The Intergenic Region Near the Right End of Bacteriophage Lambda DNA is Nonessential

by

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Submitted in Partial Fulfillment of the University
Undergraduate Fellows Program

1983 - 1984

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ABSTRACT

Viruses which infect bacteria are called bacteriophages; the bacteriophage lambda (λ) infects certain strains of the bacterium Escherichia coli K12. Near the right end of the linear DNA of bacteriophage lambda, from the coordinates 0.96 to 1.00, is a region of DNA approximately 2079 base pairs which is previously uncharacterized with respect to function; that is, no identifiable genes have been mapped in this region. In addition, no mutants have ever been isolated which contain either insertions or deletions of the region which spans the coordinates 0.96 to 0.98.

A 1576 base pair deletion of this region was constructed on a plasmid using recombinant DNA techniques and recombined back into lysogenic (dormant) phage. Once the deletion was physically characterized, the lysogens were placed under conditions which normally induce bacteriophage lambda development and maturation. The production of mature infectious viral particles was then assayed. It is shown that viable phages carrying the engineered deletion were isolated. Furthermore, the number of phage particles produced did not differ appreciably between deleted and undeleted lysogens. Thus, the introduced deletion did not affect bacteriophage lambda viability, and it is likely that if any genetic elements are contained in this region they are non-essential to lambda growth in the laboratory strain of Escherichia coli used.

INTRODUCTION

The bacteriophage lambda is one of the most extensively studied model systems for gene expression and regulation in prokaryotes. Almost the entire physical and genetic map of the lambda genome has been elucidated (8,10). In addition, sequencing of the DNA genome has been completed (2,8). This work has led to the most complete understanding of the genetic organization of any one organism. However, the lambda phage remains an enigma with respect to certain questions about its genetic composition. In the study which follows, one of these unanswered questions is addressed.

DNA extracted from mature lambda phage particles is a double-stranded, linear molecule which is 48,502 base pairs long (8) and has been completely sequenced through the combined work of Daniels, Sanger, Coulson, and Blattner (2,8). Right and left ends of the lambda DNA molecule are defined as shown in Fig. 1. The ends of the molecule are termed "cohesive" due to their ability to join, forming either circular or concatemeric molecules (3,4). In vivo circularization occurs after infection and in this form the DNA is actively transcribed into viral mRNA's. The lambda genes are transcribed into polycistronic messages containing up to twenty-four genes on a single mRNA. The polycistronic messages os lambda typically encode clusters of functionally related genes. Temporally the last of these mRNA's to be synthesized is referred to as the "late" transcript. The late transcript is initiated from a promoter (p'R) which is found at the right end of the linear

map (see Fig. 1) and is extended across the joined cohesive ends (called the cos site) through most of the left arm. The late transcript encodes four distinct functional domains of genes. The first is the cell lysis region, composed of the last three known genes of the right arm (S, R, and Rz); the next three domains follow the cos site on the left arm and are responsible for DNA packaging, "head" assembly, and "tail" assembly (Fig. 1). Most of the late transcript is arranged very compactly, end to end, with one major exception. Between the cell lysis gene Rz and the cos site lies an intergenic (i.e. between genes) region of 2079 base pairs; the function of this region is unknown (1). This right end region from gene Rz to the cos site (coordinates 0.96 to 1.00) does not appear by sequence analysis to code for any new late genes; that is, there are no significantly long open reading frames (1). In addition, a specific region near the right end (0.96 to 0.98 map units) appears to be highly conserved. While many types of lambda phage mutants have been isolated with deleted non-essential regions of DNA, no lambda phage has ever been described which has an insert into or deletion of this 1047 base pair region (2, 10). Here I describe the construction of such a deletion in the lambda right end DNA and investigate whether it is essential to the production of viable lambda progeny.

MATERIALS AND METHODS

Construction of Plasmid Containing Lambda Insert: A total of 2 ug purified lambda C1857 DNA (obtained from Ry Young, Dept. Medical Biochemistry, Texas A&M University) was first treated so that the linear molecules would be circularized by ligation at the cos site. Ligation was carried out using 1 unit of T4 ligase [Bethesda Research Laboratories] in a 15 ul reaction mixture containing 66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP with overnight incubation at 15°C. The ligated DNA was ethanol precipitated by adding sodium acetate to 0.3 M and 2 volumes of 95% ethanol with overnight incubation at -20°C. The DNA was pelleted by centrifugation at 13,000 x g for 15 min, dried, and resuspended in 30 ul medium salt restriction endonuclease reaction mixture containing 50 mM Tris-HCl, 10 mM $MgCl_2$, and 50 mM NaCl at pH 7.5 (7). Two ug of plac Q (Fig. 2, obtained from Tom Edlind, Dept. Medical Biochemistry, Texas A&M University) were added to the lambda DNA solution. The combined DNA was cleaved with 5 units each of Bam Hl and Eco Rl [Bethesda Research Laboratories] with 2 h incubation at 37°C. Half the sample was ethanol precipitated as described above; the remaining half was electrophoresed through a 0.7% agarose gel containing 40 mM Tris-HCl, 20 mM sodium acetate, 18 mM NaCl, 2 mM EDTA at pH 7.5 and visualized under UV light after staining with 1 ug/ml ethidium bromide in order to monitor cleavage by the restiction endonucleases. The ethanol precipitated, cleaved DNA was pelleted as above, resuspended in 15 ul ligase reaction mixture, and ligated with 1 unit ligase overnight at 15°C.

