J. Biosci., Vol. 7, Number 1, March 1985, pp. 39–47. © Printed in India.

Isolation, characterization and mapping of temperature-sensitive mutants of mycobacteriophage I3

CHANCHAL SADHU, MOHAN LAL GOPE, KRISHNA SADHU and K. P. GOPINATHAN

Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore 560 012, India

MS received 20 August 1984; revised 23 November 1985

Abstract. Eighteen temperature-sensitive mutants of mycobacteriophage I3 have been isolated and partially characterized. All the mutants were defective in vegetative replication. Based on temperature shift experiments with the temperature sensitive mutants, the thermosensitive phase of the phage development period has been characterized for each mutant. The genes have been mapped by recombination analysis. The early, continuous and middle genes seem to cluster on the genetic map.

Keywords. Mycobacteriophage I3; temperature sensitive mutants; recombination analysis; gene mapping.

Introduction

Mycobacteriophage I3 is one of the very few transducing phages of the genus *Mycobacterium* (Sunderraj and Ramakrishnan, 1970; Gopinathan, 1981). It has a growth cycle of 5h consisting of 180–210 min of latent period and 60–90 min of rise period (Nagaraja and Gopinathan, 1980). The macromolecular syntheses in mycobacteriophage I3-infected *Mycobacterium smegmatis* cells have been studied in our laboratory (Nagaraj a and Gopinathan, 1981 a,b; Nagaraj a, 1981). However, no gene has been identified for any of these functions.

Use of conditional lethal mutants for such purposes is well known. In this paper, we report the isolation and characterization of 18 temperature-sensitive (ts) mutants of phage I3. These mutations have been mapped by recombination analysis.

Materials and methods

Bacteria and phage

M. smegmatis SN2 and mycobacteriophage I3 were from our laboratory stocks; either the wild type phage or a clear plaque mutant, C5 (Gopinathan *et al.,* 1978) was used.

Abbreviations used: ts, Temperature-sensitive; moi, multiplicities of infection; pfu, plaque forming unit; NTG, N-methyl-N'-nitro-N-nitrosoguanidine; KCN, potassium cyanide.

Growth of bacteria and phage

M. smegmatis was grown in the minimal medium (Karnik and Gopinathan, 1980) at 35°C for 14–16 h (approximately 10^8 cells/ml) on a rotary shaker. For the preparation of phage lysate, the host *M. smegmatis* was infected with the phage at multiplicities of infection (moi) 5–10 in the presence of 1 mM CaCl2 and the shaking continued. After 24 h at 35°C, a few drops of chloroform were added to the lysate and the mixture was shaken for complete lysis of bacteria. The lysate was then clarified by centrifugation (8000 *g*, 20 min). The phage titers were determined as plaque forming unit (pfu) by the soft agar overlay method.

Isolation of ts mutants of phage I3

M. smegmatis cells (10^8 cells/ml) were infected with phage I3 (wild type I3 or C5) at a moi of 5 in the presence of 1 mM $CaCl_2$; N-methyl-N′-nitro-N-nitrosoguanidine (NTG, 500μg/ml) was then added to this, and the culture was shaken at 35°C for 1 h. The cells were pelleted by centrifugation, washed with and resuspended in the original amount of growth medium containing $CaCl_2$, and incubated overnight at 35°C to prepare the phage lysate. Appropriate dilutions of the mutagenized lysate were plated and incubated first at 35°C for 12-14 h and then at 40°C for 24 h. Under this condition, the ts mutants produced tiny plaques while the wild type phage produced larger plaques. The suspected mutants were further tested by replica plating at 35°C and 40°C. The mutants which formed plaques at 35°C but not at 40°C were selected. Each mutant was isolated from an independent treatment.

