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THE ISOLATION AND PRELIMINARY GENETIC CLASSIFICATION OF TEMPERATURE-SENSITIVE MUTANTS OF BLUETONGUE VIRUS

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ABSTRACT

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Temperature-sensitive mutants of bluetongue virus were isolated and classified in 6 genetic recombination groups. The frequency of recombination varied both within and between groups. The 4 mutagens used viz. nitrous acid, N-methyl-N-nitroso-N-nitroguanidine, proflavine and 5-fluoro-uracil were found to differ in their efficacy. The period of incubation required for maximum recombination was 48 h at 28 °C.

Résumé

L'ISOLEMENT ET CLASSEMENT GÉNÉTIQUE PRÉLIMINAIRE DE MUTANTS THERMOSENSIBLES DU VIRUS DE LA PESTE OVINE

Des mutants du virus de la peste ovine sensibles aux variations de la température ont étés isolés et classés en 6 groupes génétiques en les combinant de nouveau. La fréquence de cette combinaison a varié dans les groupes et entr'eux. L'efficacité des 4 mutagènes utilisés, l'acide nitrique, l'N-methyl-N-nitrose-N-nitroguanidine, la proflavine et la 5-fluoro-uracil a été différente. La durée d'incubation nécessaire à la combinaison maximale est 48 h à 28 °C.

INTRODUCTION

Bluetongue virus (BTV) contains a double-stranded RNA (dsRNA) genome that migrates as 10 distinct segments on polyacrylamide gels (Verwoerd, Louw & Oellermann, 1970). These 10 segments can be transcribed into 10 species of single-stranded RNA (ssRNA) (Huismans & Verwoerd, 1973). The segments probably represent 10 separate cistrons, although to date only 7 virus-specific polypeptides have been identified (Verwoerd, Els, De Villiers & Huismans, 1972).

The 3 remaining virus-specific polypeptides have still to be identified, and further investigation should be made into the function of all 10 of the BTV-specific polypeptides. Such information would be of especial importance for the elucidation of the virulence of the virus as well as for the possible modification of this virulence in BTV temperature-sensitive mutants with vaccine production in view. Since the use of these mutants is basic to all the proposed investigations, a start has been made on their isolation. No previous studies of this nature have been carried out on BTV, although similar work has been done with the closely related reovirus (for review, see Joklik, 1974).

We report here the isolation of temperature-sensitive mutants of BTV and their genetic classification in recombination groups. In addition, some genetic implications are discussed.

MATERIALS AND METHODS

Cells

L cells: NCTC clone 929 obtained from the American Type Culture Collection USA was used throughout. These were grown as monolayers in Roux flasks and maintained as described by Verwoerd, Oellermann, Broekman & Weiss (1967). Eagle's medium was prepared according to the modified prescription of Macpherson & Stoker (1962) and as described by Verwoerd (1969).

BHK cells: BHK-21 (C-13) from the American Type Culture Collection were used. Growth and maintenance were the same as for L-cells.

Virus

BTV: Type 10A was employed. Propagation was routinely carried out by the inoculation of BHK monolayers with 5–10 plaque-forming units (PFU) per cell, and the cells and medium were harvested after 48 h incubation at 38 °C.

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Virus titration

BTV was titrated according to the method of Howell, Verwoerd & Oellermann (1967). L-cell monolayers were prepared by dispensing 5 ml cell suspensions (5×10^5/ml) into 60 mm Petri dishes and incubating them overnight at 38 °C. After the monolayers had been rinsed in Eagle's medium, the virus (0,1 ml) was allowed to adsorb for 30 min. The cell cultures were then overlaid with a 0,5% agarose suspension in Eagle's medium. The dishes were further incubated, in an atmosphere of 5% CO_2 and 70% humidity, for 5 days at 38 °C or for 10 days at 28 °C. Plaques were stained for 4 h with a 0,03% neutral red solution in a 0,2 M phosphate buffer pH 6,5.

