Structure and Physical Map of *Rhodopseudomonas sphaeroides* Bacteriophage RS1 DNA

**TIMOTHY J. DONOHUE, JOANNE CHORY,† TERI E. GOLDSAND,‡ STEVEN P. LYNN,§ AND SAMUEL KAPLAN*‡

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received 10 January 1985/Accepted 27 March 1985

We analyzed, by restriction endonuclease mapping and electron microscopy, the genome of the lytic *Rhodopseudomonas sphaeroides*-specific bacteriophage RS1 and characterized it as a linear molecule of approximately 60 to 65 kilobases. When the DNA from purified phage particles was examined by several independent methods, considerable size heterogeneity was apparent in the RS1 DNA. This size heterogeneity was concluded to be of biological origin, was independent of the specific host strain used to propagate virus, and was not due to the presence of host DNA within or nonspecifically associated with purified virions. In addition, treatment of RS1 DNA with either BAL 31 nuclease or DNA polymerase I Klenow fragment revealed that several distinct regions exist within the viral chromosome which contain free 3' hydroxyl groups. A restriction endonuclease map of the RS1 genome was constructed by using the restriction endonucleases EcoRI, *ClaI*, KpnI, BamHI, MluI, Smal, and BclI; thereby allowing the positioning of some 40 restriction sites within the viral genome. The results are discussed in terms of the significance and the possible biological origin of the unique features discovered within the phage RS1 DNA.

Members of the family Rhodospirillaceae represent a group of gram-negative facultatively photosynthetic bacteria uniquely suited to studies of the environmental regulation of gene expression due to the ability of these organisms to grow under a variety of defined physiological conditions (21). The DNA of these bacteria is characterized by a relatively high mole percent guanine plus cytosine content (approximately 65 to 70% [22]), and differences exist, at least in the transcriptional regulation of gene expression, between these and other gram-negative bacteria such as *Escherichia coli* (4). Therefore, elucidating the factors responsible for the differential expression of specific groups of physiologically controlled genes in these organisms has significance not only for the photosynthetic bacteria themselves but also as a model for other organisms such as *Caulobacter* spp. (23), *Myxococcus* spp. (27), and members of the genus *Streptomyces* (2), which have similar DNA base compositions.

The development of a cell-free transcription-translation system from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* has shown that DNA from the *R. sphaeroides*-specific phage RS1 is expressed with fidelity by these extracts but that a similar cell-free system from *E. coli* does not express RS1 DNA (4). We have continued our studies with the *R. sphaeroides* lytic bacteriophage RS1 to develop this as a model system with which to study the factors controlling the temporal regulation of gene expression during phage infection. Although relatively little is known of the molecular events surrounding infection by RS1 (1), early and late phage gene products have been identified during RS1 infection (4). Further, the gene products synthesized in vitro by using an *R. sphaeroides* cell-free transcription-translation system derived from uninfected cells coincide with those present early in phage infection as judged by two-dimensional electrophoresis (T. J. Donohue, J. Chory, and S. Kaplan, unpublished observations).

In an attempt to identify specific regions of the RS1 genome which code for temporally regulated gene products, we have analyzed the phage RS1 DNA and constructed a restriction endonuclease map of the viral chromosome. Both restriction endonuclease analysis and electron microscopy indicated that the RS1 genome is a linear duplex molecule of approximately 60 to 65 kilobases (kb). During the course of this analysis we also observed that there was considerable complexity within the DNA molecules packaged into virions during RS1 infection. Our studies have indicated that the DNA isolated from highly purified RS1 phage particles exists as a heterogenous population of differently sized molecules which has regions containing single-stranded nicks or gaps nonrandomly located throughout the genome. To our knowledge, this is the first report of such a phenomenon within wild-type bacteriophage DNA.

**MATERIALS AND METHODS**

Growth of bacteria and bacteriophage. Table 1 lists the bacterial and phage strains used in this study. *R. sphaeroides* strains were grown aerobically in Sistrom minimal medium (4), either on a Gyrotory shaker or by sparging a liquid culture with a mixture of 25% O2, 74% N2, and 1% CO2. When necessary, adenine was included in the medium at a final concentration of 50 µg/ml for growth of *R. sphaeroides* 7001.

Phage titers were estimated by plating virus under aerobic conditions with soft agar (0.7%) overlays of media containing (per liter) 5 g of yeast extract, 3 g of peptone, 0.1 g of NaCl, 0.6 g of MgSO4·7H2O, 0.22 g of CaCl2·2H2O (final pH 7.0). Approximately 5 × 107 CFU of *R. sphaeroides* was used per plate, and phage were allowed 15 to 20 min for absorption before the addition of molten top agar for plating. Liquid lysates were prepared essentially as described by Abeliiovich and Kaplan (1) except that the initial multiplicity of infection was 0.1 to 0.2 and each liter of phage-infected (12 to 16 h postinfection) cells were treated with 10 ml of...
TABLE 1. Bacterial strains and bacteriophage

