Mapping and cloning of Eco RI-fragments of bacteriophage T5+ DNA

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ABSTRACT

The Eco RI-fragments of bacteriophage T5 DNA were mapped using a technique which involves primarily length measurements of molecules observed in the electron microscope. Since Eco RI cleavage generates termini with 4-nucleotide long cohesive ends, fragments of complete and partial Eco RI digests were covalently circularized with DNA ligase at dilute DNA concentrations before measuring relative to internal length standards. This established the order of the internal Eco RI fragments. The two external Eco RI fragments, which had only one Eco RI terminus, were positioned relative to the internal fragments by identifying the location of some of the naturally-occurring nicks in partially denatured linear Eco RI fragments. An attempt was made to clone each of the internal Eco RI-fragments of T5 DNA via transformation into E. coli after ligation in vitro with the plasmid pMB 9. Only one fragment could be cloned and this fragment did not specify any new polypeptides in mini-cells of either the E. coli EK1 host, X1411, or the EK 2 host, X1776.

INTRODUCTION

The construction of a restriction enzyme cleavage map of a given DNA molecule generally involves the analysis of partial digestion products after they have been resolved by agarose or acrylamide gel electrophoresis. The complete digestion of the partial products allows one to determine adjacent restriction enzyme fragments and if enough partial products can be obtained, overlapping regions which encompass all of the sites of a limit digest can be ordered with respect to each other. Occasionally, mutant forms of the DNA which have lost one or more of the cleavage sites are available to aid in the mapping of the wild type molecule.

We were interested in studying the expression of specific regions of the bacteriophage T5+ genome when cloned in E. coli via recombinant DNA techniques. In order to do so, it was first necessary to construct a map of the cleavage sites on T5 DNA of one or more restriction enzymes. We began with the restriction enzyme, Eco RI. However, preliminary gel electrophoresis experiments showed only two small and several large Eco RI fragments of T5 DNA indicating only a few cleavage sites which were rather asymmetrically located throughout
the genome. Since some of the Eco RI fragments were too large to be resolved from each other on agarose gels, we sought an alternative technique to the conventional separation procedures used in mapping cleavage sites. We took advantage of the fact that (i) Eco RI cleavage products have 4 nucleotides long cohesive ends which can be sealed in vitro by DNA ligase and (ii) T5 DNA when isolated from the virus particle has several naturally-occurring nicks whose relative locations have been determined\textsuperscript{7,8,9,10,11}.

Partial digestion products were circularized, sealed with DNA ligase, separated from linear fragments and their lengths measured in the electron microscope. This ordered the internal restriction enzyme fragments. The two outside fragments were positioned by partial denaturation of an Eco RI digest so that the location of the naturally-occurring nicks could be observed relative to the cleavage sites. The Eco RI cleavage sites were confirmed by comparing the pattern of denatured T5 DNA fragments on an agarose gel prior to and after Eco RI digestion.

Unfortunately, only one of the Eco RI fragments could be cloned in \textit{E. coli} using the plasmid, pMB 9, as a vector; the others probably coded for the expression of a product lethal to the cell. Recombinant plasmids containing this fragment were transformed into two mini-cell producing strains, an EK 1 host, XI411, and the recently-constructed EK 2 host, X1776. In both hosts, the recombinant plasmids specified the synthesis of only the same three polypeptides as did the parent plasmid, pMB 9. No extra polypeptides were observed.

**MATERIALS AND METHODS**

**Materials**

DNA from the bacteriophages T5\textsuperscript{+} and ØX 174\textsuperscript{2} and the \textit{E. coli} plasmid DNAs, pMB 9\textsuperscript{13} and pDM 4\textsuperscript{14} were isolated and purified as previously described. The enzymes, Eco RI and T4 DNA ligase, were obtained from Miles Laboratory. \textsuperscript{[35S]} methionine was purchased from New England Nuclear. The \textit{E. coli} mini-cell producing strains, XI411 and X1776, were obtained from Dr. Roy Curtiss III and his colleagues.

