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Restriction Endonuclease Analysis of Bacteriophage P1 DNA and Its Derivative Hybrid DNAs

Gregory Alan Schulz
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RESTRICTION ENZYMASE ANALYSIS OF
BACTERIOPHAGE PJ DNA AND ITS
DERIVATIVE HYBRID DNAs

by

Gregory Alan Schulz

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Masters of Science
August
1977
ACKNOWLEDGEMENTS

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The author would also like to acknowledge the students and faculty of the Department of Microbiology for advice, instruction, and helpful discussion throughout his entire graduate studies.
VITA

The author, Gregory Alan Schulz, is the son of Robert William Schulz, Sr., and Stella (Haas) Schulz. He was born July 24, 1951, in Alton, Illinois.

His elementary education was obtained at St. Mary's Grade School, Alton, Illinois, and his secondary education was obtained at Marquette High School, also in Alton, Illinois.

In September, 1969, he entered Loyola University of Chicago, and in June, 1973, received the degree of Bachelor of Science with a major in biology.

In June, 1974, he joined the Department of Microbiology of Loyola University. He presently holds the position of Research Assistant at Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois.

Mr. Schulz is co-author on one publication: Integration Sites of Foreign Genes in the Chromosome of Coliphage P1: A Finer Resolution. 1976. Virology 73:299-302.
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LIST OF ABBREVIATIONS

argF - Escherichia coli K12 genes controlling ornithine transcarbamylase synthesis.

cat - Genes of R plasmid origin controlling resistance to chloramphenicol.

cat-gene 2 - Genetic region of the P1 chromosome found to be associated with aberrant recombination.

dalton - The mass of one hydrogen atom = 1.67 \times 10^{-27} \text{ grams}.

lac - Escherichia coli K12 genes controlling lactose catabolism.

md - $1 \times 10^6$ daltons.

mcp - Minichromosomal population.

str - Genes of R plasmid origin controlling resistance to streptomycin.

sul - Genes of R plasmid origin controlling resistance to sulfonamides.
CHAPTER I

INTRODUCTION

A. Bacteriophage Pl.

Pl is a temperate bacteriophage which lysogenizes a few coliform bacterial species. Since its discovery and isolation (4), bacteriophage Pl has proven to be a valuable tool to the bacterial geneticist because it mediates generalized transduction (15) in which the transducing particles carry a defined length of deoxyribonucleic acid (DNA) (11). This characteristic has enabled the use of Pl to define linkage groups within the bacterial chromosome (12), locate chromosomal loci of integrated prophage (21), and observe the effect that the presence of foreign genes has upon replication of the bacterial chromosomes (3).

Most Pl virions (PlB) produced following lytic infection of the host contain linear, duplex DNA having a molecular weight of $66 \times 10^6$ (29). In the lysogenic state, Pl exhibits several interesting characteristics not seen in other coliphage, such as those of the lambdoid group. It exists in the cytoplasm of its host as a circular, duplex DNA molecule of $52 \times 10^6$ daltons (11). There is only one copy of the plasmid per host chromosome, and no covalent association between the prophage Pl and its host chromosome has been shown (11). The lytic cycle of Pl is believed to involve the formation of concatamers (end to end genomic
oligomers) perhaps proceeding through a rolling circle mechanism of replication and/or recombination of monomeres. Encapsidation of phage DNA proceeds through the action of a precessive, volume dependent, headful packaging system on concatamers. A randomly permuted collection of phage DNA molecules are sequentially encapsidated into phage heads, each containing more than one unit phage genome of DNA. This packaging system was first hypothesized for bacteriophage T4 by Streisenger et al. (25) from purely genetic data. A recombination between the redundant ends of the virion DNA is believed responsible for converting the linear, infecting DNA, into circular P1 plasmid DNA. This concomitant elimination of genetic redundancy accounts for the lower molecular weight of the plasmid DNA.

Within a lysate of P1 virions, there also are a certain percentage of P1 small capsid morphology variants (P1S). The P1S comprise about 20% of the total virion population, and are well separated from P1B in isopycnic CsCl density gradients (respective densities 1.42 and 1.47) (27).

The P1 genetic map, constructed through bacteriophage crosses, is linear, as opposed to a circular map one would expect from a purely random, permuted population of virion DNAs (22). This suggests that the ends of the P1 genetic map are due to the existence of a high frequency recombinational region known as a hot spot. The above observations suggest that other systems are functioning in conjunction with a volume dependent, headful
chromosome packaging system.

B. Specialized transducing derivatives of P1.

Another characteristic of bacteriophage P1 is its ability to carry out specialized transduction of some genes of *Escherichia coli* (*E. coli*) and R factors. A number of these specialized transducing derivatives have been isolated. P1CM (*P1cat*) was isolated by Kondo and Mitsuhashi (14) during studies of the ability of phage P1 to transduce genes from a drug resistance plasmid. *P1cat* carries chloramphenicol resistance genes (*CM*<sup>R</sup>), and expresses all normal P1 functions.

The isolation of specialized *argF* transducing bacteriophage has been accomplished in three laboratories (8, 13, 24). These *argF* derivatives carry a segment of *E. coli* genes which control ornithine transcarbamylase synthesis. The lambdoid and P22 derivatives are deficient in some bacteriophage functions and must accordingly be propagated with the assistance of helper phage. The bacteriophage *P1argF* derivatives were isolated through a one-step transduction procedure: a bacteriophage P1 mediated low frequency transduction from *E. coli* K12 *argI<sup>+</sup>* pro<sup>+</sup>*arg<sup>+</sup>* lac<sup>+</sup> to P1cl.100 lysogenic *E. coli* argI<sup>+</sup> proA-argF-lac deletion XIII (24). As in the case of *P1cat*, the *P1argF* derivatives thus far isolated show no defects in normal P1 functions. Thus, *P1argF* is a stable hybrid of bacteriophage P1 DNA and *E. coli* *argF* genes, without altering any P1 functions.
Iida et al. (10) have been analyzing a family of P1 derivatives with drug resistance genes from R plasmids and provided us with the lysogen 3507, Pldcatsulstr (Picss). This specialized transducing derivative is a hybrid of P1 and genes from an R plasmid controlling antibiotic resistance to chloramphenicol, the sulfonamides, and streptomycin.

Of the few specialized transducing derivatives of P1 thus far isolated, Pldlac has been most extensively investigated. Luria and co-workers first detected Pldlac upon transduction of Shigella dysenteriae lac- with virions produced in E. coli lac+ (16). The frequency of occurrence of these Pldlac transductants is over one hundred fold less than those produced by simple lac integration. Once formed, Pldlac promotes specialized transduction of the lactose catabolizing genes at a high frequency. Further investigation of Pldlac by Rae and Stodolsky (20) provided genetic evidence that the Pldlac chromosomes can be too large to be encapsidated genetically intact. A test of this deduction is included in this thesis.

Mapping data on some P1 specialized transducing derivatives has been analyzed in order to locate the position on the P1 chromosome where foreign genetic material is integrated. Genetic crosses have previously shown that the cat-gene 2 region of the P1 chromosome is involved in the aberrant recombinational events underlying the de novo formation of the genetically hybrid, specialized transducing derivatives (26). In each case studied,
at least one of the union sites between P1 and foreign gene sequences is in the cat-gene region.

C. Restriction enzyme analysis of DNA.

The primary analytical tool used was electrophoretic fractionation of minichromosomal populations (mcp) produced by restriction endonucleases. This procedure is known as cleavage analysis and has enabled the identification of chromosomal alterations of many hybrid derivatives of P1. The isolation and subsequent exploitation of restriction enzymes, as an analytical tool in molecular biology research, has a lengthy history. Observations made in the early 1950's by Luria and Bertani led to the development of the primary analytical system used here (17, 5). They found that systems exist for the destruction of foreign chromosomes absorbed by bacteria. Basically, these initial observations showed that the host range of a given bacteriophage depended in part on the bacterial strain on which the phage had last propagated. This affect was called host controlled variation and was shown to be a purely host function, that is exerted on foreign DNA which has gained entrance to the bacterial cell. This system is now known as host restriction-modification (R-M) and has been detected in many microbial species (18).

