Intercistronic Regions in \( \phi X174 \) DNA

I. Construction of Mutants with Altered Intercistronic Regions between Genes J and F

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Viable mutants of \( \phi X174 \) with modified J-F intercistronic regions were constructed using a novel technique for site-specific mutagenesis. A HaeIII site located in the J-F intercistronic region of the single-stranded viral DNA (+ strand) was made double-stranded by hybridization with the (−) strand of a 404 base-pair TaqI restriction fragment from \( \phi X \) replicative form. Subsequent cleavage with HaeIII specifically at the J-F junction converted the circular partially duplex molecule to a linear form with double-stranded ends. In vitro insertion of pBR322 DNA fragments to or removal of nucleotides from these ends resulted in modification of the sequence comprising the intercistronic region. The ligated molecules were treated with HaeIII to render inactive for transformation all molecules with a regenerated HaeIII site. This step was extremely powerful in reducing the level of wild-type phage since only mutants of the anticipated form were isolated in the subsequent transfection. A total of 13 phages, all mutants in the J-F intercistronic region, were isolated; ten were insertions and three were deletions. The DNA sequences and biological properties of these mutants are described in the following paper (Müller & Wells, 1980).

Also, a procedure was developed to determine the maximum size of inserts tolerated by \( \phi X174 \) in its J-F intercistronic region. The largest insert found was 163 base-pairs.

1. Introduction

Determination of the complete nucleotide sequences of phages \( \phi X174 \) (Sanger et al., 1977) and G4 (Godson et al., 1978a) revealed the existence of untranslated, or intercistronic, regions. Although the remainder of the genetic information is very efficiently utilized (i.e. overlapping genes), \( \phi X \)† contains four intercistronic regions and G4 has five. Several investigators have speculated on the possible role of these untranslated regions (Godson et al., 1978a,b; Fiddes, 1976; Fiddes & Godson, 1978; Sanger et al.,

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‡ Abbreviations used: RF, replicative form DNA; bp, base-pairs; \( \phi X, \phi X174; \) PEG, polyethylene glycol; wt, wild-type.
1977); the potential of these regions, or their transcripts, to form hairpin loop structures has, in general, had an important role in these postulates. In spite of considerable variation in their nucleotide sequences, it is possible to draw similar types of loop arrangements for the two viral DNAs (Godson et al., 1978b).

The isolation of mutant phages with modified or deleted sequences in these regions was undertaken in an effort to rigorously evaluate the biological role of intercistronic regions. We also wished to determine if the potential for hairpin loop structures was necessary. Previous work (reviewed by Wells et al., 1980) has demonstrated the existence of duplex regions at certain loci in some "single-stranded" DNAs.

Two techniques have been employed by other laboratories for site-directed mutagenesis in φX174. Oligonucleotides of seven or more bases in length were synthesized, which were identical in sequence to a stretch of the φX (−) strand with the exception of one base change (Hutchison et al., 1978; Razin et al., 1978; Kössel et al., 1979). When used as primers for negative strand synthesis, point mutants in the lysis gene were created in vitro, converting φX174 am3 mutants to the wt form.

A different approach was reported recently by Humayun & Chambers (1979). Melting of a φX174 RF fragment in the presence of φX (+) strand DNA and subsequent renaturation gave rise to some partially double-stranded (+) strand molecules, which allowed for specific cleavage with a restriction endonuclease and removal of part of the sequence coding for gene G. Also, a φX RF fragment which contains the whole gene G sequence was cloned into an Escherichia coli plasmid (Humayun & Chambers, 1978). The expression of this inserted sequence provided a permissible host with endogenous gene G product, which was used for propagation of the mutant phage.

Both of these techniques relied on powerful biological selection procedures to identify the desired mutant. The construction of mutants in the J-F intercistronic region by conventional genetic techniques was hampered by the lack of information on the necessity of the untranslated regions. Hence, we designed a biochemical approach. The success of our method depended (a) on the construction of sufficiently large amounts of mutant DNAs in order that all reaction steps could be monitored biochemically and (b) on the destruction of infectivity of wt molecules so that only mutant phages would be propagated.

We have generated a collection of φX174 mutants with modifications of the J-F intercistronic region, which prove that at least part of this region is not essential for maintenance of lytic growth under laboratory conditions.