<table>
<thead>
<tr>
<th>Bacterium or phage</th>
<th>Relevant genotype or phenotype</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. sphaeroides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4.1</td>
<td>Wild type</td>
<td>W. R. Sistrom</td>
</tr>
<tr>
<td>Y</td>
<td>Wild type</td>
<td>H. Gest</td>
</tr>
<tr>
<td>CU-1</td>
<td>Wild type</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>630</td>
<td>Wild type</td>
<td>J. Pemberton</td>
</tr>
<tr>
<td>7001</td>
<td>Sm', ade' res',</td>
<td>J. Pemberton</td>
</tr>
<tr>
<td></td>
<td>B800-850 derived</td>
<td></td>
</tr>
<tr>
<td></td>
<td>from strain 630</td>
<td></td>
</tr>
<tr>
<td>Bacteriophage RS1</td>
<td>Wild type</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>E. coli MO</td>
<td>F', isogenic with Hfr</td>
<td>J. Gardner (9)</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Bacteriophage λcI857</td>
<td>Temperature-sensitive</td>
<td>J. Gardner (29)</td>
</tr>
<tr>
<td></td>
<td>cl gene</td>
<td></td>
</tr>
</tbody>
</table>

chloroform before the removal of cell debris by centrifugation at 10,000 × g for 10 min. High-titer bacteriophage RS1 stocks were prepared either by ultracentrifugation (1) or by precipitation of phage from liquid lysates with 10% polyethylene glycol 6000 and 0.5 M NaCl (15). The pelleted phage particles were suspended in a measured minimal volume of SM buffer (15), solid CsCl was added to 0.8 g/ml of phage suspension, and the phage were purified by flotation to their apparent density of 1.50 g/ml (1) via two successive equilibrium CsCl gradients (45,000 rpm for 20 h in a Beckman 50 Ti rotor). CsCl was removed by dialysis at 4°C against two 2-liter changes of 10 mM Tris (pH 8.0)–10 mM MgSO4.

Bacteriophage λcI857 was propagated on E. coli MO in either liquid lysates or soft agar overlays with a modified LB medium (15) supplemented with 10 mM MgSO4. High-titer liquid lysates were prepared, and phage particles were purified from polyethylene glycol precipitates by successive equilibrium CsCl gradients, followed by dialysis as described above.

DNA isolation. Bacteriophage DNA was routinely isolated from CsCl-purified phage preparations by phenol extractions (15). The phage DNA was then dialyzed against three 2-liter changes of 10 mM Tris (pH 8.0)–10 mM NaCl–0.1 mM EDTA. In some instances, dialyzed, CsCl-purified phage preparations were treated with a mixture of pancreatic DNase and RNase A (final concentrations, 5 and 1 μg/ml, respectively) at 37°C for 30 min before DNA isolation. Where indicated in the text, phage DNA was isolated by treating CsCl-purified phage with either a mixture of proteinase K-sodium dodecyl sulfate (SDS) (15) or SDS-EDTA (6).

Bulk R. sphaeroides DNA was prepared as previously described (18). DNA concentrations were estimated by absorbance measurements at 260 nm, assuming that 1 U of optical density at 260 nm is equivalent to 50 μg of duplex DNA (6).

Analysis of DNA preparations. DNA molecules were routinely separated by electrophoresis in either 0.6 or 1.0% agarose gels (6). Treatment of DNA samples with restriction endonucleases was performed as described in the specifications of the manufacturers. The sizes of RS1 restriction fragments were estimated by comparison to λcI857 DNA standards digested with EcoRI, HindIII, or EcoRI and HindIII (28). By convention, the RS1 restriction fragments have been designated alphabetically by size, with A being the largest fragment. When necessary, the size of smaller RS1 restriction fragments (<2.0 kb) was estimated on 5% polyacrylamide gels with Rsal, Hinfl, or Alul digests of pBR322 as standards (28).

Labeling of phage DNA preparations (1 μg of DNA per assay) was accomplished by incubation with DNA polymerase I Klenow fragment (ca. 1 U) at 37°C for 15 min in the presence of a mixture containing 10 mM Tris hydrochloride (pH 7.9), 10 mM MgCl2, 50 mM NaCl, 1 mM β-mercaptoethanol, 5 μCi of either [α-32P]dATP or [α-32P]dCTP, and 1 mM each of the appropriate unlabelled deoxyribonucleoside triphosphates. Samples previously digested with EcoRI were phenol extracted, ethanol precipitated, and heated at 70°C for 5 min before labeling to denature any cohesive ends. The labeling reaction was terminated by phenol extraction, and the labeled DNA was purified by ethanol precipitation before analysis on a 1% agarose gel. Autoradiography of the intact agarose gel was accomplished at −76°C with the use of an intensifying screen.

Phage DNA samples were treated with BAL 31 nuclease in the buffer recommended by the manufacturer at a DNA concentration of 0.1 μg/μl and 1.2 U of BAL 31 nuclease per μl. At the times indicated, 20-μl samples (2 μg of DNA) were removed from the reaction mixture, and the assay was terminated by the addition of EDTA and SDS to final concentrations of 10 mM and 0.5%, respectively. EcoRI-digested phage DNA samples were treated identically, except that the EcoRI was inactivated by phenol extraction and the DNA was concentrated by ethanol precipitation before BAL 31 nuclease treatment.

Radioactively labeled DNA probes were prepared with [α-32P]dCTP to a specific activity of approximately 108 cpm/μg by using a nick-translation kit supplied by Bethesda Research Laboratories, Gaithersburg, Md., and were purified through a column of Sephadex G-50 fine before use (15). For construction of the phage RS1 restriction map, individual restriction fragments, purified either by extraction from low-melting point agarose (31) or by electrophoresis onto DEAE paper (8), were hybridized to various restriction digests of phage RS1 DNA which had been electrophoretically separated on 1% agarose gels and electrophoretically transferred to Gene Screen (New England Nuclear Corp., Boston, Mass.). Hybridization to and washing of these filters was performed as described in method I of the instructions of the manufacturers. Hybridization of nick-translated phage DNA to R. sphaeroides 2.4.1 bulk DNA or itself, discussed in the legend to Fig. 4, was accomplished by using DNA transferred via capillary action to Gene Screen. For these experiments, the hybridization and washing conditions were as described in method III of the instructions of the manufacturers.

