

QR
342
R85x
1983

ABSTRACT

Perry Walker Russell. ENZYMATIC CONSTRUCTION OF BACTERIOPHAGE ØX174
MUTANTS WITH INCREASED GENOME SIZES. (Under the direction of Dr. Uwe R.
Muller, Department of Microbiology, E.C.U. School of Medicine)
Department of Biology, 1983.

The icosahedral bacteriophage ØX174 is a single-stranded DNA virus with a genome containing 5,386 nucleotides. It codes for 10 genes and has four intergenic or intercistronic regions which are transcribed but not translated. One of these regions located between the two major capsid component genes J and F has been proven non-essential. Insertion of additional nucleotides into a portion of this region did not disrupt normal phage growth.

The described work made use of this information to investigate how much DNA could be inserted into the ØX174 genome before phage viability was lost. This was attempted experimentally using modern recombinant DNA methodology to construct ØX174 genomes with increasing genome sizes *in vitro* and introducing these into the appropriate host cell. Before this could be accomplished, it was necessary to devise a biochemical scheme for the construction and isolation of such mutants.

The scheme involved site-specific mutagenesis of the J-F intercistronic region of a previously constructed insertion mutant of ØX174. Cleavage of a unique site in the J-F region of this mutant with a restriction endonuclease allowed for insertion of DNA fragments of varying sizes by an enzyme which ligates DNA ends together. Successful

insertions are a relatively rare event in this reaction, but since the original unique restriction site is lost in these cases, the desired mutant genomes could be enzymatically selected for. By using the appropriate combination of restriction enzymes, genomes without an insert (i.e. parental) were fragmented and rendered non-infective. Subsequent infection of *E. coli* host cells resulted in propagation of mutant bacteriophages which could be isolated and studied. This scheme was used in three separate insertion experiments resulting in several mutants with genomes larger than any previously constructed ØX174 phage. The largest of these contains an insert which increases its genome approximately 340 nucleotides above that of wt ØX174.

The conclusions drawn from this study are as follows:

- a) The genome of ØX174 can be enlarged by at least 6.3% without loss of viability.
- b) The maximum number of nucleotides which can be inserted remains to be determined but appears to be less than 550 (10.2% of the genome).
- c) The insertion mutants obtained appear to grow less efficiently than the parental strain.
- d) The system of overlapping genes in the single-stranded icosahedral DNA phages must have evolved due to factors other than physical constraints in packaging the DNA into the phage head.

ENZYMATIC CONSTRUCTION OF BACTERIOPHAGE ØX174
MUTANTS WITH INCREASED GENOME SIZES

A Thesis

Presented to

the Faculty of the Department of Biology
East Carolina University

In Partial Fulfillment
of the Requirements for the Degree
Master of Sciences in Biology

by

Perry Walker Russell

May 1983

ENZYMATIC CONSTRUCTION OF BACTERIOPHAGE ØX174
MUTANTS WITH INCREASED GENOME SIZES

by

Perry Walker Russell

APPROVED BY:

SUPERVISOR OF THESIS

Uwe R. Muller
Uwe R. Muller, Ph.D.

THESIS COMMITTEE

Wendall E. Allen
Wendall E. Allen, Ph.D.

W. James Smith
W. James Smith, Ph.D.

Takeru Ito
Takeru Ito, Ph.D.

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Charles E. Bland
Charles E. Bland, Ph.D.

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette
Joseph G. Boyette, Ph.D.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. Uwe R. Muller for his guidance and patience during this study. His friendship and advice are greatly appreciated.

I am also grateful to Dr. Wendall E. Allen, Dr. W. James Smith and Dr. Takeru Ito for their patience during the preparation of this manuscript.

The author gratefully acknowledges the support of the East Carolina University Medical School which provided a student research assistantship and the money which made this study possible.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	v
INTRODUCTION	1
MATERIALS AND METHODS	11
RESULTS	19
DISCUSSION	61
LITERATURE CITED	65

LIST OF FIGURES

FIGURE	Page
1. Scheme for the construction of ϕ X174 <i>am</i> 3 mutants with increased genome sizes	20
2. Ligation for Insertion Experiment I	24
3. Pvu II - Sst II restriction analysis of mutant RF DNAs from Insertion Experiment I	29
4. Hae III restriction analysis of insertion mutants from Insertion Experiment I	31
5. Hpa II restriction analysis of insertion mutants from Insertion Experiment I	34
6. Hha I restriction analysis of insertion mutants from Insertion Experiment I	37
7. Ligation for Insertion Experiment II	40
8. Hae III restriction analysis of insertion mutants from Insertion Experiment II	45
9. Hae III restriction analysis of the largest insertion mutant from Insertion Experiment II	47
10. Ligation for Insertion Experiment III	51
11. Hae III digest of isolated RF DNAs from Insertion Experiment III	56
12. Triple digest with Pvu II, Sst II and Pst I	58

LIST OF TABLES

TABLE	Page
1. HPLC fractionation of Hae III digested pBR322 DNA	17
2. Transfection results of Insertion Experiment I	26
3. Transfection results of Insertion Experiment II	42
4. Transfection results of Insertion Experiment III	53
5. Summary of Insertion Experiments I, II and III	60

INTRODUCTION

ØX174 is a member of a family of small, tail-less, icosahedral bacteriophages whose genomes consist of circular single-stranded DNA (14). The ØX genome is composed of a 5,386 nucleotide positive strand (71), which is packaged in a capsid only 25 nm in diameter. The genome codes for 10 genes, three of which are overlapping genes (5, 21, 88). Extensive research has been done on the products of these genes in order to elucidate their functions. Gene A codes for two proteins designated A and A^{*}. The A protein is a site specific endonuclease necessary for progeny RF DNA synthesis (18, 23, 38). Evidence also suggests that the A protein cleaves unit length viral DNA at a specific site during single-stranded DNA synthesis and packaging (25, 38, 47). A^{*} is initiated in gene A within the same reading frame as the A protein (50). Its function is not well understood, yet there is an indication that it is involved in the shutoff of host DNA synthesis (16, 49, 52). A^{*} is also found associated with a precursor structure in phage maturation and may be involved in morphogenesis (26, 86). The products of genes B, C and D have all been proven essential components in the assembly of progeny virus particles (27, 57, 74) while gene E protein is required for lysis (40, 64). Genes J, F, G and H code for the capsid proteins (11). J and F proteins constitute the main structure of the capsid while proteins G and H compose the spikes (15). An analysis of the capsid structure has suggested that there are 60 copies each of the F and G proteins and 12 copies of H (11). On the basis of this a model was derived in which the F proteins aggregate into 12 pentamers with

each pentamer forming a vertex of the icosahedron. The G proteins also form 12 pentamers and associate with the 12 vertices. Since the J protein is a major capsid component, it is believed to be made in equimolar amounts to that of protein F (84). Its orientation on the capsid is unclear but it has been proven essential in capsid assembly (1). Each of the 12 spikes is thought to contain one copy of the H protein. In addition to being a spike protein, the gene H product may also have several other functions. One is recognition and binding to the host lipopolysaccharide receptor site (41, 43). The G spike protein also seems to be involved in this reaction (89). After attachment, the H protein may act as a pilot protein by guiding the single-stranded viral DNA through the cell membrane to its replication site (43, 44). Other studies indicate a function of the H protein in morphogenesis (75, 78). A function for gene K protein has yet to be found and there are still some virus specified functions that have yet to be ascribed to a particular gene product.

A brief description of the ϕ X174 life cycle begins with conversion of the single-stranded genome into a circular double-stranded replicative form (RFI) by host replication enzymes (46, 73). Formation of this double-stranded template allows transcription of the phage genes by host RNA polymerase, initiating at 3 promoter sites (65). Only the newly synthesized negative strand is transcribed (24). Translation of the message and accumulation of the A protein leads to nicking of the + strand of the supercoiled RFI molecule, allowing progeny RF DNA molecules to form by the rolling circle model of replication (30).

Towards the end of the infectious cycle, rolling circle intermediates become complexed with viral proteins involved in morphogenesis leading to synthesis and packaging of single-stranded viral genomes (29). Finally, the E protein causes lysis of the cell releasing new virus particles.

The morphogenetic pathway of ØX174 is a complex process in which synthesis of single-stranded viral DNA is closely coupled with capsid assembly. A model has been proposed for this process involving the ØX encoded proteins A, A^{*}, B, C and D as well as the capsid components (29). In the capsid model mentioned earlier, proteins F and G were each postulated to aggregate into pentameric forms (11). *In vivo* studies confirmed that these aggregates exist as precursor particles sedimenting at 9S (F pentamer) and 6S (G pentamer) (85). Precursor particles sedimenting at 12S were also found. These were determined to be formed by a protein B mediated aggregation of the 9S and 6S particles (74). With gene D product acting as a scaffold protein (27, 29, 90), 12 of these 12S particles are assembled into a 108S complex. Up to this stage, assembly is independent of DNA replication. However, at this point protein C is believed to bind RFII DNA (nicked circle) molecules preventing their conversion to the closed RFI form (27). This allows the 108S precursor structure to form a 50S complex with the RFII DNA. ØX proteins A, A^{*}, J and H are also associated with this complex (25). It is within the 50S complex that simultaneous synthesis and packaging of viral DNA is believed to occur. Viral DNA synthesis only undergoes one round of replication within the 50S complex, at which point unit length viral DNA is cleaved by the gene A protein (26). This cleavage

dissociates the 50S complex producing an RFII DNA molecule and a capsomeric structure containing circular single-stranded viral DNA. The RFII DNA molecule can be recycled and used for viral DNA synthesis within another 50S complex. In this model the encapsidated DNA would be expected to be linear. Yet, such an intermediate has never been observed indicating a very rapid ligation of the ends of the linear single-stranded DNA after A protein cleavage. A recent study suggests that the A^{*} protein may be responsible for this rapid single-stranded ligation (17). Upon circularization of the viral DNA, the capsomeric structure becomes a 132S particle. Analysis of this particle indicates that it is composed of the capsid constituents J, F, G and H in association with protein D (28, 90). There is no *in vivo* evidence that the 132S particle is converted into a mature 114S particle but under the appropriate *in vitro* conditions the D protein can be removed resulting in a mature 114S virion (28, 90). This suggests that the 132S particle may be the final intracellular product of ØX morphogenesis which upon lysis is converted to the 114S virus particle (29). Possible functions for ØX gene products A, B, C, D, F and G are included in this ØX174 morphogenetic model. Although essential, the function of the gene J product in the maturation process is still unclear due to a lack of gene J mutants. The function of gene H product is also ill defined. However, studies on amber H mutants of ØX174 (75, 76) have shown the production of two kinds of defective particles. One, sedimenting at 110S, was not infectious but contained infectious viral DNA. The other particle sedimented at 70S and contained non-infectious linear single-

stranded DNA shorter than genome length. Based on this, it has been proposed that the absence of a properly functioning H protein results in defective phage particles whose DNA has been left open to attack by nucleases (42). A more recent study using gene H mutants supports this view (78).

The *in vivo* studies that contributed to the development of the ØX model for morphogenesis have made it possible to develop *in vitro* systems that can form mature virions from precursor elements. These studies were first directed towards developing a system that mimicked the *in vivo* mechanism of viral DNA synthesis (58, 81). In 1977, a system using *in vitro* complementation for proteins B and C was used to synthesize infectious ØX174 particles (59), confirming the necessity of ØX proteins B and C. Under the electron microscope, the *in vitro* particles looked the same as *in vivo* 114S particles. Recently, single-stranded plasmid DNAs carrying the A protein recognition and cleavage site of ØX174 were packaged inside a ØX174 capsid using the same components needed for phage synthesis from ØX174 RFI DNA (2). This study proved that the protein A recognition sequence is the only part of the ØX174 sequence necessary for single-stranded DNA synthesis and packaging as long as all necessary proteins are provided. More than that, it establishes an effective *in vitro* system for determining the amount of DNA that can be packaged into the ØX174 capsid. Preliminary results from experiments using this system indicate the possibility of packaging plasmid DNA 540 nucleotides larger than the ØX174 genome (3).

G4 is a single-stranded DNA phage closely related to ØX174 and is believed to have similar mechanisms of replication and assembly (33).

The G4 life cycle is very similar to the ϕ X life cycle with the exception that G4 requires fewer host enzymes for parental RF DNA synthesis (95, 96). Sequencing of the G4 genome revealed that it codes for the same 10 genes as ϕ X174 and that their genetic maps are virtually identical (32). Comparative sequence analysis also reveals a 67% conservation of amino acid and nucleotide sequences between these two phages (33). G4 genes J, F, G and H also code for the capsid components and their functions along with the other G4 proteins are considered to be the same as their ϕ X174 counterparts (13). Complementation experiments using the G4 capsid proteins F, G and H have in fact shown that these proteins can be used in the ϕ X174 virion assembly system (10). Therefore it appears that the G4 and ϕ X capsids are very similar in size, yet the G4 genome contains 5,577 nucleotides (32) which is 191 nucleotides more than that of ϕ X. This raises the question of whether it is possible to package an additional 191 bases into the ϕ X capsid and still obtain a viable mutant phage. This question may be answered by an experiment designed to insert additional DNA into the ϕ X genome without disruption of any essential functions. Since all of the genes appear to be essential, additional DNA would have to be inserted in between two genes. Such a system has been developed in a different group of single-stranded DNA phages having filamentous capsids.

The filamentous bacteriophages differ greatly from the icosahedral viruses in that their capsids are α -helical rod-like structures. Also, their mode of host entry as well as their morphogenetic pathway are completely different from the isometric phages (13, 53, 66). A property

of the filamentous phages (M13, fd etc.) is that the size of the virion is determined by the amount of DNA packaged (19, 35, 70, 94). It is this property which has made the filamentous phages so useful as cloning vehicles and transducing particles (39, 54). Most of these kinds of studies involved *in vivo* or *in vitro* insertions into essential regions of the genome, requiring the use of helper phages for propagation of the mutants. However, in more recent experiments DNA has been inserted into non-essential areas of intergenic regions (56, 67). One of these studies involved site directed *in vitro* insertion of foreign DNA into a cleaved intergenic restriction site (56). In this experiment, a portion of the *E. coli lac* - operon, containing the regulatory region and part of the β -galactosidase gene, was inserted into M13 RF DNA. The mutant constructed (M13 mp1) was viable and expressed the part of the β -galactosidase gene inserted. This allowed for biological identification of the mutant by α -complementation of β -galactosidase resulting in blue plaques (48). By creating a unique Eco RI restriction site in the β -galactosidase portion of M13 mp1 a new mutant (M13 mp2) was developed that could be used as a cloning vector or for insertion and study of regulatory elements (55). This or similar mutants could also be used for studies on the packaging capacity of the filamentous phage capsid. In contrast to the filamentous phages, the icosahedral capsid of ϕ X174 as well as the other isometric viruses places physical constraints on the amount of DNA that can be packaged (93).