One step growth experiment

The one step growth experiment was done by the classical method established for coliphages (Adams, 1959) but with the modifications necessary for mycobacteria. To the 12 h old culture of *M. smegmatis* (5×10^7 cells/ml), potassium cyanide (KCN) was added to a final concentration of 1 mM. The cells were infected with phage at a moi of 0·5 and incubated at 35°C for 10 min for adsorption. The cells were pelleted by centrifugation, resuspended in the original volume of fresh medium, diluted 10^5 fold and incubated at appropriate temperature with shaking. Samples were withdrawn at different times and infective centers or phages were assayed using *M. smegmatis* as the plating bacteria. The burst sizes were calculated as pfu produced per infective center.

Temperature shift experiment

This was done by a modification of the one step growth experiment. The infected bacteria were incubated at 40°C or at 35°C. At different intervals, samples were transferred from one temperature to the other and incubated till the end of one life cycle.

Complementation and recombination

Titers of each mutant phage were adjusted to 2×10^5 pfu/ml and then mixed in all possible combinations of two phages in 1:1 ratio. A drop from each mixture along with

individual mutants to serve as controls, were spotted on the bacterial lawn and incubated at 40°C. Growth of phages on the spot was taken as positive complementation.

Crosses to measure recombination frequencies were performed essentially according to Edgar and Lielausis (1964) described for coliphage T_4. *M. smegmatis* cells (5×10^7 cells/ml) were infected with two mutant types at an moi of 5 for each mutant in the presence of 1 mM $CaCl_2$ and 1 mM KCN. After 10 min, the infected cells were pelleted, resuspended in the original volume of fresh medium and dilutions (10^4 and 10^5) were incubated at 35°C with shaking. After 5 h chloroform was added to the samples, and the total pfu (by plating at 35°C) and the wild phenotypes (by plating at 40°C) were scored. Recombination frequencies were calculated by the expression, (pfu at 40°C × 2 × 100)/(pfu at 35°C).

Results

Bacteriophage I3 multiplies on *M. smegmatis* with a relatively long growth cycle (nearly 5 h). It has a latent period of 180–210 min and a rise period of about 60–90 min (Nagaraja and Gopinathan, 1980). Due to this slow growth, the development of phage I3 can be monitored at wider intervals.

Phage output is dependent on incubation temperature

Wild type phage I3 formed plaques of 2–3 mm diameter and grew equally well at temperatures between 30 and 40°C on both nutrient agar and Luria agar plates. However, at 42°C, the plaques formed were very minute. The effect of temperature on phage I3 growth was most significant when propagated in liquid medium. Burst size of the phage was maximum at 37°C in both nutrient broth and minimal medium. At 42°C, the average burst was about 3–4 % of that at 37°C in both the media even though the growth of the host was unaffected at this temperature. Temperature sensitivity of growth was similar in the case of the wild type (turbid plaque) and C5 mutant. When the phage suspension was incubated at 42°C for 5 h there was no significant reduction of phage titer which indicated that the phage particles maturing at 37°C were not susceptible to inactivation at 42°C.

Isolation of ts mutant

The ts mutants of phage I3 were isolated using NTG. While screening for the ts mutants we could eliminate a large number of wild type phages because of their larger plaque size, observed after the incubation at 40°C. The tiny plaques were screened by replica plating and the ts mutants were obtained at a frequency of approximately 10^{-2}. Some of the mutants proved to be leaky and were discarded. In all 18 mutants were studied further.

The 12 mutants of G series were from I3 wild type (turbid plaque) and the 6 mutants of S series were from I3 C5 (clear plaque mutant). The letters G and S stand for Gope and Sadhu, the workers who isolated the mutants. Reversion frequencies of these mutants were determined by plating out high titer stocks at 40°C. Of the mutants

employed, G2 showed the highest reversion frequency ($6{\cdot}7 \times 10^{-4}$), S1 the lowest (3×10^{-7}) and the others in between.

