Identification of an early positive regulatory gene of mycobacteriophage L1

Hirock J. Datta† and Nitai C. Mandal

Department of Biochemistry, Bose Institute, Acharya J. C. Bose Birth Centenary Building, P-1/12, CIT Scheme VII M, Calcutta 700 054, India

Among 14 temperature-sensitive, growth-defective mutants of mycobacteriophage L1 showing a lysis-defective phenotype at 42 °C, six are, in addition, defective in phage DNA synthesis at 42 °C. In the present study, we show that one of the latter six mutants, L1G27ts901, is also defective in the synthesis of both an L1-specific exonuclease (a representative delayed early protein), and of RNA in both the delayed early and late periods but not in the immediate early period. The results of a temperature-shift experiment suggest that the synthesis of L1 exonuclease is regulated by G27 at the level of transcription. Furthermore, the temperature-sensitive defect in delayed early and late RNA synthesis could be largely overcome when the L1G27ts901-infected culture was shifted from 32 to 42 °C at 10 min but not at zero time post-infection. These results suggest that the primary effect of the G27ts901 mutation is to make the phage defective in transcription of delayed early genes at 42 °C, and the defect in late RNA synthesis by this mutant is a secondary effect which is caused by its inability to express regulatory gene products. We conclude that G27 is involved in the positive regulation of expression of the delayed early genes of L1 at the transcriptional level.

Introduction

Expression of early, delayed early (or middle) and late genes of a phage is sequentially activated at the transcriptional level during its programmed growth (Calendar, 1970; Geiduschek, 1991; Herskowitz, 1973). The phage early genes are usually transcribed by host RNA polymerase (Calendar, 1970; Herskowitz, 1973; Rabussay & Geiduschek, 1977), with the exception of coliphage N4 which carries within its virion a phage-encoded RNA polymerase that is carried into the host cell during infection and is used for the transcription of N4 early genes (Falco et al., 1980). The transcription of delayed early genes of phage is dependent on one or more products of early genes (Chamberlin et al., 1970; Chelm et al., 1982; Costanzo & Pero, 1984; Das, 1993; Maitra, 1971; March-Amegadzie & Hinton, 1995; Rabussay, 1983; Zehring & Rothman-Denes, 1983), and transcription of late genes requires certain delayed early gene functions (Geiduschek et al., 1983; Herendeen et al., 1989; Kassavetis & Geiduschek, 1984; Roberts, 1975). A temperate phage, besides having the above temporal regulation, also exhibits negative regulation of transcription from its early promoter(s) by repressor, and the transcription of the gene encoding the repressor requires delayed early gene functions (Herskowitz & Hagen, 1980; Neufing et al., 1996). Thus a mutation in an early positive regulatory gene of a phage may affect transcription of the genes for DNA synthesis, recombination and late regulation (which are all delayed early functions and are required for phage lytic growth). Therefore, a mutation in an early positive regulatory gene of a phage may be identified by screening those lysis-defective mutants which are also defective in DNA synthesis.

L1 is a temperate mycobacteriophage (Doke, 1960). It can lysogenize different mycobacterial species, and in a lysogen its 50 kb double-stranded DNA remains integrated at a specific site in the host chromosome (Snapper et al., 1988). However, the mechanism of temporal regulation of the expression of L1 genes during lytic growth is not known. To investigate this, temperature-sensitive mutations in its 28 different genes essential for lytic growth and in the gene encoding the repressor were isolated, mapped and preliminarily characterized (Chaudhuri et al., 1993). Based on whether those growth-defective L1ts mutants could lyse the host cell at...
42 °C, the authors classified them into two groups, each containing 14 mutants. Of the 14 mutants showing lysis-defective phenotype at 42 °C, six were also temperature sensitive in phage DNA synthesis. This suggested that the expression of lysis (late) genes of L1 is dependent on DNA replication. Also, out of 14 is mutations showing a lysis-defective phenotype at 42 °C, 10 are clustered between coordinates 62 and 83% in the L1 genome (Chaudhuri et al., 1993). In the present study, by screening the above DNA synthesis-defective ts mutants of L1, we have identified a gene, G27, which qualifies as an early positive regulatory gene of this phage.