In 1977, sequencing of the ϕ X174 genome allowed for determination of its genetic map and revealed the existence of 4 intergenic regions

(71). Since then, several other viral genomes have also been sequenced and shown to have intercistronic regions varying in their size and number depending on the genome being examined. The ØX related phage G4 has 6 of these regions (32) while the eucaryotic viruses SV40 (22, 68) and polyoma (12) each contain 2. Bacteriophages fd and M13 (6) contain 6 intercistronic regions. In M13, one of these regions was used as the target site for cloning (56), as described above. The detailed function of intercistronic regions remains largely unknown, but since they are transcribed and not translated, they are postulated to be of regulatory significance. This is supported by two findings. First, they have been shown in several cases to contain regulatory signals such as promoters or terminators of transcription, ribosome binding sites or an origin of replication (33, 73, 91). Second, these regions generally have a larger potential to form stable secondary structures than the translated regions (60, 61). In the genome, secondary structures have been proposed to have an important role in the regulation of replication (77, 80), transcription (69, 79) and translation (34) by acting as potential binding sites for proteins. Despite their possible regulatory importance, there is evidence that parts of at least some intercistronic regions in ØX174, G4 and other viruses are not essential (62, 63, 72, 87).

Next to the M13 system, the most extensively studied intercistronic regions have been those of ØX174. One such study dealt with the region between genes J and F (62, 63). This region is 36 nucleotides long and contains in addition to the F gene ribosome binding site a potential hairpin structure implicated to function as a terminator of

transcription (20, 23). The absence of an assay for this putative function was overcome by using a biochemical rather than biological screening procedure for the construction and identification of mutants in this region. Within the J-F hairpin structure is a sequence recognized by restriction endonuclease Hae III. Starting with single-stranded phage DNA and a cloned fragment containing this sequence, it was possible to construct and cleave this site specifically. DNA fragments were ligated into this site to construct insertion mutants. The success of this technique depended upon having sufficient amounts of DNA to monitor the reaction steps and on the enzymatic destruction of infective wt ϕ X DNA molecules. The results of this experiment indicated that it is possible to make changes in part of the J-F region without destroying the essential function of the gene F ribosome binding site. Furthermore, it became clear that additional DNA could be inserted into the ϕ X genome without loss of viability. The largest mutant obtained in this study was ϕ X174 *ins*11 containing 163 additional bases. Although less efficient than wt ϕ X174, it was still viable (63).

Although the study in the previous paragraph was not designed to test how much additional DNA can be packaged into an isometric capsid, it did show that DNA insertion into ϕ X174 is possible without disrupting any vital function of the life cycle. This finding plus the larger genome size of the closely related phage G4 and preliminary results from *in vitro* packaging experiments with ϕ X174 suggest the feasibility of inserting and packaging even more DNA into ϕ X174 than has been done so previously. The question of how much DNA can be packed into an isometric capsid is relevant in light of the potential use of virus

vectors in eucaryotic genetic engineering. SV40 may possibly be used as such a vector and its capsid is very similar in size to that of ØX174.

The thesis work presented here represents an attempt to determine how much additional DNA can be inserted and packaged into the isometric capsid of ØX174. The experiments are based on a newly devised scheme for construction and selection of insertion mutants of ØX174. This scheme is dependent upon the availability of a previously constructed ØX insertion mutant containing a unique restriction site in the J-F intercistronic region. Cleavage of this site allows for ligation of increasingly larger DNA fragments into the ØX genome. Because of the fixed volume of an isometric capsid, there should be a limit in the genome size which can be packaged and exceeding this limit should result in the loss of viability. It was hoped that a plot of virus viability vs. genome size would result providing useful information for studies with isometric eucaryotic viruses such as SV40 and polyoma.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The plasmid pBR322 (82) was propagated in *E. coli* C600 R⁻M⁻ (36). *E. coli* HF4714 (7) containing an amber suppressor was the permissive host for all phage strains used in this study. The non-permissive host used was *E. coli* C.

The bacteriophage strains used in this study were ØX174 *am*3 (40) and several mutant strains derived from it by either insertion or deletion of nucleotides within the J-F intercistronic region (62). Strain ØX174 J-F *ins*11 contains an insert of 163 bp. Strains ØX174 J-F *del*3 and ØX174 J-F *del*2 have lost 27 bp and 4 bp respectively. Strain ØX174 J-F *ins*6 has a fragment from pBR322 of 115 bp inserted into the J-F intercistronic region. This fragment contains a Pvu II restriction site which is unique to ØX174. Because of this unique restriction site, strain ØX174 *ins*6 was chosen as the parental strain in this work.

Media and buffers. TYE media contained 10 g tryptone, 5 g yeast extract (both from Difco), 5 g NaCl and 3 g KCl per liter. TYE agar plates contained 1.5% (w/v) agar in TYE medium; for soft agar the concentration was reduced to 0.7% (w/v). The constituents of M9 supplemented medium have been described elsewhere (9). M9 supplemented medium containing 100 µg/ml ampicillin was used for growth of pBR322 transformed *E. coli* C600 R⁻M⁻ cells.

Standard buffer consisted of 10 mM Tris-HCl and 10⁻³ mM EDTA, pH 8.2. TAE buffer contained 40 mM Tris, 5 mM sodium acetate and 1 mM EDTA, pH 8.2. Peacock's buffer was composed of 90 mM Tris, 5 mM NaOH, 90 mM Boric acid and 2.2 mM EDTA, pH 8.3.

Bromophenol blue solution (8), consisted of 5 mg bromophenol blue, 1.0 ml SDS (1% solution), 1.0 ml EDTA (0.5 M), 0.3 ml peacock's buffer (10X) and 2.7 ml glycerol in a total of 5 ml.

Enzymes. Restriction digests with restriction endonucleases Pvu II, Pst I, Sst II, Hae III, Hpa II and Hha I were done in the buffers specified by the manufacturer (Bethesda Research Laboratories, Inc.). T4 DNA ligase was also purchased from BRL and used in the buffer specified. Ribonuclease A (Boehringer Mannheim GmbH) was boiled 10 min before use to destroy contaminating DNases. Lysozyme was obtained from Sigma and was generally mixed with 50 mM Tris (pH 8.0) to a concentration of 10 mg/ml.

Transfection and transformation. Transfection of *E. coli* HF4714 with RF DNA and transformation of *E. coli* C600 R⁻M⁻ with plasmid DNA were both carried out using a modification of a procedure described for the transformation of CaCl₂-treated cells (36). In the transfection experiments, 0.1 µg to 0.3 µg of RF DNA were added to CaCl₂-shocked cells and after a 1 min heat shock (37°C), these transfection mixes were incubated for 90 min on ice. Portions of the mixes were then added to 2.0 ml of soft agar at 47°C and plated onto TYE plates. Usually seed cells (100 µl), grown in culture to an optical density of O.D._{550nm}=0.5 were added to the soft agar along with each portion of the transfection mix. In transformation experiments, 10 µg of plasmid DNA were used to transform CaCl₂-shocked cells. Transformation mixes were also incubated for 90 min on ice. However; before the cells were plated, they were pelleted by centrifugation, resuspended in 0.5 ml of TYE medium (without ampicillin) and incubated for 45 min at 37°C. Portions of the cells

were finally spread onto TYE plates containing 100 g/ml ampicillin. Ampicillin resistant colonies were screened for the presence of pBR322 plasmid as described previously (4).

Preparation of phage stocks from plaques. Individual plaques were cut from TYE plates with a sterile pasteur pipette and placed into 1.0 ml cultures of *E. coli* HF4714 grown to an optical density $O.D._{550\text{ nm}} = 0.2$. These cultures were shaken at 37°C until lysis occurred. An additional 5 ml of *E. coli* HF4714 ($O.D._{550\text{ nm}} = 0.35$) were added to the 1.0 ml lysates and incubation continued until lysis occurred. Finally, 15 ml of *E. coli* HF4714 ($O.D._{550\text{ nm}} = 0.5$) were added to the 6 ml lysates. Once these 21 ml cultures had lysed completely, 10 ml were removed and stored under chloroform. Thirty ml of *E. coli* C ($O.D._{550\text{ nm}} = 0.5$) were added to the remaining 11 ml lysates. Eight minutes after addition of these cells, chloramphenicol was added to a final concentration of 100 µg/ml. Incubation was continued an additional 2 h and then the cells were pelleted by centrifugation. The pellets were either stored by freezing or immediately used in the miniprep procedure.

Miniprep procedure. The miniprep procedure is based on a previously described method for the preparation of plasmid DNA from small cultures (45). In short, the cells were resuspended in 0.5 ml of 50 mM Tris (pH 8.0) and then treated with lysozyme (1.0 mg/ml) for 15 min at room temperature. After lysing the cells, 0.5 ml of phenol (equilibrated with 50 mM Tris, pH 8.0) was added, the tube gently inverted and then immediately centrifuged for 15 min in a microfuge. The top aqueous layer was removed and extracted two more times with phenol. After addition of 30 µl sodium acetate (final concentration of

0.3 M) and approximately 1.0 ml of 95% ethanol, the tube was placed in a dry ice-ethanol bath for 5 min. The DNA was then pelleted by centrifugation for 5 min in a microfuge, resuspended in 0.3 M sodium acetate (100 μ l), extracted twice with ether and ethanol precipitated again. After a final ethanol wash (0.3 ml), the DNA was dried under vacuum and resuspended in 100 μ l of water. A 10 μ l sample was usually mixed with 5 μ l of bromophenol blue solution containing 0.5 mg/ml Ribonuclease A. After incubation for 15-20 min at 37°C, the sample was electrophoresed on an agarose gel (1.4%) containing ethidium bromide (1 μ g/ml). From the intensity of the DNA bands on the gel, the total amount of DNA was approximated. The remaining 90 μ l of DNA were used for restriction enzyme digests.

Gel electrophoresis. Agarose gel electrophoresis was done on horizontal slab gels (37) using TAE buffer in both the gel and the buffer reservoirs. Generally the gels contained 1.4% agarose (w/v). The voltage and length of time for running a gel was variable, depending upon the type of samples loaded. Most gels were stained in an ethidium bromide solution (5 μ g/ml) for 15 min and then destained in water for 15 to 30 min. After ethidium staining, the DNA bands were visible under ultraviolet light. Gels were photographed with Polaroid high speed black and white film (Type 57).

Vertical polyacrylamide slab gels were used to separate smaller DNA fragments (51). Gels were generally continuous, containing 4% polyacrylamide (w/v) and 25% glycerol. Peacock's buffer was used in the gels and in the buffer reservoirs. Gels were usually run at 80 V for 16 h and then stained, destained and photographed as described above.

Determination of restriction fragment size. The migrational distance for each restriction fragment was measured on the photographs and plotted versus the fragment size (in base pairs) on semilog graph paper. Restriction fragments of known lengths were used as size markers. Using these plots, the sizes of new fragments in mutant RF DNA digests could be determined with an accuracy of ± 5 bp.

Preparation of Pvu II linearized parental RF DNA stock. ØX174

6 RF DNA was purified as described before (36). Sixty-two μg of this parental RF DNA were digested to completion with 62 units of restriction endonuclease Pvu II. After digestion, the linearized RF DNA was phenol extracted twice in order to remove the enzyme and any contaminating exonucleases. The phenol was removed by 3 ether extractions and the DNA was then dialyzed against water. The dialyzed DNA was dried under vacuum and then resuspended in 100 μl of sterile water. This stock of Pvu II linearized parental RF DNA was used in each of the succeeding insertion experiments.

Purification and Hae III digestion of pBR322 DNA. 20 l of M9 supplemented media containing ampicillin (100 g/ml) were inoculated with a 250 ml overnight culture of pBR322 transformed *E. coli* C600 R⁻M⁻ cells. The culture was incubated at 37°C and aerated by bubbling air through the media with a pump. Once the cells had grown to an O.D._{550 nm} of 0.65, chloramphenicol was added to a final concentration of 100 $\mu\text{g/ml}$ and incubation continued for another 15 h. The cells were pelleted by centrifugation and then lysed with lysozyme. Purification of the DNA, using established procedures (36), yielded 3.5 mg of pBR322 DNA. 1.5 mg of this DNA was digested to completion with restriction endonuclease

Hae III (200 units). The digested DNA was purified by several phenol-ether extractions and dialysis against water.

Purification and isolation of Hae III restriction fragments of pBR322.

a) HPCL fractionation. 490 μ g of Hae III digested pBR322 DNA (stock digest) were fractionated by high pressure liquid chromatography (92). The DNA was suspended in 100 μ l of 200 mM KCl before being injected into the column. The column was packed with RPC-5 analog (BRL) and equilibrated with a 10 mM Tris-HCl, 10 mM EDTA buffer, pH 7.2, containing 200 mM KCl. Packing of the column, loading of the sample and elution were done under pressure (approximately 1,000 lb/inch²). Fractionation was achieved by eluting the column with a 40 ml KCl gradient (0.6 M to 0.64 M) mixed in the above buffer. Fractions (0.4 ml) were collected and the optical density of the effluent monitored at 260 nm on an Isco model UA-5 absorbance monitor. Fractions were also checked for their DNA content by agarose gel electrophoresis (20 μ l samples). An overlap of similar sized fragments was obtained in most of the fractions, usually with a certain fragment predominating over the others. Fractions containing similar sized fragments were pooled as shown in Table 1.

b) Electrophoretic fractionation. A 422 μ g sample of Hae III digested pBR322 DNA was electrophoresed on a discontinuous polyacrylamide gel. The bottom layer (20 ml) was composed of 10% polyacrylamide to prevent the loss of smaller fragments. The top layer (80 ml) was composed of 4% polyacrylamide in order to separate larger fragments. The DNA fragments were loaded in a 1.0 ml sample containing 250 μ l of bromophenol blue solution and electrophoresed at 80 V for 16 h. After staining the gel

Table 1. HPLC fractionation of Hae III digested pBR322 DNA.

Fragment number	Fragment length (bp)	Fragment pools [†]
1	587	
2	540	
3	504	
4	458	
5	434	
6	267	
7	234	
8	213	
9	192	
10	184	
11a	124	
11b	123	
12	104	
13	89	
14	80	
15	64	
16	57	
17	51	
18	21	
19	18	
20	11	
21	7	

The diagram illustrates the assignment of DNA fragments to three pools (A, B, and C) based on their size. A vertical dashed line represents the separation boundary. Fragments 8 through 17 are grouped into Pool C (top), fragments 13 through 17 are grouped into Pool B (middle), and fragments 18 through 21 are grouped into Pool A (bottom). Solid lines indicate predominant fragments, while dotted lines indicate fragments present in lower concentrations.