Restriction-modification systems consist of two related enzymes. One enzyme, the restriction enzyme, cleaves foreign,
unmodified DNA at specific nucleotide sequences which then enable non-specific, cellular exonucleases to further digest the foreign DNA. The second enzyme, a modification enzyme, protects host DNA from attack by its own restriction enzyme by modifying the host DNA, often by methylation of specific bases at susceptible DNA sequences. The site specificity of the two enzymes is identical. Many sites are 4-6 base pairs long and possess two-fold rotational symmetry. For example, the cleavage site for the restriction endonuclease Eco•R1, from *E. coli* is 5'---G/A-A-T-T-C ---C-T-T-A-A/G-S' (6).

Once such enzymes were identified and purified in quantity, *in vitro* chromosomal analysis was revolutionized (19). These endonucleases produce genetically defined fragments, herein called minichromosomes, which are unique to a particular genome both in number and molecular weight. Minichromosomes can be fractionated by a number of analytical techniques. Analytical agarose gel electrophoresis has been the chief tool used in the fractionation of mcp produced by restriction enzymes (23). Uniform agarose gels provide an easy, inexpensive, and relatively accurate method for determining the molecular weights of a mcp of DNA molecules produced through the use of restriction endonucleases. For a given concentration of agarose, DNA, in general, will migrate with a velocity inversely proportional to its logarithmic molecular weight. A plot of the distance migrated versus the log molecular weight is thus linear. Given a DNA population of standard molecular weight
markers, molecular weight estimates can be made for an unknown mcp by comparing the electrophoretic migration distances of the unknowns with those of the standards. The migration distances of the standards, plotted against their log molecular weights, yields a straight line whose slope is unique for a given gel concentration. The migration distances of the unknown mcp can also be measured in the same gel, and molecular weights assigned to each band by observing where a given migration distance intercepts the standard plot.

In actuality, however, for a given agarose concentration, there is only a set molecular weight range of DNA whose molecular weights can be measured from migration distances. The distance migrated for DNA above this range, plotted against the log molecular weight, is not linear, and thus accurate molecular weight estimates cannot be made. DNAs whose molecular weights are below this range simply migrate through and off of the gel by the time higher molecular weight DNA has had a chance to migrate sufficiently. Therefore, in order to determine the molecular weights of a broad size-range mcp, a variety of agarose concentrations must be employed.

The major purpose of this research has been to further define that area of the P1 chromosome involved in the acquisition of foreign genetic material. Cleavage analysis has been employed as the chief analytical tool. A number of possibilities existed. Acquisition of foreign DNA could occur randomly, at one common
site, or at a few unique sites on the P1 chromosome. Such chromosomal regions would be considered hot spots for recombination. As compared with a P1 control, differences in electrophoretic patterns among different hybrid mcp reveal how the intact chromosome is altered by the presence of foreign genes.
CHAPTER II

MATERIALS AND METHODS

A. Bacterial strains.

Strains of *Escherichia coli* lysogenic for bacteriophage P1 were obtained from the collection of M. Stodolsky and other P1 investigators and are listed in Table 1. All lysogenic strains used for bacteriophage production contained a temperature sensitive, thermally inducible P1 prophage.

B. Media.

Luria broth (1 broth: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 5 g NaCl per l, pH 7.2) was used for propagation of bacterial lysogens and bacteriophage production.

C. Bacteriophage production and purification.

For large scale virus production, the method of Yamamoto and Alberts was used with slight modification (28). Overnight starter cultures of *E. coli* lysogens were grown in L broth at room temperature with no agitation for approximately 16 h. A 1:20 dilution was made from the starter cultures into a 2-l flask containing 500 ml of L broth prewarmed to 30 C. Freshly inoculated cultures appeared slightly turbid, and never exceeded 5 Klett units (at 540 nanometers (nm) using a Klett-Summerson Photoelectric Colorimeter).
Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Prophage</th>
<th>Host Characteristics</th>
</tr>
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<tbody>
<tr>
<td>AB 259</td>
<td>-</td>
<td>Hfr Hayes</td>
</tr>
<tr>
<td>CA7033</td>
<td>-</td>
<td>HfrH ΔAll1 str^R</td>
</tr>
<tr>
<td>921</td>
<td>-</td>
<td>F^-res^-mod^-</td>
</tr>
<tr>
<td>SC1800</td>
<td>-</td>
<td>F^-ΔAll1 str^R*</td>
</tr>
<tr>
<td>SC1701</td>
<td>-</td>
<td>F^-pro^+lac^+arg^+</td>
</tr>
<tr>
<td>PL0110</td>
<td>Pldlac</td>
<td>CA7033</td>
</tr>
<tr>
<td>921</td>
<td>P1cl</td>
<td></td>
</tr>
<tr>
<td>1801</td>
<td>P1cl.100</td>
<td>SC1800</td>
</tr>
<tr>
<td>1802</td>
<td>P1CMcl.100</td>
<td>SC1800</td>
</tr>
<tr>
<td>D1</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D2</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D3</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D4</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D5</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D6</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D7</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D8</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D9</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>D10</td>
<td>P1argF</td>
<td>SC1800</td>
</tr>
<tr>
<td>3507</td>
<td>Pldcatsulstr</td>
<td>921</td>
</tr>
</tbody>
</table>

*Other characteristics: his^-metB^-arg_I^-xyl^-md^-ΔAll1 = proA-argF-lac deletion XIII.
Culture incubation proceeded at 30 C on a rotary shaker, and growth was followed by Klett readings. Thermal induction was initiated when, in logarithmic phase, cultures reached 40-50 Klett units (corresponding to about 2 x 10^8 E. coli per ml). Cultures were removed from the shaker and heated with swirling over an open flame, to rapidly raise the temperature to 40 C. Cultures were then transferred to a 40 C gyrotory waterbath shaker (New Brunswick Scientific Co.), and briskly agitated to assure sufficient aeration throughout induction. If excessive foaming occurred, anti-foam spray (Dow-Corning) was used to eliminate this problem. Induction proceeded for 20 min at 40 C, after which time cultures were transferred back to the 30 C rotary shaker, where growth and subsequent bacterial lysis was measured spectrophotometrically. During this period, MgCl₂ was added to cultures to obtain a final concentration of 0.01 M in order to stabilize the mature virions.

Following induction, and cell lysis, whole cells and cellular debris were pelleted at 4 C in 500-ml centrifuge bottles, using the Sorval GS-3 rotor at 9,000 rpm (13,700 g) for 20 min (Sorval RC2-B refrigerated centrifuge). The supernatant containing the mature virions was decanted back into 2-l flasks and made 0.5 M in NaCl and 10% (w/v) in Polyethylene Glycol 6000 (PEG 6000) in the cold. Lysates were left at 4 C overnight.

Culture volumes used in phage production were dictated mainly by convenience in concentrating the phage in existing
laboratory equipment. When larger culture volumes could not be conveniently handled by centrifugation, the following changes were made. For induction, cultures were pooled into a large flask and a sparger was inserted for aeration. Warm, sterile L broth (65°C) was added to the culture to quickly raise the temperature to 40°C. The flask was then submerged into a 40°C waterbath. Following a 20 min induction period, 500-ml culture volumes were aliquoted from the pooled cultures. Growth and subsequent lysis occurred in the 30°C rotary shaker. Following lysis, cultures were filtered through 1 inch Hyflo-Super Cell (Fisher Scientific) using a Buchner funnel and a large suction flask. Filtrate containing the mature virions was then made up to 0.5 M NaCl and 10% (w/v) PEG 6000 as described above. The remaining procedures for phage purification were identical.