Identification and Isolation of Plasmids: The ligated DNA was transformed into frozen competent cells (6). The cell strain was CQ3 [Cold Spring Harbor Laboratories] which is lac I^q. Transformation was carried out for 30 min at 0°C followed by heat-shock at 43°C for 1 min and incubation at 37°C for 1 h in 5 ml Luria broth (7). Approximately one fifth of the culture was plated on Luria broth agar plates containing 50 ug/ml ampicillin, to select for the plasmid, and incubated overnight at 37°C. Two hundred colonies were replica plated on agar plates containing either 50 ug/ml ampicillin or 15 ug/ml tetracycline, in order to monitor loss of tetracycline resistance; that is, insertion into the tetracycline resistant region of plac Q. Approximately 40% of the ampicillin resistant cells were also tetracycline sensitive. All of the tetracycline sensitive cells were further screened by a transactivation assay; cells were inoculated into Luria broth containing 0.4% maltose and 10 mM $MgCl_2$ and incubated on a roller at 37°C for 4 h. Each culture was then plated with 2 ml each soft agar (0.7% agar) containing 0.4% maltose and 10 mM $MgCl_2$ on agar plates with 50 ug/ml ampicillin. The soft agar overlays were then spotted with dilutions of lambda 81 which has an R gene double amber mutation and lambda S7 which has an S gene amber mutation and incubated at 37° overnight. Transactivants were inoculated into 1 ml Luria broth and incubated at 37° overnight on a roller. Rapid plasmid preparations as described by Holmes and Quigley (5) were carried out. The cells were pelleted at 13,000 xg for 1 min. and resuspended in 0.1 ml STET buffer containing 8% sucrose, 5% Triton-X100, 50 mM EDTA, 50 mM Tris HCl at pH 8.0. The

suspensions were then boiled for 40 sec and the cell debris was pelleted for 10 min in an Eppendorf microcentrifuge. The supernatant was precipitated with 1 volume of isopropanol for 2 h at -20°C. The plasmid DNA's were then pelleted for 10 min in an Eppendorf microcentrifuge and desiccated for 15 min. The DNA pellets were resuspended in 30 ul meduim salt restriction endonuclease reaction mixture and each was cleaved with 2 units of Bam Hl and Eco Rl for 2 h at 37°C. These samples were then electrophoresed through 0.7% agarose. For a size standard, a sample containing 0.5 ug of purified lambda DNA was cleaved as above with Bam Hl and Eco Rl and electrophoresed in an adjacent lane.

Construction of the Right End Deletion: A 500-fold scaled up rapid plasmid preparation of the plasmid was performed as described above except the plasmid DNA pellet was resuspended in 10 mM Tris, 1 mM EDTA at pH 7.5 to a concentration of approximately 1 mg/ml and stored at 4°C. Approximately 2 ug of the plasmid isolated above was transformed into GM119 cells (obtained from Ry Young) which have a dam mutation and produce unmethylated plasmid DNA. Selection was carried out in the presence of 50 ug/ml ampicillin and a large scale rapid plasmid preparation was performed with final resuspension of DNA in 10 mM Tris, 1 mM EDTA at pH 7.5. Then 2 ug of the unmethylated DNA was cleaved with Bcl 1 in meduim salt restriction endonuclease reaction mixture for 2 h at 37°C. Half of the sample was ethanol precipitated as above and half was electrophoresed to monitor the cleavage. The precipitant was pelleted as above and treated with

1 unit of the ligase at 15°C overnight. The ligated DNA was then transformed in CQ3 cells as above and colonies were picked and rapid plasmid prepped as previously described. The purified plasmids were then cleaved with Bam Hl and Eco Rl followed by electrophoesis as above.

Characterization of Lambda Lysogens: Lysogens constructed from W cells and containing Lambda DNA with S and A gene amber mutations were obtained from Ry Young. The W(Lambda S^- A^-) lysogens are also C1857 mutants which are heat inducible at 42°C and viable at 30°C. The lysogen was streaked out on two agar plates and incubated at 42°C and 30°C, respectively. In addition, a 15 ml culture of the lysogens inoculated from a single colony of the plate incubated at 30°C above was incubated with shaking in a 250 ml Erlynmeyer flask to a cell density of approximately 1.2 $O.D._{550}$ at which point it was incubated at 42°C for 15 min followed by shaking at 37°C for 2 h. The culture was then frozen at -80 °C for 1 h and thawed at 37°C for 15 min; the cell debris was spun out by centrifugation for 10 min at 11,000 rpm. Dilutions of 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} were mixed with 0.2 ml of W cells (approx. 5 x 10^8 cells/ml) incubated at 37°C for 5 min and plated on agar plates with 2 ml soft agar. The plates were incubated at 37°C overnight.