Electron microscopy. RS1 DNA was prepared either by an aqueous spreading technique (7) or by a modified formamide spreading technique (5) with essentially identical results. The results presented are from DNA samples prepared for microscopy by the aqueous spreading method. Cytochrome c was used as a basic protein film for both procedures, and Parlodion grids containing phage RS1 DNA were shadowed with platinum-palladium and visualized by using a Phillips 300 electron microscope. Contour length measurements of DNA preparations were obtained by using a Numonics Graphics Calculator (Landsdale, Pa.) interfaced to a Wang programmable calculator. The sizes of the phage RS1 DNA...
molecules were estimated by comparison to the contour length of $\phi$X174 relaxed replicative form (form II) DNA, which was assumed to be 5,386 base pairs (15).

Materials. All restriction endonucleases and nucleic acid-modifying enzymes were obtained from Bethesda Research Laboratories or from New England Biolabs, Beverly, Mass., and were used as described in the specifications of the manufacturers. DNA polymerase I Klenow fragment was the product of Boehringer Mannheim Chemicals, Indianapolis, Ind., and form II $\phi$X174 DNA was from New England Biolabs. DEAE-cellulose paper was the product of Whatman, Clifton, N.J. [\(\alpha\)-\(^{32}P\)]dATP and [\(\alpha\)-\(^{32}P\)]dCTP (800 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. With the exception of phenol, which was distilled before use, all other reagents used were of reagent grade purity and were used without further purification.

RESULTS

Restriction analysis of RS1 DNA. As an initial step in characterizing the phage RS1 DNA, the susceptibility of the phage DNA to a variety of restriction endonucleases was tested. Of the restriction endonucleases tested with six base recognition sequences, the enzymes ApaI, BglII, Sall, SphI, PstI, and XhoI did not cleave the RS1 DNA. The restriction digest patterns of RS1 DNA obtained with 10 of the restriction endonucleases are shown in Fig. 1 (lanes 2 through 11) along with a sample of undigested RS1 DNA (lane 12) and HindIII-digested $\lambda$X1857 DNA size standards (lane 1). With the exception of the RS1 HindIII digest (lane 9), the patterns of restriction fragments produced by the restriction endonucleases shown was relatively simple.

The sizes of the individual restriction fragments shown in Table 2 were determined either by direct comparison of their electrophoretic mobilities with those of restriction fragments of known size (see above) or, in the case of the larger (>15-kb) RS1 restriction fragments, by analysis of the restriction fragments produced when the RS1 DNA was sequentially treated with more than one restriction enzyme (see restriction map in Fig. 7). The average size of the RS1 genome calculated from the restriction digests summarized in Table 2 is 65.8 ± 1.8 kb. For reasons discussed below, these estimates were obtained by considering only those restriction fragments which have ethidium bromide fluorescence indicative of their presence in relatively high amounts and not those restriction fragments which would appear to represent incomplete RS1 DNA digestion products (such as the faint high-molecular-weight band above restriction fragment A in the MluI digest of RS1 DNA in Fig. 1, lane 5). Despite the fact that the relative fluorescence of several individual restriction fragments derived from RS1 DNA (e.g., EcoRI restriction fragment E) might suggest the presence of more than one copy of this restriction fragment per RS1 genome, we have (for reasons summarized below) only considered these as representing a single restriction fragment in our estimates of the RS1 genome size.

Close examination of the sample of undigested phage RS1 DNA in lane 12 of Fig. 1 revealed considerable UV fluorescence material present as discrete, lower-molecular-weight DNA molecules separable on a 1% agarose gel. Size heterogeneity of this nature has been present in every preparation of phage RS1 DNA we have isolated with no significant variation in the extent of the heterogeneity from one phage lysate to the next (data not shown).

Considerable background fluorescence, similar to that noted in the undigested RS1 DNA sample, is evident as discretely sized DNA molecules in several of the digested RS1 DNA samples (most notably lanes 5, 6, 8, 10, and 11 in Fig. 1). These samples represent those in which the restriction endonuclease used has few cleavage sites within the phage RS1 DNA. The UV fluorescent intensity of the molecules in these samples plus several others (Fig. 1, lanes 3, 4, and 7) could be due to incomplete digestion of the phage RS1 DNA molecules. However, given the precise nature of the size heterogeneity found within the undigested phage RS1 DNA (to be discussed in more detail below), it is probable that these molecules do not represent partial digestions of full-sized phage RS1 DNA: rather, we believe that those molecules with less fluorescence are the result of the action of the restriction endonuclease in question on the less-than-full-sized RS1 DNA molecules present in various

---

**TABLE 2.** Restriction digests of phage RS1 DNA

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Size (kb) of fragment resulting from digestion by the following restriction enzyme:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EcoRI</td>
</tr>
<tr>
<td>A</td>
<td>20.0</td>
</tr>
<tr>
<td>B</td>
<td>15.5</td>
</tr>
<tr>
<td>C</td>
<td>9.2</td>
</tr>
<tr>
<td>D</td>
<td>7.0</td>
</tr>
<tr>
<td>E</td>
<td>4.4</td>
</tr>
<tr>
<td>F</td>
<td>3.8</td>
</tr>
<tr>
<td>G</td>
<td>2.0</td>
</tr>
<tr>
<td>H</td>
<td>1.8</td>
</tr>
<tr>
<td>I</td>
<td>1.5</td>
</tr>
<tr>
<td>J</td>
<td>1.3</td>
</tr>
<tr>
<td>K</td>
<td>0.4</td>
</tr>
<tr>
<td>L</td>
<td>0.3</td>
</tr>
<tr>
<td>M</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* The average size of the RS1 genome from the analysis with the seven restriction enzymes shown is 65.8 ± 1.8 kb.
copy numbers within the Rs1 DNA population. These observations suggest that the lower-molecular-weight DNA in the undigested Rs1 DNA sample contains a representative or random distribution of the restriction sites contained within a full-length Rs1 genome (see below).