2. Materials and Methods

(a) Bacterial and phage strains

E. coli C600 SF8 (rK- mK- recBC- lop 11 lig+) (Hardies & Wells, 1979), E. coli HF4714 (Benbow et al., 1971), and the non-permissive host E. coli C were used as described previously (Hardies et al., 1979; Dodgson et al., 1976). Bacteriophage φX174 am3 (Hutchison & Sinsheimer, 1966) carries an amber mutation in the lysis gene, and is referred to as the parental strain herein.

(b) Media and buffers

TYE medium contained 10 g tryptone, 5 g yeast extract (both from Difco), 5 g NaCl and 3 g KCl per liter. TYE plates contained 1-5% (w/v) agar in addition. Soft agar contained TYE medium and 0.7% agar.
Standard buffer contained 10 mM-Tris, $10^{-3}$ mM-EDTA, pH 8.2. TAE buffer contained 40 mM-Tris, 5 mM-sodium acetate and 1 mM-EDTA, pH 8.2. TEN buffer was composed of 50 mM-Tris, 5 mM-EDTA and 50 mM-NaCl, pH 8.0. Phage dilution buffer consisted of 20 mM-Tris, 10 mM-MgSO$_4$, 10 mM-CaCl$_2$ and 50 mM-NaCl, pH 7.4.

(c) Enzymes

EcoRI, HaeIII, HindII, TaqI and AluI were prepared and characterized in this laboratory as described previously (Blakesley et al., 1977; Hardies et al., 1979); reaction conditions were as reported previously. T4 DNA ligase (800 units/ml) was purchased from Miles and diluted 10-fold with 0.05 M-KCl, 0.01 M-Tris (pH 7.4), 0.001 M-dithiothreitol, 0.0001 M-EDTA, 50% glycerol, and 0.5 mg bovine serum albumin per ml before use. Micrococcus luteus DNA polymerase was prepared as described (Harwood et al., 1970), and was a gift from J. E. Larson. Ribonuclease A (Worthington Biochemical Corp.) was kept at 100°C for 10 min at a concentration of 5 mg/ml before use. Lysozyme was as described (Harwood et al., 1970).

(d) CX(+) strand and RF DNAs

ϕX174 viral DNA was isolated essentially as described (Pagano & Hutchison, 1971). For preparation of RF, 1-l cultures of E. coli C were infected at a multiplicity of infection of 5 and treated with chloramphenicol (100 μg/ml); the cells were harvested 2 h after infection and RF DNA was isolated according to the procedure used for the isolation of plasmid DNA (Hardies et al., 1979; Hardies & Wells, 1979). The final dialysis was against standard buffer.

(e) Isolation and propagation of phage in small cultures

For isolation of mutants, plaques were cut out of the agar plate with a sterile 200-μl glass pipette and placed into 1.0 ml of E. coli HF4714 culture, grown to an O.D.$_{550}$ value of 0.25. Cultures were incubated under shaking until cleared. 1 ml of an E. coli C culture (O.D.$_{550nm} = 0.5$) was then added, and incubation continued for 2 h. Cells were harvested by centrifugation, resuspended in 1 ml 0.1 M-sodium tetraborate and incubated at 22°C with 50 μl lysozyme (10 mg/ml) for 10 to 20 min. 0.15 ml EDTA (0.15 M) was added, cell suspensions sonicated for 10 s with a Branson Sonifier model W185 (microtip, setting 3), and cell debris was removed by centrifugation. Supernatants were made 10% in PEG (Carbowax 6000), 0.5 M-NaCl and stored at 4°C for at least 2 h. Precipitates were pelleted and resuspended in 1 ml sodium tetraborate for storage or in 0.5 ml dilution buffer for immediate use in the "miniprep" procedure.