Following overnight storage at 4°C the phage-PEG 6000 aggregate of the treated lysates was pelleted at 4°C in 500-ml centrifuge bottles, using the Sorval GS-3 rotor at 9,000 rpm (13,700 g) for 20 min (Sorval RC2-B refrigerated centrifuge). The pellets were suspended in phage buffer (P buffer: 0.1 M Tris-HCl, pH 7.2, 0.01 M MgCl₂) whose volume never exceeded 5 ml per a 500-ml starting culture volume.

The final step in phage purification employed CsCl step gradients. CsCl gradients were prepared in 12 ml polyallomer centrifuge tubes (14.5 X 96 mm: International Equipment Co.) by layering 2 ml of a 1.6-density CsCl solution in P buffer
underneath 5 ml of a 1.4-density CsCl solution, also in P buffer. After gently layering the suspended phage-PEG aggregate on top of the gradient, mineral oil was used to fill the tubes. Equilibrium centrifugation was carried out at 4°C in an International SB 283 rotor at 35,000 rpm (180,000 g) for approximately 16 h, in an International B 35 or B 60 preparative ultracentrifuge. Sharp bands were clearly visible following centrifugation, and phage were collected by using a needle and syringe either from the top of the tube, or by piercing the side.

Phage were immediately put into prepared dialysis tubing. Preparation of dialysis tubing involved boiling in 1% Na₂CO₃ for 1 h, rinsing thoroughly with distilled water, and storing in 70% ethanol in the cold. Phage were dialyzed against 5 one-1 changes of P buffer which effectively removed all cesium chloride. Phage were stored at 4°C.

D. Purification of bacteriophage DNA.

Purification of phage DNA involved a detergent disruption of phage with sodium dodecyl sulfate (SDS), followed by a phenol extraction of the disrupted phage proteins. All purified bacteriophage were diluted to an optical density of 20 at 260 nm. For more dilute phage, DNA extraction was performed at the existing concentration. The entire extraction procedure was most easily performed in 10-ml Corex centrifuge tubes. Phage suspended in disruption buffer (D buffer: 0.1 M sodium phosphate,
pH 7.2, 25 mM EDTA) were heated at 65°C in a prewarmed waterbath for 10 min and then chilled on ice. Solutions were then made 0.3 M KCl and put on ice to precipitate the SDS. A low speed centrifugation was performed (3,000 g for 10 min) to pellet the SDS. The viscous supernatant was then decanted into another 10-ml Corex tube where phenol extraction of the disrupted phage proteins was performed.

Redistilled phenol was used in all extractions. An appropriate volume of phenol was saturated with an equal volume of D buffer, and the organic phase was made 0.08% (w/v) 8-hydroxyquinoline sulfate (Schwartz-Mann) in order to inactivate any residual peroxides in the phenol. All preparations were extracted 3 times with an equal volume of phenol. Each extraction was performed at room temperature by gentle mixing for 15 min. After each extraction a low speed centrifugation was performed (3,000 g for 10 min) in order to separate aqueous and organic layers. The interface was extracted twice, but removed before the final extraction. Following this final extraction, DNA was gently pipetted into dialysis tubing to avoid shear breakage. Extensive dialysis was performed in order to remove all traces of phenol. Phenol absorbs at 270 nm, so the dialysis buffer (0.01 M Tris-HCl, pH 7.2, 1 mM EDTA) was monitored and changed until its absorbance at 270 nm was under an optical density of 0.05. This usually required 5 to 6 one-1 changes of dialysis buffer. DNA was assayed for purity and concentration
spectrophotometrically at 260 and 280 nm, respectively. A 260:280 ratio of approximately two was required. DNA concentrations were computed based on the standard that at 260 nm, one optical density unit corresponds to a DNA concentration of 50 ug per ml.

E. Restriction endonucleases.

The majority of restriction enzymes were obtained from Dr. G. Haywood, University of Chicago. Some enzymes were also purchased from New England Biolabs, Beverly, MA. All restriction enzymes used, along with their appropriate reaction conditions, are listed in Table 2.

F. Enzymatic cleavage of DNA.

For each enzyme used, it was empirically determined the quantity needed to give a limit digest of a given amount of DNA. This was done by digesting a known quantity of DNA with successive 2-fold dilutions of enzyme until a partial digest was obtained. Digestion was assayed through agarose gel electrophoresis as described below. Hamilton syringes (Hamilton Co., Reno, Nev.) were used in all manipulations of DNA, enzyme, and reaction mixtures.

DNA to be cleaved was placed in a small disposable tube, heated at 65 C for 5 min in order to inactivate any residual nuclease which may have escaped phenol extraction, and then chilled on ice. The appropriate amount of 10 X reaction buffer
and enzyme (Table 2) were then added, and slight vortexing was used to assure proper mixing. Reactions usually proceeded for 2 h and were terminated by heating at 65°C for 5 min. If DNA was not immediately used, it was stored at -40°C with no deleterious effects.

G. Electrophoresis of DNA minichromosomes.

Electrophoresis was carried out in either agarose and/or acrylamide gels. Uniform agarose gels were most commonly used. Gradient pore agarose and acrylamide gels were employed for high resolution, and to simultaneously observe a wide molecular weight range of restriction products.

1. Uniform agarose gels. Uniform agarose gels were prepared by the method of Sharp et al., with modifications made necessary because of the electrophoresis apparatus used (23). An EC 490 electrophoresis apparatus was used for all uniform agarose gels (EC Apparatus Corp., Jacksonville, Fla.). Slab gels run in this apparatus are 17 X 13 cm X 3 or 6 mm. A 10% (w/v) acrylamide plug was used to seal the bottom of the gel chamber. This plug was formed by using a 20% (w/v) solution of Cyanogum-41 (EC Apparatus Corp.) in electrophoresis buffer (E buffer: 0.09 M Tris, pH 8.3, 0.09 M boric acid, 2.8 mM EDTA) containing 0.1% (v/v) NNN'N' tetramethylenediamine (TMED). Polymerization was promoted with 0.1% (w/v) ammonium persulfate. A 50-ml plug was used for a 6-mm thick gel, and a 30-ml plug was used for a 3-mm
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<th>Microorganism</th>
<th>Eco-R1</th>
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<td></td>
<td><em>Escherichia coli</em> RY13</td>
<td><em>Streptomyces albus</em> G</td>
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<tr>
<td>Cleavage Sequence</td>
<td>G+AATTTC(6)</td>
<td>?</td>
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<tr>
<td>(5'-3')</td>
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<tr>
<td>Reaction Conditions</td>
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<td>6 mM Tris, pH 7.4, 6 mM 2-mercaptoethanol, 6 mM MgCl₂ 37 C</td>
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<tr>
<td>Cuts in lambda DNA</td>
<td>5(7)</td>
<td>2(1)</td>
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thick gel. Agarose concentrations employed varied from 0.35\% (w/v) - 1.0\% (w/v), depending upon the size of the fragments being resolved. Agarose (Seakem) was dissolved in E buffer by boiling for 5-10 min, and then cooled to 50°C before pouring into the gel chamber. A comb-like slot former, previously soaked in Photo-flo (Kodak Chemical Co.) was inserted into the molten agarose to form sample slots. The gel was allowed to cool and solidify completely, at which time the slot former was carefully removed. The buffer chambers were then filled with E buffer, and a recirculating buffer pump was activated. Gels were prerun at 50 V for one h in the cold using cold tap water as a coolant for the cooling plates of the apparatus. Following the prerun, samples were introduced into the preformed slots using a long stem pasteur pipette. Before addition to sample slots for electrophoresis, the appropriate volume of cleaved DNA was heated at 65°C for 5 min in order to separate any fragments whose cohesive ends may have associated during cooling and storage. Bromphenol blue in 60\% (w/v) sucrose was added sparingly to each sample; the dye serving as a tracking substance and the sucrose increasing the density of the samples so that they seeded under buffer in the sample slots. Samples were initially pulled into the gel at 150 V for 10 min after which the running voltage was established. Running voltage of the gels varied from 20 to 60 V and gels were run overnight for approximately 16 to 24 hr or until the tracking dye had reached the agarose-acrylamide interface. Gels were
carefully removed and stained in the dark with 0.5 \text{ug} per ml ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) in E buffer.

