Characterization of Mycobacteriophage 18 and its Unrelatedness to Mycobacteriophages 11, 13 and 15

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(Accepted 3 December 1986)

SUMMARY

Homology among the genomes of mycobacteriophages 11, 13, 15 and 18 has been studied. Based on restriction endonuclease cleavage patterns, dot blot hybridization and Southern blot hybridization analysis, the DNAs of phages 11, 13 and 15 have been shown to be homologous and indistinguishable, but entirely different from phage 18. Unlike the others, the 18 genome does not harbour any single-strand interruptions. The DNA is 43 kb in length with limited cyclic permutations and has a G+C content of 54%. The presence of 5-methylcytosine in 18 DNA was indicated from the restriction patterns of \( MspI \) and \( HpaII \). The number of sites and fragment sizes for several restriction enzymes on 18 DNA has been determined. Phage 18 has a replication cycle of 300 min, with a latent period of 180 min, a rise period of 120 min and a burst size of 100. The viability of phage 18 is significantly reduced by treatment with organic solvents.

INTRODUCTION

Although a large number of phages have been isolated for mycobacteria, their genomes have not been characterized in detail. Only the G+C contents and molecular weights of some of the phage DNAs have been reported (Menezes & Pavilanis, 1969; Soloff et al., 1978; Drapier et al., 1978). Recently we have characterized the genome of phage 13 (Reddy & Gopinathan, 1986a), a temperate mycobacteriophage. Phage 13 DNA is 135 kb long, circularly permuted and rich in G+C content (67%). The genomic DNA of phage 13 harbours random single-strand gaps in both strands (Reddy & Gopinathan, 1986b). For comparative purposes, here we have carried out investigations on the genomes of a few more mycobacteriophages, namely phages 11, 15 and 18, which were isolated in our laboratory (Sunderraj & Ramakrishnan, 1971). Phages 11, 13, 15 and 18 differed from each other in their host range. Further, phage 18 formed clear plaques on all the host strains tested, unlike the others which formed turbid plaques on some of them.

Analysis of restriction endonuclease cleavage patterns (Dean et al., 1976; Chaney et al., 1983), dot blot hybridization (Roberts et al., 1984), and a combination of these two techniques (Cochran & Faulkner, 1983) have been very useful in studying the genomic relatedness among groups of phages and plant and animal viruses. In this paper we report the results of DNA homology studies among mycobacteriophages 11, 13, 15 and 18. Characterization of phage 18 and its genomic DNA are also presented.

METHODS

Growth of bacteria and phages. Mycobacterium smegmatis SN2, mycobacteriophages 11, 13, 15 and 18 and Escherichia coli K-12 strain C600 were from our laboratory stocks. Phages were propagated on M. smegmatis SN2 grown in synthetic medium (Nagaraja & Gopinathan, 1980). E. coli was grown in Luria broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2).

DNA extraction and base composition. Phage purification and extraction of DNAs were carried out as described earlier (Reddy & Gopinathan, 1986a). The bacterial DNAs were extracted as described by Sadhu et al. (1984). Base composition of phage DNAs was determined by thermal melting analysis (Mandel & Marmur, 1968) using a Beckman DU8B spectrophotometer.
One-step growth experiment. A 12 h culture of \textit{M. smegmatis} SN2 (5 × 10^7 cells/ml) was infected with phage I8 (m.o.i. 0.5) in the presence of 1 mM-KCN and incubated at 37 °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 37 °C with shaking. Samples were withdrawn at different times and phage titres were determined using \textit{M. smegmatis} SN2 as the plating bacteria.

Organic solvent treatment of the phage. The phage lysate (7 × 10^{10} p.f.u./ml) was treated with 33\% (v/v) of the organic solvents chloroform, ether, benzene and butanol at 37 °C for 45 min. The samples were centrifuged at 4 °C to obtain phase separation, and the organic phase was discarded. The samples were left in Petri plates allowing evaporation of the remaining traces of solvents and the phage titre was determined.