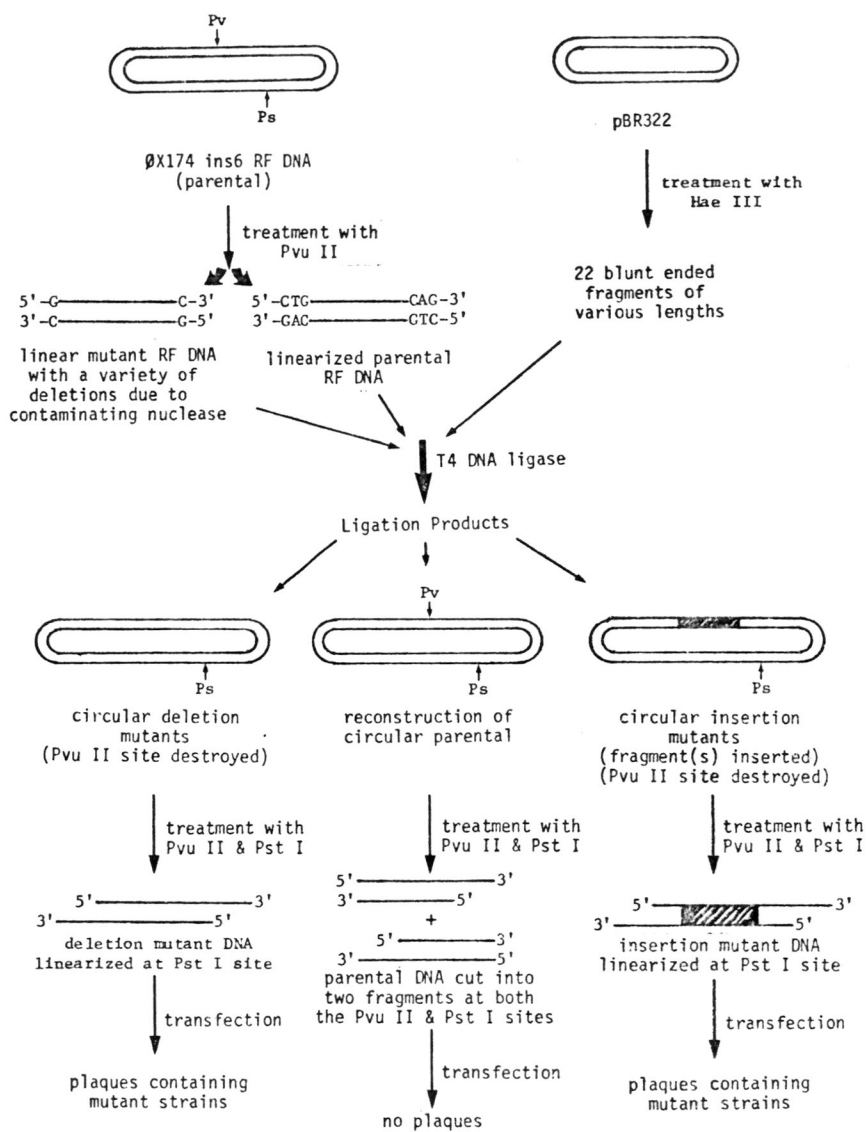
[†]Solid lines indicate predominant fragments in the pool; dotted lines indicate fragments present in lower concentrations.

with ethidium bromide, the DNA bands were visualized under UV light. Hae III fragment bands 1-6 were individually cut out and placed into dialysis bags with 1.0 ml of peacock's buffer. The dialysis bags were submerged in peacock's buffer and the DNA electroluted for several hours at 150 V. After electrolution, each DNA preparation was phenol-ether extracted and dialyzed against water. After dialysis the DNA fragments were dried under vacuum and then resuspended in 30 μ l of water.

RESULTS

The overall strategy of this study requires an efficient method of constructing insertions within the J-F intercistronic region of ϕ X174. Since this region is not translated and no biological assay is available to identify mutations in it, isolating mutants by standard genetic techniques would be difficult. This made it necessary to devise a biochemical rather than biological scheme for the construction and isolation of insertion mutants. Fig. 1 shows a general outline of this scheme as it was used in this study. The parental strain was the previously constructed insertion mutant ϕ X174 *ins* 6 (62). It was chosen because its entire sequence is known (63) and its insert sequence contains a unique Pvu II restriction site. Pvu II cleaves the sequence 5'-CAGCTG-3' and leaves blunt ends into which blunt ended DNA fragments can be inserted. Since its entire sequence has been established (82) and because it is relatively easy to isolate in large quantities, the plasmid pBR322 was used as the source of DNA fragments for insertion. Hae III treatment of pBR322 yields 22 blunt ended fragments ranging in size from 7 to 587 bp (84), (see Table 1). The Hae III ends on these fragments can be joined to Pvu II ends without regenerating new Pvu II sites. Furthermore, pBR322 contains only one Pvu II site, so only one of these fragments can be cleaved with Pvu II. Both of these considerations are important because Pvu II restriction is an essential part of the biochemical screening procedure used in the scheme as described below.

Fig. 1. Scheme for the construction of ϕ X174 *am3* mutants with increased genome sizes. Pv stands for a restriction site recognized by the enzyme Pvu II. Ps stands for a restriction site recognized by the enzyme Pst I.



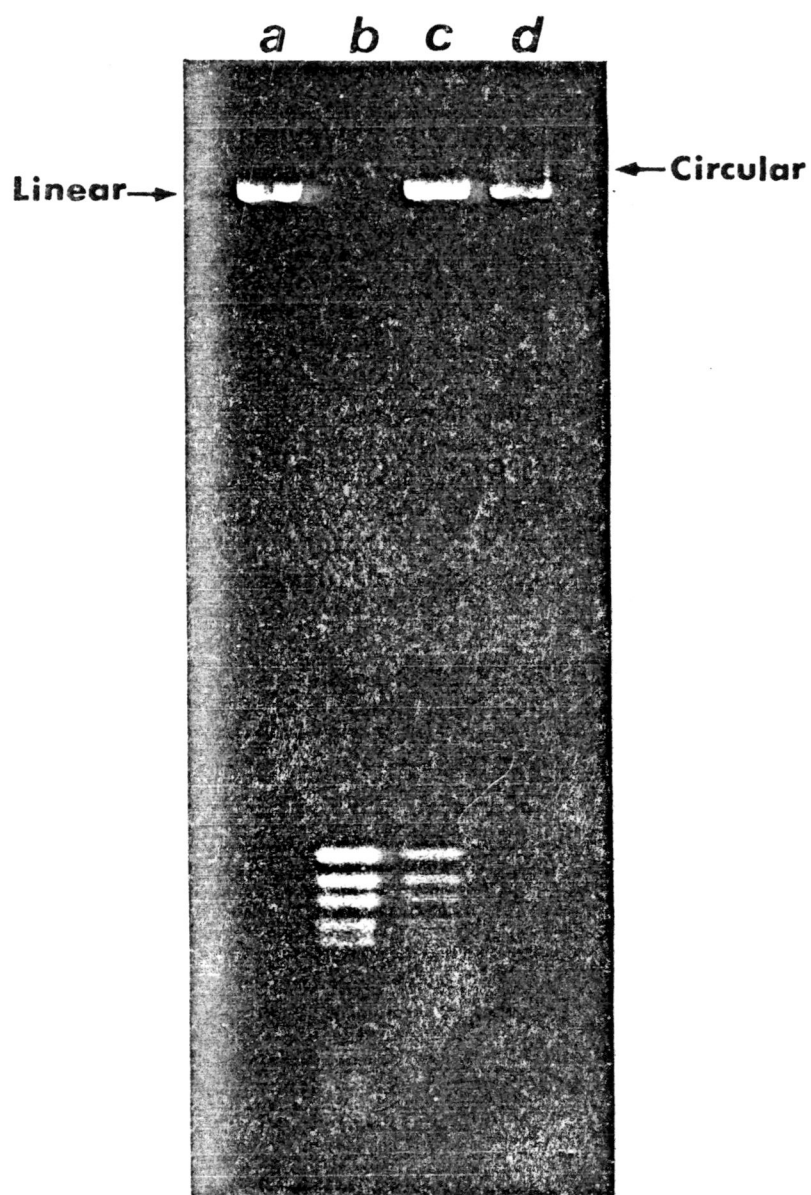
ØX174 *ins6* RF DNA is digested to completion with restriction endonuclease Pvu II to obtain linear blunt ended RF DNA molecules. During this reaction, minor nuclease contaminants in the commercial enzyme preparation attack the exposed Pvu II ends of some of the linearized parental molecules making deletions of various sizes. Ligation of the Pvu II linearized parental RF DNA with T4 DNA ligase in the presence of pBR322 Hae III fragments results in ligation products consisting mainly of circular parental RF DNA molecules with reconstructed Pvu II sites. In some cases, however, circular RF DNA molecules are generated with one or more Hae III fragments inserted into the Pvu II site. Due to the nuclease contamination mentioned above, some circular deletion mutant RF DNA molecules are also formed having one or more bases deleted from the Pvu II site. The insertion of a Hae III fragment or the removal of even one base effectively destroys the Pvu II site in the mutant DNAs. A major portion of the infective centers formed and the plaques obtained upon transfection of CaCl₂-shocked *E. coli* with the ligation products would consist of the parental phage strain. In order to prevent this and enhance the chances of isolating the desired insertion mutants, it is necessary to destroy the parental RF DNA without destroying the infectivity of the mutant DNAs. This is achieved by screening the ligation products enzymatically with the restriction endonucleases Pvu II and Pst I. Treatment of the ligation products to completion with Pvu II will result in linearization of any parental RF DNA molecules, while any mutant RF DNA molecule will remain circular. Although linearizing the parental DNA molecules lowers their transfection efficiency, it does not destroy their infectivity

(62). For this reason, it is necessary to treat the ligation products with another restriction enzyme that recognizes a unique site on the ϕ X genome. Pst I was used in this study and subsequent treatment of the ligation products to completion with this enzyme results in the fragmentation and loss of infectivity of the parental RF DNA molecules. Mutant RF DNAs are only linearized and maintain their infectivity. Transfection of *E. coli* with the enzymatically screened ligation products should therefore result in plaques containing either insertion or deletion mutant strains of ϕ X174 *ins6*. In this study, this scheme was used in three separate insertion experiments.

Insertion Experiment I. The largest insertion mutant of ϕ X174 known at the beginning of this study was ϕ X174 *ins11*, containing 163 additional nucleotides in the J-F intercistronic region (62). Since strain ϕ X174 *ins6* contains only 115 additional bases, it should be possible to insert 48 additional nucleotides into its J-F region without loss of viability. Based on this assumption, experiment I was undertaken to test the efficiency of the construction and isolation scheme. A second objective of this experiment was to determine if it was possible to exceed the number of nucleotides contained in the J-F intercistronic region of ϕ X174 *ins11*.

The experiment was carried out by ligating Pvu II linearized ϕ X174 *ins6* RF DNA in the presence of all 22 Hae III fragments of pBR322 DNA. A sample of this ligation mixture was analyzed on a 1.4% agarose gel as shown in Fig. 2 (lane d). Comparison of this sample with the unligated controls in lanes a, b and c demonstrates that a portion of the DNA was

Fig. 2. Ligation for Insertion Experiment I. Hae III digested pBR322 DNA (4 μ g total) was added to 1.8 μ g of Pvu II linearized ϕ X174 *ins*6 RF DNA in 20 μ l of ligase buffer. After addition of T₄DNA ligase (1.0 unit), the reaction was incubated for 16 h at 15°C. A sample (7 μ l) of this ligation mixture was applied to a 1.4% agarose gel (lane d) and electrophoresed for 11 h at 60 V. As controls, Pvu II linearized ϕ X174 *ins*6 RF DNA (0.6 μ g) was run in lane a, the Hae III fragments (4 μ g) of pBR322 in lane b, and the combination of linear parental DNA (0.6 μ g) and the fragments (2 μ g) in lane c. Both linear and circular ϕ X174 *ins*6 RF DNA are indicated by arrows.



converted from the linear to the circular form. The smear in the lower part of lane d is due to ligation of the fragments. The remaining portion of the ligation reaction was treated with excessive amounts of Pvu II and Pst I for 12 h to "screen" out non-mutant molecules. Approximately 0.15 μ g of this restricted DNA was used for transfection of CaCl₂-shocked *E. coli* cells and the result is summarized in Table 2. Equivalent amounts of circular, linear and screened parental DNAs were used in the transfection as controls. The circular and linear DNA controls demonstrate the greater efficiency of circular DNA in transfecting cells (62). A control using screened ϕ X174 *ins* 6 RF DNA was necessary to determine the efficiency of the screening procedure, e.g. the number of plaques formed due to infective parental DNA molecules surviving the screen. This number also includes deletion mutants formed by removal of nucleotides from the Pvu II site by contaminating nucleases as shown in Fig. 1.

Comparison of the plaque number obtained using linear DNA with the number obtained in the screened control indicates an 8-fold decrease. This suggests an efficiency of at least 87% in the destruction of infective parental RF DNA using the Pvu II-Pst I screening procedure. Hence, seven out of every eight plaques obtained from the screened ligation products were expected to contain mutant phages. However, there was no obvious difference found in the size or morphology of the plaques to distinguish mutant strains from parental. Therefore it was necessary to test individual plaques by examining the DNA of the phages. Phage titers and RF DNAs were prepared from plaques by the miniprep procedure. RF DNAs from 55 different plaques were prepared and tested for the

Table 2. Transfection results of Insertion Experiment I.

Type of DNA	Amount of DNA per transfection mix (μ g)	% of transfection mix plated	Number of plaques
Circular ϕ X174 <i>ins6</i> RF	0.146	1.7	> 300 ^c
		6.7	> 300 ^c
		33.3	> 300 ^c
		58.3	> 300 ^c
Pvu II linearized ϕ X174 <i>ins6</i> RF	0.155	1.7	67
		6.7	102
		33.3	220
		58.3	> 300 ^c
ϕ X174 <i>ins6</i> RF screened control ^a	0.155	1.7	0
		6.7	0 ^d
		33.3	25 ^d
		58.3	48 ^d
Screened ligation products ^b	0.155	1.7	3
		6.7	7
		33.3	240
		58.3	300 $\pm$ 30

^aAs a control, ϕ X174 *ins6* RF DNA was linearized with Pvu II, religated with T₄ DNA ligase and then cleaved with Pvu II and Pst I to completion.

^bRefers to treatment of the ligation products with Pvu II and Pst I (Fig. 1).

^cMore than 300 plaques per plate were uncountable due to confluent lysis.

^dNo attempts were made to determine whether these plaques contained parental phage or deletion mutants.

absence of a Pvu II site with Pvu II-Sst II double digests. Shown in Fig. 3 are 15 samples examined with this double digest. Parental DNA was digested as a control (lane b) to indicate the fragments expected of a sample containing an intact Pvu II site. Migration of a DNA sample to the linear position was indicative of a mutant DNA with a destroyed Pvu II site. Fourteen of these first 15 samples were identified as mutants, while one sample (lane l) displayed the two fragments characteristic for parental DNA. Further analysis showed that 48 of the 55 isolated RF DNAs were from mutant strains. To determine which of these were deletions and which were the desired insertion mutants, samples from all 48 mutant DNAs were digested with Hae III and electrophoresed on agarose gels. This digest indicated that only two of the isolated DNAs were from insertion mutants (Data not shown). Of the remaining 46 mutant RF DNAs, 31 were from deletion mutants while the rest were either mixtures of more than one type of mutant or a contamination. Sizing of the deletion mutants demonstrated that only one retained part of the original parental insert. The rest had deleted the entire parental insert plus additional nucleotides. One of these appeared equivalent to strain ØX174 *del2* while the other 29 all had deletions equivalent to strain ØX174 *del3*, which contains the largest possible deletion from the J-F region (62, 63). The significance of this finding will be discussed later.