2. Gradient pore agarose gels. A 3-mm thick agarose gel was used for all gradient gels and a 10\% (w/v) acrylamide plug was also employed as previously described.

A gradient former was constructed from two 100-ml graduated Nalgene cylinders. In one cylinder, two holes of approximately 3 mm in diameter, diametrically opposing one another, were drilled into the side of the cylinder as close to the base as possible. In the second cylinder, one 3 mm diameter hole was also drilled into the side as close to the base as possible. Three glass tubes, 5 mm long and 3 mm in outside diameter were cemented into the holes, being careful not to allow them to protrude into the cylinders. These glass fittings served as connections for rubber tubing. A piece of Nalgene tubing about 1 inch long was cemented to two glass fittings connecting the bases of both cylinders. The two cylinders were then permanently mounted on a square of plexiglass and the final glass connection was fitted with a long piece of rubber tubing, ending in a Y joint, hooked to 2 small bore plastic tubes. This allowed for two outlet ports for the molten agarose. Each cylinder had a magnetic stir bar placed in the bottom.

Two agarose solutions were prepared corresponding to the lower and upper gradient limits of agarose concentration. These solutions were also made 10 and 30\% (w/v) sucrose, respectively.
Both sucrose and agarose were dissolved in E buffer by boiling for 5 min. Eighty ml of each solution (160 ml total) was sufficient to fill the gel chamber. The lower limit concentration of agarose employed was 0.35% (w/v) and the upper limit was 1.7% (w/v). All gradients were made between these two concentrations. The gradient former was placed in a 4-l beaker and lead weights were used to anchor it. The beaker was placed on a stirring plate and filled with warm water (50 C). The tube connecting the base of the two cylinders was clamped off and the cylinders were then filled with the two molten agarose solutions; the higher concentration in the rear cylinder. The electrophoresis apparatus was prewarmed to 50 C by running warm tap water through the cooling coils. This was done in order to allow the gradient to stabilize before solidifying. The gel chamber was set in a vertical position and E buffer was poured into the bottom of the chamber in order to provide some back pressure to inhibit leakage of the molten agarose past the acrylamide plug. The two stir bars were set in vigorous motion, and the two outlet hoses were put into the bottom of the gel chamber just above the acrylamide plug. The gradient was then formed from bottom to top in the following manner. Agarose was forced out of the front (light) chamber by air pressure before the clamp between the two cylinders was released. Care was then taken to release the clamp in such a manner that there was no back flow of agarose from the front chamber to the rear chamber. The flow rate into the
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electrophoresis chamber was adjusted by a clamp on the outlet hose to an approximate total pouring time of 15 min. The molten agarose gradient was allowed to stabilize for another 15 min by keeping warm water flowing through the cooling coils. Solidification was then achieved by forcing cool tap water (25 C) through the cooling coils, displacing the warm water.

After complete solidification, a third agarose solution was prepared in E buffer with a concentration of agarose slightly below that of the lower gradient concentration. This solution was prepared by boiling in the same manner as the other solutions. This solution was poured boiling onto the top of the gel chamber in order to affect a seal with the upper portion of the gradient gel. This proved necessary so that the gel sections would not separate during prolonged electrophoresis. This gel was allowed to cool to 45 C, and then a slot former was introduced in the same manner as described earlier for uniform percentage agarose gels. After solidification of this upper gel, the slot former was removed and the gel was prepared for electrophoresis as previously described. Gradient pore agarose gels were usually run at a voltage slightly higher than straight percentage gels. Gels were stained as previously described.

3. Gradient pore acrylamide gels. Gradient pore acrylamide slab gels were purchased from Pharmacia Fine Chemicals (Uppsula, Sweden) and ranged in concentration from 5 to 30% (w/v) acrylamide. These gels were run on a Pharmacia slab gel
electrophoresis apparatus, and all methods concerning the use of these gels were consistent with the above methods for agarose gels.

H. Photography and densitometric tracings.

Fluorescence of DNA-ethidium bromide complexes were viewed under long wavelength ultraviolet light (Ultraviolet Products, San Gabriel, Calif.). All photographs were taken with a Polaroid MP-3 Land camera using a yellow filter (Kodak No. 9 Wratten gelatin filter). Polaroid Type 57 high speed film was used for the majority of prints. Kodak Tri-X Pan film was used for the majority of negatives. Polaroid Type 55 P/N film was used for both prints and large negatives. Some of these large negatives were used for densitometric tracings (Joyce-Loebel microdensitometer) to determine relative molar quantities of DNA in resolved bands.
CHAPTER III

RESULTS

A. Fractionation of virions of P1 and P1 hybrid derivatives on cesium chloride density gradients.

Some qualitative information has been obtained from the visual comparison of virion band thickness after fractionation of P1 and P1 hybrid lysates on isopycnic CsCl density gradients. Cesium chloride gradients were used in the final step of phage purification, following initial co-precipitation with PEG-6000, as described in Materials and Methods. At the last step of purification virions are visible as bands, P1B and P1S. Visual comparisons of four populations of genetically hybrid P1 virions revealed some interesting facts with respect to relative distribution P1B and P1S, when compared to the P1 control.

P1cat and P1argF are indistinguishable from the P1 control. In contrast, P1dcss and P1dlac have an excess of small capsid morphology variants (P1S). P1dlac has an approximate 3:1 ratio of P1B to P1S. P1dcss has about equal thickness of small and normal virion bands. P1dcss and P1dlac, therefore, both have mutations in virion capsid size distribution.

B. Migration of DNA in uniform agarose gels as a function of agarose concentration.
Figure 1 is semilogarithmic graph of relative migration distance versus molecular weight of DNA over a range of agarose concentrations. Helling et al. have constructed a similar plot using Eco·RI restriction products from a variety of DNAs (7). Figure 1 was constructed using Eco·RI restriction products from bacteriophage lambda, P1 and P1 hybrid DNAs. Figure 1 clearly shows how the use of differing percentages of agarose allows for molecular weight estimations of a much broader molecular weight range of DNA. Relatively large molecular weight DNA, $10 \times 10^6$ daltons (10 md), is best resolved on low percentage gels, and relatively small molecular weight DNA, one md, is best resolved on higher percentage gels. Figure 1 also shows that for a given agarose concentration, the plot of relative mobility versus log molecular weight is linear only up to a given molecular weight of DNA.

C. The Eco·RI limit digest of bacteriophage P1 DNA.

The limit digest of P1 virion DNA by the restriction endonuclease Eco·RI produced 17 minichromosomes ranging in molecular weight from 9.6 to 0.56 md.

Figure 2 graphically depicts the complete Eco·RI cleavage pattern of bacteriophage P1 and lambda DNAs. The lambda DNA Eco·RI bands serve as standards for determining molecular weights of other cleavage products run on the same gel. Figure 2 also illustrates the log molecular weight versus mobility plot of P1 and lambda Eco·RI minichromosomes run on a 0.7% (w/v) agarose
Fig. 1. Relative electrophoretic mobility versus molecular weight of noncircular, double-stranded DNA as a function of agarose concentration. Mobilities were determined, and curves were plotted using restriction products from bacteriophage lambda, PI and PI derivative hybrid DNAs. The Eco-R1 lambda fragments, and some Eco-R1 PI products were used as DNA molecular weight standards (7).
gel. Some of the molecular weights are taken from the first description of an Eco•R1 digest of P1 DNA (7). All molecular weights of minichromosomes depicted in Figure 2 were assessed by employing a variety of gel concentrations, ranging from 0.35 to 1.0% agarose, along with differing amounts of DNA ranging from one to 10 ug. Larger amounts of DNA were used with higher percentage gels to resolve small, quickly migrating minichromosomes. Smaller quantities of DNA were used with lower percentage gels to resolve large, slowly migrating minichromosomes. Note in Figure 2 that the log molecular weight versus mobility plot is linear for only a given molecular weight range of minichromosomes, thus necessitating the use of differing percentage gels for complete assessment of the population.