**Methods**

- **Chemicals.** All fine chemicals were purchased from Sigma. [3H]Uracil and [3H]thymidine were purchased from BRIT, Trombay, Bombay, India.
- **Media and solutions.** The compositions of Middlebrook 7H9 broth, enriched 7H9 broth, 7H9 hard agar, soft agar and phage dilution medium are all given by Chaudhuri et al. (1993). The composition of tryptone broth is given by Chattopadhyay & Mandal (1982) and those of SSC, SM and TE buffers by Sambrook et al. (1989). Enzyme assay buffer: 67 mM Tris–HCl buffer; 3 mM MgCl₂ pH 7.0. Denaturing solution for RNA isolation: 4 M guanidium thiocyanate; 25 mM sodium citrate pH 7.0; 5% sarcosyl; 0.1 M 2-mercaptoethanol.
- **Bacteria and bacteriophage strains.** *Mycobacterium smegmatis mc²6* was obtained from B. Bloom (Albert Einstein College of Medicine, Bronx, NY, USA) and *Escherichia coli* strain 394 (Gal- Lac+ Thi- StrR Su- F-) from M. Lieb (Dept of Microbiology, USC School of Medicine, Los Angeles, CA, USA). *Mycobacteriophage strains L1Δc*, L1ΔG18ts892, L1ΔG19ts892, L1ΔG20ts714, L1ΔG21ts815, L1ΔG26ts282 and L1ΔG27ts901 (the latter six are defective in phage DNA synthesis and host cell lysis at 42 °C) are described by Chaudhuri et al. (1993). The map positions of these ts mutant genes are shown in Fig. 1.
- **Growth of bacteria and bacteriophage L1.** Mycobacterial cultures were routinely grown with shaking at 37 °C in enriched 7H9 broth. Growth was monitored by measuring OD₆₅₀. L1 lysate was prepared by confluent lysis on a plate, and the phage was quantified by plaque assay on the mc²6 host.
- **Preparation of DNA.** *E. coli* strain 394 DNA was labelled with [3H]thymidine (1 µCi/ml, 37 Ci/mmol) as described previously (Mandal et al., 1974); the DNA was isolated by the method of Marmur (1961) and stored in TE buffer at 4 °C.
- **Bacteriophage L1 DNA was prepared by the procedure described by Chaudhuri et al. (1993).**
- **Preparation of L1-infected bacteria for phage-specific RNA and exonuclease synthesis.** *M. smegmatis mc²6* culture was grown at 37 °C in enriched 7H9 broth without Tween 80 and containing 2 mM CaCl₂ to an OD₆₅₀ of around 0.5. The culture was mixed with the desired phage at a m.o.i. of 5 and kept at 30 °C for 20 min without shaking to allow phage adsorption. In all the kinetic studies using phage-infected cells, the end of the above 20 min adsorption period was taken as zero time. No phage-specific expression could be detected during the 20 min of phage adsorption under static conditions (our unpublished results). The culture was then divided into two equal parts: one was allowed to grow at 32 °C, the other at 42 °C, both with shaking.

For labelling RNA, aliquots of these cultures were pulsed at different times with [3H]uracil for 4 min. Uninfected cultures were used as control.

These were then chilled, and the cells were harvested, washed and used for the determination of phage-specific RNA.
- **Isolation of RNA.** The labelled cells were suspended in 0.2 M NaCl containing 0.01 M Tris–HCl pH 7.6, containing 0.001 M 2-mercaptoethanol and sonicated as described above. The extract was centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was used to assay exonuclease by the method of Radding (1964), except that the pH of the assay mixture was 7.0 (optimum pH for the L1-encoded enzyme; P. Mandal & N.C. Mandal, unpublished results). Moderately sonicated [3H]thymidine-labelled *E. coli* DNA (sp. act. ≈ 2 c.p.m. per pmol of nucleotide) was used as substrate. One unit of exonuclease was defined as the amount of enzyme which could release 1 nmol of acid-soluble nucleotide in 30 min under the conditions of the assay.
- **DNA–RNA hybridization.** A solution of L1 DNA at 1·5 µg/ml in 0.1 M NaOH containing 0.5 M NaCl was denatured in a boiling water bath for 15 min and chilled immediately. Nitrocellulose membrane filters (25 mm, 0.45 µm) were loaded with around 7 µg of the denatured DNA as described by Sambrook et al. (1989). The filters were then treated to 42 °C and baked at 80 °C in vacuum for 1 h. The vials were then slowly cooled to room temperature under vacuum, capped tightly and stored.