For further identification of the two insertion mutants, larger amounts of RF DNA were prepared (36) and digested with restriction enzymes. Fig. 4 shows a Hae III digest of these two new mutant DNAs

Fig. 3. Pvu II - Sst II restriction analysis of mutant RF DNAs from Insertion Experiment I. RF DNAs (0.5-1.0 μ g) were digested with Pvu II and Sst II for 8 h at 37°C. The samples were electrophoresed on 1.4% agarose gels for 12 h at 40 V. A control digest of ØX174 *ins*6 RF DNA (0.6 μ g) shown in lane b, indicates the expected fragments when an intact Pvu II site is present. Lane a contains undigested circular, supercoiled parental DNA (0.6 μ g). Lanes c to q contain digested RF DNAs of isolated mutant candidates. The positions of linear, circular and supercoiled RF DNA are indicated by arrows.

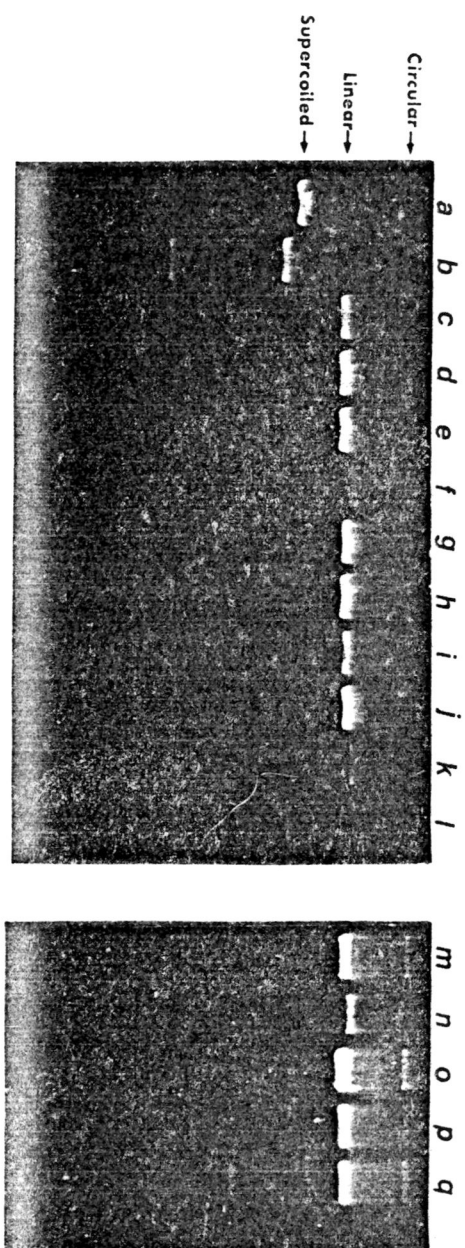
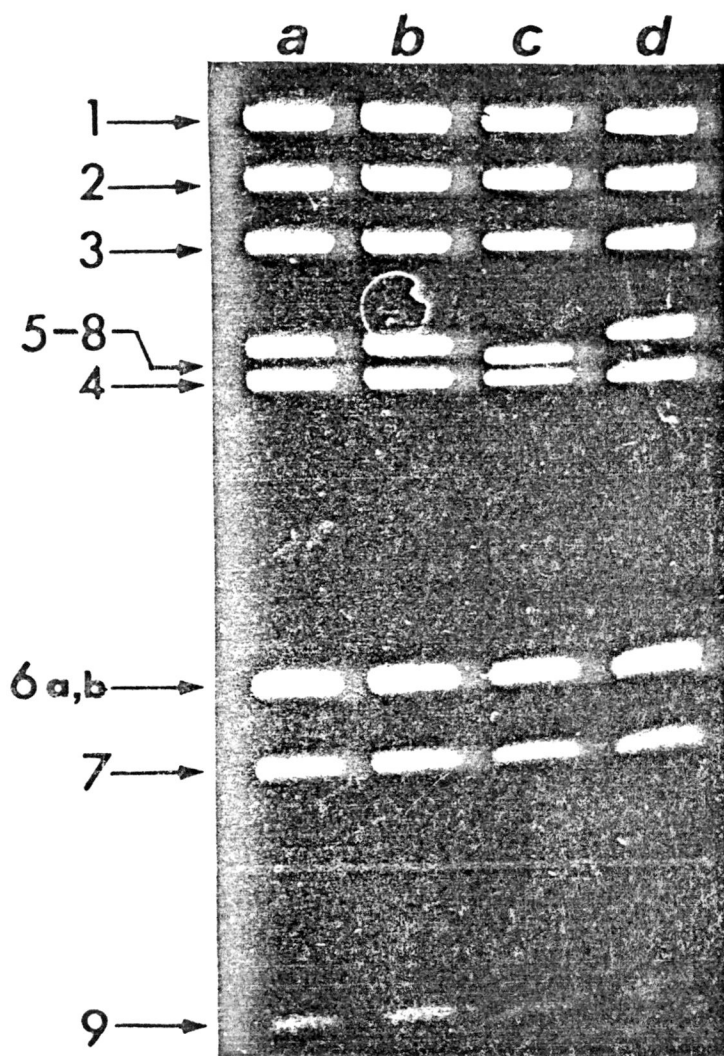
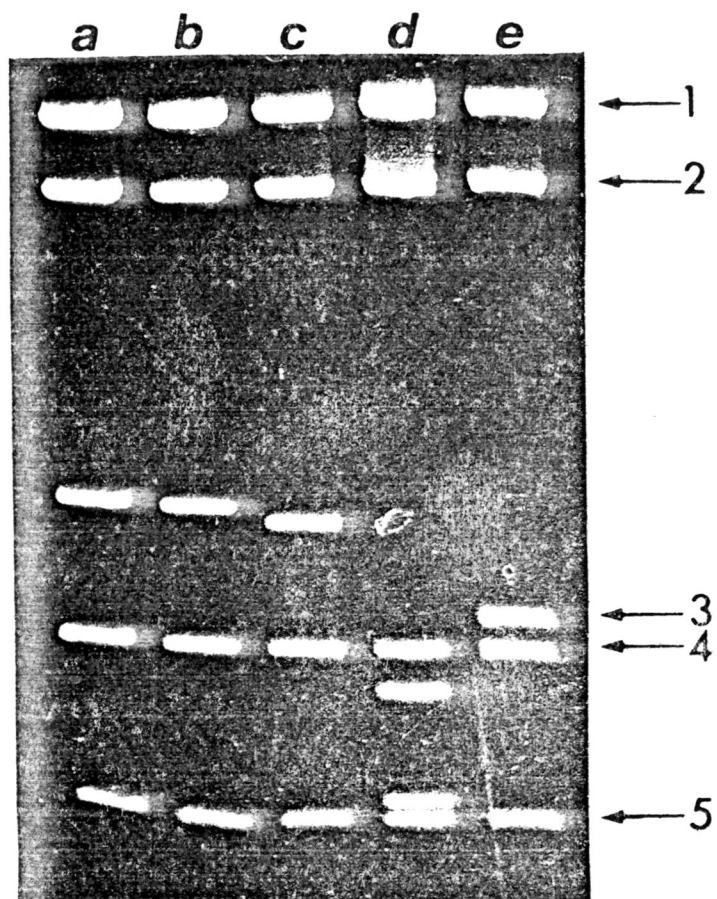


Fig. 4. Hae III restriction analysis of insertion mutants from Insertion Experiment I. RF DNAs (6 μ g) were treated with restriction endonuclease Hae III (6 units) for 8 h at 37°C, and the products were separated on a 4% polyacrylamide gel (16 h, 80 V). The arrows indicate the positions of Hae III fragments characteristic for the parental strain ØX174 *ins6* (lane c). Digested strain ØX174 *ins11* RF DNA was run as a control in lane d. The digested DNA of two newly isolated insertion mutants was run in lanes a and b respectively.



(lanes a and b). The characteristic Hae III fragments obtained by digestion of parental RF DNA are shown in lane c. Hae III fragment band 5-8 is a fusion band created when the original Hae III site between Hae III fragments 5 and 8 of ϕ X174 *am3* was destroyed by the insertion of the 115 nucleotides contained in ϕ X174 *ins6* (62). This means that Hae III fragment band 5-8 is the indicator band for determining whether an insertion or deletion has occurred because it contains the ϕ X174 *ins6* Pvu II site. Therefore, an increase in the size of band 5-8 is indicative of an insertion. For both new insertion mutants this increase was determined graphically to be approximately 18 bp (Materials and Methods). Consequently, neither mutant contains an insert larger than ϕ X174 *ins11* (lane d). This was confirmed in a second digest using restriction endonuclease Hpa II (Fig. 5). In this digest the five characteristic Hpa II fragments of ϕ X174 *am3* RF DNA are shown in lane e. A marked increase in the size of fragment 3 is shown in lane c containing digested ϕ X174 *ins6* RF DNA. This indicates that fragment 3 has the ϕ X174 *ins6* insert containing the Pvu II site. Both mutant digests (lanes a and b) show a further increase in the size of fragment 3 due to insertion of DNA. Graphic determination of fragment 3 indicated that both mutant DNAs contained approximately 18 more base pairs than ϕ X174 *ins6*. ϕ X174 *ins11* (lane d) behaves differently in this digest in that fragment 3 is cleaved into two new fragments due to insertion of an additional Hpa II site. A final digest of the mutant DNAs was carried out using restriction endonuclease Hha I. This enzyme was chosen because it provides a very sensitive means of determining the size of insertions within the J-F intercistronic region of ϕ X174 *am3*,

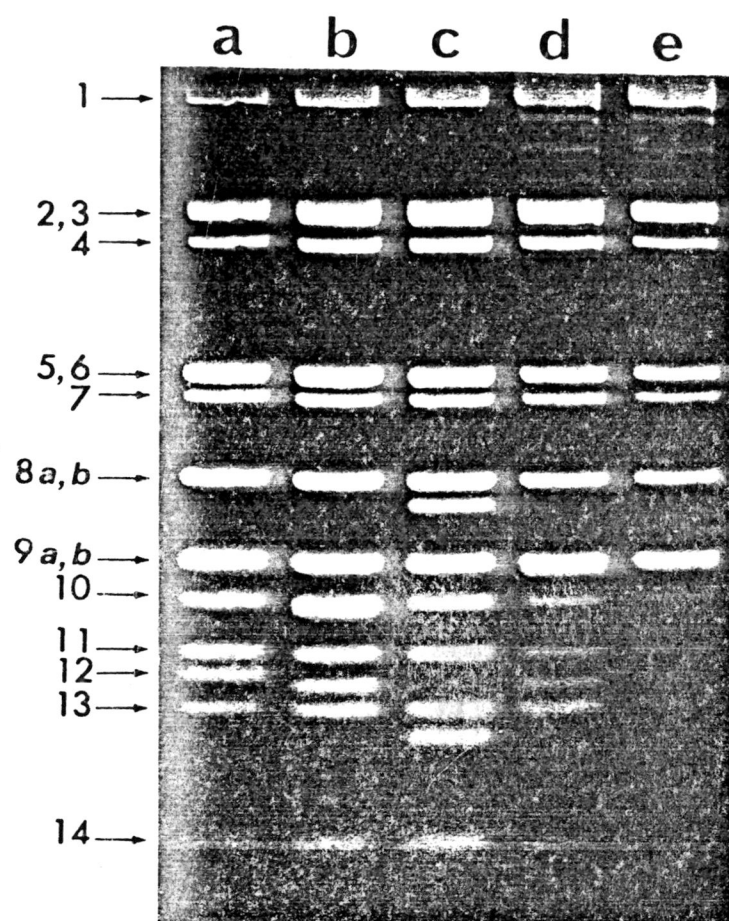
Fig. 5. Hpa II restriction analysis of insertion mutants from Insertion Experiment I. RF DNAs (6 μ g) were treated with 6 units of restriction endonuclease Hpa II for 8 h at 37°C and the fragments were separated on a 4% polyacrylamide gel (80 V, 16 h). The arrows mark the 5 Hpa II restriction fragments characteristic for strain $\emptyset$ X174 *am*3 RF DNA (lane e). Digested RF DNAs of parental strain $\emptyset$ X174 *ins*6 (lane c) and strain $\emptyset$ X174 *ins*11 (lane d), both of which are insertion mutants of $\emptyset$ X174 *am*3, were run as controls. Digested RF DNAs of the two newly isolated insertion mutants were run in lanes a and b respectively.



since this whole region is contained within a relatively small Hha I fragment of 93bp. The characteristic Hha I fragments for ØX174 *am3* (lane a) are indicated by the arrows in Fig. 6. In comparison, the fragment bands for ØX174 *ins6* (lane b) show the disappearance of fragment 12 and the appearance of two new fragments. This is due to a new Hha I site contained within the insert of ØX174 *ins6*. One of these new fragments can not be resolved from fragment 10 and both appear on the picture as one bright band. In the new insertion mutants (lanes d and e), this new fragment comigrates with Hha I fragments 9a and 9b, creating a band of triple intensity. The difference in the size of this fragment between the mutant and parental strains was graphically determined to be approximately 20 bp.

Overall the restriction analyses of the two new insertion mutants indicate that both have genome sizes approximately 18 to 20 bp greater than strain ØX174 *ins6*. In relation to ØX174 *am3*, this means a total insert size of 134 bp in the J-F intercistronic region. The insert size points to pBR322 Hae III fragments 18, 19 or a combination of 20 and 21, (see Table 1). However, the Hae III analysis indicated no new Hae III site which eliminates the double insertion of fragments 20 and 21. Likewise, Hae III fragment 18 was eliminated as a possibility by the Hpa II analysis, since this fragment contains an Hpa II site. This leaves Hae III fragment 19 as the most likely fragment inserted. Although both mutants appeared to have the same insert, DNA sequencing may show a difference in the direction of fragment insertion or a difference in size by 1 or 2 base pairs. Therefore after restriction analyses, these

Fig. 6. Hha I restriction analysis of insertion mutants from Insertion Experiment I. Hha I digests of RF DNA (7 μ g) were performed with 6 units of restriction enzyme for 8 h at 37°C and the products separated on an 8% polyacrylamide gel (16 h, 80 V). Hha I fragments characteristic for ϕ X174 *am3* (lane a) are indicated by arrows. Digested RF DNAs of strain ϕ X174 *ins6* (lane b) and strain ϕ X174 *ins11* (lane c) were used as controls. Digests of the two new insertion mutants were run in lanes d and e.