To ascertain whether the size heterogeneity within the Rs1 DNA preparation is of biological origin, DNA from Rs1 and λc1857 was isolated separately and in combination by the procedures described above. These samples were subsequently analyzed on 0.6% agarose gels to more accurately visualize the extent of the size heterogeneity within the DNA preparations. The size heterogeneity within the phage Rs1 DNA is pronounced, with a series of discrete-sized DNA molecules as small as 20 kb detectable by UV fluorescence alone (Fig. 2). The size heterogeneity within the phage Rs1 DNA was independent of the DNA extraction protocol used (phenol extraction versus proteinase K-SDS versus SDS-EDTA; Fig. 2), and no size heterogeneity was observed when λc1857 DNA was prepared by the same extraction protocols. Control experiments showed that colysis of λc1857 and Rs1 phage particles did not affect the Rs1 DNA, nor did it result in degradation of the λc1857 DNA (data not shown). Thus, it would appear that the size heterogeneity within the Rs1 DNA was not due to degradation of or damage to the Rs1 DNA during phage DNA extraction.

An alternative explanation is that there is nucleic acid absorbed to the Rs1 virion which remains associated with the phage particles during purification. When intact λc1857 or Rs1 virions were treated with DNase and RNase before extraction of the phage DNA with phenol, no significant change in the electrophoretic mobility of either DNA sample was observed (Fig. 3). The amount of DNase used was sufficient to digest 3 μg of purified λc1857 DNA during a 30-min incubation period (Fig. 3, lane 3). Additional experiments showed that the nuclease treatment had no detectable effect on the EcoRI digestion of either λc1857 or Rs1 DNA derived from nuclease-treated virions (data not shown). Finally, these results indicate that the Rs1 capsid is not leaky to external nucleases. Therefore, we have concluded that the size heterogeneity of the Rs1 DNA is of biological origin.

A sample of Rs1 DNA was nick translated and hybridized to intact Rs1 DNA, EcoRI-digested Rs1 DNA, and bulk R. sphaeroides 2.4.1 (host) DNA digested with different restriction endonucleases to ascertain whether the source of the heterogeneity within the Rs1 DNA was from contaminating chromosomal DNA packaged into virions during infection. The results (Fig. 4) show that phage Rs1 DNA contained no detectable sequences homologous to host DNA even on the long exposure of the Southern blot shown. However, the undigested Rs1 DNA contained discrete DNA fragments as small as 2.6 kb which were not visible as UV fluorescent material but which were detectable on this or longer exposures of the Southern blot (Fig. 4B, lane 8). These smaller DNA molecules would appear to be present in low copy number by this type of analysis. However, conclusions on the relative abundance of these or any other species of DNA within the Rs1 DNA population cannot be made from this experiment alone (see below). Control experiments have shown that hybridization patterns identical to those presented in Fig. 4 were obtained when nick-translated Rs1 DNA was digested with different restriction enzymes. This observation has led to the conclusion that the size heterogeneity in the Rs1 DNA population is not due to contamination with chromosomal DNA.

A more detailed analysis of the size heterogeneity of the Rs1 DNA population was performed by restriction enzyme digestion and Southern blot hybridization. A series of discrete-sized DNA molecules of λc1857 DNA was resolved by agarose gel electrophoresis and Southern blot hybridization with nick-translated Rs1 DNA (Fig. 5). The results showed that the size heterogeneity in the Rs1 DNA population is due to the presence of a series of discrete-sized DNA molecules of λc1857 DNA. The relative proportion of these discrete-sized DNA molecules is determined by the size of the lambda DNA molecule.

The results shown in Fig. 5 indicate that the size heterogeneity in the Rs1 DNA population is due to the presence of a series of discrete-sized DNA molecules of λc1857 DNA. The relative proportion of these discrete-sized DNA molecules is determined by the size of the lambda DNA molecule.
DNA (prepared via phenol extraction) was hybridized to RS1 DNA prepared by the other isolation techniques described in the legend to Fig. 2 (data not shown). When a similar analysis was performed with nick-translated λ1857 DNA, no degraded λ1857 DNA was detectable when λ1857 virions were lysed alone or concomitantly with RS1 virions (data not shown).

**RS1 host specificity.** In the initial description of *R. sphaeroides* bacteriophage RS1, Abeliovich and Kaplan (1) reported that phage propagated in strain 2.4.1 [hereafter referred to as RS1 (2.4.1)] were able to infect wild-type *R. sphaeroides* L but were unable to plaque on strains such as M29-5. We have extended the analysis of RS1 host range specificity, and Table 3 lists the relative titers of various bacteriophage preparations on several *R. sphaeroides* strains.