Comparison of growth properties at permissive and non-permissive temperatures

The one step growth patterns of the individual mutants were determined at the permissive (35°C) and non-permissive temperatures (40°C) and the results are summarized in table 1. At 35°C, the burst sizes of the mutant phages did not show any significant difference from that of the wild type. In fact, some of the mutants (S3 and S6) showed higher burst sizes. However, at 40°C, all of them showed much reduced burst. At 35°C, burst sizes of the ts mutants derived from C5 were higher than the burst sizes of mutants of I3 wild type parent.

Table 1. Comparison of burst sizes of the I3 ts mutants[a] at 35° and 40°C

Phage	Plaque[b] morphology	Burst at 35°C	Burst at 40°C
Mutants			
S1	C	36·0	0·09
S2	C	46·0	0·06
S3	C	79·0	0·01
S4	C	41·0	1·40
S5	C	40·5	0·03
S6	C	65·5	0·01
Parental I3 (C5)	C	43·0	36·00
Parental wild type I3	T	25·0	21·00
Mutants			
G2	T	18·0	0·50
G3	T	20·0	0·01
G4	T	17·0	0·01
G5	T	24·0	0·10
G6	T	19·0	0·15
G7	T	21·0	0·09
G8	T	16·0	0·32
G9	T	15·0	0·08
G10	T	18·0	0·23
G11	T	26·0	0·34
G12	T	18·0	0·12
G13	T	21·0	0·87

[a] All the S series mutants also carried C5 mutation.

[b] C, Clear plaque; T, turbid plaque

Thermostability of ts mutants

When the mutant virions were incubated at 40°C for 5 h and then assayed at 35°C, it was observed that the ratio of titers of the phage suspension kept at 40°C to that at 4°C (control) varied from 0·68-0·93 for different mutants (data not shown). This ratio for

the wild type phage was 0·80. These results suggest that the stability of the ts virions is not affected at nonpermissive temperature.

Effect of temperature shift on the growth of ts mutants

The phage development proceeds through a temporal sequence of events and the functions of different gene products are required at different stages of development (Brewer, 1978). To determine which stage of the phage development requires which gene product, temperature shift experiments were performed with individual mutants.

Results of such experiments indicated that all the mutants could be broadly classified into four different groups showing four distinct types of effect on growth of the mutants following temperature shift. Typical results with one mutant from each such group are depicted in figure 1. Temperature shift experiments with G2, G4, G6, G7, G8, G9, G10, Gl1, G12 and G13 indicated that these were early mutants (figure 1A). Their development was affected by shift up at the initial periods. An early exposure of the cells infected by these mutants to nonpermissive temperature and then shift to permissive temperature did not result in the recovery of phage progeny. Growth of the mutants G7, G12 and G13 were rapidly inhibited within a span of 30 min, early in the development period. The inhibition of G4 was even faster (within 10 min).

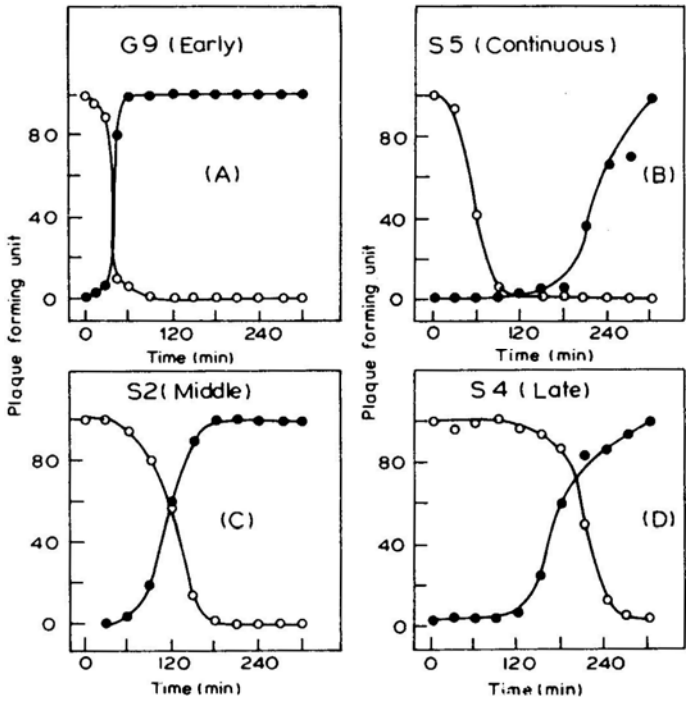

Figure 1. Temperature shift up (●) and shift down (O) profiles of early, continuous, middle and late ts mutants.