Assaying Lambda Phage Production With and Without the Deletion: Both the deleted and undeleted plasmids called pP'_R-B and pP'_R-BΔBcl, respectively, were transformed as above into competent W(Lambda S^-A^-) lysogens, and plated on agar plate with 50 ug/ml ampicillin; incubation was carried out at 30°C overnight. One colony each of W(Lambda S^-A^-),

W(Lambda S^-A^-)/ pP'R-B and W(Lambda S^-A^-)/ pP'R-BΔBcl was inoculated into 1 ml appropriate Luria broth (i.e. 50 ug/ml ampicillin in the media for the plasmid containing cells) and incubated at 30°C overnight on a roller. The overnight cultures were then diluted to approximately 5×10^7 cells/ml and incubated with shaking at 30°C for approx. 2.5 h until cell density was approx. 1.0 $O.D._{550}$ at which time they were incubated with shaking at 42°C for 15 min followed by incubation with shaking at 37°C for 2 h. The cultures were then frozen at -80°C for 1 h and thawed at 37°C for 15 min. The cell debris was spun out for 10 min at 11,000 rpm. Dilutions of 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} were mixed with W cells (laboratory strain) and incubated at 37°C for 5 min followed by plating with 2 ml soft agar plus 0.4% maltose on an agar plate. The plates were then incubated at 37°C overnight. The above titrations were repeated with soft agar plus 0.4% maltose and 10 mM $MgCl_2$.

Purification and Characterization of Lambda DNA: The rapid lambda DNA purification described by Silhavy was performed (9). Plaques obtained above were streaked out for isolation; then individual plaques were picked and incubated with 0.1 ml of a two-fold concentrated overnight culture of MH1160 cells (obtained from Ry Young) suspended in 10 mM $MgCl_2$ for 5 min at room temperature. Then 2 ml of Luria broth was added and the culture was rolled at 37°C for approx. 6 h until the cells lysed. After lysis 0.1 ml chloroform was added with vigorous mixing and cell debris was spun out for 10 min at 7,000 rpm. The supernatant was extracted 1:1 with 75% DE 52 cellulose and 15% Luria

broth; the solid fraction was pelleted for 30 sec in an Eppendorf microcentrifuge. The supernatant was then spun again to remove any remaining DE 52. Then phenol extraction was carried out twice, 1:1 using distilled washed phenol saturated in 10 mM Tris-HCl and 1 mM EDTA at pH 7.5. From this 10 ul was placed in medium salt restriction endonuclease reaction mixture and cleaved with 5 units each of Bam Hl and Eco Rl, incubated at 37°C for 2 h. Samples were electrophoresed as above.

RESULTS

Experimental Approach and Stategy

To explore the role of the right end of lambda DNA, I set out to isolate a deletion of the region and determine its effect on phage viability. Previous attempts by others have failed to isolate such a deletion *in vivo* (10); for this reason, I decided to construct the deletion *in vitro*. It would have been extremely difficult to engineer a deletion in the entire lambda genome using recombinant DNA techniques. Instead, I decided to isolate out a relatively small fragment of the right and left end region and place it in a plasmid (Fig. 3). Using this plasmid, the construction of the desired deletion would be straightforward. The deletion could then be recombined *in vivo* into an entire lambda DNA molecule. For this purpose a lysogen would be used; a lysogen is a cell containing a resident lambda DNA integrated into the host cell's chromosome. The lambda DNA remains dormant and is passed vertically, generation to generation, until some environmental stimuli causes induction of the lambda DNA to become active at which time it initiates expression of viral genes. Using a lysogen, I was able to introduce plasmids containing deletions and allow the cells to mediate recombination between the plasmid and the lysogen lambda DNA. This permitted me then to assay the production of mature lambda phage particles upon induction. A non-deleted plasmid was also used in order to make a comparison of the production of undeleted to deleted lambda phage.