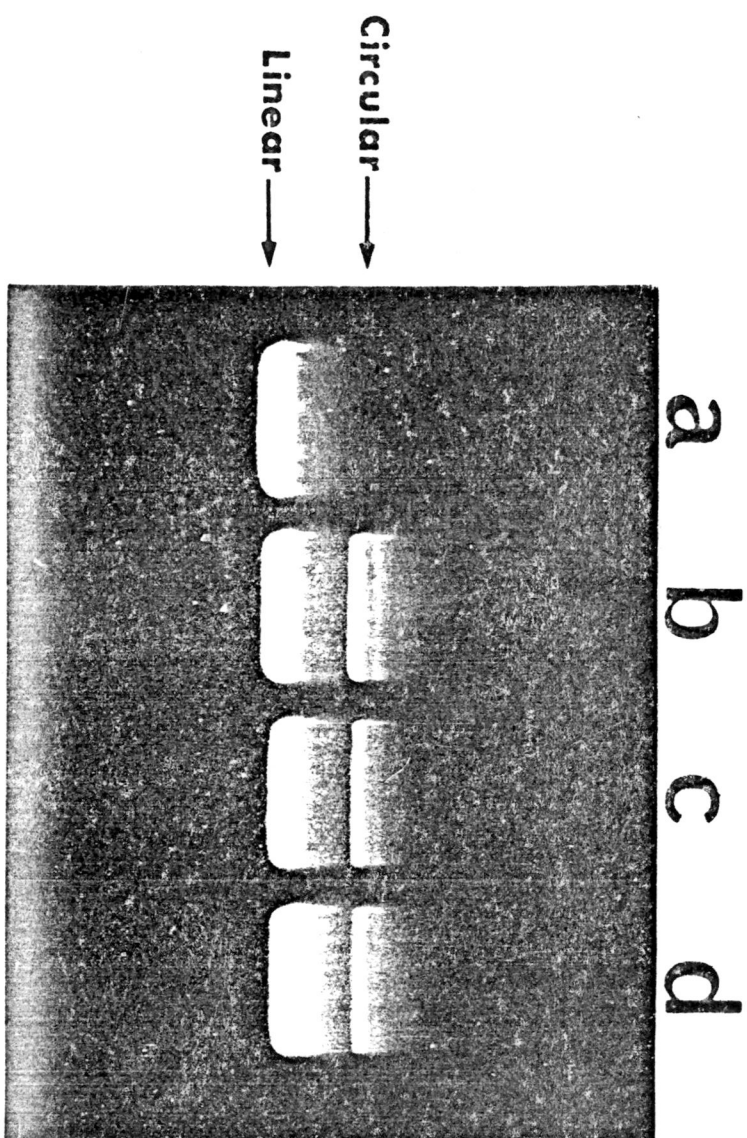
two new mutants were designated ØX174 J-F *ins* 61 and ØX174 J-F *ins* 62 respectively.

Insertion Experiment II. Even though only two insertion mutants were obtained, Insertion Experiment I proved that it was possible to construct and isolate mutants with insertions in the J-F intercistronic region of ØX174 *ins* 6 by using the scheme outlined in Fig. 1. However, the results did not allow any conclusions as to whether it was possible to increase the genome size beyond that of strain ØX174 *ins* 11.

Insertion Experiment II represents an attempt to increase the genome size beyond that of ØX174 *ins* 11. Hae III fragments of pBR322 were fractionated by high pressure liquid chromatography (Materials and Methods), and specific fractions were combined to make three separate pools. The predominating fragments of each pool increased in size moving from pool A to C (Table 1). Thus, it was possible to use pools with fragments of defined size ranges for the insertion experiment. Pool A containing fragments 18 and 19 was used as a control because fragments of this size range (18-21 bp) had already been inserted successfully into ØX174 *ins* 6 in the previous experiment.

In the ligation reaction, 1.2 µg of Pvu II linearized ØX174 *ins* 6 RF DNA was ligated in the presence of at least a 40-fold molar excess of the Hae III fragments contained in either pool A, B or C. A sample was taken from each ligation mixture and the extent of ligation analyzed on an agarose gel. Fig. 7 shows that 25 to 30% of the RF DNA was converted from the linear to the circular position, as judged from the intensity of the bands. As expected, ligation products from the fragments were not visible, since an insufficient amount of fragment DNA was contained in

Fig. 7. Ligation for Insertion Experiment II. Aliquots of either pool A ($\sim 0.3 \mu\text{g}$), B ($\sim 0.8 \mu\text{g}$) or C ($\sim 1.5 \mu\text{g}$) containing purified Hae III fragments of pBR322 (Table 1), were added to $1.2 \mu\text{g}$ of Pvu II linearized $\phi\text{X174 } ins6$ RF DNA in $20 \mu\text{l}$ of ligase buffer. After addition of T_4 DNA ligase (4 units), the reaction mixtures were incubated for 16 h at 15°C . Samples of each ligation were analyzed on a 1.4% agarose gel (3 h, 120 V). Lane a contains Pvu II linearized $\phi\text{X174 } ins6$ RF DNA ($0.6 \mu\text{g}$) as a linear marker. Lanes b, c and d contain $10 \mu\text{l}$ aliquots of the ligation products A, B and C respectively.



each ligation mixture to present a visible band for each of the many possible products. Like Insertion Experiment I, a major product of the ligation reaction was expected to be recircularized parental RF DNA molecules. Therefore, each mixture of ligation products was treated with a large excess of the screening enzymes Pvu II and Pst I to insure destruction of parental RF DNA. Samples from each mixture were then used to transfect CaCl_2 -shocked *E. coli* and the results are shown in Table 3. Comparison of the number of plaques obtained from the unscreened linear control DNA with those obtained from parental screened control DNA indicated over 99% reduction of infective parental DNA molecules due to the screening procedure. This suggested that at least 99% of the plaques obtained from each screened ligation mixture were mutant strains. Comparison of plaque numbers from each of these transfections also reveals that the larger the fragment size used for insertion, the fewer the number of plaques obtained. Furthermore, inspection of the plaque size indicated an increased percentage of smaller plaques concomittant with the increase in size of the insertion fragments. These two findings may point to decreasing phage viability with increasing insertion size. Since it was possible that the smaller plaques contained less viable mutant strains, they were used for isolation of phage and subsequently of RF DNA by the miniprep procedure (Materials and Methods). RF DNAs from a total of 23 plaques were prepared and used for restriction analysis with restriction endonuclease Hae III. Separation of these digests on agarose gels revealed that 20 of the RF DNAs stemmed from insertion mutants and 3 from deletion

Table 3. Transfection results of Insertion Experiment II.

Type of DNA	Amount of DNA per transfection mix (μ g)	% of transfection mix plated	Number of plaques
Circular ϕ X174 <i>ins6</i> RF	0.15	1.7	> 300 ^c
		6.7	> 300 ^c
		33.3	> 300 ^c
		58.3	> 300 ^c
Pvu II linearized ϕ X174 <i>ins6</i> RF	0.15	1.7	83
		6.7	167
		33.3	300 $\pm$ 30
		58.3	> 300 ^c
ϕ X174 <i>ins6</i> RF screened control ^a	0.15	1.7	0
		6.7	0
		33.3	1 ^d
		58.3	0
Screened _b ligation products A	0.15	1.7	4
		6.7	14
		33.3	100
		58.3	300 $\pm$ 30
Screened _b ligation products B	0.15	1.7	4
		6.7	18
		33.3	47
		58.3	152
Screened _b ligation products C	0.15	1.7	1
		6.7	8
		33.3	22
		58.3	75

^a As a control ϕ X174 *ins6* RF DNA was linearized with Pvu II, religated with T₄ DNA ligase and then cleaved with Pvu II and Pst I to completion.

^b The ligation reaction contained Pvu II linearized ϕ X174 *ins6* RF DNA and purified Hae III fragments 18 and 19 (A), 11a-17 (B), and 8-17 (C) from the plasmid pBR322 (Table 1). Screening involved treatment with Pvu II and Pst I.

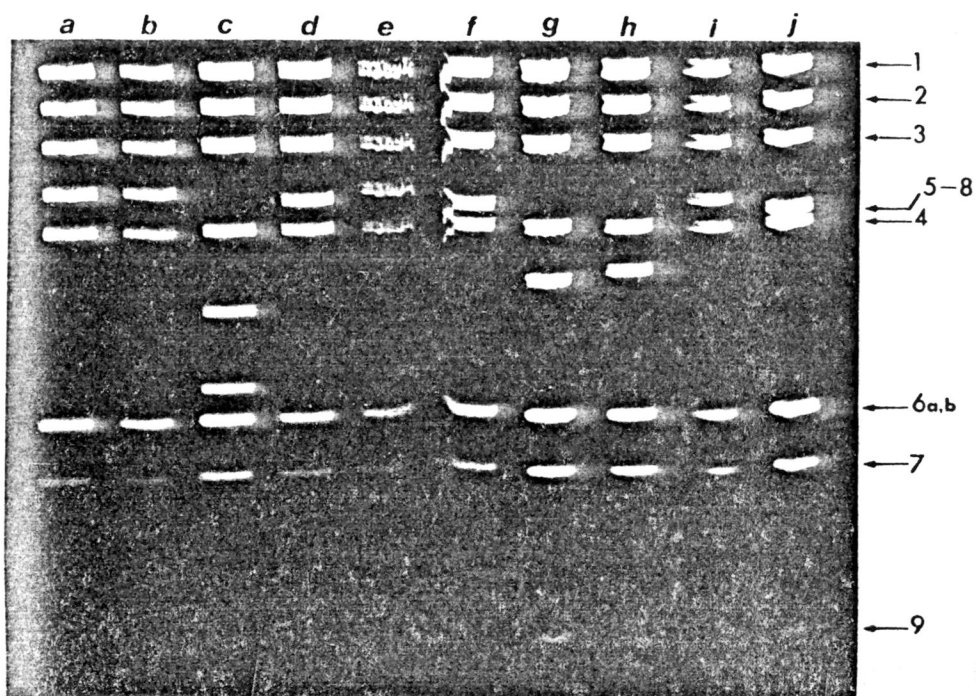
^c More than 300 plaques per plate were uncountable due to confluent lysis.

^d No attempt was made to determine whether this plaque contained parental phage or a deletion mutant.

mutants. None of the isolated RF DNAs were from a parental phage. Since the insertion mutants were of predominant interest, the Hae III digests of the 12 largest were further characterized on polyacrylamide gels (Fig. 8a and 8b). Determination of the fragment sizes revealed that 11 of the 12 new insertion mutants were larger than $\emptyset$ X174 *ins*11. Some of the new mutants appeared to have more than one Hae III fragment inserted (Fig. 8a, lane c and Fig. 8b, lanes a, c, e and f). This regenerated a Hae III site between the inserted fragments. Consequently, upon Hae III digestion the characteristic fragment 5-8, which contains the new insertion, is cleaved into two new fragments. Mutants having only one Hae III fragment inserted maintained an intact but enlarged fragment 5-8, (Fig. 8a, lanes a, b and d to f and Fig. 8b, lanes b and d). The amount of DNA inserted into each of these mutants was estimated by graphically determining and summing the fragment sizes. Mutant C-7 (Fig. 8b, lane a) was found to have a total of 226 ± 5 bp inserted and was the largest strain obtained. Since $\emptyset$ X174 *ins*6, the parental strain, is already 115 nucleotides larger than wt $\emptyset$ X174, the genome of this new mutant is approximately 341 nucleotides larger than that of wt $\emptyset$ X174. This was confirmed by comparing a Hae III digest of mutant C-7 RF DNA with a digest of $\emptyset$ X174 *am*3 RF DNA as well as two other size markers (Fig. 9). The insert in mutant C-7 contains a Hae III site and is therefore composed of two pBR322 fragments originally derived from pool C (Table 1). Length determination of these fragments suggests that the inserted DNA contains pBR322 fragments 12 and either 11a or 11b. This is not surprising because these three fragments are the predominant ones of pool C (Table 1). Fragment 12 contains the Bam HI site of pBR322 and

Fig. 8. Hae III restriction analysis of insertion mutants from Insertion Experiment II. RF DNAs (5 to 8 μ g) were treated with 12 units of restriction endonuclease Hae III for 6 h at 37°C and the products were analyzed on 4% polyacrylamide gels (15 h, 80 V). In both Fig. 8a and Fig. 8b, the arrows indicate the Hae III fragments characteristic for strain $\emptyset$ X174 *ins*6 (lane j). Digested RF DNAs of strains $\emptyset$ X174 *ins*11 (lane i), $\emptyset$ X174 *del*2 (lane h) and $\emptyset$ X174 *del*3 (lane g) were used as size markers. Lanes a to f contain digested RF DNAs of newly isolated insertion mutants.

8a



8b

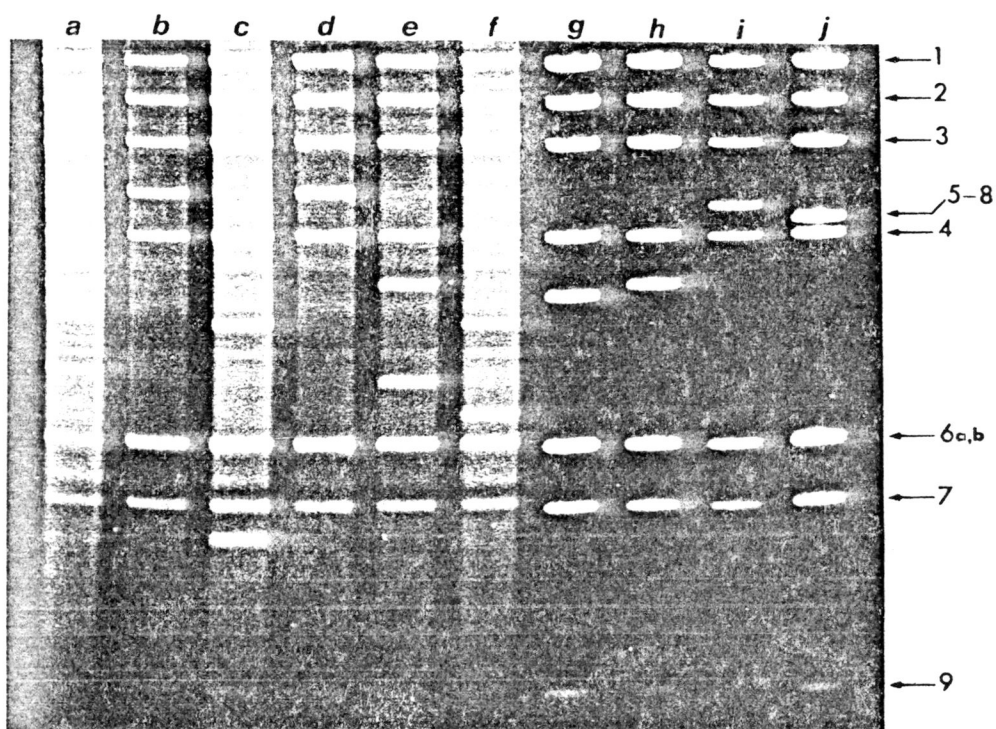
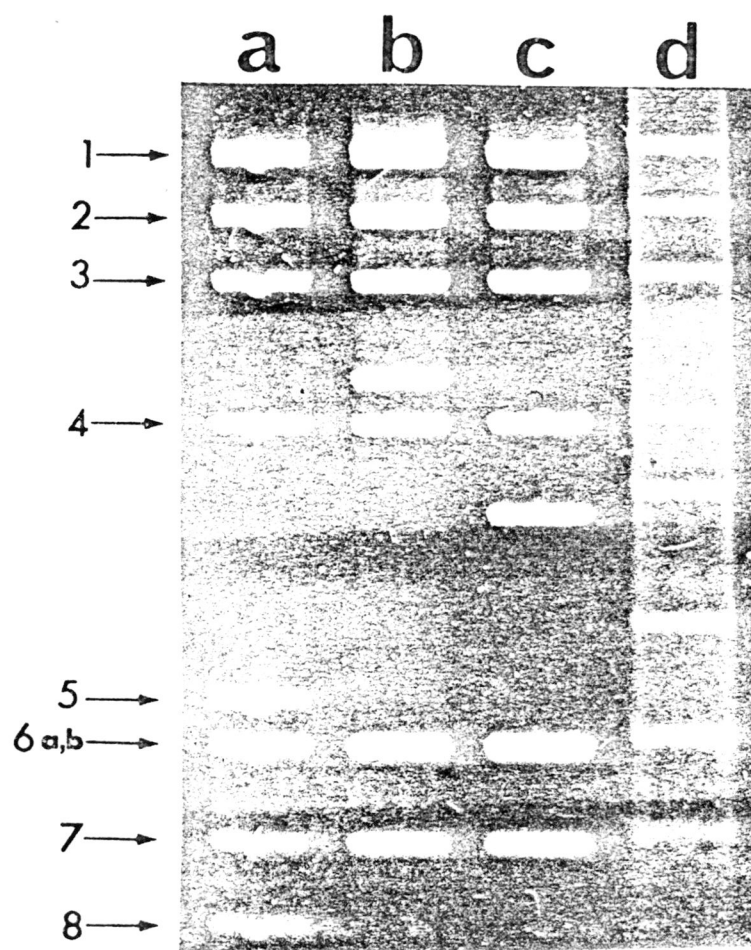


