	11	221
III G1.....	0,7	0,5	1,0	120	0,4	90
G304.....	1,9	3,2	3,5	295	8,0	203
IV G345.....	11,0	25	9,7	245	150	12,0
V F210.....	1,4	1,2	11,0	204	6,0	138
G262.....	0,8	0,5	5,1	165	4,9	141
VI F73.....	0,9	22,8	6,0	378	17,4	330

T0: Virus titre at commencement of experiment

T24 (28 °C): Virus titre after 24 h incubation at 28 °C

T48 (38 °C): Virus titre after 48 h incubation at 38 °C

T48 (28 °C): Virus titre after 48 h incubation at 28 °C

T24 Shift-up: Virus titre after first an incubation for 24 h at 28 °C followed by 24 h incubation at 38 °C

T24 Shift-down: Virus titre after initial incubation at 38 °C for 24 h followed by incubation at 28 °C for 24 h

A shift to the non-permissive temperature after an incubation time of 24 h at 28 °C (shift-up) resulted in a marked reduction in the expected viral titre in the case of 4 t-s mutant groups (II, III, V and VI). This suggests that for these mutants the critical temperature-sensitive stage during viral replication occurs later than 24 h post-infection.

For the mutants of Group I and IV, an incubation period of 24 h at the non-permissive temperature prior to the permissive temperature (shift-down) caused a fall in expected viral titre. For these mutants the t-s lesion is most likely expressed within the first 24 h of virus replication. Other studies have shown that this critical period is between 20 and 24 h post-infection (Shipham, 1977).

RNA synthesis

It has been demonstrated that synthesis of BTV ssRNA takes place in both the early and late stages of the replication cycle (Huisman & Verwoerd, 1973), while maximum synthesis of dsRNA occurs 8–10 h post-infection (Huisman, 1970). In the functional characterization of t-s mutants it was important to establish if any mutants were defective in their ability to synthesize either ssRNA or dsRNA at the non-permissive temperature. Similar studies with reovirus t-s mutants showed that all RNA negative mutants lacked the ability to synthesize dsRNA at 38 °C.

Preliminary investigations at 28 °C showed that RNA synthesis could successfully be demonstrated from 16 h post-infection onwards (Shipham, 1977). For practical reasons, a labelling period of 20–22 h post infection for the 4 mutants, F207, P9, G1 and F73, used was selected for this study. Experiments were carried out as described under Materials and Methods and the RNA product analysed on sucrose gradients. The radio-activity for each fraction was scored and the results of 2 of these are shown in Fig. 2.

An uninfected cell culture was included as a control to indicate residual cell RNA synthesis. Three of the

4 mutants tested (F207, P9 and G1) were able to synthesize RNA at both 28 °C and 38 °C and the results for 1 of these mutants (F207) are shown in Fig. 2. The sedimentation behaviour of the newly synthesized RNA is in agreement with that recorded for BTV RNA (Huisman, 1970). Mutant F73, however, was not able to synthesize RNA at the non-permissive temperature (38 °C), although, in identical cultures pulse-labelled at 28 °C, RNA synthesis could be demonstrated (Fig. 2). The mutant F73 may therefore be classified as an RNA negative mutant under the conditions specified.

In order to investigate whether the product synthesized by F73 under the conditions described was single-stranded or double-stranded RNA, the sensitivity of this product to pancreatic RNase was tested. Both BTV wild type and F73 virus were inoculated onto BHK cells and the RNA synthesized was fractionated on sucrose gradients as previously described. In this experiment the larger samples collected resulted in a smaller number of fractions collected compared to the RNA sucrose gradient analysis described previously. Each fraction was incubated in the presence of pancreatic RNase (2 µg/ml) and 300 mM NaCl for 15 min. at 37 °C. The reaction was stopped by precipitating with excess 10% TCA and acid precipitable material collected on millipore filters. The precipitable radio-activity in each fraction was determined and the results (cpm) are given in Fig. 3.

Since the product synthesized by F73 is sensitive to the action of pancreatic RNase, it may be concluded that this mutant is temperature-sensitive for the synthesis of ssRNA at a late stage in the replication cycle.