Analysis of many gels has revealed the 1.6-md P1 band to be either an unresolved doublet, or to contain DNA fragments from a chromosomal duplication. This observation has subsequently been confirmed (2). The combined molecular weights of the 17 P1 Eco•R1 minichromosomes, 0.56 md or above (assuming that the 1.6-md band is a doublet) is approximately 52 md. Eight lower molecular weight minichromosomes have also been detected using gradient pore acrylamide gels, as will be presented later.

D. Eco•R1 limit digests of some genetically hybrid P1 DNAs.

With an established Eco•R1 cleavage pattern for bacteriophage P1 DNA, comparisons can now be made with the Eco•R1
Fig. 2. Relative electrophoretic mobility versus molecular weight plot of the Eco•RI PI DNA restriction products (●) taken from a 0.7% agarose gel. The Eco•RI lambda restriction products (△) are used as standards for molecular weight determinations (7). The Eco•RI PI bands are graphically depicted along with their approximate molecular weights in $10^6$ dalton units.
cleavage patterns of some of the genetically hybrid Pl DNAs. In Figure 3, agarose gel electrophoresis patterns of the Eco·R1 mcp of 4 hybrid P1 DNAs and wild-type P1 DNA are compared. Figure 3 was constructed in order that actual photographs could be viewed in comparing P1 and P1 hybrid DNA cleavage patterns. The central gel of Figure 3, however, does not resolve all relevant features. All hybrid minichromosomes could not be compared on one gel for two main reasons: the molecular weight range of the P1 Eco·R1 produced mcp is too broad, and there is a wide range of DNA content per band in some of the hybrids (30 fold for P1dlac).

High molecular weight features are best resolved in the upper photograph of Figure 3. To clearly show lower molecular weight features of the P1dlac digest, photographs of differing amounts of DNA have been joined together. All molecular weight labels of the bands were established as previously described.

The far right portion of Figure 3 depicts all relevant data extrapolated from the photographs, in order that alterations of P1 genetic material, observed in the hybrid digests, can be viewed free of less relevant data. The figure should be referred to for information concerning sample quantities of DNA, and for electrophoresis conditions.

1. Eco·R1 limit digest of P1cat virion DNA

An Eco·R1 limit digest of P1cat virion DNA is shown in Figure 3. Eighteen minichromosomes of 0.56 md or larger are resolved. Compared to the P1 Eco·R1 digest, the P1cat digest
Fig. 3. Comparison of Eco-R1 digests of P1 and derivative hybrid DNAs in uniform agarose gels. All fragment molecular weight labels are in 10^6 dalton units. The lower Pldlac photograph is a composite derived from photographs of gels through which 2, 10, and 30 ug of DNA was fractionated. The following electrophoretic conditions were employed: top right, 2 ug of DNA at 30 V for 36 h through a 0.5% gel; top left, 5 ug of DNA for 24 h at 40 V through a 0.6% gel; bottom, 5 ug of DNA for 18 h at 40 V through a 0.7% gel. All gels were run at 4 C. The chart to the right shows the molecular weight designations of the novel Eco-R1 digest products of P1 derivative hybrid DNAs shown in the photographs. Molecular weight designations are in their approximate electrophoretic positions as compared to the Eco-R1 P1 digest control.
lacks only the 4.3-md P1 band. Two additional bands of 3.3 and 2.2 md, respectively, are also resolved. The 1.6-md Plcat band, as in the P1 digest, appears to be a doublet. The total molecular weight of all Eco·Rl Plcat minichromosomes seen in Figure 3, assuming the 1.6-md band is a doublet, is 54 md.

2. **Eco·Rl limit digest of PlargF virion DNA**

An Eco·Rl limit digest of PlargF virion DNA is shown in Figure 3. Nineteen minichromosomes are resolved of 0.56 md or larger. The PlargF digest, as in the Plcat digest, lacks only the 4.3-md P1 band. Three additional bands, 10.5, 8.0, and 1.9 md, respectively, are also resolved. The 1.6-md PlargF band also appears to be a doublet. The total molecular weight of all minichromosomes shown is approximately 68 md.

Ten PlargF hybrid DNAs have been characterized by their Eco·Rl limit digests. The digest shown in Figure 3 is the only PlargF Eco·Rl digest which has revealed an overlarge chromosomal element, such that one unit phage genome cannot be encapsidated genetically intact. The other digests characterized are all missing the 4.3-md P1 band, and show only one additional minichromosome band of 1.9 md. Digests of two of these hybrids are pictured in Figure 4. The only other difference in these digests from the one pictured in Figure 3, is that the observed fluorescent intensity of the 9.6-md PlargF bands indicates that it may contain an excess of DNA for its molecular weight. Widely varied fractionation conditions, including gel percentage,
Fig. 4. Eco•R1 limit digests of three PlargF hybrid DNAs. Three, 1, and 0.5 μg of DNA, respectively, were fractionated at 25 V for 23 h at 4°C, through a 0.7% agarose gel. Molecular weight labels are in 10^6 dalton units and were determined as described in the text using Eco•R1 lambda fragments as standards (7).
amount of DNA fractionated, and length and running voltage of electrophoresis, failed to resolve more than one band which migrated as 9.6 md. Assuming that the 9.6-md band is a singlet, and the 1.6-md band is a doublet, the PlargF hybrids in Figure 4 both have a total molecular weight of approximately 50 md. However, the 9.6-md band is believed to contain an excess of DNA; a problem which will be taken up later.

3. Eco·R1 limit digest of Pldcatsulstr virion DNA

An Eco·R1 limit digest of Pldcatsulstr (Pldcss) virion DNA is also shown in Figure 3. Nineteen minichromosomes are resolved of 0.56 md or larger. The Pldcss digest, as opposed to the P1cat and PlargF Eco·R1 digests, lacks both the 9.6- and 0.78-md P1 bands, but retains the 4.3-md P1 band. Four additional bands are resolved of 10.5, 3.3, 2.7, and 2.3 md, respectively. The 1.6-md band appears to be a doublet as in the P1, P1cat, and PlargF Eco·R1 digests. The combined molecular weight of all the Pldcss minichromosomes pictured in Figure 3 is approximately 70 md.

4. Eco·R1 limit digest of Pldlac virion DNA

The Pldlac Eco·R1 limit digest pictured in Figure 3 resolves 25 minichromosomes ranging in size from 15 to 0.56 md. The Pldlac cleavage pattern lacks the 9.6- and the 0.78-md P1 bands, as observed in Pldcss. In addition, the Pldlac Eco·R1 digest also lacks one member of the 1.6-md P1 doublet. Ten additional bands are resolved with the following molecular weights:
15, 12, 10, 8.3, 4.8, 3.3, 3.0, 2.9, 1.5, and 1.4 md. The 3.0- and 2.9-md bands are both doublets. To unambiguously establish that there is no Pldlac fragment at the 9.6-md position, Pldlac and Pldlac plus P1 digests are compared in the upper left photograph of Figure 3. The appearance of the 9.6-md fragment in the combined digest shows that the Pldlac fragment with the 8.3-md designation is indeed novel.