**Methods**

**Eco RI Digestions.** Bacteriophage T5 DNA (60 µg) was digested to completion with Eco RI (2000 units) by incubating for 30 minutes at 37° in 225 µl of 100 mM tris-\(\text{HCl},\) pH 7.5, 50 mM NaCl, 5 mM \(\text{MgCl}_2\), and 2 mM \(\text{β}-\text{mercaptoethanol}.\) A partial digestion was conducted under the same conditions with one-tenth the amount of enzyme after which EDTA was added to 20 mM and the mixture heated to 65° for 10 minutes to inactivate the enzyme. The DNA was then diluted to
2 μg/ml in 30 mM tris-HCl, pH 8.1, 6 mM MgCl₂, 4 mM dithiothreitol, 0.2 mM ATP, 50 μg/ml bovine serum albumin and 20 units of T4 DNA ligase and incubated for 18 hours at 16°. The ligated circular DNA molecules were separated from linear molecules according to Schachat and Hogness. The DNA ligase mixture was adjusted to 50 mM EDTA, 200 μg/ml ethidium bromide and pH 8.8 with tris base. Solid CsCl was added to a density of 1.51 gm·cm⁻³ and 40 ml of this solution centrifuged at 10° in a Beckman-Spinco 60 Ti rotor at 40,000 rpm for 48 hours. The lower band containing circular DNA was spread directly for electron microscopy.

Construction and Screening of PMB9-T5 DNA Hybrids. Eco RI digests of pMB9 DNA (1 μg) and T5 DNA (10 μg) were incubated for 18 hours at 16° in 500 μl containing 10 units of T4 DNA ligase in the above ligase reaction mixture. The CaCl₂ transformation procedure was used to introduce the ligated DNA into the E. coli strain, HB101, which were then selected for the ability to grow on agar plates containing 20 μg/ml tetracycline. Individual colonies were screened for the presence of T5 DNA by the in situ filter hybridization procedure of Grunstein and Hogness using radioactive T5 complementary RNA (T5 cRNA) synthesized in vitro with E. coli RNA polymerase (a gift of Elliot Rosen) as described by Wensink et al. Plasmid DNA was extracted from 200 ml overnight cultures (L-broth and 20 μg/ml tetracycline) of individual colonies to which T5 cRNA hybridized. A sample of the DNA was digested to completion with Eco RI, and run on 0.85% agarose gels to see which of the Eco RI fragments of T5 DNA had been inserted.

Some of the pMB9-T5 DNA hybrid plasmids were subsequently transformed into the EK1 host X1411, or the EK2 host, X1776, using the same CaCl₂ procedure except that cells of X1776 were grown at 32° in L-broth supplemented with diaminopimelic acid (100 μg/ml, Sigma) and thymidine (10 μg/ml, Sigma). Strain X1776 had between 2%-40% of the transformation efficiency of strain X1411.

Labeling the 3'-Termini. T5 DNA fragments were labeled at the 3'-termini using E. coli DNA polymerase I and [α-³²P]dGTP as described previously except that the incubation was at 6°C for 30 minutes. The reaction was stopped by adding EDTA to 20 mM. The unreacted [α-³²P]dGTP was removed by dialyzing the incubation mixture first against 2 liters of 1 M NaCl, 100 mM tris-HCl, pH 7.5, 1 mM EDTA for 12 hrs and then against 2 liters of the Eco RI reaction mixture (minus MgCl₂) for 12 hours.

Polypeptide Synthesis in Bacterial Mini-Cells. Mini-cells from individual transformants of the two E. coli strains, X1411 and X1776, were isolated and
incubated with $[35S]$ methionine as described by Miller et al.20. Mini-cell lysates were then electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels followed by autoradiography.

Electron Microscopy. DNA molecules were prepared for electron microscopy by either the aqueous or formamide technique described by Davis et al.16. Photographs were taken with a Hitachi model HV-125E electron microscope and molecules measured directly on prints using a Hewlett Packard calculator and digitizer.