Plasmid Isolation and Characterization

The previously described plasmid was constructed; it contained a lambda DNA Eco Rl—Bam Hl fragment which spans the S gene to the B gene across the cos site (Fig.3). The fragment was inserted into the tetracycline resistance region of plac Q (Fig.2). The plasmid was characterized by cleaving it with Bam Hl and Eco Rl then comparing it to Bam Hl-Eco Rl cleaved lambda DNA after electrophoresis (Fig.4). The plasmid was thus shown to carry the proper lambda insert. This insert contains the right end region of interest which conveniently contains two Bcl l sites, one located in the Rz gene the other approximately 1576 base-pairs away in the right end intergenic region (Fig.3). Cleaving the plasmid with Bcl l and then ligating together the two sites served to remove the portion of lambda DNA cooresponding to coordinates 0.956 to 0.988. This plasmid was characterized to have the appropriate deletion as above (Fig.4). During the characterization of these plasmids it became apparent that a spontaneous deletion of the plasmid had occured of approximately 1000 base-pairs. Further restriction characterization revealed that the deletion had occured in the plac Q portion of the plasmid and included the lac promoter and operator, the Q gene and most of the sequences just prior to the lambda late promoter p'R (Fig.2 and 3). the deletion did not disturb the Eco Rl-Bam Hl lambda insert, however, so it was not deleterious to our purposes.

Introduction of Deleted DNA Sequences Into the Lambda Genome

To introduce the characterized deletion into the lambda genome I made use of a temperature sensitive lysogen which is thermally induced

at 42°C. The particular lysogen used was W(Lambda S^-A^-); the host cell is W, and the resident lambda has two mutant genes S and A located on either side of the right end intergenic region. Upon heat induction all the viral gene products were synthesized except for genes S and A. Gene S is required for lysis of the cell and liberation of mature phage; gene A functions to package mature lambda DNA into immature heads (proheads). As a result the cells did not lyse except when freeze-thawed at -80°C and then 37°C and then only proheads, unattached tails, and free lambda DNA were obtained (Fig.5). After the lysogens were transformed with the plasmid (pP'R-B) a small percentage of the plasmids inside the lysogens underwent homologus recombination with the resident lambda DNA. In a small number of these recombinants the two crossover points spanned the genes S and A (Fig.5) and included the right end intergenic region. Phage produced from these recombinants had thus acquired functional S and A genes and presumably the intergenic region as well. Upon thermal induction then, some viable lambda phage were produced and titered (Fig.5 and Table 1). Approximately 6.07×10^5 plaque forming units (PFU)/ml were produced. Next, the deleted plasmid (pP'R-BΔBcl) described previously was transformed into a W(Lambda S^-A^-) lysogen and the experiment above was repeated. Approximately 4.96×10^5 PFU/ml were produced. The number of recombinant phage produced in the two situations did not differ appreciably (Table 1).

In order to prove that the region in question was indeed exchanged, loss of the Rz phenotype was demonstrated and DNA from presumed recombinants purified and shown to be deleted in the right end.

In the absence of the Rz gene phage are still produced normally except in the presence of 10 mM $MgCl_2$ when many cells fail to lyse (10). Titering of deleted phage was carried out in the presence of 10 mM $MgCl_2$ with a two-fold decrease in the number of plaques observed, approximately 2.91×10^5 PFU/ml ; no difference was obseved in the undeleted phage (Table 1). Finally, plaques produced from phage presumed to be deleted were used to prepare purified lambda DNA (9). Approximately 0.1 ug of the purified DNA was cleaved with Eco R1 and Bam H1 and examined by agarose gel electrophoresis. The results comfirm that the lambda DNA isolated was indeed deleted by approximately 1576 base-pairs in correspondance with the restriction fragment where it should have occured (Fig.6).

DISCUSSION

I constructed a plasmid using recombinant DNA techniques bearing the right end region of lambda DNA (Fig.3). This region, carried on the plasmid, recombined with full length lambda S^-A^- DNA *in vivo*, producing viable lambda S^+A^+ phage (Fig.3). Using this approach, I have demonstrated that a plasmid deleted in the intergenic region of the right end can also recombine with full length lambda S^-A^- DNA *in vivo*, again producing viable lambda S^+A^+ phage. Approximately the same number of phages were produced in both instances. I have shown by both physical (Fig.6) and genetic (Table 1) evidence that the lambda phage obtained are products of the recombination event described above.

I conclude, having constructed a deletion of a previously uncharacterized region of lambda DNA (1,10) spanning coordinates 0.96 to 0.98 near the right end, that this region is non-essential to phage production. I cannot, ofcourse, exclude the possibility that this region may serve an essential function in other strains of *E. coli*.

The implications of this discovery with respect to lambda's utility as a cloning vector are significant. Lambda phages have approximately 20,000 base-pairs of DNA which can be removed and replaced with "foriegn" DNA without affecting lambda viability (7). This property along with lambda's ability to integrate into bacterial chromosomes and transfer genetic information from host to host has made lambda bacteriophages universally employed cloning vectors. The discovery made here increases the total replacable DNA in lambda to approximately 21,600

base-pairs.