(f) Rapid phage screening procedure

The recently described "miniprep" procedure (Klein et al., 1979) was adapted to the ϕX system as follows. 10 ml cultures of E. coli C (O.D.$_{550nm} = 0.5$) were infected with 0.5 ml plaque-purified phage (see above) and chloramphenicol was added to a final concentration of 100 μg/ml 4 to 6 min later. After 2 h shaking at 37°C, cells were pelleted and DNA was prepared as described for the preparation of plasmid DNA from small cultures of cells (Klein et al., 1979). Generally, the DNA obtained after the last ethanol precipitation was dried under vacuum, resuspended in 90 μl water and mixed with 10 μl HaeIII salts (Blakesley et al., 1977), 2 μl HaeIII (1–2 units) and 2 μl RNAse A (5 mg/ml). The 2 to 4 h reaction (37°C) was terminated by the addition of 20 μl bromphenol blue solution (Blakesley et al., 1977), and samples were loaded onto acrylamide slab gels.

(g) Cloning of the 404 bp TaqI fragment containing the J-F intercistronic region

25 μg ϕX RF DNA was digested with TaqI and fractionated on 4% (w/v) tubular acrylamide gels. A slice containing the third largest fragment was minced, incubated with 1 ml 0.5 M-ammonium acetate, 0.01 M-magnesium acetate, 1% sodium dodecyl sulfate
and 0.1 M-EDTA (pH 7.4), for 24 h at 37°C. After filtration through glass wool, the clear filtrate was extracted with phenol. 4 ml ethanol were added and the mixture was kept in a solid CO2/ethanol bath for 15 min. After centrifugation at 15,000 revs/min for 10 min, the precipitate was dried under vacuum. This DNA was used for ligation into the EcoRI site of pBR322 vector DNA (0.25 µg), after both the fragment and the vector sticky ends were filled in with *M. luteus* DNA polymerase. Reaction conditions for the DNA polymerase and ligase reactions were as described (Hardies *et al.*, 1979).

(h) Transfection and transformation

Transfection of *E. coli* HF4714 with φX (+) strand DNA and transformation of *E. coli* C600 SF8 was performed as described for transformation of CaCl2-treated cells (Hardies *et al.*, 1979). In transfection experiments, portions of the transfection mixture were plated with 2.5 ml soft agar on TYE plates. In transformation experiments, 200 µl of transformed cells were spread on TYE plates containing 100 µg ampicillin/ml. Ampicillin-resistant colonies were screened essentially as described by Barnes (1977).

(i) Hybridization of (−) strand of 404 bp TaqI fragment to φX viral DNA and subsequent linearization with HaeIII

The general hybridization procedure was as follows. A mixture of φX (+) strand (1 µg in 10 µl HaeIII buffer (Blakesley *et al.*, 1977)), (−) strand (pool B† from above) of the 404 bp TaqI fragment (0.125 µg in 50 µl standard buffer) plus 2 µl of 5 M-NaCl was kept at 65°C for 1 h, and 90 µl HaeIII buffer was then added. The kinetics and extent of the hybridization were monitored as described in Results.

The DNA was linearized specifically at the HaeIII site in the J-F intercistronic region by addition of HaeIII (2 units in 1 µl) and the reaction was performed at 65°C for 10 min. Samples (20 µl) were mixed with bromophenol blue solution (5 µl) and analyzed by 1.4% agarose gel electrophoresis.

When the linearized DNA was used in a DNA ligase reaction, the HaeIII reaction mixtures were extracted with phenol and dialyzed against standard buffer.

3. Results

(a) Scheme for construction of mutants

Figure 1 shows a general outline of the approach used to construct insertion and deletion mutants in the J-F intercistronic region of φX174 DNA. A HaeIII site (G-G-C-C) is present at position 978 (Sanger *et al.*, 1977), which is approximately in the center of this intercistronic region and is located in a hypothetical hairpin structure (Godson *et al.*, 1978b). In order to cleave the φX DNA with HaeIII specifically in the J-F intercistronic region without affecting the other nine HaeIII sites, we constructed a partially double-stranded molecule, where only the HaeIII site of interest was in the double-stranded form. This was achieved by hybridizing the (−) strand of the 404 bp TaqI fragment to the circular φX (+) strand isolated from the phage. The circular partially double-stranded DNA was then treated with HaeIII under conditions (65°C) where completely single-stranded φX DNA was only cleaved very slowly (Blakesley *et al.*, 1977). The partially double-stranded region at the J-F intercistronic region was cleaved efficiently, converting the circular partially duplex molecule into a linear form with double-stranded ends.