RESULTS AND DISCUSSION

The Location of Eco RI Cleavage Sites in T5 DNA. Preliminary agarose gel electrophoresis of Eco RI digested T5 DNA established that T5 DNA possesses an unusual distribution of cleavage sites which would make it difficult to use gel electrophoresis to separate the products of a partial cleavage. For example, there are three fragments greater than 25 kb and two of about 3 kb. Since fragments larger than 20 kb do not resolve well on agarose gels, it seemed unlikely that partial products could be separated by this technique. So, instead, electron microscopy was used to determine the locations of the Eco RI cleavage sites. Figure 1A shows a histogram of length measurements of the linear DNA fragments generated by a complete Eco RI digestion. Six different fragments were observed ranging from $2.4 \pm 0.1$ kb to $44.1 \pm 1.1$ kb in length. The three smallest fragments (fragments 1, 2 and 3) were observed in approximately equal quantities in random photographs indicating that there is one of each such fragment per T5 DNA molecule. The three largest fragments were occasionally tangled into "flower" configurations making it more difficult to determine their relative molar ratios, but their size alone suggested that they occur only once per T5 DNA molecule. In addition to these six fragments, some very small fragments were observed which were too small to accurately measure and are discussed later.

To determine which of the restriction fragments were the result of 2 internal Eco RI cleavages, a complete Eco RI digest was diluted to 2 µg DNA/ml and incubated with DNA ligase at 16°. Under these conditions, DNA ligase catalyzes the intramolecular circularization of the internal restriction fragments by covalently sealing the two cohesive ends generated by Eco RI. Since the DNA concentration is low, very few dimer molecules will be formed which, if ligated a second time to yield circular dimers, might confuse the interpretation of the data. After ligation the DNA was then spread for length measurements in the electron microscope. The arrows in Figure 1A indicate those restriction fragments which were capable of forming circles in the
Figure 1(A). A histogram showing the length distributions of fragments generated by Eco RI cleavage of T5 DNA. An aqueous spread of the linear fragments for electron microscope observation was made in the presence of 2 internal length standards, circular φX 174 RF DNA (5.37 ± 0.2 kb, B.G. Barrell, personal communication) and circular pDm 4 plasmid DNA (21.84 ± 0.19 kb, reference 14). Six fragments (1-6) are observed whose mean lengths and standard deviations in kb are respectively 2.4 ± 0.1, 3.1 ± 0.1, 14.0 ± 0.3, 25.5 ± 0.4, 29.9 ± 0.3 and 44.7 ± 1.1 for a combined length of 119.5 ± 1.9 kb. The lengths of the smallest 3 fragments agreed with the lengths determined from their migration on agarose gels in the presence of standard length molecules. The arrows indicate those fragments which formed covalently closed circles upon treatment with DNA ligase (see text). (B) Length measurements of circular molecules which were formed in vitro by ligation of a partial Eco RI digest of T5 DNA and subsequently purified from the remaining linear fragments by equilibrium centrifugation in a CsCl-ethidium bromide gradient. Measurements were taken from photographs similar to that shown in Figure 2 except that the DNA concentration was less. Note that circular fragments 1, 2, 3 and 4 (the internal Eco RI fragments) are present as well as 3 new circular molecules of 16.6 ± 0.3 kb, 28.8 ± 0.3 kb and 39.6 ± 2.0 kb (arrows) which correspond in length to fragments 3 + 1, 2 + 4 and 3 + 4 respectively. Since the 39.6 ± 2.0 kb circular partial product had a large standard deviation, it is possible that a few molecules were measured which possess 3 Eco RI fragments, 1 + 3 + 4 or 2 + 3 + 4.

presence of DNA ligase and hence are the internal fragments.