The relative plating efficiency of RS1 (2.4.1) phage particles on wild-type strains Y and CU-1 were consistent with restriction of the RS1 (2.4.1) DNA by these strains. When high-titer phage stocks from *R. sphaeroides* Y and CU-1 were used, they plaqued with an efficiency of approximately 1 on all of the strains tested with the exception of *R. sphaeroides* 630 (Table 3). *R. sphaeroides* 630 contains both a restriction endonuclease (RsrI) which is an isoschizomer of EcoRI (26) and a recently described restriction endonuclease (RsrII) with a novel heptanucleotide recognition sequence (19). *R. sphaeroides* 7001 is a restrictionless derivative of strain 630 (J. Pemberton, personal communication) which preliminary experiments show is severely depleted if not totally devoid of RsrI restriction endonuclease activity in crude lysates (S. P. Tai and S. Kaplan, unpublished data). Infection of strain 7001 with RS1 (2.4.1) resulted in a plating efficiency of approximately 1 (Table 3). The results shown in Table 3 would be expected if the inability of RS1 (2.4.1) to plaque on strain 630 were due in part to the presence of the RsrI restriction system. Although the data presented in Table 3 are typical of the response produced by host restriction of bacteriophage infection (11), RS1 DNA isolated from virus propagated on *R. sphaeroides* 7001 appeared identical to that obtained from RS1 (2.4.1) when analyzed on 0.6% agarose gels (data not shown), suggesting that the size heterogeneity within the RS1 DNA described above was independent of the host restriction system (14).

**Electron microscopy of RS1 DNA.** Figure 5 shows an electron micrograph of a preparation of RS1 DNA. In agreement with previously discussed experiments, electron micrographs such as the one shown indicate that there was size heterogeneity within the RS1 DNA. It was also revealed that the RS1 genome was linear, with the size of the largest molecule shown in the micrograph in Fig. 5 being 62.2 kb.

Figure 6 shows a size frequency histogram generated from measuring the contour length of approximately 318 DNA molecules (representing approximately 2,281 kb of RS1 DNA).

### Table 3. Plating efficiencies of various RS1 preparations

<table>
<thead>
<tr>
<th>Bacterial host</th>
<th>Plating efficiency of the following phage preparation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RS1 (2.4.1)</td>
</tr>
<tr>
<td>2.4.1</td>
<td>3 x 10^-6</td>
</tr>
<tr>
<td>Y</td>
<td>3 x 10^-6</td>
</tr>
<tr>
<td>CU-1</td>
<td>2 x 10^-6</td>
</tr>
<tr>
<td>630</td>
<td>ND</td>
</tr>
<tr>
<td>7001</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* The phage stocks used for these experiments had a titer of at least 10^9 PFU/ml.
* ND, Not detectable at a multiplicity of infection of 1 or less. At higher multiplicities of infection, confluent lysis of the bacterial cells occurred.
DNA) wholly contained within electron micrographs similar to that shown in Fig. 5. In general, the approximate sizes of those DNA molecules present at higher frequencies coincided with the sizes of small DNA molecules which gave rise to the discrete hybridization signals in the Southern blot analysis shown in Fig. 4B, lane 8 (see arrows to right of panel). Finally, the average size of the seven largest RS1 DNA molecules (>59 kb) was 61.4 ± 2.5 kb, which agrees well with the value of 65.8 ± 1.8 kb obtained for the RS1 genome size from the restriction endonuclease analysis summarized in Table 2.

The electron microscopic analysis revealed a prevalence of relatively small molecules (<5.0 kb) within the RS1 DNA sample. It should be noted that this analysis only includes molecules wholly contained within randomly selected fields at magnifications high enough to yield reliable contour length measurements of the φX174 replicative form DNA. This fact introduces a bias in the analysis of the DNA molecules present within a given population of RS1 DNA molecules since it is more probable at this magnification to have smaller DNA molecules wholly contained within randomly selected fields than to have full-length RS1 DNA molecules. In addition, despite the fact that the seven full-length RS1 DNA molecules measured represented only 2% of the total DNA molecules analyzed, they accounted for 19% of the total length of RS1 DNA analyzed by this technique.

Physical map of RS1 DNA. Figure 7 shows a restriction endonuclease map of the RS1 genome for eight restriction endonucleases with six base recognition sequences. Sequential restriction endonuclease digestions were used to place individual restriction sites for one restriction enzyme within a given restriction fragment until most of the larger restriction fragments were mapped relative to each other. In addition, nick-translated RS1 EcoRI restriction fragments were hybridized to Southern blots of RS1 DNA digested with the restriction endonucleases shown in Fig. 7 to confirm the order and presence of restriction sites internal to the remaining restriction fragments.

The identity of the ends of the RS1 genome was confirmed by treating EcoRI-digested RS1 DNA with T4 DNA ligase at dilute DNA concentrations which favored circularization of the individual restriction fragments upon themselves (15). Analysis of the ligated restriction fragments relative to control EcoRI digestions of RS1 DNA on 1% agarose gels showed that only EcoRI restriction fragments B and D did not exhibit an altered mobility after ligase treatment (data not shown). These results indicated that the EcoRI B and D restriction fragments had only one EcoRI end and as such were not able to be ligated under these conditions. Attempts to further characterize the EcoRI B and D restriction frag-
ANALYSIS OF RS1 DNA

FIG. 7. Restriction endonuclease map of the RS1 genome. The top line indicates distance along the RS1 genome as a fraction of the complete viral chromosome (approximately 65.6 kb). The techniques used for construction of this map are detailed in the text. The experiments performed to date have not allowed precise positioning of the Clal-J and Smal-L and -M restriction fragments (Table 2) on this map.

ments as the ends of the RS1 genome by more traditional techniques such as end labeling with T4 polynucleotide kinase (data not shown) or treatment with the nuclease BAL 31 (see below) were unsuccessful. However, the results of these experiments (presented below) were enlightening for their analysis of the physical state of the RS1 genome.