Progeny output profiles of S5 and G3 during the shift up and shift down indicated that the mutated function was needed continuously throughout the life cycle (figure 1B), though to different extents. These mutants attained 100 % yield in a shift up, only after 300 min at permissive temperature.

The shift up of cells infected with mutants S1, S2, S3, S6 and G5 to the nonpermissive temperature during initial 1 h produced less than 10% progeny, as compared to the control (figure 1C). The yield of the progeny increased gradually attaining almost 100 % if the shifting up to nonpermissive temperature was delayed progressively towards the end of the latent period. In the shift down experiments, on the other hand, the phage progeny decreased as the samples were transferred to permissive temperature after longer time periods at nonpermissive temperature. These mutants seem to be defective in delayed early or middle functions.

The development of mutant S4 was not affected at the elevated temperature upto the initial 120 min (in a shift down experiment) beyond which there was loss in progeny output. In the shift up experiment this mutant yielded 100 % progeny only when shifted at the end of the growth cycle. Shift up at any time during latent period resulted in reduced phage production (figure 1D). These results revealed that S4 is a late mutant.

The nature of thermosensitive lesion

To know whether or not the thermal inactivation of the mutant proteins was reversible, one set of samples were allowed to grow at permissive temperature for one complete life cycle (5 h) following the shift down and the average burst obtained in each case was compared to that in the samples incubated at nonpermissive temperature for 200 min. It was observed that S1, S3, S4, S6, G2, G3, G6 and G10 did not show, any significant difference under the above two conditions indicating their reversible nature. S2 and Gl 1 showed partial reactivation, while others were found to be irreversibly inactivated (data not presented).

Complementation

The S and G series of mutants were studied for complementation separately. All of the S mutants complemented with one another. In the G series, there was complementation in most of the cases, except in G7: G8: G9 and G4: G 12: G 13. Possibly G7, G8 and G9 and G4, Gl2 and Gl3 formed two separate complementation groups, which are different from all other mutants.

Mapping of the ts mutations

The ts mutations of phage I3 were mapped by determination of recombination frequencies. Approximately 100–300 plaques (in triplicate) were counted in each cross. Data presented here is from the mean of 3 individual crosses and the variation observed was in the order of 10 %. The results of recombination of S mutants are shown in table 2. The frequency of recombination was maximum between S4 arid S5 and between S4 and S6 which were 6·9 and 6 respectively. Considering the large size of the I3 genome, this value indicated that the S-designated mutations are distributed over a relatively short stretch of the phage genome. The map shown in figure 2A is the best fit of the recombination data for S mutants.

Table 2. Recombination frequencies between the S mutants.

	S1	S2	S3	S4	S5	S6
S1	—	0·54	0·81	3·21	1·61	2·66
S2		—	0·54	3·58	2·23	2·09
S3			—	3·95	3·35	1·99
S4				—	6·92	6·00
S5					—	5·00
S6						—

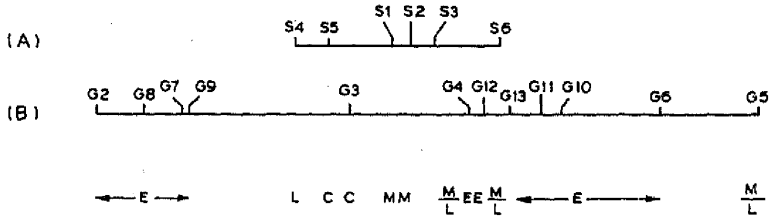

Figure 2. Genetic map of I3. S series of ts mutants (A) and G series of ts mutants (B). E, Early; M, middle; C, continuous; L, late. Recombination frequency between S4 and S5 (6·92) does not match with the above map. However, considering the other gene distances, this map is the best fit.