DISCUSSION

The results of complementation studies with t-s mutants of BTV, recorded in Table 1, are inconclusive, as in most cases only low indices of complementation could be demonstrated. The fact that no complementation at all could be demonstrated between any 2 t-s mutants of the same recombination class, suggests a compatibility between complementation results and the previously defined recombination classes. Such a correlation could be interpreted to mean that each BTV recombination class is representative of a t-s lesion in a specific genome segment, and that each genome segment represents 1 cistron.

Similar inconclusive results have also been reported for complementation studies carried out with reovirus t-s mutants (Fields & Joklik, 1969), although complementation has been readily demonstrated between defective mutants and t-s mutants of reovirus (Spandido & Graham, 1975 a; Spandido & Graham, 1975 b). Complementation studies have, however, proved to be an effective method for the genetic classification of t-s mutants of influenza virus, where 7 complementation groups have been established (Spring, Nusinoff, Tierney, Richman, Murphy & Chanock, 1975; Spring, Maassab, Kendal, Murphy & Chanock, 1977). This classification was compatible with the grouping of the same t-s mutants into specific recombination classes (Sugiura, Tobita & Kilbourne, 1972).

The growth rate of BTV t-s mutants at 28 °C was very similar to that of the BTV WT, since both required a lag phase of 16–20 h, while maximum virus titres could be demonstrated from 40–48 h post-infection (Fig. 1). A single exception to this growth pattern was found in the case of mutant F211 which

