ISOLATION OF COXSACKIEVIRUS B3 TEMPERATURE-SENSITIVE MUTANTS AND THEIR ASSIGNMENT TO COMPLEMENTATION GROUPS

M.D. TROUSDALE, R.E. PAQUE and C.J. GAUNTT
Department of Microbiology
The University of Texas Health Science
Center at San Antonio
San Antonio, Texas 78284

Received April 11,1977

SUMMARY: Ten temperature-sensitive (ts) mutants of Coxsackievirus B3 were isolated from a parent strain capable of replication to similar yields at either 34° or 39.5°. Eight mutants were isolated following mutagenesis with 5-fluorouracil and two were spontaneous mutants. Complementation tests permitted assignment of the ts mutants into three non-overlapping groups with complementation indices of 12.4 to 2.0. One mutant was not assigned to a complementation group.

Conditional lethal mutants of the ts type have been obtained with a variety of animal viruses (1, 2). The initial genetics study of the Picornaviridae was given impetus by the search for avirulent strains of poliovirus because ts mutants of some viruses were less pathogenic than the parental strain from which they are derived. This property of reduced pathogenicity has frequently appeared to be directly correlated to temperature-sensitivity, i.e. failure to grow at supraoptimal temperatures, and, therefore, helpful in the search for attenuated viruses for development of live virus vaccines (3, 4, 5).

Theoretically, ts mutants in one complementation group possess defective lesion(s) in the same gene, but not necessarily at identical locations (2). This capability enables a determination of the total number of genes in the viral genome and possibly the relationship of gene function with capacity for virulence or attenuation.

Ts mutants from other members of the Picornaviridae family, such as poliovirus, foot-and-mouth-disease (FMD) virus, and mengovirus, have been isolated and assigned to complementation groups (6, 7, 8, 9). This report presents information on the isolation of ts mutants from Coxsackievirus B3 and the separation of these ts mutants into complementation groups.

ISSN 0006-291X

MATERIALS AND METHODS

Propagation of Coxsackievirus B3 and ts mutant stocks and plaque assays were carried out in HeLa cells using Autopow-MEM (Flow Laboratories, Rockville, Md. 20852) supplemented with 2% fetal bovine serum, 2 mM L-glutamine and 20 µg/ml neomycin sulfate (10). The nutrient agar overlay for the plaque assay consisted of 1% Bactoagar, MEM with 1% heat inactivated fetal bovine serum, 100 µg/ml diethyl-aminoethyl-dextran, and neomycin sulfate. Coxsackievirus B3 (obtained from J. F. Woodruff, Cornell Univ. Med. College, New York, N.Y.) was plaque purified five times, and a large virus stock was prepared at 34°C. The identity of this parent virus was verified by virus neutralization with commercial typing antiserum (Type B-3 Coxsackie antiserum, bovine, NIH Research Reagent, NIAID, Bethesda, Md.). Mutagenized parent virus stocks were prepared by growing virus in the presence of 2 or 16 mM 5-fluorouracil (Sigma Chemical Co., St. Louis, Mo. 63178) for 18 hr at 34° (11, 12). Field's "shift-up method" was used to select presumptive ts mutant plaques (13). Briefly, agar-overlayed virus inoculated monolayers were incubated at 34° in a 5% CO_2 incubator until plaques were clearly visible (usually about 48 hr). The outer circumference of isolated plaques was outlined on the plastic dish with a fine-tip marker and the monolayer cell cultures were then "shifted-up" to 39.5° for an additional 24 hr. Plaques not appearing to increase in size at 39.5° were picked and virus stocks prepared for confirmational studies.

Each ts mutant was classified according to its ability to complement other mutants in pairwise mixed infection at a nonpermissive temperature. Multiplicity of infection (MOI) was 10-20 with photosensitized virus (passed five consecutive times in the presence of 4 µg/ml of neutral red). Virus adsorption was at room temperature (under Wratten AO filtered 15 watt safelight, total radiant energy <0.16 mW/cm^2) for 40 min after which the inoculum was removed. The cell monolayers were rinsed with phosphate buffered saline (PBS) and incubated in total darkness at 39.5° for 9 hr in 5% CO_2. Cultures were frozen and thawed three times, diluted 1:10 in PBS and irradiated with 375 W Photoflood

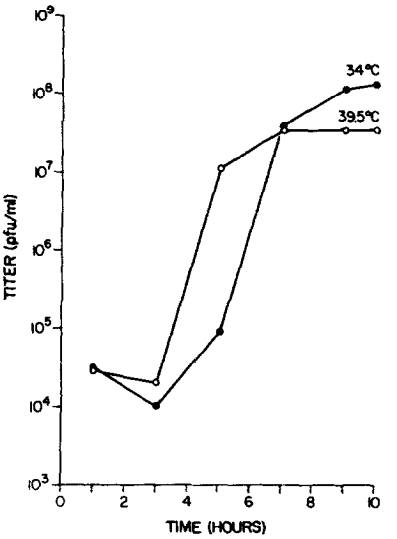

Figure 1. One step growth curves of Coxsackievirus B3 in HeLa cells. Cells were inoculated with 20 plaque forming units (pfu) per cell and virus allowed to adsorb for 30 min at 25°. The inoculum was removed, the monolayers rinsed three times prior to addition of maintenance medium and incubated in 5% CO_2 at the appropriate temperature.