Restriction endonuclease digestion and hybridization procedures. Restriction digestions were carried out as recommended by the suppliers (New England Biolabs) and the DNA fragments were analysed by electrophoresis on agarose gels in 40 mM-Tris–acetate pH 8.0 containing 2 mM-EDTA (Maniatis \textit{et al.}, 1982). The specific DNA fragments were purified from preparative agarose gels by the electro-elution method (Maniatis \textit{et al.}, 1982). Fragments of DNA or intact phage DNA were labelled \textit{in vitro} with [α-32P]dCTP by nick translation with \textit{E. coli} DNA polymerase I (Rigby \textit{et al.}, 1977). Southern blot hybridization (Southern, 1975) was carried out at 42 °C in the presence of 6 × SSC, 5 × Denhardt's solution, 50\% formamide and 100 µg/ml of calf thymus DNA. After hybridization, the nitrocellulose filters were washed twice in 2 × SSC containing 0.1 % SDS at room temperature and in 0.1 × SSC containing 0.1 % SDS at 68°C, air-dried and exposed to X-ray films at -70°C for autoradiography. For dot blot hybridization, the binding of DNA to nitrocellulose filters was done as described by Parnes \textit{et al.} (1981). The hybridization conditions were the same as in Southern hybridization.

RESULTS AND DISCUSSION

DNA homology among mycobacteriophages I1, I3, I5 and I8

The homology among the genomes of phages I1, I3, I5 and I8 was examined by restriction endonuclease analysis, dot blot hybridization and Southern hybridization analysis of the restriction fragments.

To compare the restriction patterns, phage I1, I3, I5 and I8 DNAs were individually digested by a variety of enzymes and the products were analysed by electrophoresis on agarose gels. Fig. 1 shows one such gel, where the patterns of \textit{BglII}, \textit{PstI}, \textit{HindIII} and \textit{BamHI} digests of I1, I3 and I5 DNAs are shown. The restriction patterns of these three DNAs with the enzymes did not show any differences. The cleavage patterns of I1, I3 and I5 DNA with \textit{XbaI}, \textit{SalI}, \textit{SmaI} and \textit{KpnI} were also identical, and none were cut by \textit{EcoRI} or \textit{HpaI}, indicating a great deal of similarity among these genomes. In contrast, the restriction digestion patterns of phage I8 DNA were entirely different from the others (Fig. 2a). Enzyme \textit{EcoRI} cleaved I8 DNA seven times, but I3 DNA had no sites for this enzyme (Reddy & Gopinathan, 1986a). On the other hand, \textit{SmaI} which cleaved I3 DNA 40 times had no sites on I8 DNA. Differences were also seen in restriction with \textit{PstI}, \textit{KpnI} and \textit{HindIII}. Phage I8 is therefore different from the other mycobacteriophages compared here.

The DNA sequence homology of phages I1, I3, I5 and I8 was also studied by cross-hybridization using a dot blot technique (Fig. 3). It is clear from the figure that I3 DNA hybridized well with I1 and I5 but not with I8 DNA (a), again showing a great extent of similarity among I1, I3 and I5 genomes. When I8 DNA was used as the probe, it hybridized only to I8 DNA and not the others (b) thus showing its distinctness.

Although the restriction endonuclease analysis and dot blot hybridization analysis showed an overall similarity among I1, I3 and I5 DNAs, it is still possible that there are some non-homologous regions among these three genomes which cannot be detected by dot blot hybridization. To clarify this matter, the cross-hybridization analysis was carried out against the restriction fragments of I1, I3 and I5 DNAs. All the \textit{HindIII} and \textit{BamHI} fragments of I1 and I5 DNAs hybridized with [32P]-labelled I3 DNA, showing homology between I1, I3 and I5 genomes. The \textit{HindIII} and \textit{BamHI} fragments of I1, I3 and I5 DNAs did not hybridize with [32P]-labelled I8 DNA, showing a complete absence of any homologous sequences between I8 and the other genomes.
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Characterization of phage 18 and its DNA

Since the genome of 18 was quite distinct from that of phages 11, 13 and 15, further characterization of phage 18 was undertaken. Characterization of 13, serving as a representative for 11, 13 and 15 has been described elsewhere (Reddy & Gopinathan, 1986a,b).