Virus purification

A modification of the method of Verwoerd (1969) was used to prepare partially purified virus. Infected cells were collected by centrifugation for 60 min at $3\,000\times g$, washed with 2 mM Tris-HCl buffer pH 8,0 and then suspended in the same buffer at a concentration of 5×10^7/ml. The suspension was then homogenized in an Ultra-Turrax homogenizer for 30 s. Trichloro-trifluoro-ethane (Freon 113) was added (1:3) together with Sephadex G200 (final concentration 0,5%) and the extract again homogenized for 30 s before centrifugation at $3\,000\times g$ for 10 min. The freon phase was washed twice with the 2 mM Tris buffer and the supernatants combined. When required, the partially purified virus was concentrated by pelleting at $40\,000\times g$ for 90 min. Virus pellets were suspended in 2 mM Tris-HCl buffer containing 1% bovine albumin and stored at 4 °C.

Treatment of wild type (WT) BTV with different mutagens

Nitrous Acid: The partially purified virus pellets were suspended in a 0,1 M solution of $NaNO_2$ (Merck) in 0,2 M phosphate buffer pH 5,0 to give a final virus concentration of 1×10^9 PFU/ml. At various intervals after treatment was started, aliquots were removed from the suspension, diluted 10-fold in Tris-HCl buffer pH 8,0 and dialyzed overnight before titration against the same buffer.

N-methyl-N-nitroso-nitroguanidine (MNNG): Partially purified virus at a concentration of 1×10^9 PFU/ml in 2 mM Tris-HCl pH 8,0 was treated with 10 μg/ml MNNG and left for 1 h at 20 °C with occasional shaking. The reaction was stopped by a 10-fold dilution with the same buffer and the virus was then pelleted at $40\,000\times g$ for 90 min. The pellet was rinsed twice and subsequently suspended in the Tris-HCl buffer before titration.

Proflavine: An L-cell monolayer was inoculated with BTV (10 PFU/ cell). Proflavine (BDH) was added 1 h after infection of the cells to give a final concentration of 10 μg/ml, and the infected cultures incubated for 24 hours at 38 °C. Cells were harvested by scraping and centrifuged at $1\,200\times g$ for 15 min., washed in fresh Eagle's medium and titrated for virus.

5-Fluoro-uracil: The method described for proflavine was followed except that 5-fluoro-uracil (Sigma Chemical Company) was added to a final concentration of 2 mg/ml.

Mutant isolation

L-cells in 60 mm Petri dishes were infected with mutagen-treated virus to yield not more than 10 plaques per dish. The dishes were incubated at 28 °C with an 0,5% agarose overlay in Eagle's medium for 8 days when a 2 ml agarose overlay containing 0,002% neutral red was added. After incubation for 24 h at 28 °C, the plaque size was scored and the cultures transferred to 38 °C for a further 48 h. Plaques were then examined for further growth. Where there was no further increase in size (i.e. no halo around the original plaques) plaques were aspirated off with a Pasteur pipette, suspended in 1 ml of Eagle's medium and titrated at 38 °C and 28 °C. If a 100-fold difference was found in the preliminary titre, the virus isolates were seeded on small roller bottles and, after incubation at 28 °C for 7 days, again titrated at the two temperatures. If the efficiency of plating (38 °C/28 °C) was less than 10^{-3}, the plaque isolates were considered putative mutants and were purified through 2 plaque isolation steps and stored at 4 °C.

Mutant nomenclature

Virus mutants were prefixed and numbered according to the mutagenic agent used and the number of non-halo plaque isolates tested, i.e. nitrous acid (N), MNNG (G), proflavine (P) and 5-fluoro-uracil (F). F73 is therefore the 73rd non-halo plaque tested after treatment with 5-fluoro-uracil.