Figure 8 shows the results of an experiment in which undigested and EcoRI-digested \( \lambda c1857 \) or RS1 DNA was treated with DNA polymerase I Klenow fragment in the presence of either [\( \alpha ^{-32}P \)]dATP or [\( \alpha ^{-32}P \)]dCTP and the other three nucleotide triphosphates to polymerize the DNA ends flush. The cohesive ends of \( \lambda c1857 \) DNA contain 10 guanine and 2 thymine residues (30) which can serve as 3' primers for DNA polymerase I Klenow fragment, so that as expected, the intact \( \lambda c1857 \) DNA was labeled to a greater extent with [\( \alpha ^{-32}P \)]dCTP than with [\( \alpha ^{-32}P \)]dATP (Fig. 8B, cf. lanes 1 and 2). Also as expected, when the \( \lambda c1857 \) EcoRI restriction fragments (cohesive ends, 5' AATT) were polymerized flush
in the presence of [\(\alpha^{-32}\mathrm{P}\)]dATP, all of the restriction fragments were labeled (Fig. 8B, lane 3), whereas the only \(\lambda I857\) EcoRI restriction fragments which were labeled with [\(\alpha^{-32}\mathrm{P}\)]dCTP (Fig. 8B, lane 4) were those which represent the left and right arms of the \(\lambda I857\) genome (6). When the identical experiment was performed with RS1 DNA, it was found that the undigested DNA incorporated more label with [\(\alpha^{-32}\mathrm{P}\)]dATP than with [\(\alpha^{-32}\mathrm{P}\)]dCTP (Fig. 8D, cf. lanes 1 and 2). Also, the labeling pattern obtained with the undigested RS1 DNA when either \(\alpha\)-labeled deoxynucleoside triphosphate was used mimicked the size heterogeneity within the RS1 DNA visualized by UV fluorescence, suggesting that the majority of these molecules contained regions into which deoxynucleotides could be polymerized with the 5'-to-3' activity of DNA polymerase I Klenow fragment. In addition, when EcoRI-digested RS1 DNA was treated under identical conditions, the RS1 EcoRI restriction fragments A, C, D, and E were labeled with [\(\alpha^{-32}\mathrm{P}\)]dCTP (Fig. 8D, lane 4). Since the cohesive ends produced by EcoRI activity are devoid of guanine residues, the incorporation of [\(\alpha^{-32}\mathrm{P}\)]dCTP into these restriction fragments must be due to the activity of the DNA polymerase I Klenow fragment on 3'-hydroxyl groups internal to these restriction fragments. Although the results obtained precluded the identification of the ends of the RS1 genome by this analysis, our interpretation of the results of this experiment is that the RS1 EcoRI restriction fragments A, C, D, and E contain single-stranded nicks or gaps producing 3' hydroxyl groups. The labeling pattern obtained with \(\lambda I857\) DNA prepared by the identical method suggested that the results obtained for the phage RS1 DNA are of biological significance.

Figure 9 shows the results obtained when BAL 31 nuclease was used in an attempt to identify the ends of the RS1 genome. Because of its exonuclease activity on double-stranded DNA (13), BAL 31 nuclease is commonly used to digest the ends of linear duplex DNA molecules for the purpose of creating blunt-end molecules or for mapping the ends of linear duplex DNA molecules (15). BAL 31 nuclease preparations also contain endonuclease activities specific for single-stranded DNA which digests such DNA across from a nick in a double-stranded DNA molecule (10, 12). The amount of BAL 31 nuclease used in these experiments (1.12 U/\(\mu\)g of DNA) was sufficient to hydrolyze approximately 100 base pairs of duplex DNA per min based on the specifications of the manufacturers. As expected there was very little change in the mobility of the intact \(\lambda I857\) DNA over the 10-min incubation (Fig. 9A), although it was possible to see a slight increase in the amount of lower-molecular-weight UV fluorescent material as a function of time. When EcoRI-digested \(\lambda I857\) DNA was treated under identical conditions, there was a size- and time-dependent degradation of the individual \(\lambda I857\) restriction fragments of the magnitude expected based on the amount of BAL 31 used (Fig. 9C). Similar treatment of an equivalent amount of intact RS1 DNA with the same amount of BAL 31 nuclease resulted in the very rapid disappearance (by 1.0 to 1.5 min) of the higher DNA species visualized by UV fluorescence (Fig. 9B). The kinetics of loss of RS1 DNA, when compared with the kinetics of degradation of the \(\lambda I857\) DNA, were too rapid to be explained by the action of BAL 31 nuclease from single sites at the ends of the linear RS1 DNA molecule. The products of BAL 31 nuclease treatment of RS1 DNA (Fig. 9B) appear to correspond to several of the lower-molecular-weight DNA species present in the untreated RS1 DNA sample, although neither the identity of the products nor a precursor-product relationship has been rigorously demonstrated by the experiments performed. In addition, the fact that the sum of the molecular weights of these early BAL 31 digestion products is considerably larger than the estimated genome size of RS1 DNA suggests that individual DNA molecules within an RS1 DNA population do not all have the same set of BAL 31-sensitive sites. Further experiments are necessary to test both of these hypotheses.

Finally, when EcoRI-digested RS1 DNA was treated with BAL 31 nuclease, the EcoRI restriction fragments A, C, and D were rapidly digested relative to the loss of either similarly sized \(\lambda I857\) EcoRI restriction fragments or the RS1 EcoRI fragment restriction B (Fig. 9D). Thus, the results shown in Fig. 9 precluded the eventual use of BAL 31 nuclease to identify the phage RS1 DNA ends. However, the fact that three of the four RS1 EcoRI restriction fragments which
FIG. 10. Denaturation of λc1857 and RS1 DNA. Lanes 1 and 2 contain 1 μg of EcoRI and HindIII-digested λc1857 molecular weight standards. Lanes 3 through 8 contain 1 μg of RS1 DNA treated as follows: lane 3, room temperature incubation for 1 h; lane 4, same as lane 3 but followed by phenol extraction; lane 5, control (65°C) incubation for 1 h; lane 6, same as lane 5 but followed by phenol extraction; lane 7, room temperature treatment with 50% deionized formamide for 1 h; lane 8, treatment with 50% deionized formamide at 65°C for 1 h. Lanes 9 through 14 contain 1 μg samples of λc1857 DNA treated under the same conditions described above for the RS1 DNA samples shown in lanes 3 through 8, respectively. All treatments of phage DNA samples were performed in a buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, and 0.1 mM EDTA.