To determine the relative order of these internal fragments, a partial Eco RI digest was similarly diluted to 2 μg DNA/ml and incubated with DNA ligase. However when the mixture was observed in the electron microscope immediately after ligation, very few ligated circular partial products were observed because they were such large molecules that intramolecular circularization occurred infrequently. To get a higher concentration of circles for
electron microscopy, the ligated circular molecules were separated from the remaining linear fragments by centrifugation in CsCl and ethidium bromide. Figure 2 shows a representative photograph of the separated circles of a partial Eco RI digest. The actual length measurements of these circles (shown in Figure 1B) were made from a more dilute spread of the DNA to lessen the chance of one circle overlapping another and confusing the measurement. As can be seen in the histogram of Figure 1B, three new size classes of circles occur after the ligation of a partial Eco RI digest, which are circular partial products of fragments $1 + 3$, $4 + 2$ and $3 + 4$ respectively and establishes the order of the internal restriction fragments as $2-4-3-1$ (or the equivalent order $1-3-4-2$).

This block of internal fragments was then oriented relative to the two large external fragments 5 and 6. For example, the order could be either 5-2-4-3-1-6 or 6-2-4-3-1-5. This was accomplished by taking advantage of the fact that T5 DNA has several naturally occurring nicks which can be observed directly in an electron microscope spread of partially denatured molecules and whose locations are predominately in one-half of the molecule. Table 1 gives the number of nicks observed per fragment in a spread of partially denatured linear Eco RI fragments. The external fragment 5 has the largest average number of nicks at 2.9 per molecule followed by
the internal fragment 4. The other two large fragments had less than 0.5 nicks/molecule. The occurrence of nicks is not merely a function of fragment size since the largest fragment (fragment 6) had only 0.4 nicks/molecule.

Several laboratories \(^7,8,9,10,11\) have reported that the major naturally-occurring nicks of T5 DNA are located at the following positions from one end of the 120 kb molecule; 4.3 kb, 9.9 kb, 22.1 kb, 38.0 kb and 76.4 kb. Note that 4 of the 5 nicks lie in one-half of the molecule. Since the external fragment 6 (44.1 kb) contains relatively few nicks, it originates from the half of the molecule containing the fewest nicks. Since the other external fragment (fragment 5) and the internal fragment 4 contain the largest number of nicks/molecule, they must be in the half that contains the majority of nicks. This establishes the fragment order as 5-2-4-3-1-6.

The Relative Locations of Nicks and Eco RI Cleavage Sites. Since the 5'-terminal nucleotide at the internal nicks of T5 DNA is dGMP\(^{21,22}\), the nicks of the T5 DNA fragments can be labeled by extending the 3'-termini at the nicks one residue by incubation with [\(\alpha-^{32}\)P]dGTP and E. coli DNA polymerase I\(^{21}\). To confirm the above Eco RI fragment order, T5 DNA labeled at the nicks in this manner was denatured and electrophoresed through a slab agarose gel before and after Eco RI digestion.

The solid line in Figure 3 shows the migration pattern of the 3'-terminally labeled denatured T5 DNA fragments before Eco RI cleavage. The migration of a

### TABLE 1. The Distribution of Nicks in the Eco RI-Fragments of T5 DNA.

An Eco RI digest of T5 DNA in 83% formamide was spread on top of a 53% formamide hypophase for electron microscope observation. In these conditions about 20% of the DNA is denatured and the relative positions of the naturally occurring nicks can be detected as breaks in the single-stranded denaturation "bubbles"\(^{21}\). The fragment lengths were measured and the number of nicks per fragment scored. Control experiments with \(\lambda\) DNA established that the Eco RI digestion conditions did not introduce single stranded internal nicks in the DNA.