The method of DNA–RNA hybridization on a Millipore filter was adapted from that described by Denhardt (1966) for DNA–DNA hybridization and carried out under the conditions described by Sambrook et al. (1989). After hybridization for 16 h at 68 °C, the filters were washed two or three times with 2 ¥ SSC and then treated with RNase (20 µg/ml) at room temperature for 30 min followed by washing successively with 2 ¥ SSC containing 0.5% SDS, 2 ¥ SSC containing 0.1% SDS and 0 ¥ 1 SSC containing 0.5% SDS for 2–3 min each. The filters were dried and the radioactive counts determined. All the experiments reported in this paper were repeated two to three times, each showing reproducible results. Results for one set of experiments are presented in each case.
- **Protein determination.** Protein was quantified by the method of Bradford (1976).
Radioactivity measurement. Radioactivity was determined in a Beckman liquid scintillation counter model LS 5000 using aqueous or non-aqueous cocktail as required.

Results

L1 codes for an exonuclease which is expressed during the delayed early period of its post-infection growth

The genes coding for exonuclease of certain DNA phages are members of the delayed early class, and their expression is regulated by early positive regulatory genes (Carlson et al., 1994; Luzzati, 1970). It was observed that after infection of mc<sup>6</sup> by L1, a DNA hydrolysing activity was induced which could use sonicated DNA as a much better substrate than unsonicated, polymerized DNA (results not shown). Preliminary studies with partially purified enzyme indicated that this L1-induced DNase was an exonuclease (P. Mandal & N. C. Mandal, unpublished results). The results shown in Fig. 2 reveal that this exonuclease activity could be detected as early as 10 min after L1 infection, and its level increased very sharply between 10 and 70 min. There was no significant increase of this enzyme activity in uninfected bacteria grown under identical conditions. Within a total latent period of 110 min for L1 (Chaudhuri et al., 1993), 10 min after infection may be considered as the starting point of the period of expression of delayed early genes of this phage. These results indicate that the <i>exo</i> gene of L1 belongs to the delayed early class.

Temperature-sensitive DNA synthesis-defective mutant L1G27<sup>ts</sup>901 is also defective in phage exonuclease synthesis at 42 °C

The growth-defective phage mutants, which cannot lyse the host cell under non-permissive conditions, may include those which are defective in early or late regulatory genes or in lysin genes. A phage which has mutation in the gene involved in its early regulation would be phenotypically defective in expression of delayed early genes, including those involved in DNA synthesis, recombination and late regulation (Calendar, 1970; Dove, 1966; Geiduschek, 1991). The results presented in Fig. 2 show that out of the six different DNA synthesis-defective L1Gs mutants, L1G27<sup>ts</sup>901 was defective in exonuclease synthesis at 42 °C (curve 5) but not at 32 °C (curve 4). Synthesis of this enzyme by wild-type L1 (curves 2 and 3), as well as by five other DNA synthesis-defective mutants, L1G18<sup>ts</sup>892, L1G19<sup>ts</sup>892, L1G20<sup>ts</sup>714, L1G21<sup>ts</sup>815, and L1G26<sup>ts</sup>282 (data not shown), was not affected at 42 °C.

There are two possibilities which could explain the low level of L1-specific exonuclease activity in the L1G27<sup>ts</sup>901-infected bacteria grown at 42 °C: the G27 gene of L1 may either be the structural gene for exonuclease itself, or may code for a different protein which controls the expression of exonuclease. To clarify this, thermal inactivation of exonuclease synthesized by L1<sup>+</sup> and L1G27<sup>ts</sup>901 at 32 °C was studied by preincubating these phage-infected cell extracts for 20 min at temperatures ranging from 37–60 °C and then assaying the enzyme at 37 °C. It was observed that (i) in both cases the enzyme was quite stable up to 45 °C, but was inactivated very rapidly between 45 and 50 °C at almost identical rates, and (ii) at 48 °C, the half-life of the enzyme in the two extracts was about 12 min (data not shown). So the L1G27<sup>ts</sup>901-coded exonuclease could not be differentiated from the L1<sup>+</sup>-coded enzyme in respect of their heat stability. This suggests that the mutant G27<sup>ts</sup>901 gene does not code for exonuclease.