Recombination studies

The 2 viruses to be tested for recombination were mixed prior to inoculation. Double infections of L-cell monolayers were carried out in Petri dishes at a multiplicity of infection of 10 PFU/cell for each virus. For all practical purposes there is a 100% probability of each cell being infected by both viruses under these conditions (Fields & Joklik, 1969). After the virus had been allowed to adsorb for 1 h at 20 °C, the cells were then rinsed in 5 ml of Eagle's medium and incubated with a further 5 ml of medium at 28 °C for the periods indicated under Results. Singly infected cultures were treated in the same way. Recombination frequency (RF) was determined as follows:

$$RF = \frac{[(AB)_{38} - \{(A)_{38} + (B)_{38}\}]}{(AB)_{28}} \times 10^2$$

where $(AB)_{38}$ is the titre at 38 °C of the doubly infected cultures, $(AB)_{28}$ the titre at 28 °C of the same culture, and $(A)_{38}$ and $(B)_{38}$ the yield of the singly infected cultures titrated at 38 °C.

Results

Mutant isolation

To date, 829 non-halo plaques have been isolated and tested for their ability to form plaques. Only 19 have been found to yield stable mutants with an efficiency of plating (38 °C/28 °C) of 10^{-3} or less. The 4 mutagens used differed in their efficiency. The frequency of temperature-sensitive mutants induced by MNNG, 5-fluoro-uracil, nitrous acid and proflavine, was 5%, 3%, 2% and 0,5%, respectively. Treatment of BTV with 2 M $NaNO_2$ at pH 4 for 30 min, which are the conditions prescribed for reovirus (Fields & Joklik, 1969), reduced a titre of $1,0\times10^9$ PFU/ml of BTV to a negligible level. For this reason BTV was treated for 5 min only with 0,1 M $NaNO_2$ at pH 5,0. Under these conditions, 0,01% infective virus could be demonstrated after treatment. A 1% survival rate was demonstrated after treatment of virus with MNNG for 1 h at pH 8,0. The yield of virus from proflavine-treated cultures incubated at 38 °C for 24 h was 10^5 PFU/ml while that of 5-Fluoro-uracil-treated cultures was 2×10^7 PFU/ml. Non-treated cultures infected with the same multiplicity of infection of 5 PFU/cell gave a virus yield of 5×10^7 PFU/ml.

Stability of temperature-sensitive mutants

After 2 plaque isolation steps, the clones selected normally yielded stable mutants. Persistent leaky mutants were sometimes found, however.

Kinetics of recombination

Recombination experiments were carried out at an incubation temperature of 28 °C. The yield of wild-type at both 28 °C and 38 °C was in the order of $5,0\times10^7$ PFU/ml. Since the replication rate at 28 °C was considerably slower than that at 38 °C, the period of incubation at 28 °C required for optimum recombination had still to be determined. The frequency of recombination was tested in the case of 3 pairs of mutants over an incubation period of 48 h at 4-hour intervals, the results being recorded in Fig. 1.

There is considerable variation in the period of incubation required to achieve maximum recombination, but no marked increase in the frequency of recombination could be demonstrated prior to 16 hours after infection. The frequency of recombination between F207 × F211 reached a plateau after 24 h, but the combination F207 × G1 only reached this point after an incubation period of 32 h. Maximum recombination between F207 × F73 was only recorded after 48 h. Other experiments have shown that no further increase in recombination frequency is found with incubation periods up to 72 h. For this reason a period of 48 h was taken as standard incubation time for all further recombination studies. Experiments carried out at 4 °C have shown that viral replication is a prerequisite for recombination. Double infected cultures were incubated at 4 °C and 28 °C for 48 h. Only in the case of cultures incubated at 28 °C could recombination be demonstrated.