Finally, two remaining questions must be addressed. Why hasn't this deletion been previously isolated and what possible role if any could the right end intergenic region play? It is unlikely that given the extensive investigation of lambda this deletion if naturally occuring, as is the case with other non-essential regions, has simply been overlooked. I suggest that though this region is non-essential it is not "naturally" deletable. Certain DNA sequences contain physical properties which promote natural deletion such as the presence of direct or inverted repeats of the nucleotide sequence (7). The right end intergenic region may simply lack the proper physical properties to be deleted in vivo. In terms of the possible role of this non-essential region I suggest that it may function in a very subtle way to affect not the manner but the amounts of late gene expression. Non-coding sequences of DNA can potentially play a role in affecting gene expression through the formation of nucleotide secondary structure. This region could form secondary structures such as hairpins or in the case of long-range base pairing stem and loop structures which might influence protein-RNA interactions such that levels of gene expression are enhanced or depressed. This effect though subtle in terms of our ability to measure it might have proven favorable in the course of lambda evolution.

In any case I have discovered an unknown fact involving the bacteriophage lambda and add it to the already extensive knowledge gathered on this organism.

TABLE OF FIGURE LEGENDS

FIGURE 1. The genetic,physical, and functional structure of bacteriophage lambda. the lambda map system is shown on the middle line. Genes and an indication of their functions are shown above the line. (Daniels, Sanger, and Coulson, 1983, C.S.H.S.Q.B. 47:1009)

FIGURE 2. Restriction and genetic map of plac Q. It contains the lac promoter and operator, the lambda late promoter p'R, the lambda Q gene, and the genes coding for tetracycline and ampicillin resistance.

FIGURE 3. Restriction and genetic map of the plasmid pP'R-B. The entire plasmid is approximately 11559 base-pairs long. It was constructed by cloning the indicated lambda sequences (heavy line) into the Eco Rl-Bam Hl sites of plac Q. The lambda insert is 9035 base-pairs long and contains the lysis genes S,R, and Rz; the intergenic region; the ligated cos site; and the head proteins Nul, A, W, and B. In addition, the plasmid has the genes coding for ampicillin resistance.

FIGURE 4. Restriction characterization of the plasmids pP'R-B and pP'R-BΔBcl. Both plasmids were cleaved with Eco Rl and Bam Hl and electrophoresed on agarose gel. Lambda was also cleaved with Eco Rl and Bam Hl and run along side the samples as a size marker.

FIGURE 5. Heat induction of W(Lambda S^-A^-) with and without pP'R-B. The lysogens contain a resident lambda S^-A^- DNA integrated into the host chromosome. Upon induction no viable phage are produced. In the presence of the plasmid pP'R-B (S^+A^+) recombination can occure after induction and viable lambda (S^+A^+) phage produced.

FIGURE 6. Restriction characterization of phage obtained from lysogens with pP'R-B and pP'R-BΔBcl. Purified lambda DNA's were cleaved with Eco Rl and Bam Hl. Samples were electrophoresed on an agarose gel.

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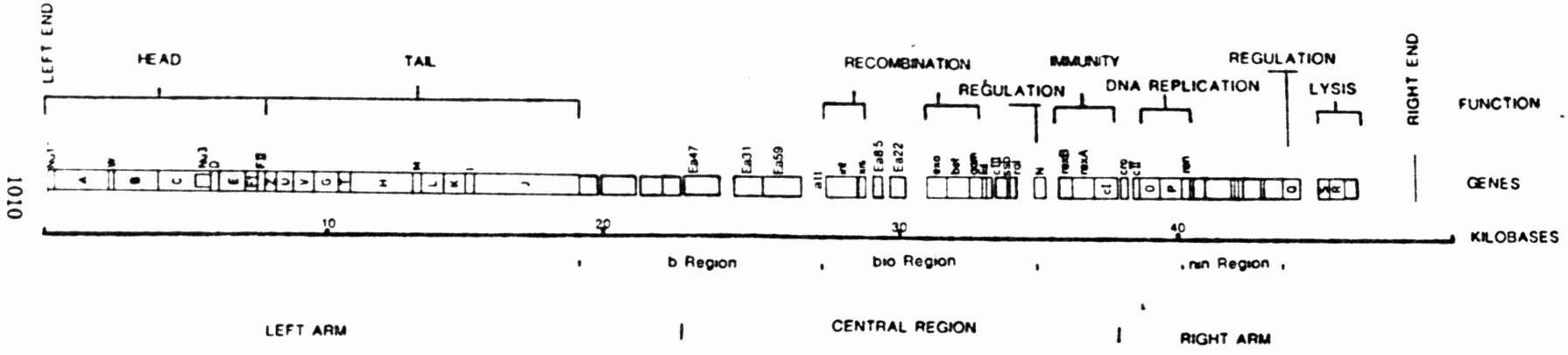
LEFT END
HEAD
TAIL
RECOMBINATION
IMMUNITY
REGULATION
REGULATION
DNA REPLICATION
LYSIS
RIGHT END
FUNCTION
GENES
KILOBASES
10
20
30
40
b Region
bio Region
nin Region
LEFT ARM
CENTRAL REGION
RIGHT ARM