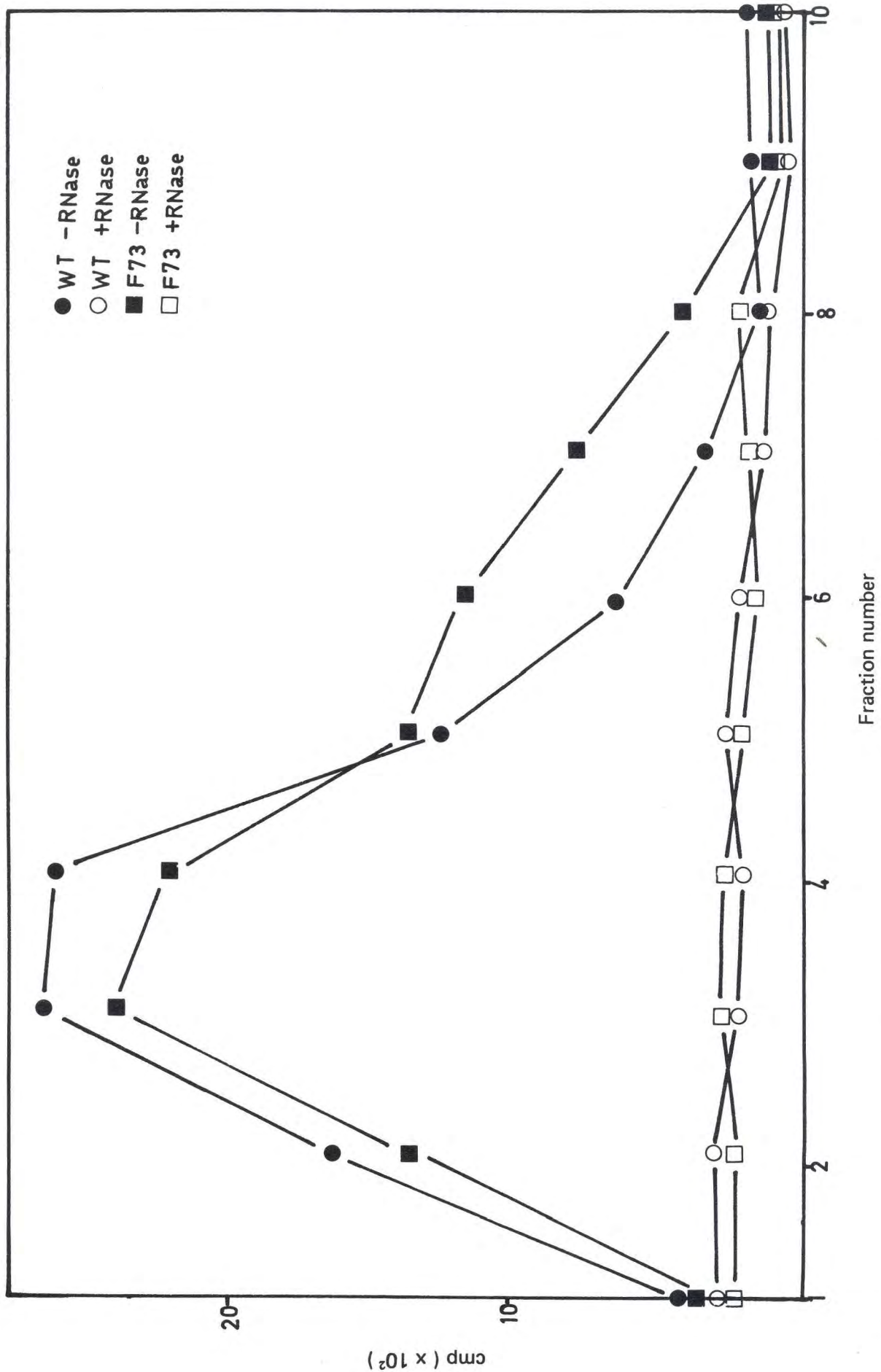


FIG. 3 Effect of RNase treatment on RNA synthesized by bluetongue virus wild type and t-s mutant F73. Sedimentation is from left to right

had a latent period of growth of 32 h. Some discrepancy between the growth rates of reovirus t-s mutants and influenza virus has also been reported (Ikegami & Gomatos, 1968; Sugiura, Tobita & Kilbourne, 1972). Although no reasons for this discrepancy in the case of BTV t-s mutants can be offered, it might be speculated that, in the case of the mutant F211, a double mutation is involved, resulting in a specific retardation of its replication. This slow growth rate is not a characteristic of all mutants of Group I (Fig. 1).

The 6 genetic recombination groups of BTV t-s mutants have been further classified into 2 groups, based on the period of time post-infection, when the t-s lesion can be demonstrated in temperature-shift experiments (Table 2). One class of mutants (Groups I and IV) expresses their t-s lesion prior to 24 h post-infection, while the 2nd class (Groups II, III, V and VI) only reaches this stage after 24 h post-infection. The fact that the majority of the t-s mutants can be classified as late mutants suggests that a defective assembly process in the production of progeny viral particles may be involved.

Reovirus RNA negative mutants have all been characterized as defective in the synthesis of dsRNA [Cross & Fields, 1972; Fields & Joklik, 1969; Ito & Joklik, 1972 (a)], which differs from the results of the RNA synthesis studies of BTV t-s mutants reported here. In these studies we have shown that at 38 °C the mutant F73 is defective in its ability to synthesize ssRNA. This defect can be demonstrated at a late stage in the replication cycle under experimental conditions where infected cultures are first incubated at the permissive temperature for 20 h. This would allow time for the production of progeny transcriptase particles prior to the testing for the synthesis of RNA at the non-permissive temperature and indicate that progeny sub-viral transcriptase particles are involved in the thermo-labile defect. These results therefore suggest that at 38 °C a functional difference exists between parental and progeny transcriptase-active particles present in F73 infected cultures, a difference which could be due to a difference in polypeptide composition as reported in the case of reovirus (Morgan & Zweerink, 1975).

A comparison of the results of temperature-shift and RNA synthesis studies in the case of the mutant F73 suggests some discrepancy as regards the time during replication at which the t-s lesion of this mutant is expressed. According to the temperature-shift results, the mutant F73 can be maintained at the non-permissive temperature for 24 h without any reduction in infective viral titres (Table 2, shift-down), yet a F73 temperature-sensitive viral function (RNA synthesis) can be demonstrated at 20 h post-infection. This apparent discrepancy could be explained if it is accepted that temperature-shift studies merely indicate the time during viral replication when the defective polypeptide component is utilized, even though it may be synthesized at an earlier stage. Similar discrepancies between RNA synthesis studies and temperature-shift results have been reported for Sendai virus t-s mutants (Portner, Marx & Kingsbury, 1974).

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