<table>
<thead>
<tr>
<th>Eco RI Fragment of T5 DNA (see Figure 1A)</th>
<th>No. of Partially Denatured Fragments Measured</th>
<th>No. of Nicks Observed</th>
<th>Average No. of Nicks per Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2.4 kb)</td>
<td>Not measured</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 (3.1 kb)</td>
<td>Not measured</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 (14.0 kb)</td>
<td>26</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>4 (25.5 kb)</td>
<td>15</td>
<td>19</td>
<td>1.3</td>
</tr>
<tr>
<td>5 (29.9 kb)</td>
<td>19</td>
<td>55</td>
<td>2.9</td>
</tr>
<tr>
<td>6 (44.7 kb)</td>
<td>9</td>
<td>4</td>
<td>0.4</td>
</tr>
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given fragment is a measure of its molecular weight$^{19,21}$. Since only the 3'-terminal nucleotide is labeled, the relative intensity of a given peak is a measure of the frequency with which that fragment occurs in a population of T5 DNA molecules. For example, the 4 major fragments (peaks) probably occur in all T5 molecules while the several minor fragments (peaks) occur in only some molecules$^{11}$. Peak I is thought to contain 2 fragments of about 44.4 kb and 38.4 kb$^9$ but from the relative intensities of the peaks, only one appears to be labeled at the 3'-terminus by DNA polymerase I and [α-32P]dGTP.

After Eco RI cleavage (dotted line) the 3'-terminal label on the fragment in Peak I migrates as though it is now on a fragment of 21.5 kb while the label in peak II appears to be on a fragment of 8.1 kb. The fragments in peaks III and IV and most of the minor fragments remain unaltered by the Eco RI digestion. Thus all of the Eco RI cleavage sites occur within the 2 fragments in peaks I and II. This is consistent with the data from the electron microscope measurements.

![Densitometer tracing of an autoradiogram of an 0.85% agarose gel through which has been electrophoresed denatured T5 DNA (solid line) and denatured T5 DNA which has been digested with Eco RI prior to denaturation (dotted line). Migration is from top to bottom. The 3'-termini of unlabeled T5 DNA fragments were labeled with $^{32}$P]dGMP as described in the text and in reference 21. From the work of this laboratory and others$^{7,8,9,10,11}$, the size of the fragments in peaks I-IV is thought to be: peak I, 2 fragments of 44.4 kb and 38.4 kb; peak II, 15.6 kb, peak III, 12.2 kb, and peak IV, 5.6 kb. The arrows show the changes in migration of peaks I and II after Eco RI cleavage.
Figure 4 summarizes the data by presenting the locations of both the Eco RI cleavage sites and the major naturally-occurring nicks. In addition to the Eco RI fragments shown in Fig. 4, a restriction fragment too small to accurately measure was observed in the electron microscope (indicated by the arrows in Figure 2) which was the result of 2 internal Eco RI cleavages since it could be circularized by DNA ligase. This fragment cannot be positioned unambiguously from the data presented here although it appears to be adjacent to the 3.1 kb Eco RI fragment and to be about 0.4 kb in length from a consideration of the data shown in Figures 3 and 4. Fragment II (Figure 3) is a 15.6 kb fragment which Eco RI cleaves into 8.1 kb and 3.1 kb fragments (Figure 4) plus one or more fragments whose sum is 4.4 kb, i.e., 15.6 kb - (8.1 kb + 3.1 kb) = 4.4 kb. The remaining length of DNA at the 5' termini of Fragment II is obtained from 25.5 kb - 21.5 kb = 4.0 kb (Figure 4). This leaves unaccounted for a length of 4.4 kb - 4.0 kb = 0.4 kb which would suggest a third Eco RI cleavage within the 15.6 kb region to generate the small fragments observed in Figure 2. Although this does not prove the location of this small fragment, it does suggest that it is located at one of the 2 positions indicated by the arrows in Figure 4. This conclusion is supported by the Eco RI cleavage maps of T5 DNA published by two other laboratories both of whom position a small restriction fragment of 0.3 - 0.4 kb just to the left of the 3.1 kb fragment. The Eco RI restriction map of T5 DNA presented in Figure 4 agrees very well with the maps determined in these previous reports.

Polypeptide Synthesis in Bacterial Mini-Cells Containing pMB9-T5 DNA Hybrids. Hybrid molecules between Eco RI-cleaved pMB 9 and the Eco RI-fragments of T5 DNA were constructed in vitro as described under Methods. Electron microscope observation of the ligated mixture indicated the presence of circular molecules of a variety of sizes ranging from 2.4 kb to over 25 kb.