were labeled with [α-32P]dCTP in the presence of DNA polymerase I Klenow fragment (Fig. 8) also exhibited hypersensitivity to BAL 31 nuclease digestion (Fig. 9) suggested that the putative single-stranded nicks or gaps within these RS1 EcoRI restriction fragments which serve as priming sites for the DNA polymerase I Klenow fragment provided additional sites for digestion of these restriction fragments by the mixture of nuclease activities present in commercial preparations of BAL 31 nuclease (10, 12, 13).

The experiments shown in Fig. 10 were performed to further analyze and characterize the putative single-stranded nicks or gaps within the RS1 DNA. The data shown indicate that heating of the RS1 DNA (65°C, 1 h, low salt) followed by phenol extraction resulted in a significant alteration of the electrophoretic mobility of the RS1 DNA on 0.6% agarose gels (Fig. 10, lanes 3 through 6). The finding that the relative amount of high-molecular-weight RS1 DNA was diminished by this treatment and resulted in an increase in the amount of low-molecular-weight RS1 DNA visualized by UV fluorescence suggests that this treatment denatures the high-molecular-weight RS1 DNA at the regions which are bounded by the single-stranded nicks or gaps identified in the experiments depicted in Fig. 8 and 9. Control experiments have shown that treatment of the same preparation of RS1 DNA with SDS (0.5%, 65°C, 1 h, low salt) or a combination of SDS and proteinase K under identical conditions did not result in an increased loss in the high-molecular-weight DNA (data not shown) and were as described for lanes 3 and 4 of Fig. 10. We interpret these results to mean that proteins are not involved in stabilizing the RS1 DNA at or near the regions of single-stranded nicks or gaps within the viral chromosome. Finally, the sensitivity of RS1 DNA, relative to that of bacteriophage λ DNA, to formamide treatment (Fig. 10, cf. lanes 7 and 8 with 13 and 14) indicated that the RS1 DNA exhibits hypersensitivity to formamide denaturation. Thus, all the results are consistent with the existence of regions of single-stranded nicks or gaps within both strands of the RS1 genome which are stabilized, presumably, by complementary base-pairing.

DISCUSSION

We have analyzed the genome of the R. sphaeroides lytic bacteriophage RS1 and found it to be a linear molecule of approximately 60 to 65 kb. To our knowledge, although several other phages specific for the photosynthetic bacteria have been identified (see references 16 and 20 for recent reviews), this report represents the first analysis of the DNA from a bacteriophage which infects a member of the family Rhodospirillaceae.

The data presented point to several unusual properties of the RS1 genome. The size heterogeneity within RS1 DNA preparations was not due to the nonspecific association of nucleic acid with purified phage particles, to phage capsids which are permeable to external nucleases, or to the packaging of chromosomal DNA into phage heads during infection. This latter observation is consistent with our inability to use RS1 as a generalized transducing phage for R. sphaeroides (unpublished data). The size heterogeneity of the RS1 DNA

an ability to use
was not due to the method of DNA isolation, since identical results were obtained regardless of the means of phage lysis. Likewise, the isolation of λI857 DNA alone or together with RS1 never resulted in detectable degradation of the λI857 DNA. All the data presented are thus consistent with the hypothesis that the size heterogeneity of the RS1 DNA is of biological origin.

Recent experiments with E. coli bacteriophage T4 have shown that several mutants of T4 package discretely sized DNA fragments of between 2 and 20 kb into phage heads (3). Genetic analysis has implicated specific nucleases in fragmentation of the T4 genome, and some sequence specificity within the fragments has also been identified (3). The biological origin of the size heterogeneity within the packaged RS1 DNA is not known at this time, and the answer to this question possibly awaits the isolation of a battery of RS1 mutants. Questions of sequence specificity within the smaller DNA fragments contained within RS1 virions remain to be analyzed rigorously. The observation that the size heterogeneity within the RS1 DNA was not significantly affected by the bacterial host used suggests that this heterogeneity was not due to a specific host restriction endonuclease system within R. sphaeroides. In addition, results of preliminary experiments in which several of the cloned RS1 EcoRI restriction fragments (C and E through J) were nick translated and hybridized to Southern blots containing undigested RS1 DNA suggest that there is no preference for specific regions of RS1 DNA to be represented in the smaller DNA fragments (data not shown). The pattern of UV fluorescent material obtained with several of the restriction endonuclease used to analyze the RS1 DNA in Fig. 1 also suggests a lack of sequence specificity within the smaller RS1 DNA molecules present in virions.