The one-step growth curve of phage 18 on *M. smegmatis* SN2 has been determined. It has a relatively long growth cycle (300 min), comprising an eclipse period of 120 min, a latent period of 180 min, a rise period of 120 min and a burst size of approximately 100. The one-step growth curve of phage 13 was also similar except that its burst size was much lower (Nagaraja & Gopinathan, 1980). Phage 18 showed a loss in titre on treatment with various organic solvents. Maximum inactivation was observed with butanol (10^{-7} survivors) and methanol (10^{-5} survivors). Benzene, chloroform and hexane were much less effective (>10\% survivors). Thus, like other mycobacteriophages (Gope & Gopinathan, 1982; Sellers & Tokunaga, 1970; Soloff et al., 1978), inactivation of 18 by organic solvents suggests the presence of lipids essential for phage viability.

The G + C contents of 11, 13, 15 and 18 DNAs were determined by thermal melting analysis and found to be very similar (67 ± 0.3\%) for the former three phages. At a \(T_m\) value for 18 in
Fig. 2. Restriction endonuclease analysis of phage I8 DNA. (a) Various restriction digests of I8 were analysed on 0.8% agarose gel. Lanes 1 to 6, EcoRI, HindIII, XbaI, BamHI, XhoI and SalI. Lanes 8 to 11, KpnI, BglII, Smal and PstI. Lane 13, undigested I8 DNA. Lanes 7 and 12 contain λ DNA Smal and EcoRI M, markers. (b) HpaII (lane 2) MspI (lane 3) digests of I8 and a HpaII (lane 1) digest of φX174 DNA were subjected to electrophoresis on 1.8% agarose gel.

Fig. 3. Cross-hybridization among I1, I3, I5 and I8 DNAs by dot blot hybridization. DNA samples (10 μg each) were denatured with 1 M-NaOH and neutralized with a solution containing 0.5 M-Tris–HCl pH 8.0, 1 M-HCl, 1 M-NaCl and 0.3 M-sodium citrate and spotted on nitrocellulose filters. The filters were washed in 6 × SSC, baked and used for hybridization (conditions given in Methods). The probes used for hybridization were 2 μg each of (a) I3 and (b) I8 DNA 32P-labelled in vitro. The DNAs in different spots were E. coli (used as a control); 2, I3; 3, I1; 4, I5; 5, I8.

0.1 × SSC of 76.2°C, the G+C content was calculated to be 54% (Fig. 4a). The transition width, defined as the temperature span from 17 to 81% of hyperchromaticity of the profile, was 3.2°C for I8 DNA; that is, much sharper compared to phage λ DNA (Yabuki et al., 1971), and comparable to I3 DNA (Reddy & Gopinathan, 1986a), indicating a random base distribution. The melting of I8 DNA was also monitored at higher resolution (0.2°C intervals) and the first
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Fig. 4. Thermal melting analysis of I8 DNA. The thermal denaturation of I8 DNA (1.5 A260/ml of DNA in 0.1 x SSC containing 0.2 mM-EDTA, pH 7.0) was monitored at (a), 1.0 °C and (b), 0.20 °C intervals in a Beckman DU8B spectrophotometer fitted with a programmable heating cuvette system. The stabilization time between each temperature rise was 2 min. Using E. coli DNA as the standard, the G+C content of I8 DNA was calculated from the expression \( \Delta G + C = \Delta T_m \times 2.44 \) (Mandel & Marmur, 1968).

dervative plot of the melting data is shown in Fig. 4(b). The number of subtransitions was less in the I8 DNA profile compared to \( \lambda \) DNA (Yen & Blake, 1980). This provided further support for the random distribution of bases in I8 DNA.

In order to check for the presence of methylated bases in I8 DNA, restriction of the DNA with the isoschizomeric enzymes MspI and HpaII was attempted and the results are presented in Fig. 2(b). MspI digested I8 DNA more extensively than did HpaII, showing the presence of 5-methylation in the internal deoxycytosine of some of the 5'-CCGG-3' sequences (thus inhibiting HpaII). However, phage 13 DNA, grown on the same host, \( M. \ smegmatis \) SN2, did not have any methylation at this sequence (Reddy & Gopinathan, 1986a). These results demonstrated that the methylase responsible for I8 DNA methylation was a phage-influenced (coded) function, rather than a host-imposed property. The MspI and HpaII patterns of I1 and I5 DNAs were identical to I3 DNA patterns (data not shown), another property common to I1, I3 and I5 DNAs.