For construction of mutants, this linear, partially duplex DNA was ligated with *Alu*I fragments of pBR322, which led to the occasional insertion of one or more of these fragments into the HaeIII site and thus into the intercistronic region J-F.

† See legend to Fig. 3.
pBR322 was chosen as a source for inserting "non-specific" fragments because its sequence is established (Sutcliffe, 1978), an AluI digest provides a large number of fragments ranging from 11 to 910 bp, it is easy to isolate in quantity, and since blunt end ligation of a HaeIII site (G-G-C-C) with an AluI site (A-G-C-T) does not regenerate a HaeIII site. Since we wished to reduce the background in the transfection step by including a "re-cleavage" step with HaeIII, the last consideration was important.

Due to low level contaminating exonuclease activity, an inherent problem in ligation reactions, we also observed removal of nucleotides at both ends of the linearized hybrid molecule, resulting in circular molecules with deletions of at least some of the nucleotides in the HaeIII site and adjacent nucleotides. Hence, the HaeIII site was destroyed also in these cases.

The mixture of molecules obtained in the ligation reaction was then treated with HaeIII under the same conditions as before. Only molecules that contained a double-stranded HaeIII site were linearized in this step, while those with a defective HaeIII site due to deletion of nucleotides or insertion of AluI fragments (which did not contain an internal HaeIII site) remained in the circular form. This step was necessary in order to enhance the frequency of finding mutant viruses, since the majority of the products in the ligase reaction is due to re-closure of the wt φX DNA with no insertions or
deletions. Since circular φX DNA has a higher transfection efficiency than linear DNA, subsequent transfection of a suitable \textit{E. coli} host with these DNAs resulted in the isolation of only mutants of the desired types.

(b) Cloning and isolation of 404 bp fragment containing J-F intercistronic region

It was unclear at the outset whether the desired mutations would impair the life cycle of the phage. Thus, it was necessary to carry out each step in the construction of these mutants with sufficient quantities of DNA to enable monitoring of the reac-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2}
\caption{Restriction analyses of cloned 404 bp TaqI fragment on 8\% polyacrylamide gels. Approx. 0.5 µg fragment DNA was digested with \textit{HaeIII} or \textit{HindII} and about 1 µg was digested with \textit{HhaI}. Marker DNAs were a \textit{HaeIII} digest of pRZ2 (marker A) (Hardies et al., 1979), and a \textit{HaeIII} digest of pBR322 (marker B). The length (in bp) of some of the marker fragments is indicated. The \textit{HindII} digest was terminated before completion of the reaction to show the migration rate of the undigested TaqI fragment.}
\end{figure}
tions by gel electrophoresis. This required a relatively large amount of the purified 404 bp TaqI fragment.

The 404 bp TaqI fragment of ϕX was cloned into the EcoRI site of the pBR322 as described in Materials and Methods using the general procedures described previously (Hardies et al., 1979). pBR322 DNA was cleaved with EcoRI and, after heat inactivation of the enzyme, was mixed with the 404 bp TaqI fragment (purified by gel electrophoresis) in a molar ratio of fragment : vector = 50. Both the protruding 5′ ends of the vector as well as those of the fragment were filled in with M. luteus DNA polymerase and the products were joined with T4 DNA ligase. Blunt end ligation of a filled-in TaqI end to a filled-in EcoRI end should regenerate an EcoRI site. It was therefore anticipated that a plasmid could be constructed which would release the desired TaqI fragment upon treatment with EcoRI. Transformation of E. coli and screening of ampicillin-resistant colonies for the presence of the desired plasmid is described in Materials and Methods. Fifty-six colonies were screened, and 12 appeared to contain a plasmid of the expected size. Further characterization of these candidates (as described below), led to the isolation of two plasmid types, both of which contained an insert of approximately the expected size, but in opposite orientations, which could be released upon treatment with EcoRI.