TABLE 1. SAMPLING OF COXSACKIEVIRUS B3 POPULATIONS FOR TEMPERATURE-SENSITIVE MUTANTS

Mutagen	Total number plaques examined	Number acceptable ts mutants	Fraction of ts mutants	Relative efficiency of ts mutant induction
None	1,304	2	1/652	1.00
5-fluorouracil	200	1	1/200	3.26
5-fluorouracil with neutral red	1,270	7	1/181	3.60

lamp (36.8 mW/cm^2) for 200 sec (14, 15). Progeny virus was assayed at 34° and virus yields expressed as pfu/ml.

RESULTS

One step growth curves of the parent Coxsackievirus B3 at 34° and 39.5° are presented in Fig. 1. The eclipse phase lasted for 3 hr at both tempera-

TABLE 2. EFFICIENCY OF PLATING AND LEAKINESS VALUES FOR 10 TEMPERATURE-SENSITIVE MUTANTS OF COXSACKIEVIRUS B3

Virus	Efficiency of Plating[a]	Leakiness[b]
ts1	2.3×10^{-3}	$<2.0 \times 10^{-5}$
ts3	5.3×10^{-4}	$<3.3 \times 10^{-6}$
ts4	9.4×10^{-2}	$<9.1 \times 10^{-8}$
ts5	$<4.4 \times 10^{-4}$	5.0×10^{-3}
ts6	8.2×10^{-5}	1.3×10^{-4}
ts7	$<1.0 \times 10^{-7}$	1.5×10^{-6}
ts8	7.4×10^{-6}	2.6×10^{-4}
ts9	4.4×10^{-6}	1.1×10^{-4}
ts10	1.7×10^{-5}	3.1×10^{-4}
ts11	1.2×10^{-5}	6.0×10^{-4}
CB3 (parent)	0.50	1.17

[a]For efficiency of plating determinations, virus is propagated at 34° and the yields assayed at both 34° and 39.5° (i.e., 39.5° titer/34° titer).

[b]For leakiness determinations, virus is propagated at 39.5° and the yields assayed at both 34° and 39.5°.

tures, however final infectious virus yields were obtained slightly earlier (2 hr) at 39.5°, but yields were 10-50% higher at 34°.

From a total of 2774 plaques examined, ten ts mutants with good genetic stability were selected for further study (Table 1). Two ts mutants were spontaneous and eight were isolated following mutagenesis with 5-fluorouracil. The fraction of plaques from non-mutagenized virus stocks found to contain ts mutants was 1 per 652. This fraction increased to 1 per approximately 200 plaques tested following mutagenesis with 5-fluorouracil.

Table 2 gives the efficiency of plating (EOP) and leakiness values for the ten acceptable ts mutants of Coxsackievirus B3. The EOP for the 10 ts mutants

TABLE 3. COMPLEMENTATION INDICES OF COXSACKIEVIRUS B3 TEMPERATURE-SENSITIVE MUTANTS[a]

ts Mutant	1	3	4	5	6	7	8	9	10	11	Parent
1		7.84	0.66	4.35	0.67	0.88	1.63	0.89	0.53	1.05	0.52
3			2.45	1.67	12.40	10.40	8.73	1.23	2.96	2.10	0.49
4				2.00	0.76	3.66	0.89	0.65	1.21	0.45	0.31
5					5.70	2.56	4.16	1.42	0.24	0.52	0.31
6						0.18	1.85	1.43	0.86	3.21	1.44
7							0.37	0.91	1.07	1.04	0.40
8								0.14	0.45	1.04	0.29
9									0.05	0.05	0.17
10										0.02	0.11
11											0.08
Parent											

[a]Complementation index was calculated by the equation of Burge and Pfefferkorn (16).

ranged from 9.4×10^{-2} for ts 4 to $<1.0 \times 10^{-7}$ for ts 7. The range of leakiness values was 5.0×10^{-3} for ts 5 to $<9.1 \times 10^{-8}$ for ts 4.

Complementation indices calculated from all possible pairwise crosses of the 10 ts mutants under nonpermissive conditions (39.5°) are shown in Table 3. Fourteen of these complementation indices were found to be equal to or greater than 2.00, which is significantly different at the 95% confidence level ($p=.05$). In Table 4 these complementation indices are arranged in order of descending values from which ts mutants can be assigned to three non-overlapping complementation groups (I, II or III).