The restriction digests of I8 DNA were analysed on different concentration agarose gels to resolve all the fragments and the number of sites for various enzymes has been determined. The enzymes KpnI, EcoRI, BglII, BamHI, SalI and PstI, have two, seven, four, seven, nine and six sites respectively. XbaI and Smal have no sites on this DNA, indicating either the absence of the corresponding recognition sequence or its methylation. The fragment sizes in various restriction digests of I8 DNA are presented in Table 1. By summation of the fragment sizes of individual enzymes (total of molar fragments plus the largest submolar fragment) the molecular size of I8 DNA was estimated to be a minimum of 43 kb. Not all the submolar fragments of the restriction enzymes were considered in estimating the size of the DNA, because they arise due to cyclic permutations (see below). Thus the genome of I8 is much smaller than that of I3 (135 kb), (Reddy & Gopinathan, 1986a). Further, unlike the DNAs of I3 (Reddy & Gopinathan, 1986b), I1 and I5, denaturation of I8 DNA did not result in fragmentation (data not shown), demonstrating the absence of single-strand interruptions in the latter.
Fig. 5. Circularly permuted nature of I8 DNA. EcoRI, SalI and PstI digests of I8 DNA were subjected to electrophoresis on 0.7% agarose gel, transferred to nitrocellulose filters and hybridized with an I8 SalI fragment (11.2 kb submolar) \( ^{32}P \)-labelled \textit{in vitro}. The ethidium bromide-stained gels (left) and autoradiograms (right) for each digest are aligned in parallel.

Several of the restriction digests of I8 DNA showed the presence of a few faint (submolar) bands (Fig. 2a). The presence of such faint bands suggests circularly permuted phage DNA. The submolar fragments having homologous sequences should be located at the ends of the DNA molecule, if they represent a limited number of cyclic permutations of the genome (Ramsay & Ritchie, 1980; Jackson et al., 1978). When I8 DNA was 5' end-labelled, only the submolar bands received the label (data not shown) showing that these fragments were located at the ends of the molecule. The 11.2 kb fragment (submolar) of SalI-digested I8 DNA was purified from a preparative agarose gel, labelled \textit{in vitro} by nick translation and used as the probe to hybridize to the EcoRI, PstI and SalI restriction fragments (Fig. 5). Apart from self-hybridization, the probe also hybridized with one other faint band (7.62 kb) in the SalI digest, as well as with the faint bands (end fragments) present in EcoRI and PstI digests of I8 DNA. These results showed that the end fragments of I8 DNA had homologous sequences (terminally redundant) and were present in submolar concentrations, a general feature of phage DNAs having a limited number of cyclic permutations.

The extent of submolarity of the faint bands provides a measure of the number of genomic phage DNAs that were derived from a single concatemer (Ramsay & Ritchie, 1980). By quantifying the intensities of the fragments of SalI, PstI and BglII digests of I8 DNA with a laser microdensitometer, it was found that two to three molecules of I8 DNA were formed from a concatemer. We believe that as in some other circularly permuted phage DNAs (Ramsay &
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Table 1. Restriction digestion of phage I8 DNA

<table>
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<tr>
<th>Fragment</th>
<th>BglI</th>
<th>EcoRI</th>
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<th>PstI</th>
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<td>9.20</td>
<td>7.62</td>
<td>7.20</td>
</tr>
<tr>
<td>4</td>
<td>6.10</td>
<td>6.70</td>
<td>7.00</td>
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<tr>
<td>5</td>
<td>5.5†</td>
<td>6.20</td>
<td>6.00</td>
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</tr>
<tr>
<td>6</td>
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<td>3.30</td>
<td>2.60</td>
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</tr>
<tr>
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<tr>
<td>Total</td>
<td>51.62</td>
<td>52.90</td>
<td>52.12</td>
<td>53.52</td>
</tr>
</tbody>
</table>

* Faint (submolar) band.
† Doublets.

Ritchie, 1980), phage I8 DNA packaging also proceeds by a headful mechanism initiated from a unique site on concatamers of limited length.

We are grateful to the Department of Science and Technology, Government of India for financial support and the Council of Scientific and Industrial Research, New Delhi for providing a research fellowship to A.B.R.

REFERENCES


*(Received 17 July 1986)*