Fig. 9. Hae III restriction analysis of the largest insertion mutant from Insertion Experiment II. RF DNAs (6 to 8 μ g) were treated with restriction endonuclease Hae III (8 units) and then electrophoresed on a 4% polyacrylamide gel (16 h, 80 V). The Hae III fragments characteristic for strain $\emptyset$ X174 *cam* 3 (lane a) are indicated by arrows. Digested RF DNAs of strain $\emptyset$ X174 *ins* 11 (lane b) and $\emptyset$ X174 *del* 3 (lane c) were used as size markers. A digest of RF DNA from the largest insertion mutant isolated was run in lane d.



a Bam HI digest confirmed the presence of this fragment in mutant C-7 RF DNA (Data not shown). Fragments 11a and 11b differ in size by only 1 bp and neither have any known restriction sites. Thus, without DNA sequencing the exact composition of this insert can not be determined.

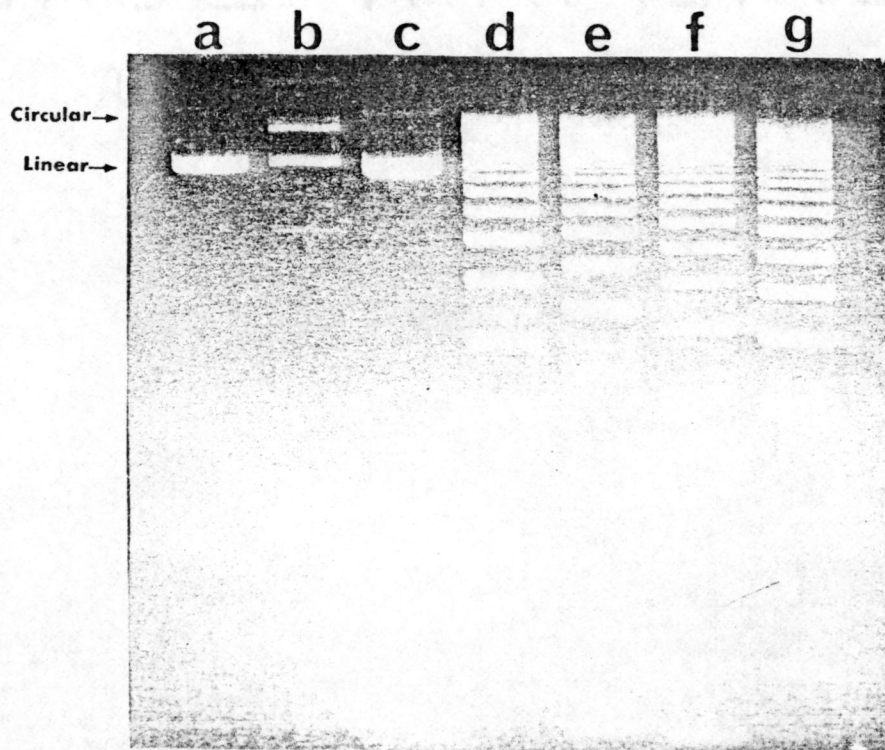
Only a few of the other insertion mutants were chosen for further analysis. One of these (C-5; Fig. 8b, lane b) was formed by the insertion of approximately 64 bp into the parental strain resulting in a genome size approximately 181 nucleotides greater than the wt ϕ X174. This suggests the insertion of Hae III fragment 15, a minor component of pool C which is 64 bp in length (Table 1). Mutants B-4 and B-9 (Fig. 8a, lanes c and f) appeared to have inserts of approximately 110 bp and thus have genome sizes which are approximately 227 bp larger than that of wt ϕ X174. Assuming a maximal error of 5 bp in determining the length of the inserted fragments, there are two candidates as potential inserts, pBR322 Hae III fragments 16 and 17. Further restriction analysis is necessary to characterize the insert in these strains accurately. The second largest insertion mutant obtained in this experiment was C-1 (Fig. 8b, lane e), having two Hae III fragments inserted. Determination of their size points to Hae III fragments 13 and either 11a or 11b as the inserts. This means that the C-1 genome is approximately 326 nucleotides longer than the genome of wt ϕ X174.

RF DNAs of the 3 deletion mutants were also Hae III digested and analyzed by agarose gel electrophoresis. Their deletions appeared to be of varying size but in no case did they exceed the number of bases deleted in ϕ X174 *del3*, the smallest available J-F intercistronic mutant of ϕ X174.

Insertion Experiment III. In the preceeding experiment a viable ØX174 mutant strain with a genome of 5726 ± 5 nucleotides was isolated. This was approximately 340 nucleotides larger than the genome of wt ØX174 and the insert was more than twice the size of the previously constructed largest insertion mutant ØX174 *ins11*. However, studies involving the *in vitro* packaging of plasmid DNAs into the ØX capsid (3) have indicated that it might be possible to isolate a ØX174 mutant with close to a 10% increase in genome size e.g. an insert of approximately 540 nucleotides. This was attempted in Insertion Experiment III using pBR322 Hae III fragments 1, 3, 4 and 5 (Table 1), which upon insertion would increase the genome size by 10% or more.

The DNA fragments were purified by electrophoretic fractionation as described in Materials and Methods. Pvu II linearized ØX174 *ins6* RF DNA was ligated in the presence of a 30-fold molar excess of either of these purified Hae III fragments. Fig. 10 shows the results of these ligation reactions. Linearized parental RF DNA before and after treatment with T4 DNA ligase was run in lanes a and b respectively, in order to show the position of circularized RF DNA. A mixture of Pvu II linearized parental RF DNA plus the four Hae III fragments was run in lane c to mark the position of each fragment before ligation. Lanes d to g contain approximately half the amount of RF DNA after ligation with Hae III fragments 1 (lane d), 3 (lane e), 4 (lane f) and 5 (lane g). The formation of multimeric forms of each of the Hae III fragments as well as the almost complete shift of the RF DNA away from the linear position is indicative of a very good ligation. In fact, the ligation control in

Fig. 10. Ligation for Insertion Experiment III. Approximately 2 μ g each of purified Hae III fragments 1, 3, 4 and 5 of pBR322 were separately mixed with 0.6 μ g of Pvu II linearized ϕ X174 *ins6* RF DNA in 20 μ l of ligase buffer. After addition of T₄DNA ligase (4 units), each ligation reaction was incubated for 16 h at 14°C. Samples of each ligation reaction containing equal amounts of RF DNA were run on a 1.4% agarose gel for 12 h at 40 V. As a control, Pvu II linearized ϕ X174 *ins6* RF DNA (0.6 μ g) was run before ligase treatment (lane a) and after ligase treatment (lane b). An unligated mixture of linear ϕ X174 *ins6* RF DNA (0.6 μ g) and all four Hae III fragments (1.0 μ g each) was run in lane c. Lanes d to g contain Pvu II linearized ϕ X174 *ins6* RF DNA ligated in the presence of Hae III fragments 1, 3, 4 and 5 respectively (also see Table 4).



lane b indicates at least a 70% shift from linear to circular form, as judged by band intensity.

As before, the ligation products were treated successively with Pvu II and Pst I to destroy infective parental RF DNA molecules. For convenience the resulting reaction mixtures were labelled A, B, C and D as outlined in Table 4. The equivalent of 0.1 μ g of input genomic DNA from each of these reaction mixtures were used for transfection and the results are summarized in table 4. Comparison of the number of plaques obtained with linear control DNA with the plaque number for screened control DNA indicated at least a 10-fold reduction of infective parental DNA molecules due to the screening procedure. Thus, one could assume that among the plaques resulting from the screened ligation reactions not more than 10% would be parental phage. One would also expect to find at least as many plaques resulting from these screened ligation samples as were obtained from the screened parental DNA control. However, the number of plaques derived from each of the screened ligation products was well below the parental control, (approximately 15-fold less). This suggested that the insertion of Hae III fragments 1, 3, 4 or 5, as well as the screening procedure, rendered the majority of the RF DNA molecules in the ligation products non-viable. Therefore, the majority of the plaques obtained from each of the screened ligation products were expected to contain either parental phage or deletion mutants of the parental strain. The only indication that there might be insertion mutants present was the finding of 5 smaller sized plaques.

A total of 30 plaques, including the 5 smaller ones, were isolated from the transfected ligation products and RF DNAs prepared by the

Table 4. Transfection results of Insertion Experiment III.

Type of DNA	Amount of DNA per transfection mix (μ g)	% of transfection mix plated ^c	Number of plaques
Pvu II linearized ϕ X174 <i>ins6</i> RF	0.11	1.7	59
		3.3	102
		5.0	167
		6.7	206
		16.7	300 $\pm$ 30 ^d
		66.6	> 300 ^d
ϕ X174 <i>ins6</i> RF screened control ^a	0.11	1.7	1 ^e
		3.3	1 ^e
		5.0	3 ^e
		6.7	5 ^e
		16.7	20 ^e
		66.6	60 ^e
Screened _b ligation products A	0.11	1.7	0
		3.3	0
		5.0	0
		6.7	0
		16.7	0
		66.6	2
Screened _b ligation products B	0.11	1.7	0
		3.3	2
		5.0	1
		6.7	0
		16.7	1
		66.6	6
Screened _b ligation products C	0.11	1.7	0
		3.3	0
		5.0	2
		6.7	2
		16.7	3
		66.6	4
Screened _b ligation products D	0.11	1.7	1
		3.3	2
		5.0	0
		6.7	0
		16.7	1
		66.6	7

^a As a control, ϕ X174 *ins6* RF DNA was linearized with Pvu II, religated with T₄ DNA ligase and then cleaved with Pvu II and Pst I to completion.

^b Ligation reactions contained Pvu II linearized ϕ X174 *ins6* RF DNA and individually purified Hae III fragments 5 (A), 4 (B), 3 (C), and 1 (D) of the plasmid pBR322. Screening involved treatment with Pvu II and Pst I.

^c The transfection mix was plated directly without additional seed cells to allow for large plaque formation.

^d More than 300 plaques per plate were uncountable due to confluent lysis.

^e No attempts were made to determine whether these plaques contained parental phage or deletion mutants.

miniprep procedure (Materials and Methods). The preliminary designations for the corresponding phage strains were 5A-1, 4B-1 to 4B-9, 3C-1 to 3C-9 and 1D-1 to 1D-11, whereby the first number stands for the Hae III fragment used in the ligation and the letters refer to the transfection mixture from which the strain was isolated. The isolated RF DNAs were treated with restriction endonuclease Hae III and analyzed by agarose gel electrophoresis. A representative gel is shown in Fig. 11 in which there are 6 deletion mutants and one insertion mutant. Analysis of all 30 plaques revealed only 3 insertion mutants, all of which contained greater lengths of inserted DNA than strain ØX174 *ins* 11. None of these insertion mutants contained as much extra DNA as strain C-7 constructed in Insertion Experiment II. This is particularly significant since the smallest fragment used for insertion was 434 bp long and if inserted would result in a mutant genome much larger than that of C-7. Thus the 3 insertions obtained may have been degradation products of the pBR322 fragments. This will be discussed in detail later. Of the 27 remaining samples, 3 were ØX174 *am* 3 contaminants, 12 had genomes similar in size to the parental strain and 12 were deletion mutants. Of these, 10 had deletions equal to that of strain ØX174 *del* 13, and are shown in Fig. 11, lanes b, c, f and g. The other two deletion mutants lacked only a part of the parental insert sequence. A closer examination of those samples similar in size to the parental strain was undertaken by testing for the presence of a Pvu II site with a Pvu II - Sst II - Pst I triple digest. This analysis demonstrated that two of the samples were mutant DNAs with destroyed Pvu II sites (Fig. 12, lanes b and c). Since both mutants also have Hae III

Fig. 11. Hae III digest of isolated RF DNAs from Insertion Experiment III. RF DNAs (6-8 g) were digested with Hae III (5 units for 12 h, 37°C) and 2.5 g aliquots were electrophoresed on a 1.4% agarose gel (3 1/2 h, 120 V). The arrows indicate some of the bands characteristic for strain ØX174 *am3*. In lane e, the band between fragments 3 and 4 migrates slower than the corresponding band of the parental phage ØX174 *ins6* in lane d. This identifies this strain as an insertion mutant. In all of the other samples this band migrates faster than the parental fragment, which is characteristic of deletion mutants.