When fragments of a substrate chromosome are present in equimolar quantities, there is a uniform decrease in the DNA content of resolved bands with the molecular weight of the fragments. While this trend has been evident in the P1 digest, and the digests of the P1 hybrid derivatives thus far shown, the Pldlac Eco·RI digest presents a striking contrast. The partially resolved 15-, 12-, and 10-md Pldlac triplet contains less DNA than expected for its aggregate molecular weight, as observed from fluorescent intensity of these bands. The 4.8-md Pldlac band is also DNA poor. Densitometric tracing made from negatives of photographed gels have confirmed these visual observations. This point is mentioned here so as not to complicate later interpretation of these results. A meaningful estimate of the size of the Pldlac chromosome cannot be made from the Eco·RI digest shown here, without a more detailed, genetic description of the Pldlac chromosome. A molecular weight estimate of the Pldlac chromosome will be reserved for the Discussion.
E. Fractionation of low molecular weight Eco·R1 minichromosomes of P1 and derivative hybrid DNAs.

Gradient pore acrylamide gels (Pharmacia) have been employed to detect low molecular weight minichromosomes produced through Eco·R1 digests of P1 and P1 hybrid DNAs. These low molecular weight minichromosomes could not adequately be resolved on agarose gels.

In order to detect and resolve low molecular weight minichromosomes, gradient pore acrylamide gels were employed. Acrylamide is the favored material for low porosity gels. Acrylamide can be used at much higher concentrations than agarose, and thus enables the resolution of much lower molecular weight DNA molecules than agarose. Gradient pore acrylamide gels provide even greater resolution of low molecular weight DNA molecules because of the added self-sharpening quality of the decreasing porosity gel.

Figure 5 is a gradient pore acrylamide gel ranging from 5 to 30% (w/v) acrylamide. Low molecular weight Eco·R1 minichromosomes of P1, P1cat, P1argF, P1dcss, and P1dlac DNA have been resolved. One ug of DNA was used for fractionation of all digests except P1dlac where 1.5 ug was employed. Molecular weight estimates of minichromosomes are not given because of lack of low-md references for molecular weight extrapolations. The arrow on either side of the photograph in Figure 5 indicates the position of the 0.56-md minichromosome present in all P1 and
Fig. 5. Linear gradient pore acrylamide gel of Eco-R1 digest products of lambda, P1 and P1 derivative hybrid DNAs. The gel shown ranges in acrylamide concentration from 5 to 30%. One ug of DNA for each sample was fractionated at 40 V for 16 h at 4 C. The upper gel photograph resolves the overexposed bands of the lower photograph. The arrows indicate the approximate position of the 0.56-md P1 band shown in Figs. 2 and 3.
P1 hybrid Eco•Rl digests.

The importance of Figure 5 is two-fold: it illustrates that many lower molecular weight minichromosomes do exist in Eco•Rl limit digests of P1 and P1 hybrid DNAs and, most important, Figure 5 shows that all lower molecular weight hybrid bands do contain the low molecular weight P1 bands. It is estimated that approximately 2 md of DNA is contained in these lower molecular weight bands for each digest.

F. Sall limit digests of P1 and derivative hybrid DNAs.

Through communication with Dr. G. Haywood, University of Chicago, it was learned that a restriction enzyme was available which did not put any breaks in P1 DNA. This enzyme, Sall, was purified from Streptomyces albus G (1).

The primary concern with Sall was to assess whether this enzyme put breaks in any of the P1 hybrid DNAs. Figure 6 shows a Sall limit digest of P1, P1cat, P1argF, P1dcss, and P1dlac virion DNA. Approximately two ug of DNA was fractionated for each digest. The gel shown is a linear gradient pore gel ranging in agarose concentration from 0.5 to 1.0% agarose. A gradient gel was employed because preliminary analysis using uniform agarose gels provided poor resolution of high molecular weight bands, while low molecular weight minichromosomes quickly ran off of the gel and escaped detection. Figure 6 clearly shows that there are Sall cleavage sites within the bacterial gene regions of all P1
Fig. 6. Sall limit digests of P1 and P1 derivative hybrid DNAs. Samples consisted of 3 ug of DNA fractionated at 40 V for 16 h at 4 C, through a linear gradient pore agarose gel, ranging in concentration from 0.5 to 1.0%.
hybrid DNAs examined with the exception of Plcat.

Molecular weight estimates are not given for two reasons. First, uniform agarose gels, on which molecular weight estimates have been made from other restriction digests, failed to adequately retain or resolve the SalI restriction products. Second, gradient agarose gels, such as the one pictured in Figure 6, do not lend themselves to accurate molecular weight estimates. The main importance of Figure 6 remains, however, that there exists a restriction enzyme, SalI, which fails to alter P1 DNA, but which does alter the DNA of one of its nondefective hybrids, PlargF, and which also alters the DNA of two defective hybrids, Pldcss and Pldlac.

G. Eco•Rl-SalI combined digest of PlargF virion DNA.

Ten nondefective PlargF hybrid DNAs have been characterized through their Eco•Rl cleavage patterns. In an attempt to characterize these PlargF hybrids as a family, the Eco•Rl cleavage data has presented some problems.

The Eco•Rl limit digest of all ten PlargF hybrid DNAs are missing the 4.3-md P1 band as shown previously (Figs. 3 and 4). However, one of these digests, pictured in Figure 3, reveals an overlarge chromosomal element, as presented earlier. The other PlargF Eco•Rl digests, represented by the three pictured in Figure 4, did not reveal an overlarge chromosomal element. The only discrepancy in the PlargF digests of Figure 4 is that
the 9.6-md bands appear to contain an excess of DNA for their molecular weight.

An Eco•R1-Sall co-digest of the two PlargF hybrids shown in Figure 4 was performed in order to resolve whether the majority of bacterial gene DNA was hidden in the 9.6-md Eco•R1 band. Sall was chosen for the co-digest because it does not put any breaks in P1 DNA, but does have cleavage sites in the bacterial gene region of the PlargF hybrid DNAs. Therefore, any additional band(s) appearing in the co-digest, with a molecular weight(s) greater than 1.9 md (the only additional band observed in the Eco•R1 digests of these two PlargF hybrid DNAs) would confirm the presence of more bacterial gene DNA than observed in the Eco•R1 digests.

Figure 7 shows a comparison between the Eco•R1 and the Eco•R1-Sall co-digest of two PlargF hybrid DNAs. Note the appearance of two novel bands in the combined digests which do not appear in the single Eco•R1 digests. These two new bands, 9.0 and 8.0 md, respectively, could only have been hidden in the 9.6-md PlargF Eco•R1 band. The novel 1.9-md Eco•R1 band, which is present in all 10 PlargF Eco•R1 digests, is still present, and thus lacks a Sall cut site. The total molecular weight of combined-digest minichromosomes is approximately 67 md, thus showing the presence of an overlarge chromosomal element in all PlargF hybrids.
Fig. 7. Eco·R1 and combined Eco·R1-SalI digests of two P largF hybrid DNAs. Samples consisted of 3 ug of DNA fractionated at 30 V for 16 h at 4 C through a 0.4% agarose gel. Molecular weight labels were calculated as previously described using Eco·R1 lambda restriction products as standards (7).
CHAPTER IV

DISCUSSION

A. The Eco•R1 limit digest of bacteriophage P1 DNA.

The first fractionation of an Eco•R1 limit digest of bacteriophage P1 DNA revealed the presence of 15 fragments with a molecular weight greater than 0.6 md (7). A more detailed description of the Eco•R1 limit digest of P1 virion DNA in both uniform agarose and gradient pore acrylamide gels is presented (Figs. 1 and 3). Amendments to the original published data are as follows.

First, the 1.6-md P1 band has been recognized as an unresolved doublet. Second, the originally reported 0.67-md singlet band has been resolved as a doublet of 0.68 and 0.65 md, respectively. Third, lower molecular weight fragments have been detected employing fractionation through both uniform agarose and gradient pore acrylamide gels (Figs. 3 and 4). Minichromosomes detected on agarose gels, 0.56 md or greater, have a combined molecular weight of approximately 53 md. The lower molecular weight bands as detected on gradient pore acrylamide gels add approximately 2 md to the 17 minichromosomes detected on agarose gels.