Other experiments indicate that there are regions which contain either single-stranded nicks or gaps within the RS1 genome. The data obtained with DNA polymerase I Klenow fragment and the susceptibility of individual RS1 EcoRI restriction fragments to BAL 31 nuclease suggest that these regions occur at a minimum within EcoRI restriction fragments A, C, D, and E. These four restriction fragments represent approximately 65% of the RS1 genome. Studies are currently in progress to determine whether these 3' hydroxyl groups are localized within specific regions of these restriction fragments or on one particular strand of the RS1 DNA, as is the case for the gaps found within one strand of E. coli wild-type bacteriophage T5 DNA (17, 24). The data presented in this paper would strongly suggest that the single-stranded nicks or gaps within the RS1 DNA occur at a much higher frequency than those in either the wild type or mutants of T5 with increased frequencies of single-stranded chain interruptions (25). Unfortunately, the spreading techniques used for electron microscopy of the phage RS1 DNA did not allow analysis of the RS1 DNA at this level; however, the results of the formamide treatment of the RS1 DNA might indicate that these single-stranded regions are interspersed in both strands of the RS1 DNA. Although we are not currently in a position to precisely explain the biological source or the nature of either the size heterogeneity or the location of the 3' hydroxyl groups within individual strands of the RS1 DNA, we can make several statements relative to these phenomena. The results of both the BAL 31 nuclease and DNA polymerase I Klenow fragment end-labeling experiments suggested that the RS1 EcoRI restriction fragment B contains few internal 3' hydroxyl groups relative to the level of these groups in the remainder of the RS1 DNA. Thus, it would appear that these single-stranded nicks or gaps are not totally randomly dispersed throughout the RS1 genome. Also, the apparent periodicity in discretely sized classes of smaller RS1 DNA molecules visualized by electron microscopy, Southern blot analysis, and UV fluorescence of undigested RS1 DNA indicates that the size heterogeneity within the RS1 DNA is not due to random breaks within the RS1 genome. Thus, the data presented suggest that the RS1 genome exists as a segmented molecule within the virion. Stabilization of the phage DNA in virions is most likely via complementary base pairing, since the experiments presented in Fig. 10 would rule out the existence of protein molecules noncovalently attached at or near the internal 3' hydroxyl groups. Obviously, much more work is required to test the feasibility of all of these hypotheses.

Despite the complexity found within the RS1 DNA, we were able to map the ends of the phage genome and construct a restriction map for the eight restriction enzymes shown in Fig. 7. The precise nature of the ends of either the full-length or the smaller fragments of RS1 DNA are not known at this time, but the fact that the electrophoretic behavior of untreated RS1 DNA on 0.6% agarose gels was unaffected by heating or incubation with T4 DNA ligase (with or without prior treatment with DNA polymerase I Klenow fragment) implies that the ends of the RS1 DNA are not blunt and do not contain cohesive regions which can be annealed by rapid cooling or ligation (data not shown). This latter observation is significant, for if the segmented state of the RS1 genome were due to the action of a nuclease with sequence specificity similar to that of a type I restriction endonuclease, one would expect the electrophoretic behavior of the RS1 DNA to be appreciably altered after treatment with T4 DNA ligase.

It should be noted that the positions of many of the restriction sites shown in Fig. 7 have been independently confirmed by analysis of the RS1 EcoRI restriction fragments C and E through J which have been cloned (T. J. Donohue, J. Chory, and S. Kaplan, unpublished data). In addition, although the ethidium bromide fluorescence pattern obtained for individual restriction endonuclease digests of phage RS1 DNA might suggest that some individual restriction fragments (such as EcoRI restriction fragment E in Fig. 1) were present in more than one copy per RS1 genome, both Southern blot analysis and the analysis of at least five independent clones containing the EcoRI restriction fragment E were not consistent with this conclusion (data not shown). The unexpectedly high ethidium bromide fluorescence of some individual restriction fragments could be explained by nonequivalent intercalation of ethidium bromide within those restriction fragments which appear to have single-stranded nicks or gaps (see above).

The estimated size of the full-length RS1 genome (approximately 60 to 65 kb) determined via electron microscopy or restriction endonuclease mapping in this study is significantly larger than the value of 3.0 × 10^6 to 3.5 × 10^6 daltons (42.1 to 47.3 kb) originally determined from sucrose density gradient or thermal renaturation analysis of sheared RS1 DNA by Abeliovich and Kaplan (1). However, given the unknown complexity within the RS1 DNA sample analyzed in the previous study (size heterogeneity, regions of single-stranded nicks or gaps), it is difficult to predict how accurate these previous estimates could have been. For example, individual techniques we used to analyze the RS1 DNA are biased for the detection of molecules of different sizes (i.e., ethidium bromide fluorescence and Southern blot analysis display a bias for detection of larger DNA fragments,
whereas electron microscopy has a bias towards smaller DNA molecules, which will have a greater chance of being wholly contained within a randomly selected visual field. Thus, the analysis of the RS1 DNA performed previously may have resulted in the measurement of a size for the phage RS1 DNA which was a composite of the distribution of DNA molecules present within an average DNA population obtained from one preparation of phage particles.

In summary, we have analyzed the DNA packaged within virions of the *R. sphaeroides* lytic bacteriophage RS1 and constructed a restriction endonuclease map of this phage genome. The utility of this information to answer questions of temporal regulation of gene expression during phage infection in *R. sphaeroides* is highlighted by our recent identification of specific cloned EcoRI restriction fragments of RS1 DNA which contain promoters and structural genes for early and possibly late phage gene products by using the *R. sphaeroides* in vitro transcription-translation system (T. J. Donohue, J. Chory, and S. Kaplan, unpublished data).

ACKNOWLEDGMENTS

We thank Eric Muller for many helpful discussions and Jeff Gardner for supplying some of the restriction enzymes used in initial experiments. We are also extremely grateful to C. Malone and Lucia Rothman-Denes (University of Chicago) for their help in preparing the electron micrographs of RS1 DNA.

This work was supported by grant PCM83-17682 from the National Science Foundation and Public Health Service grants GM31667 and GM15590 to S.K. from the National Institutes of Health. J.C. was a National Institutes of Health Predoctoral Fellow under Public Health Service training grant GM07283.

LITERATURE CITED