The recombination frequencies for G mutants were experimentally determined and the mean frequencies calculated by computer programming are presented in table 3. The recombination frequency was highest between G2 and G5. The lowest frequency was observed between G4: G12: Gl3 and G7: G8: G9. This further supports that G4, G12 and G13, and G7, G8 and G9 belong to two individual complementation groups. The genetic map shown in figure 2B is the best fit of the recombination data of G series.

Table 3. Mean recombination frequencies between G mutants.

	G-3	G-4	G-5	G-6	G-7	G-8	G-9	G-10	G-11	G-12	G-13
G-2	7·70	11·72	20·26	16·06	2·48	1·42	2·56	12·70	12·71	11·94	12·54
G-3		3·62	11·02	8·68	5·95	6·72	5·83	6·76	6·45	3·92	4·94
G-4			8·03	5·33	9·26	10·04	9·19	2·92	2·14	0·42	1·29
G-5				2·62	16·24	16·37	16·22	5·23	5·17	7·74	6·62
G-6					13·15	13·40	13·17	2·67	3·32	5·20	4·56
G-7						1·04	0·32	10·81	10·82	10·05	10·64
G-8							1·15	11·65	11·66	10·89	11·49
G-9								10·70	10·71	9·94	10·54
G-10									0·85	2·71	1·86
G-11										2·07	1·33
G-12											1.00

Discussion

Amongst the several mycobacteriophages known D29 and I3 are the only ones whose developmental steps have been studied to some extent (Mizuguchi and Sellers, 1970; Nagaraja and Gopinathan, 1981a,b). Commencement of phage I3 DNA synthesis takes place around 60–70 min after infection, reaching a peak at 150 min. Considering this and its long life cycle, we have designated the development period as early, middle or late, each of 75 min duration. To identify genes involved in these steps we have isolated 18 ts mutants of phage I3.

Temperature shift of the mutants during their growth provides valuable information on the nature of function of the ts-mutant gene (Brewer, 1978). Such experiments with the ts mutants of phage I3 indicated that S1, S2, S3, S6 and G5 are the middle mutants, considering the long growth period of the phage I3. S5 and G3 had mutation in genes functioning continuously throughout the developmental stages till the end of latent period. G2, G4, G6, G7, G8, G9, G10, G11, G12 and G13 carried mutation in early genes, whereas, only S4 was found to be a late mutant.

From this distribution it is likely that the isolated mutants are defective in phage I3 macromolecular biosynthesis except probably S4. In order to determine if any of the mutants isolated were defective in DNA replication, the DNA synthesis was monitored in phage I3 infected *M. smegmatis.* The DNA synthesis in phage infected cells was always lower than the uninfected cells during the entire latent period (Nagaraja and Gopinathan, 1981b). In the study of phage specific DNA synthesis mitomycin C has been widely used to inhibit the host DNA synthesis selectively (Levine, 1961; Eisen *et al.,* 1966; Lindqvist and Sinsheimer, 1967). In *M. smegmatis*-I3 system there was strong inhibition of *M. smegmatis* growth as well as the phage production by mitomycin C. In the infected cells, the incorporation of [^{3}H]-thymine was lower compared to the uninfected cells, even in the presence of the drug (data not shown). [^{32}P]-Labelled DNA was isolated from the I3-infected and mitomycin C treated *M. smegmatis* cells. This DNA hybridized significantly to the *M. smegmatis* DNA also (data not presented) which probably indicated a simultaneous biosynthesis of both the host and phage DNAs. Hence the DNA synthesis of the ts mutants could not be studied at present.