B. Comparison of Eco•R1 limit digests of a few P1 hybrid virion DNAs.
P1 virion DNAs differ from their counterpart plasmid DNAs in being linear, permuted, and terminally redundant (11). Since all of the digests shown in Figure 3 are those of virion DNAs, the question arises whether cleavage patterns of a population of virion DNAs are an accurate assessment of the chromosomal structure of their plasmid DNAs. In order to resolve this question, an Eco•R1 digest of P1dlac plasmid DNA, supplied by J. Bornhoeft and M. Stodolsky, was performed. All plasmid digest bands detected are present in the virion DNA digests and vice versa. Thus, we are assuming for P1dlac, P1, and the other (less defective) hybrids, that cleavage patterns of a population of virion DNAs are indeed indicative of plasmid DNA digests. This assumption has subsequently been confirmed (2). By the same reasoning, absences or additions of Eco•R1 produced minichromosomes in P1 hybrid virion DNA digests, as compared to the P1 control, do reflect alterations in the corresponding plasmid chromosome.

Deletions of minichromosome(s) in hybrid digests, as compared to P1 are subject to the following interpretation. Absence of a single minichromosome band in hybrid digests implies the insertion of foreign genetic material somewhere in the corresponding P1 minichromosome. Absence of 2 or more minichromosomes in hybrid digests implies the loss of some P1 genes, with the subsequent insertion of the foreign genetic element somewhere in the genetic region defined by the missing P1 minichromosomes.

The following is a summary of deletions of Eco•R1
produced P1 minichromosomes observed in the P1 hybrid DNA Eco·R1 cleavage patterns shown in Figure 3. P1cat and P1argF lack solely the 4.3-md P1 band. P1css lacks the 9.6- and the 0.78-md P1 bands. P1dlac lacks the 9.6-, the 0.78- and one member of the 1.6-md P1 doublet.

An interpretation of these results is as follows: cat and argF elements are encoded in the 4.3-md P1 segment. The css element is encoded in the P1 genetic region defined by the 9.6- and 0.78-md bands, and lac is encoded in the P1 genetic region defined by the 9.6-, 0.78-, and one 1.6-md P1 segment.

As stated earlier, mapping data obtained through bacteriophage crosses has shown that the cat-gene 2 region of the P1 chromosome, without exception, is involved in the aberrant recombinational events underlying the de novo formation of the genetically hybrid, specialized transducing derivative of P1 (26). At least one of the sites of union of P1 and foreign genetic sequences is in the cat-gene 2 genetic region. Therefore, there has been no fine structural information on the cat-gene 2 region. Gene 2 function has been shown to be altered in P1dlacs (20), but there has been no simple assay for the presence or absence of the cat insertion locus in P1dlac. Results presented here show that there is an Eco·R1 cut site(s) separating loci of cat and argF from that of css and lac. Cat and argF are encoded to the left of this site, and css and lac to the right. An Eco·R1 cleavage map has been constructed and the order of
these P1 DNA Eco•Rl segments is 4.3, 9.6, 0.78 and 1.6 (2). 

The cat-gene 2 region of the P1 chromosome has been shown to be a hot spot for aberrant recombination (26). Based on the Eco•Rl cleavage data presented here, the following finer resolution of the cat-gene 2 genetic region has been established. First, a unique recombinational hot spot does not exist. Aside from this fact, the following possibilities do exist: either there are at least two unique hot spots on the P1 chromosome where foreign genetic elements are inserted, or there is an extended region of the P1 chromosome considered to be a hot spot for aberrant recombinational events.

For continuing studies of P1 aberrant recombination, this finer resolution of the cat-gene 2 genetic region provides great utility in P1 hybrid DNA heteroduplex studies. For instance, a heteroduplex of PlargF and Pldlac DNA should have the following structure: argF insertion loop - duplex DNA region - lac insertion loop/corresponding gene 2 deletion loop. This theoretical heteroduplex is depicted in Figure 8. Similar heteroduplexes could be constructed between Plcat and Pldlac, Plcat and Pldcss, and PlargF and Pldcss. Electron microscopic measurements would reveal the size of the two foreign genetic elements, the distance between their sites of integration, and the corresponding size of the P1 gene deletion.
C. Molecular weight estimate of the Pldlac plasmid.

As stated in Results, there are complications in estimating the size of the Pldlac plasmid chromosome from the Eco·R1 limit digest of a population of Pldlac virion DNAs shown in Figure 3.

When fragments of a substrate chromosome are present in equimolar quantities, there is a uniform decrease in the DNA content of resolved bands with the molecular weight of the fragments. While this trend is clearly evident in most of the restriction enzyme digests presented here, the Pldlac Eco·R1 digest presents some complications.

The additive molecular weight of all Pldlac Eco·R1 fragments is about 110 md. However, the 15-, 12-, 10-, and 4.8-md Pldlac Eco·R1 digest bands do not appear to be present in equimolar quantities as assessed from gel photographs. To use this information in formulating an estimate of the size of the Pldlac plasmid chromosome, recent data on the lac region of the E. coli chromosome plays an important role.

Recent electron microscopic analysis of the lac region of the E. coli chromosome has revealed a 47-md region containing genetic regions on either size of lac which are inverted repeats of one another. This region has been designated $\alpha_3\beta_3$-lac-$\beta_5\alpha_5$ where $\alpha_3\beta_3$ and $\beta_5\alpha_5$ are inverted repeats of one another (9). If this region is encoded in the bacterial region of Pldlac, the anomalous DNA content of the aforementioned Pldlac Eco·R1 bands
Fig. 8. Graphic depiction of a DNA:DNA heteroduplex of the cat-gene 2 genetic regions of Pldlac and PlargF.
could be explained as follows.

A heterogeneous population of Pldlac plasmids could arise from the process of recombinational inversion, whereby one-half of the Pldlac plasmid population would contain the above region of DNA in the order $\alpha_3\beta_3$-lac-$\beta_5\alpha_5$, and the other half would contain this genetic region in the order of $\alpha_5\beta_5$-lac-$\beta_3\alpha_3$. In the Eco•R1 digest of virion DNA obtained from this heterogeneous population of plasmid DNA, each of the $\alpha\beta$ sequences would be represented on two distinct Eco•R1 minichromosomes. However, these fragments would only be present in one-half molar quantities with respect to the other Pldlac Eco•R1 minichromosomes. Thus, the presence of an invertable repeat may be responsible for the anomalous DNA content of the 15-, 12-, 10-, and 4.8-md Pldlac Eco•R1 minichromosomes.

Two assumptions are thus being made concerning the structure of the Pldlac plasmid chromosome, based on its Eco•R1 limit digest cleavage pattern. One, the $\alpha_3\beta_3$-lac-$\beta_5\alpha_5$ E. coli chromosome segment is encoded in the bacterial gene segment of the Pldlac plasmid. Two, this segment is represented by four minichromosomes of 15, 12, 10 and 4.8 md, respectively, each present in one-half molar quantity. A minimal molecular weight estimate of the Pldlac plasmid can be made by assuming that the 15- and 4.8-md segments are present in the inverted recombinant. Thus, if the total molecular weight of all Eco•R1 minichromosomes is 110 md, a minimal size estimate of the Pldlac plasmid is
88 md \((110\-(12\+10))\).

In estimating the size of the P1 and bacterial gene segments of the Pldlac plasmid, it is not known whether or not the missing Eco•R1 P1 bands, totaling 12 md, are present in novel Pldlac bands. Therefore, the possible molecular weight range of the P1 and bacterial segments of the Pldlac plasmid are 54-42 md, and 34-46 md, respectively.

It is apparent that the data confirm deductions of Rae and Stodolsky (20) that the Pldlac plasmid chromosome is too large to be transduced genetically intact, and that even the bacterial segment of the Pldlac plasmid must frequently be split during scission of the Pldlac chromosome into virion chromosomes.