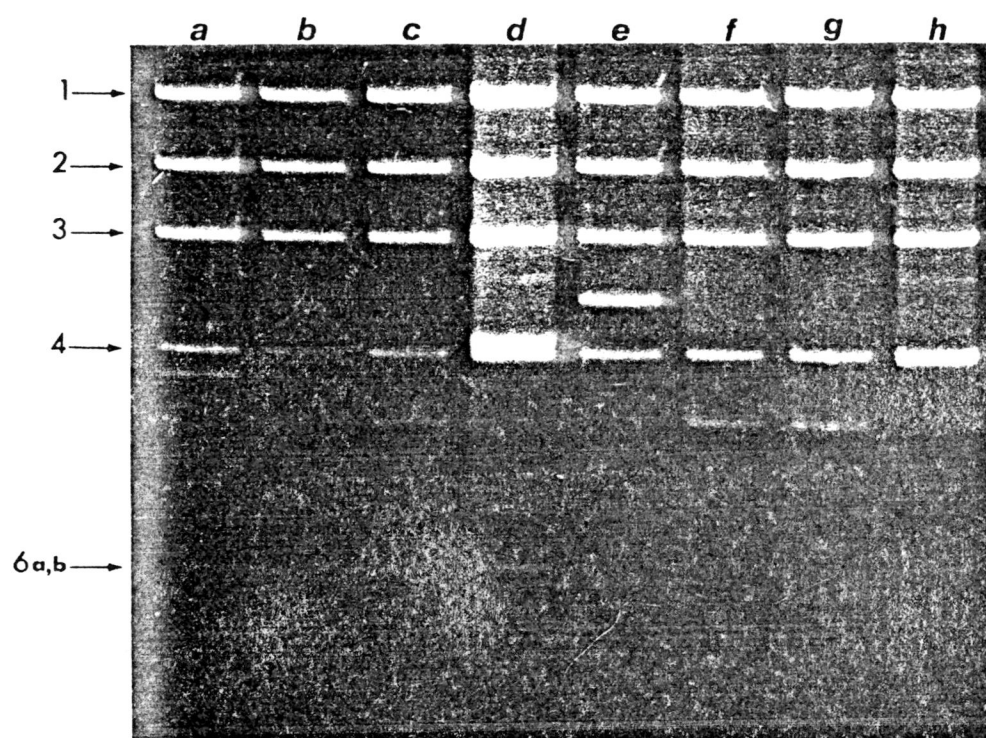
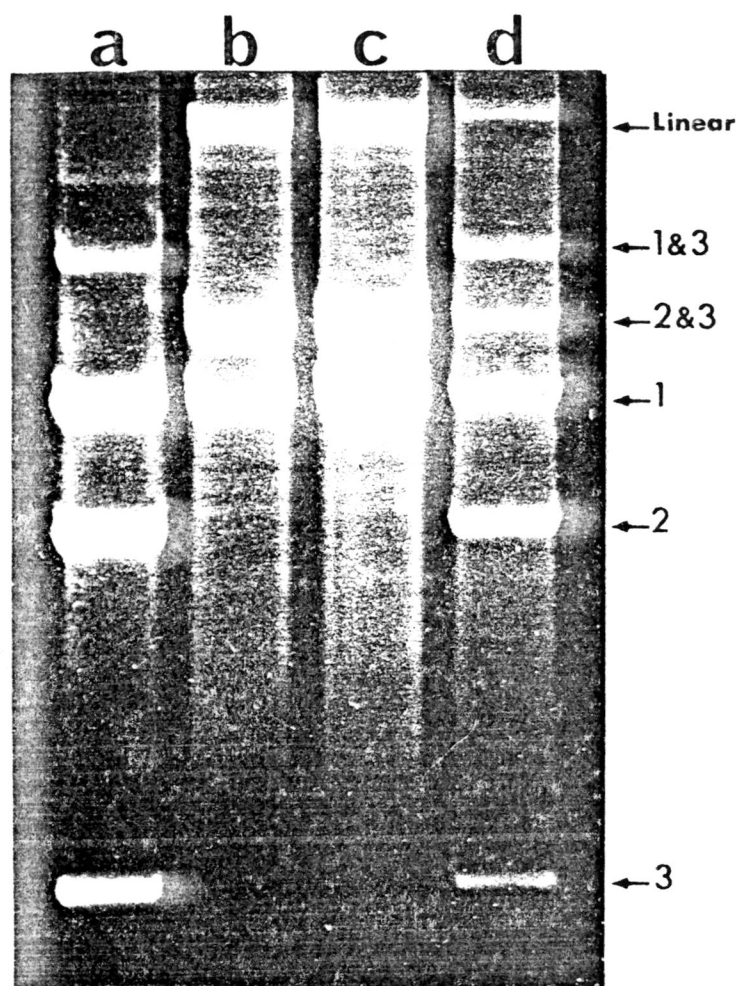


Fig. 12. Triple digest with Pvu II, Sst II and Pst I. 2 to 3 μ g of the RF DNAs were added to Pvu II buffer and then treated with Pvu II (5 units) and Sst II (6 units) for 6 h at 37°C. The DNA samples were then ethanol precipitated, resuspended in Pst I buffer and mixed with 5 units of Pst I (6 h, 37°C). Electrophoresis was on 1.4% agarose (14 h, 60 V). The arrows 1, 2 and 3 mark the fragments derived from complete digestion of strain ØX174 *ins* 6 RF DNA (lane a). The partial bands result from incomplete cleavage of the Pvu II site (2 and 3) or the Pst I site (1 and 3) respectively. The Sst II digest went to completion in each case and no 1 and 2 partial band was obtained. Complete absence of fragments 2 and 3 in lane b and c is indicative of mutant DNAs with destroyed Pvu II sites.



fragments of similar length as strain ØX174 *ins6*, they must be either deletion or insertion mutants with the loss or addition of only very few nucleotides around the Pvu II site. The results of Insertion Experiment III are summarized and compared to the two preceeding experiments in Table 5.

Table 5. Summary of Insertion Experiments I, II and III.

Experiment number	Number of plaques analyzed	% insertion mutants	% deletion mutants	% parental	% other ^a
I	55	3.6	56.4	12.7	27.3
II	23	87	13	0	0
III	30	10	46.7	33.3	10

^a This refers to plaques which seemed to contain more than one type of mutant phage or a contaminating phage.

DISCUSSION

A group of viable ØX174 mutants with genome sizes beyond that of any previously existing ØX174 strain were constructed using a unique system for site-specific modification of the J-F intercistronic region and employing a biochemical selection procedure for isolation of the desired mutants. The success of this system depended upon three factors: 1) the use of a previously constructed ØX174 insertion mutant containing a unique restriction site (62), 2) the use of sufficient quantities of materials to allow monitoring of key *in vitro* construction steps electrophoretically, and 3) successful destruction of infective parental RF DNA molecules with an enzymatic screening procedure. All three factors were crucial to the success of this work for the following reasons: a) need of a unique site for DNA insertion, b) the need to monitor ligation efficiencies and show successful construction of mutants to prove the system works and, c) the need to enhance isolation of desired mutant strains. The efficiency of this technique varied from experiment to experiment and was largely dependent on the completeness of the "screening reactions." Table 5 shows that in the best case 100% of the plaques picked were from mutant strains while in the worst case only 56% contained mutant phage.

Several mutants were isolated with genomes greater than ØX174 *ins11* and the largest of these contained a total insert of approximately 340 nucleotides. Although this is the largest ØX insertion mutant to date, experiments in which plasmid DNA was packaged into the ØX174 capsid *in vitro* suggest that larger insertions may be possible (3). In an attempt

to determine the maximum insert size possible *in vivo*, Insertion Experiment III was performed which gave two findings of significance. The first of these is shown in Table 4. The number of plaques obtained in the transfection from each of the screened ligation products was greatly reduced in comparison to that obtained with the screened parental RF DNA. This could occur if the increased molarity of "ends" produced by the addition of the fragments resulted in a lowering of the efficiency of recircularization and if the Pvu II cleavage of parental DNA was less efficient than Pst I cleavage in the screening reactions. In this way, screening of the parental DNA could possibly leave some uncleaved Pvu II sites and result in parental survivors whose number might theoretically be higher than that of the recircularized molecules in the ligations. However, as can be seen in Fig. 10, the ligation efficiency for this experiment was very good making this possibility very unlikely. This leaves only two other possible explanations for the reduction in plaque number. Either all of the large fragments used for insertion contain biologically active sites which prevented normal expression of phage genes or the insertion size, as well as the screening reaction, greatly reduced the number of viable infective centers produced upon transfection. Preliminary evidence for the latter was found upon analyzing the genomic size of mutant strains. The smallest Hae III fragment used in this experiment was 434 bp in length and if inserted would result in a genome 549 nucleotides larger than wt ØX174. The ligation results indicate that this fragment as well as the larger fragments must have been inserted. Yet, only 3 mutants containing insertions were isolated, even though almost 90% of the

plaques were analyzed. None of these had an insert as large as 549 nucleotides. This means that these 3 mutants may have originally had a larger insert which was reduced due to some form of selective pressure selecting for *in vivo* deletions. Another possible explanation is that minor contaminating nucleases in one or more of the fragment stocks may have resulted in degraded fragments which were inserted upon ligation. However, there was no evidence of substantial amounts of these fragments or measurable amounts of contaminating nucleases in the ligation reaction (Fig. 10). Therefore, if these fragments existed, their concentration must have been very small and their insertion a rare event compared to insertion of the full length fragments, present in much higher concentration. This finding in combination with the transfection results support the *in vitro* packaging results of Hayashi's lab (3) and suggest that the insertion of more than 550 nucleotides exceeds the limit for ØX174. Further studies are required to determine the exact upper size limit.

Previous comparative growth studies of the insertion mutant ØX174 *ins11* indicated that it was approximately 50% as efficient as wt ØX174 (63). This raises questions about the growth efficiency and other biological aspects of the mutants constructed in this study. In the transfection results of Insertion Experiment II, there was an indication that the efficiency of a mutant might decrease proportionately with its insert size. However, the only evidence for this was a decrease in the number and size of the plaques obtained concomitant with an increase in the size of the fragments used for insertion. Although this is not very

conclusive evidence, one would expect a further reduction in the growth efficiency of the new mutants. This remains to be tested.

Besides insertion mutants, there were also deletion mutants isolated in all three experiments. None of these mutants had a deletion greater than strain ØX174 *del3* (62). This is significant because it proves indirectly that the 27 bp deletion in ØX174 *del3* is the largest amount of DNA that can be removed from the J-F intercistronic region without disrupting an essential function (63). Furthermore, this is indirect evidence for the essentiality of gene J *in vivo*, which has not been proven so far because of a lack of gene J mutants.

The existence of ØX insertion mutants with as much as 340 additional nucleotides is interesting from an evolutionary point of view. Such an insertion is roughly equivalent to the size of ØX gene E or the combination of genes J and K. This is indirect evidence that overlapping of all of these 3 genes in ØX174 is not an absolute requirement for packaging. Rather, the evolutionary driving force for overlapping of genes may simply be improved growth efficiency due to the smaller genome. On the other hand gene overlapping may have regulatory advantages.

LITERATURE CITED

1. Aoyama, A., R.K. Hamatake, and M. Hayashi. 1981. Morphogenesis of ØX174: *in vitro* synthesis of infectious phage from purified viral components. Proc. Natl. Acad. Sci. U.S.A. 78:7285-7289.
2. Aoyama, A., and M. Hayashi. 1982. *In vitro* packaging of plasmid DNAs into ØX174 bacteriophage capsid. Nature (London) 297:704-706.
3. Aoyama, A., and M. Hayashi. 1982. Packaging of plasmid DNAs into ØX174 bacteriophage capsid *in vitro*. At the 8th biennial conference on bacteriophage assembly. Fall Creek Falls Park, Tenn.
4. Barnes, W.M. 1977. Plasmid detection and sizing in single colony lysates. Science 195:393-394.
5. Barrel, B.G., G.M. Air, and C.A. Hutchison III. 1976. Overlapping genes in bacteriophage ØX174. Nature (London) 264:34-41.
6. Beck, E., R. Sommer, E.A. Aeurswald, C. Kurtz, B. Zink, G. Osterburg, and H. Schaller. 1978. Nucleotide sequence of bacteriophage fd DNA. Nucleic Acid Res. 5:4495-4503.
7. Benbow, R.M., C.A. Hutchison III, J.D. Fabricant, and R.L. Sinsheimer. 1971. Genetic map of bacteriophage ØX174. J. Virol. 7:549-558.
8. Blakesley, R.W., J.B. Dodgson, I.F. Nes, and R.D. Wells. 1977. Duplex regions in single-stranded ØX174 DNA are cleaved by a restriction endonuclease from *Haemophilus aegyptius*. J. Biol. Chem. 252:7300-7306.
9. Bolivar, F., and K. Blackman. 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. 68:245-267.
10. Borrias, W.E., M. Hagenaar, R. Van den Brekel, C. Kuhlemeizer, and P.J. Weisbeck. 1979. Functional relationship between bacteriophages G4 and ØX174. J. Virol. 31:288-298.
11. Burgess, A.B. 1969. Studies on the proteins of ØX174. II. The protein composition of the ØX coat. Proc. Natl. Acad. Sci. U.S.A. 64:613-617.
12. Deininger, P.L., A. Esty, P. LaPorte, H. Hsu, and T. Friedman. 1980. The nucleotide sequence and restriction enzyme sites of the polyoma genome. Nucleic Acid Res. 8:855-860.

13. Denhardt, D.T. 1975. The single-stranded DNA phages. *CRC Crit. Rev. Microbiol.* 4:161-222.
14. Denhardt, D.T. 1978. The isometric single-stranded DNA phages. *Comp. Virol.* 7:1-104.
15. Edgell, M.H., C.A. Hutchison III, and R.L. Sinsheimer. 1969. The process of infection with bacteriophage ϕ X174. XXVIII. Removal of the spike proteins from the phage capsid. *J. Mol. Biol.* 42:547-557.
16. Eisenberg, S., and R. Ascarelli. 1981. The A^{*} protein of ϕ X174 is an inhibitor of DNA replication. *Nucleic Acid Res.* 9:1991-2002.
17. Eisenberg, S., and M. Finer. 1980. Cleavage and circularization of single-stranded DNA: a novel enzymatic activity of ϕ X174 A^{*} protein. *Nucleic Acid Res.* 8:5305-5315.
18. Eisenberg, S., J. Griffith, and A. Kornberg. 1977. ϕ X174 cistron A protein is a multifunctional enzyme in DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* 74:3198-3202.
19. Enea, V., and N.D. Zinder. 1975. A deletion mutant of bacteriophage ϕ 1 containing no intact cistrons. *Virology* 68:105-114.
20. Fiddes, J.C., and G.N. Godson. 1978. Nucleotide sequence of the J gene and surrounding untranslated regions of phage G4 DNA: Comparison with phage X174. *Cell* 15:1045-1053.
21. Fiddes, J.C., and G.N. Godson. 1979. Evolution of the three overlapping gene systems in G4 and ϕ X174. *J. Mol. Biol.* 133:19-43.
22. Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert. 1978. Complete nucleotide sequence of SV40 DNA. *Nature (London)* 273:113-120.
23. Francke, B., and D.S. Ray. 1971. Formation of the parental replicative form DNA of bacteriophage ϕ X174 and initial events in its replication. *J. Mol. Biol.* 61:565-585.
24. Fujimura, F.K., and M. Hayashi. 1978. Transcription of isometric single-stranded DNA phage, p. 485-505. In D.T. Denhardt, D. Dressler, and D.S. Ray (ed.), *The single-stranded DNA phages*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

25. Fujisawa, H., and M. Hayashi. 1976. Viral DNA-synthesizing intermediate complex isolated during assembly of bacteriophage ØX174. *J. Virol.* 19:409-415.
26. Fujisawa, H. and M. Hayashi. 1976. Gene A product of ØX174 is required for site-specific endonucleolytic cleavage during single-stranded DNA synthesis. *J. Virol* 19:416-424.
27. Fujisawa, H., and M. Hayashi. 1977. Functions of gene C and gene D products of bacteriophage ØX174. *J. Virol.* 21:506-515.
28. Fujisawa, H. and M. Hayashi. 1977. Two infectious forms of bacteriophage ØX174. *J. Virol.* 23:439-442.
29. Fujisawa, H., and M. Hayashi. 1977. Assembly of bacteriophage ØX174: identification of a virion capsid precursor and proposal of a model for the functions of bacteriophage gene products during morphogenesis. *J. Virol.* 24:303-313.
30. Gilbert, W., and D. Dressler. 1969. DNA replication: the rolling circle model. *Cold Spring Harbor Symp. Quant. Biol.* 33:473-484.
31. Godson, G.N. 1974. Evolution of ØX174: isolation of four new ØX-like phages and comparison with ØX174. *Virology* 58:272-289.
32. Godson, G.N., B.G. Barrell, R. Staden, and J.C. Fiddes. 1978. Nucleotide sequence of bacteriophage G4 DNA. *Nature (London)* 276:236-247.
33. Godson, G.N., J.C. Fiddes, B.G. Barrell, and F. Sanger. 1978. Comparative DNA sequence analysis of the G4 and ØX174 genomes, p. 51-86. In D.T. Denhardt, D. Dressler, and D.S. Ray (ed.), *The single-stranded DNA phages*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B.S. Singer, and G. Stormo. 1981. Translational initiation in prokaryotes. *Ann. Rev. Microbiol.* 35:365-403.
35. Griffith, J., and A. Kornberg. 1974. Mini M13 bacteriophage: circular fragments of M13 DNA are replicated and packaged during normal infections. *Virology* 59:139-152.
36. Hardies, S.C., R.K. Patient, R.D. Klein, F. Ho, W.S. Reznikoff, and R.D. Wells. 1979. Construction and mapping of recombinant plasmids used for the preparation of DNA fragments containing the *Escherichia coli* lactose operator and promoter. *J. Biol. Chem.* 254:5527-5534.