How does G27 of L1 regulate the synthesis of its exonuclease? The synthesis of an enzyme may be regulated at the transcriptional or translational level, or by post-translational modification/processing of the product of translation of the relevant mRNA. To explore this, the effect of temperature shift-up on the synthesis of exonuclease in L1G27<sup>ts</sup>901 mutant-infected bacteria was studied. The rationale was as follows: if G27 controls the transcription of the <i>exo</i> gene, then immediately after the shift of L1G27<sup>ts</sup>901-infected culture from 32 to 42 °C, the <i>is</i> protein would be inactivated, and this would result in an instantaneous halt of further transcription of the <i>exo</i> gene; however, the <i>exo</i> mRNA accumulated during prior growth at 32 °C would continue to be translated at 42 °C following the shift-up. This would increase the enzyme activity relative to the level existing at the time of the shift. However, if the above control is at the translational or post-translational level, then immediately following shift-up the synthesis of functional exonuclease would be stopped due to the inactivation of the <i>is</i> function. Hence there would be no further increase of this enzyme level during post-shift growth at 42 °C over that made at 32 °C up to the time of shift. Fig. 3 shows that the synthesis of exonuclease by the L1G27<sup>ts</sup>901 mutant was completely inhibited when the phage-infected culture was shifted from 32 to 42 °C at zero and 15 min, but was not inhibited when shifted at 60 min and later during post-infection growth. The results further show that when the L1G27<sup>ts</sup>901-infected culture was shifted up at 30 min or later and grown further for a total of 90 min at 42 °C, there was a considerable increase in the enzyme level during post-shift growth at 42 °C compared to that present at the time of shift-up (Fig. 3). These results suggest that the expression of exonuclease is regulated by G27 at the transcriptional level.

L1G27<sup>ts</sup>901 is defective in phage-specific RNA synthesis at 42 °C at both delayed early and late periods

At 42 °C, L1G27<sup>ts</sup>901 is defective in the synthesis of its DNA (Chaudhuri et al., 1993) and exonuclease (Fig. 2). This suggests that G27 may be involved in the positive control of expression of delayed early genes.

To determine whether regulation by G27 occurs at the transcriptional level, as suggested by the results of the temperature-shift experiment described above, the effect of the
Fig. 2. Kinetics of expression of L1-specific exonuclease at 32 and 42 °C. For details see Methods. Curves: 1, uninfected mc26; 2, 1 + wild-type L1 at 32 °C; 3, 2 at 42 °C; 4, 1 + L1G27ts901 at 32 °C; 5, 4 at 42 °C.

Fig. 3. Effect of temperature shift-up on the expression of exonuclease by L1G27ts901 mutant. At indicated times during post-infection growth at 32 °C, two 5 ml aliquots were taken: one was immediately chilled for the assay of exonuclease at the time of shift (curve 2), the other incubated at 42 °C in a water-bath shaker. After a total period of 90 min from the start of post-infection growth, all the shift-up samples (curve 1) were chilled and the exonuclease activity assayed. For further details see Methods. Averages of the data of two independent experiments are presented; variation in the two sets of values is indicated by vertical bars.

Fig. 4. Effect of the G27ts901 mutation on L1-specific RNA synthesis at 42 °C. For procedure, see Methods. For each hybridization with L1 DNA on the filter, the total RNA isolated from 5 ml culture labelled with [3H]uracil (0–5 μCi/ml, 5–6 Ci/mmol) was used. In the 32 °C control, the efficiency of hybridization of [3H]RNA to L1 DNA was 4–5% (4000–5000 c.p.m.) of the total input RNA at all the time periods used for labelling, except at 1–5 min when it was around 2–5% (3000 c.p.m.). For further details, see Methods. Bars: A, wild-type L1; B, L1G27ts901.