The ts mutations of phage I3 have been ordered by recombination mapping. The recombination frequencies do not show perfect additivity. In the case of phage T4, this was attributed to negative interference and to the fact that recombination frequency values have large coefficients of variation (Edgar and Lielausis, 1964). The genetic map shown in figure 2 for phage I3 is the best fit of the data and probably shows the mutants in correct order, although the map distances are only approximate. Recombination frequency between S4 and S5 (6·92) did not match with the genetic map shown in figure 2. The recombinants produced by crossing S4 and S5 showed two types of plaque morphology, normal and tiny, at 40°C. The recombination frequency was calculated counting both the plaque types. Probably due to this reason the higher frequency was observed. Three point crosses between the S series of (clear plaques) ts mutants and G series of ts mutants (turbid plaques) were done to interlink the two series of ts mutants. From our limited observation (data not included) it appears that the early, middle and continuous mutations are clustered in the I3 genetic map. This is in general agreement with the distribution of genes in the majority of the phages studied so far. However, the

only late gene reported here, *viz.* S4 is positioned in the segment between the early and continuous genes in the genetic map. Such phenomenon has been observed in the genetic map of phage T4 (Lewin, 1977).

The genome length of bacteriophage I3 being 93 kilo base pairs (Sadhu, 1984), the phage is expected to have about 100 genes. Since only 18 mutants were isolated and characterized here, mutants in the other regions of DNA remain to be isolated.

Acknowledgements

We thank Mr. Venkatesh for analysis of some of the recombination results by computer programming. Part of this work has been supported by the University Grants Commission, New Delhi, India.

References

Adams, M. H. (1959) *Bacteriophages,* (New York: Interscience).

Brewer, G. J. (1978) *Mol. Gen. Genet.,* **167,** 65.

Edgar, R. S. and Lielausis, I. (1964) *Genetics,* **49,** 649.

Eisen, H. A., Fuerst, C. R., Siminovitch, L, Thomas, R., Lambert, L., Pereira Da Silva, L. and Jacob, F. (1966) *Virology,* **30,** 224.

Gopinathan, K. P. (1981) *Curr. Sci.,* **50,** 216.

Gopinathan, K. P., Saroja, D., Gadagkar, R. and Ramakrishnan, T. (1978) in *Genetics of Actinomycetales,* (eds E. Freerkscen, I. Tarnok and J. H. Thumin) (New York: G. Fischer, Stuttgart), p. 237.

Karnik, S. S. and Gopinathan, K. P. (1980) *J. Virol.,* **33,** 969.

Levine, M. (1961) *Virology,* **13,** 493.

Lewin, B. (1977) *Gene Expression. Plasmids and Phages* (New York: John Wiley and Sons) Vol. 3, p. 565.

Lindqvist, B. H. and Sinsheimer, R. L. (1967) *J. Mol. Biol.,* **30,** 69.

Mizuguchi, Y. and Sellers, M. I. (1970) in *Host-Virus Relationship in Mycobacterium, Nocardia and Actinomycetes,* (eds S. E. Juhasz and G. Plummer) (Springfield: Thomas, C. C.) p. 65.

Nagaraja, V. (1981) *Interaction between mycobacteriophage* I3 *and its host Mycobacterium smegmatis,* Ph.D. Thesis, Indian Institute of Science, Bangalore, India.

Nagaraja, V. and Gopinathan, K. P. (1980) *Arch. Microbiol.,* **124,** 249.

Nagaraja, V. and Gopinathan, K. P. (1981a) *FEBS Lett.,* **127,** 57.

Nagaraja, V. and Gopinathan, K. P. (1981b) *Mol. Biol. Rep.,* **8,** 11.

Sadhu, C. (1984) *Characterization of mycobacteriophage* I3 *genome and its interaction with the host, Mycobacterium smegmatis,* Ph.D. Thesis, Indian Institute of Science, Bangalore, India.

Sunderraj, C. V. and Ramakrishnan, T. (1970) *Nature* (*London*), **228,** 280.