37. Hayward, G.S., and M.G. Smith. 1972. The chromosome of bacteriophage T5. I. Analysis of the single-stranded DNA fragments by agarose gel electrophoresis. *J. Mol. Biol.* 63:383-395.
38. Henry, T.J., and R. Knippers. 1974. Isolation and function of the gene A initiator of bacteriophage ϕ X174, a highly specific DNA endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* 71:1549-1553.
39. Herrmann, R., K. Neugebauer, H. Zentgraf, and H. Schaller. 1978. Transposition of a DNA sequence determining kanamycin resistance into the single-stranded genome of bacteriophage ϕ d. *Mol. Gen. Genet.* 159:171-178.
40. Hutchison III, C.A., and R.L. Sinsheimer. 1966. The process of infection with bacteriophage ϕ X174. X. Mutations in a ϕ X lysis gene. *J. Mol. Biol.* 18:429-447.
41. Incardona, N.L., and L. Selvidge. 1973. Mechanism of adsorption and eclipse of bacteriophage ϕ X174. II. Attachment and eclipse with isolated *Escherichia coli* cell wall lipopolysaccharide. *J. Virol.* 11:775-782.
42. Iwaya, M. and D.T. Denhardt. 1971. The mechanism of replication of ϕ X174 single-stranded DNA. II. The role of viral proteins. *J. Mol. Bio.* 57:159-175.
43. Jazwinski, S.M., A.A. Lindberg, and A. Kornberg. 1975. The gene H spike protein of bacteriophages ϕ X174 and S13. I. Functions in phage-receptor recognition and in transfection. *Virology* 66:283-293.
44. Jazwinski, S.M., A.A. Lindberg, and A. Kornberg. 1975. The gene H spike protein of bacteriophages ϕ X174 and S13. II. Relation to synthesis of the parental replicative form. *Virology* 66:294-305.
45. Klein, R.D., E. Selsing, and R.D. Wells. 1980. A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. *Plasmid* 3:88-91.
46. Kornberg, A. 1978. Aspects of DNA replication. *Cold Spring Harbor Symp. Quant. Biol.* XLIII:1-9.
47. Langeveld, S.A., A.D.M. van Mansfeld, P.D. Boas, H.S. Jansz, G.A. van Arkel, and P.J. Weisbeck. 1978. Nucleotide sequence of the origin of replication in bacteriophage ϕ X174 RF DNA. *Nature (London)* 271:417-420.

48. Langley, K.E., M.R. Villarejo, A.V. Fowler, P.J. Zamenhof, and I. Zabin. 1975. Molecular basis of β -galactosidase α -complementation. *Proc. Natl. Acad. Sci. U.S.A.* 72:1254-1257.
49. Lindqvist, B.H., and R.L. Sinsheimer. 1967. Process of infection with bacteriophage ϕ X174. Bacteriophage DNA synthesis in abortive infections with a set of conditional lethal mutants. *J. Mol. Bio.* 30:69-80.
50. Linney, E., M.N. Hayashi, and M. Hayashi. 1972. Gene A of ϕ X174. I. Isolation and identification of its products. *Virology* 50:381-387.
51. Maniatis, T., A. Jeffrey, and H. van DeLande. 1975. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochem.* 14:3787-3794.
52. Martin, D.F., and G.N. Godson. 1975. Identification of a X174 coded protein involved in the shutoff of host DNA replication. *Biochem. Biophys. Res. Commun.* 65:323-330.
53. Marvin, D.A., and E.J. Wachtel. 1975. Structure and assembly of filamentous bacterial viruses. *Nature (London)* 253:19-23.
54. Messing, J., and B. Gronenborn. 1977. Properties of the filamentous phage. M13 as a cloning vehicle. *Happe-Seyler's Z. Physiol. Chem.* 358:278.
55. Messing, J., and B. Gronenborn. 1978. The filamentous phage M13 as a carrier DNA for operon fusions *in vitro*, p. 449-453. In D.T. Denhardt, D. Dressler, and D.S. Ray (ed.), *The single-stranded DNA phages*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
56. Messing, J., B. Gronenborn, B. Muller-Hill, and P.H. Hofschneider. 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a Hind II fragment of the *lac* regulatory region in M13 replicative form *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 74:3642-3646.
57. Mukai, R., R.K. Hamatake, and M. Hayashi. 1979. Isolation and identification of bacteriophage ϕ X174 prohead. *Proc. Natl. Acad. Sci. U.S.A.* 76:4877-4881.
58. Mukai, R., and M. Hayashi. 1977. *In vitro* system that synthesizes circular viral DNA of bacteriophage ϕ X174. *J. Virol.* 22:619-625.
59. Mukai, R., and M. Hayashi. 1977. Synthesis of infectious ϕ X174 bacteriophage *in vitro*. *Nature (London)* 270:364-366.

60. Muller, U.R., and W.M. Fitch. 1981. Theoretical evidence for the significance of certain types of DNA secondary structure in the genomes of ϕ X174, G4, fd, SV40 and the plasmid pBR322, p. 285-297. In M.S. Dubow (ed.), *Bacteriophage assembly*. Liss, New York.
61. Muller, U.R., and W.M. Fitch. 1982. Evolutionary selection for perfect hairpin structures in viral DNAs. *Nature (London)* 298:582-585.
62. Muller, U.R., and R.D. Wells. 1980. Intercistronic regions in ϕ X174 DNA. I. Construction of mutants with altered intercistronic regions between genes J and F. *J. Mol. Biol.* 141:1-24.
63. Muller, U.R., and R.D. Wells. 1980. Intercistronic regions in ϕ X174 DNA. II. Biochemical and biological analysis of mutants with altered intercistronic regions between genes J and F. *J. Mol. Biol.* 141:25-41.
64. Pollock, T.J., I. Tessman, and E.S. Tessman. 1978. Identification of lysis protein E of bacteriophage ϕ X174. *J. Virol.* 28:408-410.
65. Rassart, E., and J. H. Spencer. 1978. Localization of *Escherichia coli* RNA polymerase binding sites on bacteriophage S13 and ϕ X174: alignment with restriction enzyme maps. *J. Virol.* 27:677-687.
66. Ray, D.S. 1977. Replication of filamentous bacteriophages. *Comp. Virol.* 7:105-178.
67. Ray, D.S., and K. Kook. 1978. Development of M13 as a single-stranded cloning vector: insertion of the Tn 3 transposon into the genome of M13, p. 455-459. In D.T. Denhardt, D. Dressler, and D.S. Ray (ed.), *The single-stranded DNA phages*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
68. Reddy, V.B., B. Thimmappaya, R. Dhar, K.N. Subramanian, B.S. Zain, J. Pau, P.K. Ghosh, M.L. Celma, and S.M. Weissman. 1978. The genome of simian virus 40. *Science* 200: 494-502.
69. Rosenberg, M., and D. Court. 1979. Regulating sequences involved in the promotion and termination of RNA transcription. *Ann. Rev. Genet.* 13:319-353.
70. Salivar, W.O., T.J. Henry, and D. Pratt. 1967. Purification and properties of diploid particles of coliphage M13. *Virology* 32:41-51.

71. Sanger, F., A.R. Coulson, T. Friedmann, G.M. Air, B.G. Barrell, N.L. Brown, J.C. Fiddes, C.A. Hutchison III, P.M. Slocombe, and M. Smith. 1978. The nucleotide sequence of bacteriophage ØX174. *J. Mol. Biol.* 125:225-246.
72. Schaller, H. 1978. The intergenic region and the origins for filamentous phage DNA replication. *Cold Spring Harbor Symp. Quant. Biol.* XLIII:401-408.
73. Shlomai, J., L. Polder, K. Arai, and A. Kornberg. 1981. Replication of ØX174 DNA with purified enzymes. I. Conversion of viral DNA to a supercoiled, biologically active duplex. *J. Biol. Chem.* 256:5233-5238.
74. Siden, E.J., and M. Hayashi. 1974. Role of the gene B product in bacteriophage ØX174 development. *J. Mol. Biol.* 89:1-16.
75. Siegal, J.E.D., and M. Hayashi. 1969. ØX174 bacteriophage mutants which affect deoxyribonucleic acid synthesis. *J. Virol.* 4:400-407.
76. Siegal, J.E.D., M.N. Hayashi, and M. Hayashi. 1968. ØX174 coat protein mutants affecting DNA synthesis. *Biochem. Biophys. Res. Commun.* 31:774-778.
77. Simms, J., D. Capon, and D. Dressler. 1979. dna G (Primase) - dependent origins of DNA replication. Nucleotide sequences of the negative strand initiator sites of bacteriophages St-1, ØK, and α3. *J. Biol. Chem.* 254:12615-12628.
78. Spindler, K.R., and M. Hayashi. 1979. DNA synthesis in *Escherichia coli* cells infected with gene H mutants of bacteriophage ØX174. *J. Virol.* 29:973-982.
79. Stahl, D.A., B. Meyhack, N.R. Pace. 1980. Recognition of local nucleotide conformation in contrast to sequence by an r-RNA processing endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* 77:5644-5648.
80. Suggs, S.V., and D.S. Ray. 1978. Nucleotide sequence of the origin for bacteriophage M13 DNA replication. *Cold Spring Harbor Symp. Quant. Biol.* XLIII:379-388.
81. Sumida-Yasumoto, C., J.E. Ikeda, E. Benz, K.J. Marians, R. Vicuna, S. Sugrue, S.L. Zipursky, and J. Hurwitz. 1978. Replication of ØX174 DNA: *in vitro* synthesis of ØX RFI DNA and circular, single-stranded DNA. *Cold Spring Harbor Symp. Quant. Biol.* XLIII:311-329.

82. Sutcliffe, J.G. 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. XLIII:77-90.
83. Sutcliffe, J.G. 1978. pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acid Res. 5:2721-2728.
84. Tessman, E.S., and I. Tessman. 1978. The genes of the isometric phages and their functions, p. 9-29. In D.T. Denhardt, D. Dressler, and D.S. Ray (ed.), The single-stranded DNA phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
85. Tonegawa, S., and M. Hayashi. 1970. Intermediates in the assembly of ØX174. J. Mol. Bio. 48:219-242.
86. Van der Ende, A., S.A. Langeveld, R. Teertstra, G.A. van Arkel, and P.J. Weisbeek. 1981. Enzymatic properties of the bacteriophage ØX174 A* protein on superhelical ØX174 DNA: a model for the termination of the rolling circle DNA replication. Nucleic Acid Res. 9:2037-2053.
87. Van Heuverswyn, H., and W. Fiers. 1979. Nucleotide sequence of the Hind-C fragment of simian virus 40 DNA: comparison of the 5' untranslated region of wild-type virus and some deletion mutants. Eur. J. Biochem. 100:51-60.
88. Weisbeek, P.J., W.E. Borrias, S.A. Langeveld, P.D. Boas, and G.A. van Arkel. 1977. Bacteriophage ØX174: gene A overlaps gene B. Proc. Natl. Acad. Sci. U.S.A. 74: 2504-2508.
89. Weisbeek, P.J., J.H. van de Pol, and G.A. van Arkel. 1973. Mapping of host range mutants of bacteriophage ØX174. Virology 52:408-416.
90. Weisbeek, P.J., and R.L. Sinsheimer. 1974. A DNA-protein complex involved in bacteriophage ØX174 particle formation. Proc. Natl. Acad. Sci. U.S.A. 71:3054-3058.
91. Wells, R.D., T.C. Goodman, W. Hillen, G.T. Horn, R.D. Klein, J.E. Larson, U.R. Muller, S.K. Neuendorf, N. Panayotatos, and S.M. Stirdivant. 1980. DNA structure and gene regulation. Prog. Nucleic Acid Res. and Molec. Biol. 24:167-267.
92. Wells, R.D., S.C. Hardies, G.T. Horn, B. Klein, J.E. Larson, S.K. Neuendorf, N. Panayotatos, R.K. Patient, and E. Selsing. 1980. RPC-5 column chromatography for isolation of DNA fragments. Methods Enzymol. 65:327-347.

93. Wheeler, F.C., and R.H. Benzinger. 1978. Abnormal forms of filamentous phage virions and phage DNA, p. 571-581. In D.T. Denhardt, D. Dressler, and D.S. Ray (ed.), The single-stranded DNA phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
94. Wheeler, F.C., R.H. Benzinger, and H. Bujard. 1974. Double-length, circular, single-stranded DNA from filamentous phage. J. Virol. 14:620-630.
95. Wickner, S., and J. Hurwitz. 1974. Conversion of ØX174 viral DNA to double-stranded form by purified *Escherichia coli* proteins. Proc. Natl. Acad. Sci. U.S.A. 71:4120-4124.
96. Zechel, K., J.P. Bouche, and A. Kornberg. 1975. Replication of phage G4: a novel and simple system for the initiation of DNA synthesis. J. Biol. Chem. 250:4684-4689.