Figure 4. A schematic diagram showing the locations of the Eco RI cleavage sites and the major naturally occurring nicks of T5 DNA. The small "x"s indicate the 3'-termini labeled with [α-32P]dGTP by DNA polymerase I. The arrows indicate one of 2 places where a 0.3 kb Eco RI fragment is thought to be located.
Figure 5. Autoradiogram of an SDS-acrylamide gel of \[^{35}\text{S}]\) labeled polypeptides synthesized in mini-cells of (A) \(E. \text{coli}\) strain, X1411, or (B) \(E. \text{coli}\) strain X1776, which contained either the parent plasmid, pMB 9, or a hybrid plasmid possessing the Eco RI-fragment \(1\) of T5 DNA (pT5 32, pT5 40, pT5 45, etc.). The mini-cell incubation with \[^{35}\text{S}]\) methionine was either in the absence (\(-\)) or the presence (+) of 20 \(\mu\)g/ml tetracycline. Using polypeptides of known molecular weight as markers in parallel slots of the gel, the 3 major bands in pMB 9 slot correspond to polypeptides of 44,000 daltons, 23,000 daltons and 16,000 daltons. The fastest moving band co-migrates with \[^{35}\text{S}]\) methionine which was not completely removed by the washing steps after the mini-cell incubation.

in length. The ligated mixture was used directly to transform the \(E. \text{coli}\) strain HB101. About 150 individual transformants were screened by the in situ filter hybridization technique\(^1\) for the presence of T5 DNA sequences. Plasmid DNA was isolated from the 14 colonies to which the T5 cRNA hybridized and electrophoresed through agarose gels after digestion with Eco RI. All 14 possessed T5 DNA fragment \(1\) (2.4 kb) plus pMB 9. None possessed any of the other 3 internal Eco RI fragments (3.1 kb, 14.0 kb, 25.4 kb) although electron microscope observation of the ligated molecules prior to transformation indicated that at least the 3.1 and 14.0 kb fragments did form hybrid circles with pMB 9. Thus it appears that this \(E. \text{coli}\) strain is not capable
of harboring hybrid plasmids containing these Eco RI fragments, possibly because when cloned, they specify a product that is fatal to the cell.

Purified plasmid DNA from 6 of the above 14 colonies were used to transform the 2 mini-cell producing strains, X1411 (an EK1 host) and X1776 (an EK2 host). Then mini-cells containing these hybrid plasmids of pMB 9 and fragment 1 were isolated, incubated with [35S] methionine and mini-cell lysates electrophoresed through SDS-acrylamide gels to separate the labeled polypeptides specified by the plasmids. Figure 5 shows a typical result for 6 pMB9-fragment #1 plasmids in X1411 and 3 in X1776. The parent plasmid, pMB 9, either in the absence or presence of tetracycline (the left-hand 2 slots) codes for 3 polypeptides that can be detected by this technique. The sum of their molecular masses (83,000 daltons) accounts for about 42% of the pMB 9 genome assuming the molecular weight of pMB 9 and the average amino acid molecular weight to be 3.3 x 10^6 daltons and 120 daltons respectively. In the colonies checked, fragment 1 did not induce the presence on the polyacrylamide gel of any new labeled polypeptides in either E. coli strain, X1411, (Fig. 5A) or strain X1776 (Fig. 5B). Thus if new polypeptides are synthesized, they occur in such small amounts that they cannot be detected by autoradiography.

Some variations from experiment to experiment were observed in the relative amounts of the pMB 9 coded polypeptides; however, no consistent pattern emerged. For example in the experiments shown, pMB 9 in X1411 codes about equal amounts of the 44,000 and 16,000 dalton polypeptides while in X1776, it codes for much more of the former polypeptide. In other experiments pMB 9 coded for about equal amounts of the 2 polypeptides in X1776.

REFERENCES