FIGURE 1

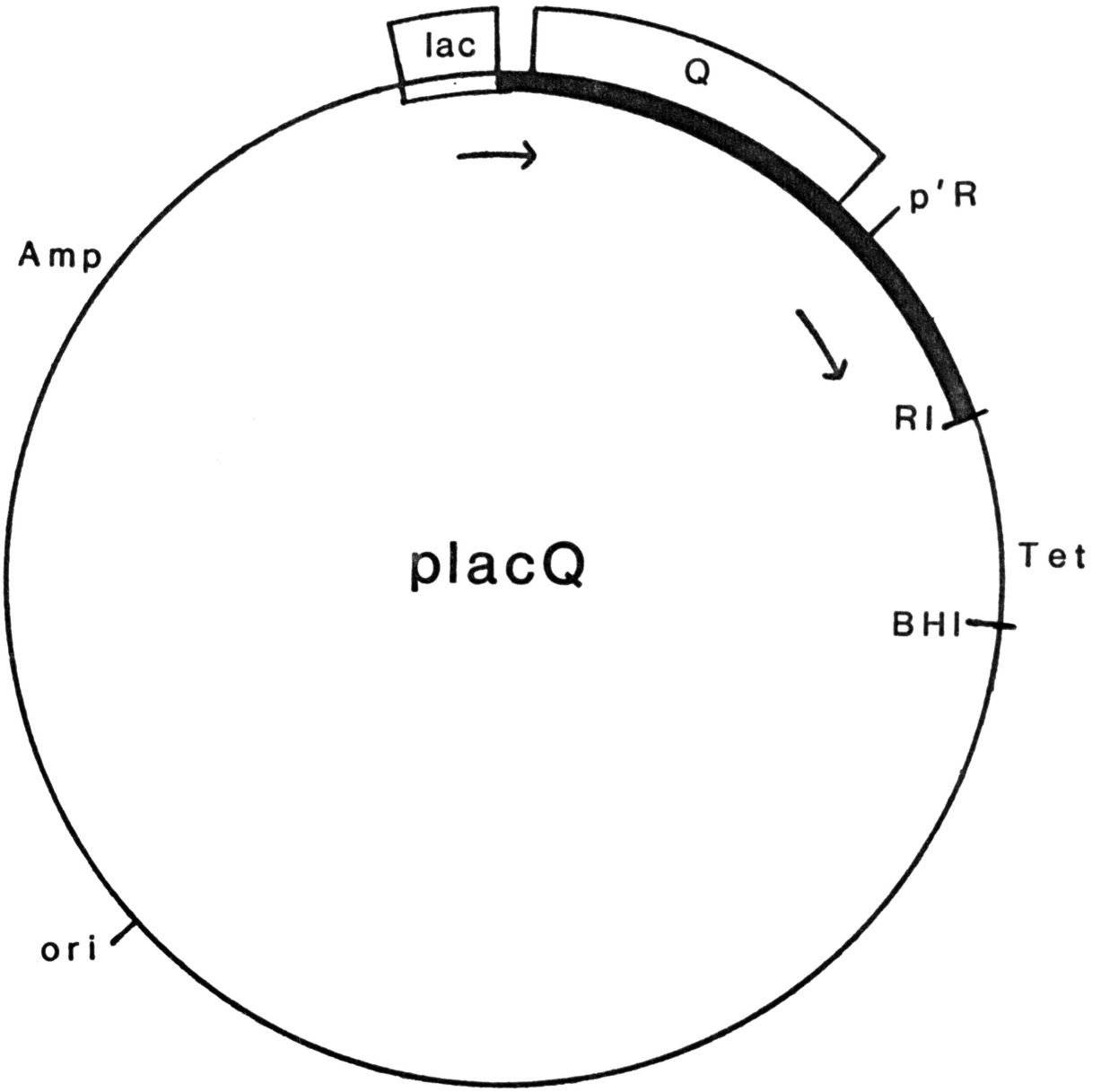

FIGURE 2

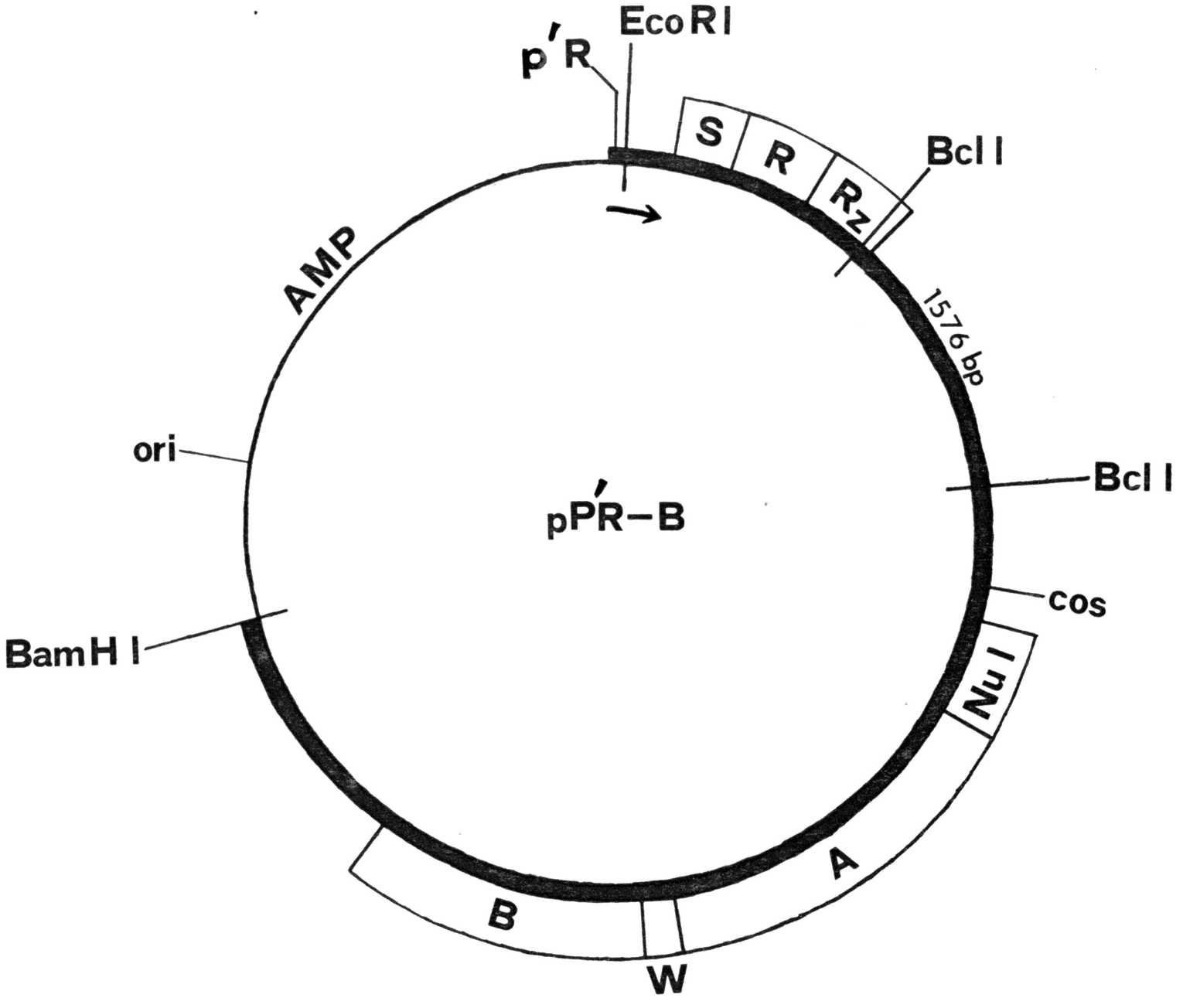

FIGURE 3

Restriction Characterization

of Plasmids

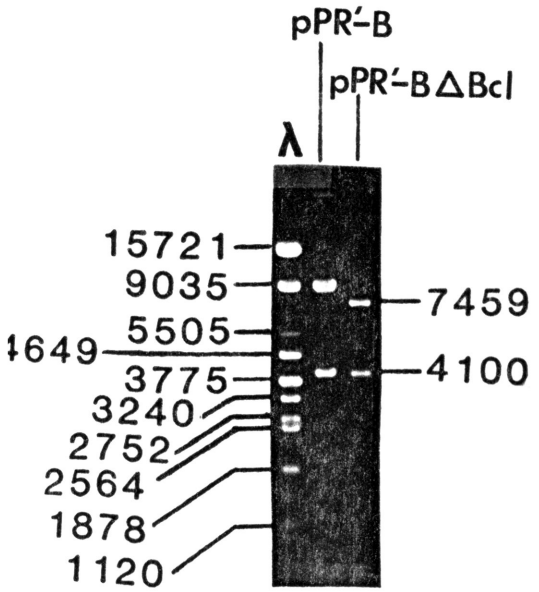

FIGURE 4

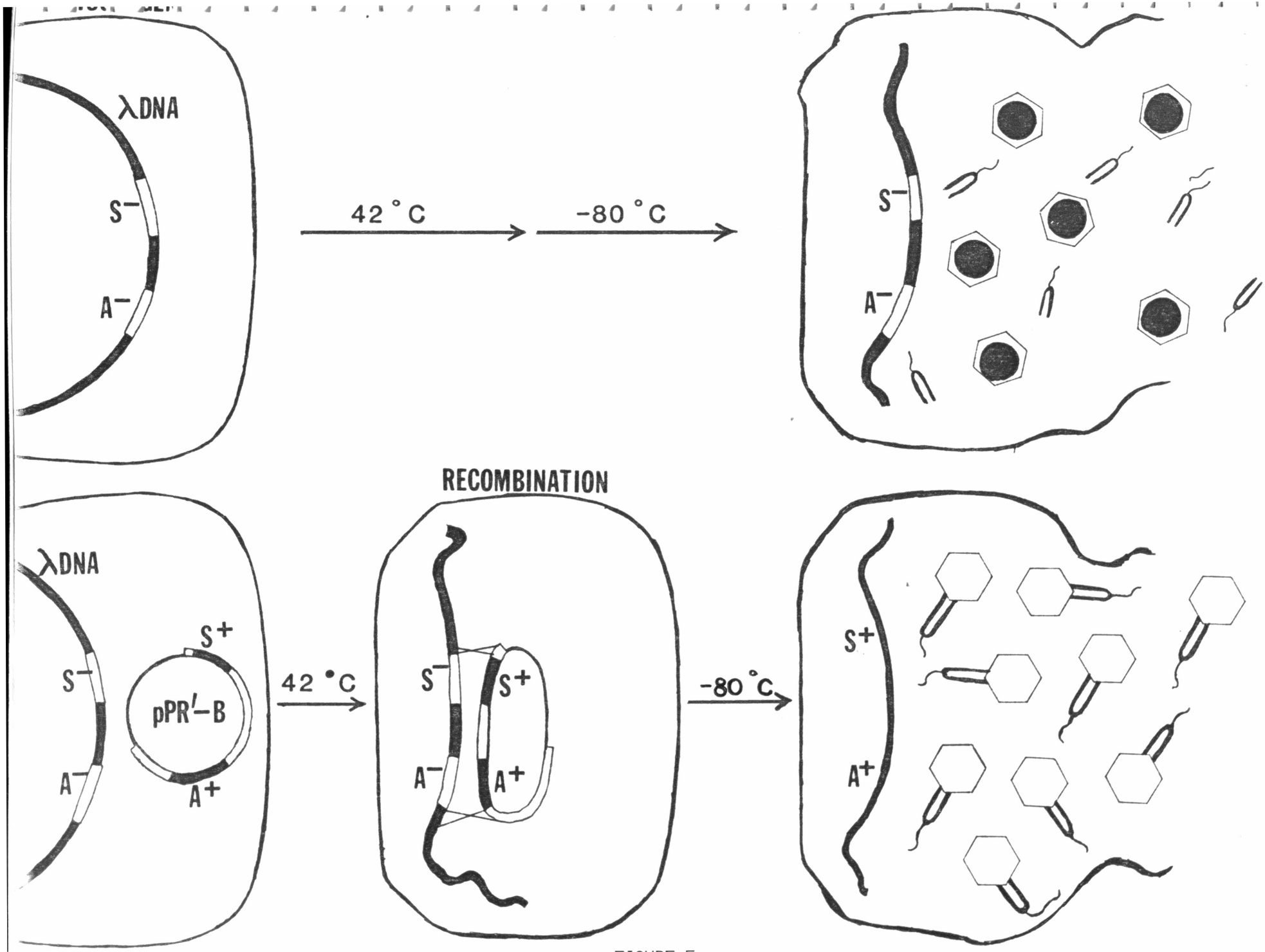