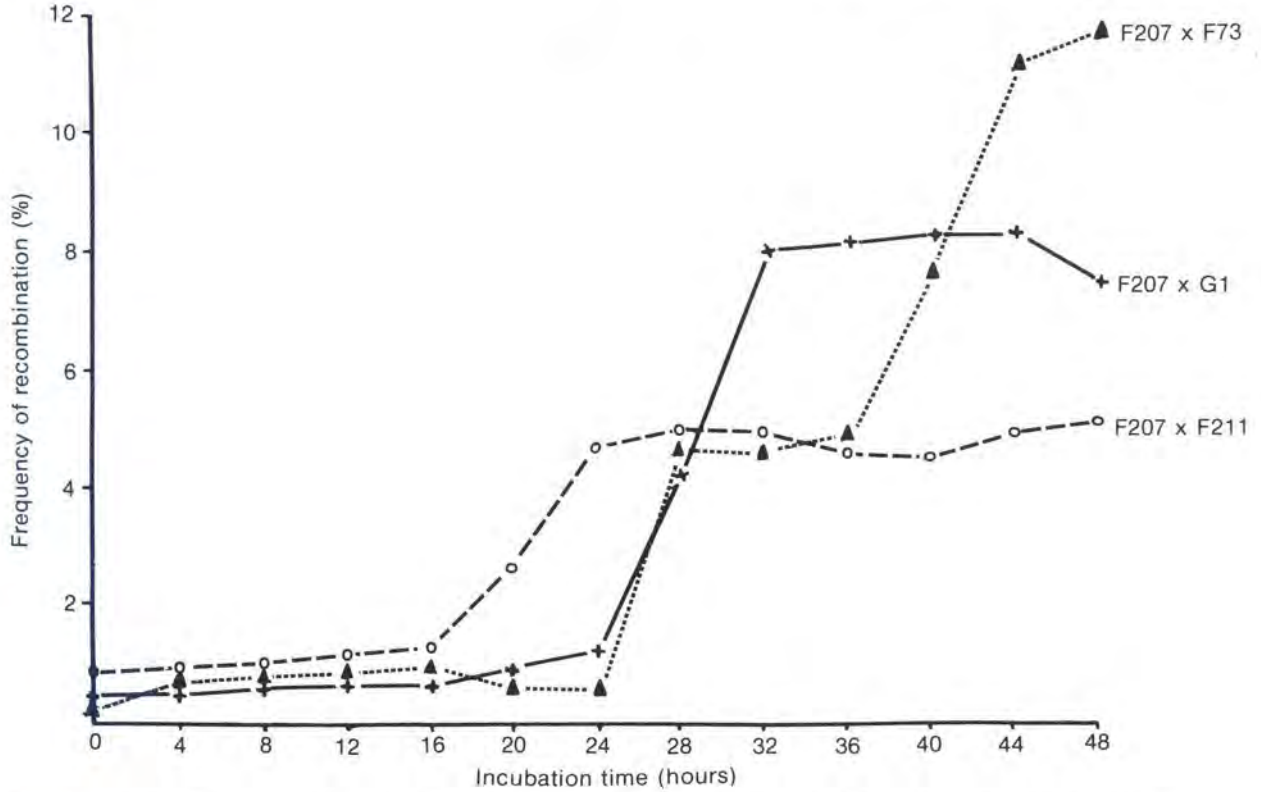

FIG. 1 The effect of incubation time at 28 °C on the frequency of recombination of BTV temperature-sensitive mutants

Recombination groups

To date, 19 mutants have been studied for recombination. The mutants have been classified in recombination classes but no attempt has been made to represent these classes on a genetic map. Two mutants are considered to fall into the same recombination class if they show no recombination with each other. All experiments were carried out in triplicate, and the average frequencies of recombination are summarized in Table 1.