One of these strains was grown in a 14 litre culture and 2.5 mg plasmid DNA was isolated (Materials and Methods). After digestion with EcoRI, the released fragment was separated from the vector by sucrose density centrifugation (Hardies & Wells, 1979), yielding 150 µg purified fragment DNA. To confirm the identity of the 404 bp TaqI fragment, the isolated DNA was characterized by restriction analyses. Figure 2 shows gel analyses of a complete HaeIII and HhaI digest and an incomplete HindII digest. As expected from the sequence, the TaqI fragment should give two HaeIII fragments (258 bp and 146 bp) each with one EcoRI end. The two HindII fragments should be 232 and 172 bp in length, each containing one EcoRI end, and the HhaI digest should yield five fragments with the following sizes: 103 bp (with one EcoRI end), 101 bp, 93 bp, 54 bp and 53 bp (with one EcoRI end). Considering that sticky ends reduce the migration rate of DNA fragments on polyacrylamide gels (Hardies & Wells, unpublished results), the observed fragment sizes are in good agreement with the predicted values (Sanger et al., 1977). The fact that the undigested 404 bp fragment in the HindII reaction migrates with a rate characteristic for a molecule of approximately 440 bp length is also due to the two EcoRI ends. The plasmid containing this fragment was named pRW401.

(c) Separation of strands of 404 bp fragment

The complementary strands of the cloned fragment containing the J-F intercistronic region were preparatively separated by RPC-5 column chromatography at high pH (Wells et al., 1979). Figure 3 shows polyacrylamide gel analyses on some of the column fractions. All fractions shown contain a DNA band migrating in approximately the same position in the gel; an additional stronger band migrating more rapidly appears in fraction 68. We conclude that fractions 52 to 66 and 70 to 72 contain mostly single-stranded TaqI fragments of either type. Where overlap of the two strands occurs as in fraction 68, the strands reanneal after neutralization of the fractions, and the DNA migrates on the gel as a double-stranded molecule.
FIG. 3. Separation of the complementary strands of the 404 bp TaqI fragment by alkaline RPC-5 column chromatography. 20 µg of the 404 bp TaqI fragment (in 0.2 ml) was loaded onto a 200 mm x 1.5 mm RPC-5 column which was equilibrated with 0.25 M-KCl in 12 mM-NaOH. Elution was carried out at 22°C with a 40 ml gradient of 1.05 to 1.25 M-KCl, all 12 mM in NaOH, and the absorbance at 260 nm was continuously recorded. 75 fractions (0.7 ml each) were collected and neutralized with 0.01 vol. 1 M-Tris, 0.1 M-EDTA (pH 7.8) and 0.012 vol. 1 M-HCl. 200 µl of fractions 52 to 68, and 50 µl of fractions 70 to 72 were evaporated to dryness, mixed with 50 µl water and 30 µl bromophenol blue solution, and analyzed on a 5% polyacrylamide slab gel (Hardies et al., 1979; Patient et al., 1979). Fractions 52 to 66 (pool A) and 70 to 72 (pool B) were combined and dialyzed against standard buffer.
This interpretation was shown to be correct when samples from each of the pools were hybridized to φX (+) strand and the products were analyzed by agarose gel electrophoresis (Fig. 4). As a control, φX (+) strand DNA was subjected to the same treatment, except that an equivalent amount of buffer was added instead of fragment DNA. The control lane in Figure 4 shows the position of linear (lower band) and circular (upper band) φX (+) strand DNA. Treatment with pool B DNA but not pool A DNA caused the appearance of a new band, migrating slower than the circular form under these conditions, with a concomitant loss of DNA in the position of the circular form. The new slow moving band is circular φX (+) strand annealed to the

![Image of gel electrophoresis](image-url)

**Fig. 4.** Effect of separated strands of 404 bp TaqI fragment on electrophoretic behavior of φX (+) strand DNA. Approx. 0.3 μg of φX (+) strand DNA in TAE buffer was mixed with either 20 μl pool A or 5 μl pool B and 1 μl 5 M NaCl in a reaction mixture adjusted to 30 μl with TAE buffer. After incubation at 65°C for 75 min, 5 μl bromophenol blue solution were added and the mixtures were electrophoresed on a 1.4% agarose slab gel at 200 V according to Johnson et al. (1979).
(-- ) strand of the 404 bp TaqI fragment. In a subsequent titration experiment, all circular \( \phi X ( + ) \) strand DNA (0.3 \( \mu g \)) could be converted to this slow moving form when 0.03 \( \mu g \) of pool B DNA were added (data not shown).