λDNA
S−
A−
42 °C
−80 °C
S−
A−
RECOMBINATION
λDNA
S−
A−
S+
pPR′−B
A+
42 °C
S−
A−
S+
A+
−80 °C
S+
A+

FIGURE 5

Restriction Characterization of Phage DNA

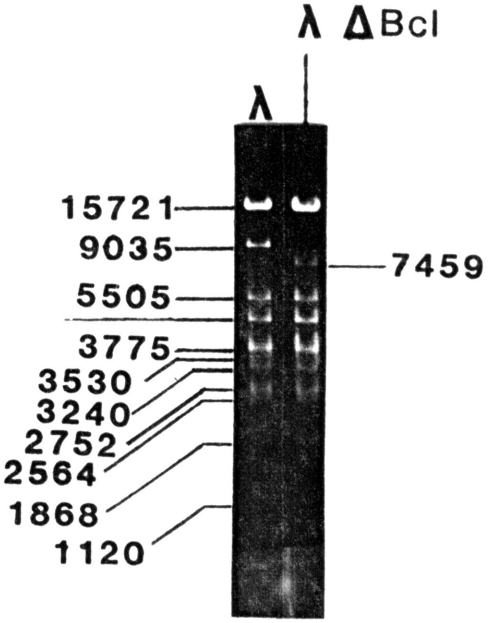

FIGURE 6

TABLE 1

THE NUMBER RECOMBINANT PHAGE PRODUCED

	# RECOMBINANT VIABLE PHAGE (PFU/ML)
W($\lambda S^- A^-$)	< 10
W($\lambda S^- A^-$)/pPR'- B	5.73×10^5
W($\lambda S^- A^-$)/pPR'- B Δ BCL	4.96×10^5
W($\lambda S^- A^-$)/pPR'- B +10 MM MGCL	5.60×10^5
W($\lambda S^- A^-$)/pPR'- B Δ BCL +10 MM MGCL	2.91×10^5