G27ts901 mutation on the synthesis of L1-specific RNA at different times during its post-infection growth at 42 °C was studied. In the absence of suitable DNA probes for the delayed early and late genes of L1, the phage-specific RNAs were quantified by their hybridization to L1 DNA instead of using Northern analysis. The results in Fig. 4 indicate that the synthesis of phage-specific RNA in L1G27ts901-infected bacteria during growth at 42 °C relative to that at 32 °C was drastically affected at around 20 min and thereafter but not at around 5 min. In a different experiment, it was observed that at 42 °C this mutant was defective in phage RNA synthesis at 8–12 min (data not shown), the time at which the start of expression of delayed early genes begins. These results suggest that gene G27 of L1 is essential for phage transcription at around 8 min and thereafter but not at 1–5 min during its lytic growth.

G27 is essential within 10 min and is dispensable during the late period of post-infection growth of L1

To determine more precisely the time boundary for the functioning of G27, the effect of a temperature shift-up on phage-specific RNA synthesis by L1G27ts901 mutant was
studies as shown in Fig. 5. These results demonstrate that the shifting of G27s901-infected culture from 32 to 42 °C at 0 min did not affect L1 RNA synthesis at around 4 min, but had a marked effect at 18 and 68 min. When the shift was done at 10 min, RNA synthesis at 18 and 68 min occurred at about 80% of the respective 32 °C control values. When shifted up at 30 min and later, phage RNA synthesis during the subsequent periods occurred nearly at control levels. These results suggest that during lytic growth of L1, the G27 gene function is essential within 10 min, is partially required between 10 and 30 min, and is not required after 30 min.

**Discussion**

In this study, we have shown that gene G27 of mycobacteriophage L1 is essential for transcription initiation between 5 and 10 min after infection. L1 phage carrying the G27s901 mutation is defective at 42 °C in the synthesis of (i) phage-coded exonuclease, a representative delayed early protein (Fig. 2); (ii) phage DNA (Chaudhuri et al., 1993); and (iii) phage-specific RNAs (Fig. 4) in both delayed early and late periods, but not in the immediate early period. Moreover, this gene function is not required after 45 min (Fig. 5). The results of [3H]methionine incorporation into phage-specific proteins in wild-type L1 and L127ts901 mutant-infected cells growing at 32 and 42 °C showed that the synthesis of phage-specific delayed early and late proteins by the latter phage was drastically affected at 42 °C but not at 32 °C (our unpublished results). All these properties of the G27s901 mutation suggest that its primary effect is to modify the transcription of delayed early genes. Its inability to transcribe late genes is a secondary effect which is caused by the inhibition of transcription of late regulatory gene(s) at 42 °C. Once the G27 protein-mediated expression of delayed early genes is achieved, this gene is dispensable during the later period of phage growth. We conclude that the G27 gene of L1 is essential for the transcription of delayed early genes during its lytic growth.

The product of early regulatory gene of a phage that activates transcription of its delayed early genes may be (i) an RNA polymerase (Chamberlin et al., 1970; Maitra, 1971; Zehring & Rothman-Denes, 1983); (ii) a protein that modifies host RNA polymerase to confer on it specificity for transcription from the delayed early promoter(s) (Chelm et al., 1982; Costanzo & Pero, 1984; March-Amegadzie & Hinton, 1995; Rabussay, 1983); or (iii) a protein that associates with host RNA polymerase and anti-terminates transcription initiated from the phage early promoter(s) (Das, 1993). It remains to be seen which of the above functions is associated with the G27 protein of L1. A 0.8 kb segment of L1 DNA which can complement the G27s901 mutation at 42 °C has been cloned in an E. coli–mycobacteria shuttle expression vector (H. J. Datta & N. C. Mandal, unpublished results). DNA sequencing, and analysis of the nucleotide sequences of this gene and the ORF translated from this sequence, may provide information about the mechanism of G27 function.

We thank Dr B. Bloom for mycobacteria and Dr M. Lieb for E. coli strains. This work was supported by an ad hoc Research Fellowship to H. J. Datta and a Research Grant from the Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi. We thank Drs D. J. Chattopadhyay and P. Sinha for critically reading the manuscript.

**References**


Received 2 April 1997; Accepted 18 August 1997