DISCUSSION

One step growth curves demonstrated that the parent strain of Coxsackievirus B3 had the capability of replicating to similar titers at either the per-

TABLE 4. ASSIGNMENT OF COXSACKIEVIRUS B3 TEMPERATURE-SENSITIVE MUTANTS INTO THREE NONOVERLAPPING COMPLEMENTATION GROUPS[a]

Complementation Index	ts Mutant in Mixed Infection	ts Mutant Group Assignment		
		I	II	III
12.40	3 x 6		3	6
10.40	3 x 7		3	7
8.73	3 x 8		3	8
7.84	3 x 1		3	1
5.70	5 x 6		5	6
4.35	1 x 5		5	1
4.16	5 x 8		5	8
3.66	4 x 7	4		7
3.21	6 x 11	11		6
2.96	3 x 10		3	10
2.56	5 x 7		5	7
2.45	3 x 4	4	3	
2.10	3 x 11	11	3	
2.00	4 x 5	4	5	

[a]ts 9 did not complement any ts mutant.

missive or nonpermissive temperatures (34° or 39.5°, respectively). Concentrations of 5-fluorouracil (2 or 16 mM) were found to reduce Coxsackievirus B3 yields by 90-99%. Mutagenesis at these levels enhanced the relative efficiency of induction of ts mutants by approximately three-fold (3.26 and 3.60) over non-mutagenized virus stocks. The criteria employed in these studies for distinguishing between acceptable and nonacceptable ts mutants was approximately 10^{-3} or less for both EOP and leakiness values. Both EOP and leakiness values were determined for all ts mutant stocks to ensure that the ts defect was genetically stable (17, 18).

A major criticism of complementation studies has been the inability to distinguish between parent and progeny virus (2). The presence of unexpressed parent virus may result in masking of complementation between two ts mutants at nonpermissive conditions. Some authors have washed pairwise infected cultures after several hr incubation in order to minimize this problem (13, 19). We attempted to solve this problem by employing ts mutants which had been made light-sensitive by repeated virus passage in the absence of neutral red. Pairwise infected cultures were harvested from nonpermissive conditions and parent virus photoinactivated (fraction of surviving light-sensitive parent virus was less than .0001) prior to assay for progeny from the complementation.

The complementation indices reported herein for Coxsackievirus B3 ts mutants (2.0 to 12.4) were similar to the indices reported for poliovirus ts mutants (6). Mutant ts 9 failed to complement the other nine ts mutants; this may be due to mutation(s) in more than one viral gene. Those complementation indices which are less than 1.0 indicate interference between the paired ts mutants. Extensive interference between different poliovirus ts mutants under nonpermissive conditions has been reported by Cooper (6). No gene order is intended by assignment of the nine ts mutants to complementation groups I, II and III. Thus far, none of the 10 ts mutants produce myocarditis in adolescent CD-1 mice, whereas revertants from 6 of 10 ts mutants produce extensive myocarditis similar to that produced by the parent virus.

ACKNOWLEDGEMENTS

We thank Stephen J. Mattingly for a critical reading of the manuscript. This investigation was supported by a research grant from the American Heart Association, Texas Affiliate, Inc. and by General Research Support Grant RR-05654-06 from the General Research Support Branch, Division of Research Facilities and Resources, National Institutes of Health to The University of Texas Health Science Center at San Antonio.

REFERENCES

1. Fenner, F. (1970) Annu. Rev. Microbiol. 24, 297-334.

2. Ghendon, Y.Z. (1972) Progr. Med. Virol. 14, 68-122.

3. Sabin, A.B., Henessen, W.A., and Winsser, J. (1954) J. Exp. Med. 99, 551-576.

4. Murphy, B.R., Baron, S., Chalhub, E.G., Uhlendorf, C.P., and Chanock, R.M. (1973) J. Inf. Dis. 128, 488-493.

5. Wagner, R.R. (1974) Infect. Immun. 10, 309-315.

6. Cooper, P.D. (1965) Virology 25, 431-438.

7. Pringle, C.R. (1970) J. Virol. 5, 559-567.

8. MacKenzie, J.S., Slade, W.R., Lake, J., Priston, R.A., Bisby, J., Laing, S., and Newman, J. (1975) J. Gen. Virol. 27, 45-59.

9. Bond, C.W., and Swim, H.E. (1975) J. Virol. 15, 288-296.

10. Dulbecco, R., and Vogt, M. (1954) J. Exp. Med. 99, 167-182.

11. Munyon, W., and Salzman, N.P. (1962) Virology 18, 95-101.

12. Cooper, P.D. (1964) Virology 22, 186-192.

13. Fields, B.N., and Joklik, W.K. (1969) Virology 37, 335-342.

14. Crowther, D., and Melnick, J.L. (1961) Virology 14, 11-21.

15. Hiatt, C.W. (1972) Res. Progr. Org. Biol. Med. Chem. 3, 320-331.

16. Burge, B.W., and Pfefferkorn, E.R. (1966) Virology 30, 214-223.

17. Burge, B.W., and Pfefferkorn, E.R. (1966) Virology 30, 204-213.

18. Simpson, R.W., and Hirst, G.K. (1968) Virology 35, 41-49.

19. Echart, W. (1969) Virology 38, 120-125.