D. Sall cleavage of P1 and P1 hybrid DNA.

A restriction enzyme from the bacterial species Streptomyces albus G, Sall, has been shown to leave normal P1 DNA virtually unaltered. The nondefective P1 hybrid derivative, P1cat, has also been shown not to possess any Sall susceptible genetic sequences. Three specialized transducing derivatives of P1, the nondefective PlargF, and the defective hybrids P1css and P1dlac, have been shown to possess genetic sequences which are susceptible to cleavage by Sall. These results have been presented in Figure 5.

Two important facts emerge from these Sall digests. First, it is indeed striking that a DNA molecule the size of P1
plasmid DNA (53 md) does not contain any Sall susceptible cleavage sites. Second, it is equally as important that a non-defective hybrid derivative of P1, P1argF, does contain Sall susceptible sites in its bacterial gene region. This second point requires some expanded explanation.

As presented earlier, P1argF lysogens yield a population of virions which are indistinguishable from P1 in all normal functions, including net virion yield and relative production of normal and small capsid morphology variants. Therefore, Sall can be used to convert a circular P1argF plasmid into a linear molecule with all P1 genes left unaltered. This is essentially the same as putting a single, site specific break in P1 plasmid DNA, converting it from a circular to a linear molecule, without altering any P1 functions. This fact is of enormous potential importance and some valuable information concerning the plasmid will aid in an explanation.

P1dlac has been shown here, by analytical procedures, and previously by purely genetic data (20), to be overlarge with respect to the size of P1 plasmid. One unit phage genome of P1dlac DNA cannot be encapsidated genetically intact. However, the P1dlac plasmid has been shown to be extremely stable, and to replicate faithfully in conjunction with the host chromosome. Therefore, the P1 plasmid is certainly capable of maintaining and replicating excess foreign DNA which has been inserted into its genome. The problem with P1dlac lies in the fact that some P1 genes are missing as a result of lac insertion, and important
P1 functions are thus either altered or missing.

Now that methods are available for putting a single break in P1 DNA, through the use of SalI and PlargF, foreign genetic material can be inserted into P1 DNA, sealed with ligase, and put back into a permissive host with subsequent maintenance and faithful replication. In essence, any DNA can be inserted into the P1 genome and faithfully passed to successive generations.

E. Combined Eco•RI-SalI digests characterizing all PlargF hybrids as a family.

Ten PlargF hybrid DNAs have been characterized by their Eco•RI cleavage patterns. In an attempt to classify these hybrids as a PlargF family, the Eco•RI cleavage data have presented some problems. All Eco•RI PlargF DNA digests revealed the absence of the same P1 genetic material of 4.3 md. However, all digests were not consistent in the relative amounts of bacterial gene DNA resolved.

Figure 3 contains an Eco•RI limit digest cleavage pattern of DNA from virions of one PlargF hybrid lysate. Nineteen minichromosomes with a combined molecular weight of approximately 70 md are resolved. Thus, for this PlargF hybrid, the Eco•RI data reveals an overlarge chromosomal element, when compared to the P1 control, meaning the corresponding PlargF plasmid contains too much DNA to be transduced genetically intact. However, for the other 9 PlargF hybrids characterized, represented by the
three Eco·Rl limit digests in Figure 4, the Eco·Rl data resolves no overlarge chromosomal elements. Thus, these PlargF hybrid derivatives initially appeared to contain a small enough amount of DNA to be transduced genetically intact.

One difference, however, is evident when the Eco·Rl digests of the PlargF hybrids pictured in Figure 4 are compared to the P1 control. The 9.6-md PlargF band appears, by eye, to contain an excess of DNA. Some PlargF bacterial gene DNA could possibly be hidden in this 9.6-md band, although differing fractionation conditions were unable to resolve more than one band.

To resolve this possibility an Eco·Rl-SalI combined digest was performed. SalI was used, as stated earlier, because it had been shown not to put any breaks in P1 DNA, but to have cleavage sites in the argF bacterial gene region. Thus, if the combined digest revealed more DNA than represented by the 1.9-md PlargF band present in all hybrid digests, some bacterial gene DNA must have been hidden in some of the normal P1 bands.

The combined Eco·Rl-SalI digest of the two PlargF hybrid DNAs whose single Eco·Rl digests are depicted in Figure 4, are shown in Figure 7. Excess DNA was revealed by the combined digests amounting to approximately 17 md, not revealed in the single Eco·Rl digests. Two new bands were resolved of 9.0 and 8.0 md, respectively. This DNA could only have been hidden in the 9.6-md Eco·Rl digest band. The combined molecular weight of all co-digest minichromosomes is approximately 68 md.
All 10 PlargF hybrids can thus be characterized as a family. All are nondefective in P1 functions, and each hybrid chromosome is overlarge with respect to the P1 control. Therefore, their entire chromosome cannot be transduced genetically intact.
CHAPTER V

SUMMARY

There exists a region on the P1 chromosome which has been shown, without exception, to be involved in the aberrant recombinational processes underlying the \textit{de novo} formation of the genetically hybrid P1 derivatives. This genetic region has been named \textit{cat-gene 2}. In an attempt to further define this genetic region, on a purely analytical basis, the technique of cleavage analysis has been employed. Agarose and acrylamide gel electrophoresis have been the chief tools used in identifying genetic differences between respective P1 hybrid minichromosomal populations produced by restriction enzymes. Some qualitative information has also been obtained through fractionation of P1 and its derivative virions on cesium chloride density gradients.

Four P1 hybrid derivatives were analyzed by equilibrium density centrifugation with respect to the relative distribution of small (P1S) and normal size (P1B) capsid virions. \textit{P1cat} and \textit{PlargF} lysogens are identical to P1 in virion capsid size distribution. In contrast, both \textit{Pldcss} and \textit{Pldlac} lysogens show excessive production of small capsid morphology variants. Thus, both \textit{Pldcss} and \textit{Pldlac} lysogens have mutations affecting virion capsid size.

The cleavage analysis of these four P1 hybrids has mainly
involved the use of the restriction enzyme Eco·R1. Eco·R1 limit
digests of P1, P1cat, PlargF, Pldccs, and P1dlac virion DNAs were
fractionated on both agarose and acrylamide gels. Comparison of
the hybrid digests with that of the P1 control revealed that
there exists an Eco·R1 susceptible site separating the loci of
integration of argF and cat, from that of lac and css. There-
fore, there does not exist a unique site in the cat-gene 2
region of the P1 chromosome where aberrant recombinational
processes occur.

Molecular weight estimates were also made in agarose gels
from Eco·R1 limit digests of P1 and its hybrid DNAs. The molecu-
lar weight estimates revealed that PlargF, Pldccs, and P1dlac
plasmid DNAs are overlarge with respect to the P1 control, such
that one unit phage genome of DNA cannot be transduced geneti-
cally intact. P1cat plasmid DNA is not overlarge and one unit
phage genome can be transduced genetically intact.

In addition to Eco·R1, the restriction enzyme Sall was
also used for cleavage analysis. Sall does not put any breaks in
normal P1 DNA. Sall limit digests of P1 and derivative hybrid
DNAs were performed. All hybrid DNAs were shown to possess Sall
susceptible cleavage sites with the exception of P1cat DNA. Of
greatest importance here is the fact that a nondefective P1
hybrid, PlargF, possesses Sall cleavage sites in its bacterial
gene region. Thus, Sall and PlargF plasmid DNA can affectively
be used to put a single break in P1 plasmid DNA, thus allowing
for the insertion of foreign DNA into the P1 plasmid chromosome.

An Eco·R1-Sall combined digest was performed on some PlargF hybrid DNAs whose single Eco·R1 digests failed to adequately resolve any excess of DNA as compared to the P1 control. The combined digests did reveal an excess of DNA which allowed all ten PlargF hybrids used to be classified as a family.
LITERATURE CITED


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