TABLE 1 Frequency of recombination of temperature-sensitive mutants of BTV

Mutant	I (F211)	II (F207)	III (G1)	IV (G345)	V (F210)	VI (F73)
F211	—	0,5	0,9	0,5	0,1	7,6
G280	0	0,3	0,2	1,4	N.D.	4,2
G339	0	3,2	N.D.	0,4	N.D.	3,9
G361	0	3,2	4,3	N.D.	1,5	2,7
F16	1,3	0	3,0	N.D.	2,6	2,5
F207	0,6	—	3,3	3,2	5,9	4,9
G231	3,6	0	3,5	N.D.	5,8	4,8
N115	1,2	0	1,5	N.D.	5,1	4,2
N175	1,5	0	4,4	N.D.	N.D.	9,4
P9	1,4	0	5,3	N.D.	1,5	5,4
F71	1,3	2,1	0	N.D.	1,0	3,2
G1	0,9	3,3	—	0,2	7,0	3,6
G296	4,0	1,3	0	1,7	N.D.	14,5
G304	N.D.	1,3	0	N.D.	0,9	2,1
G320	N.D.	2,5	0	1,6	5,1	9,0
G345	0,9	3,1	0,2	—	5,6	19,0
F210	0,1	5,9	7,0	5,6	—	12,3
G262	1,0	0,2	2,6	8,0	0	1,0
F73	7,6	4,9	3,6	19,0	12,3	—

N.D.—not done

As it was impracticable to carry out all possibe crosses, a representative was chosen from each group and tested against as many of the other members of a group as possible. In the 6 recombination groups demonstrated, the frequency of recombination varied from 0,1% to 19%. In some cases a 10-fold or even greater variation in the frequency of recombination was found within groups, but certain groups showed on the average a higher or lower frequency of recombination. For example, with any of the other mutants, the frequency of recombination of F73 was always higher than the frequency of recombination of F211 with the same mutant.

Discussion

It was shown that stable temperature-sensitive mutants of BTV can be isolated. N-methyl-N-nitroso-N-nitroguanidine, 5-fluoro-uracil and nitrous acid proved to be effective mutagens. Proflavine, on the other hand, was found to be very ineffective, yielding only a single mutant from 200 non-halo plaques. The spontaneous mutation rate of BTV was not tested, but it is possible that this single mutant could be due to a non-induced mutation, since the frequency of spontaneous mutation of the closely related wild-type reovirus is of the same order (0,1–0,3%) (Fields & Joklik, 1969). The low efficiency of proflavine to increase the mutation rate of BTV contrasts with its effect on reovirus, since in this case it proved to be the most efficient mutagen of the mutagens used (Fields & Joklik, 1969). Leakiness is a characteristic of many of these mutants, the cause of which at this point is not known.

The fact that the incubation period required for recombination of BTV mutants varied greatly (24–48 h), could be attributed to differences in their

growth patterns, particularly as such differences in growth cycle have been observed for a number of BTV mutants (unpublished results).

Since the variation in recombination frequencies found both within and between groups makes impracticable a statistical analysis of the results recorded in Table 1, no conclusion about the possible linear gene sequence of the BTV genome can be reached, and hence no genetic map has been formulated. In any case, theoretical considerations of the replication pattern of the closely related reovirus indicate that there can be no direct correlation between the structure of the viral genome and its recombination frequency (Gomotos, 1967; Schonberg, Silverstein, Levin & Acs, 1971). From studies of the replicative mechanisms of reovirus, it is evident that no exchange of genetic material can take place at the dsRNA level (Acs, Klett, Schonberg, Christman, Levin & Silverstein, 1971; Zweerink, Ito & Matsuhisa, 1972), and recombination must therefore, take place during viral replication by the exchange of ssRNA. This would mean that the molar ratios of the different ssRNA species could play a role in the frequency of recombination. Thus independent assortment would be a more appropriate term than recombination for the mechanism in the *Reoviridae*.

A similar deduction can be made for BTV if the replication cycle of BTV is similar to that of reovirus. It has been shown in the case of BTV that the smaller ssRNA species are present in higher relative amounts than the larger species (Huismans & Verwoerd, 1973). Recombination between mutants involving exchange of smaller ssRNA species would seem, therefore, to be a more likely event than those involving the larger species. This may well explain the difference in the recombination frequencies of F73 and F211. In the case of F73 the mutation could possibly be present in a small genome segment, whereas for F211 a mutation of a larger segment may be involved.

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