Thus, pool B DNA contains the (-- ) strand of the 404 bp TaqI fragment. Also, annealing the (-- ) strand of the fragment to the \( \phi X ( + ) \) strand causes an observable shift of this DNA to a slower migrating form under this gel condition.

(d) \textit{HaeIII linearization of partially double-stranded circular \( \phi X ( + ) \) strand DNA}

Figure 5 shows gel analyses on the products of the reaction of \textit{HaeIII} with the partially double-stranded DNA described above. Treatment of the partially duplex \( \phi X \) DNA (shown in lane (b) as a control) with \textit{HaeIII} efficiently converted it into a more rapidly migrating species (lane (d)). Lane (a) is a control of \( \phi X ( + ) \) DNA and lane (c) is the same after treatment with \textit{HaeIII} showing that the totally “single-stranded” DNA was not cleaved under these conditions. If a second cleavage occurred at a restriction site within the single-stranded portion of the linear molecule, it would have been converted into two smaller fragments which would migrate at a faster rate, and consequently remove it from its characteristic position. Since little or no such loss occurred, we conclude that \textit{HaeIII} linearization of the partially double-stranded \( \phi X ( + ) \) strand molecule is due to specific cleavage of the double-stranded \textit{HaeIII} site located in the J-F intercistronic region.

It should be noted that use of these specific electrophoresis conditions (high current) are necessary to achieve the resolution shown in Figure 5 of the linearized \( \phi X \) viral DNA with duplex segments at the termini (the products of the \textit{HaeIII} reaction) from the partially duplex circular \( \phi X \) DNA (lane (b)). Similar gels run at a lower current but for longer time periods will not provide this degree of resolution. Under the high voltage conditions (200 V in TAE buffer) of Figure 5, the gels become somewhat warm (approx. 45°C), which may be a factor in the improved separation.

(e) \textit{Infectivity of circular and linear \( \phi X ( + ) \) strand DNA}

\( \phi X ( + ) \) strand DNA and the partially double-stranded form before and after treatment with \textit{HaeIII} were used for the transfection of \textit{E. coli} HF4714, the permissive host. Before treatment with the restriction enzyme, both forms of DNA give rise to approximately \( 5 \times 10^4 \) plaque-forming units per \( \mu g \) DNA. After treatment with \textit{HaeIII} under the described conditions, the yield of plaque-forming units is reduced approximately tenfold for the \( \phi X ( + ) \) strand and approximately 300-fold for the double-stranded form (data not shown). Since only a very small amount of linearization of the \( \phi X ( + ) \) strand occurs when treated with \textit{HaeIII} under the above described conditions (i.e. see Fig. 5 lanes (a) and (c)), the tenfold reduction in infectivity must be due to factors other than cleavage of the phosphodiester DNA backbone. Previous work (Fiers & Sinsheimer, 1962) has documented the sensitivity of the infectivity of \( \phi X \) viral DNA to heat treatment; thus, this tenfold reduction is not unexpected, since the DNA went through a heat treatment step. We presume that the additional 30-fold or more reduction in infectivity of the partially double-stranded form is due to the \textit{HaeIII}-specific linearization.
Fig. 5. *Hae*III linearization of the circular partially double-stranded φX (+) strand DNA at the *J-F* intercistronic region. The 404 bp *Taq*I fragment (−) strand was annealed to the φX (+) strand DNA and portions were subsequently treated with *Hae*III. φX (+) strand DNA before (lane (a)) and after (lane (c)) treatment with *Hae*III. The partially double-stranded φX (+) strand DNA before (lane (b)) and after (lane (d)) treatment with the same enzyme. The position of the circular φX (+) strand as isolated from the phage is shown as a circle. A partially double-stranded circle shows the position for the φX (+) strand when hybridized to the 404 bp *Taq*I fragment (−) strand. A partially double stranded line identifies the position where linear φX (+) strand and partially double-stranded linear φX (+) strand comigrate. The horizontal slab 1.4% agarose gel was run at 200 V in TAE buffer. Other details are in Materials and Methods.
Fig. 6. Ligation of AluI fragments of pBR322 into the J-F intercistronic region. 10 μg of φX (+) strand was hybridized to 125 μg (−) strand of the 404 bp TaqI fragment and subsequently treated with HaeIII until all material was linearized as judged by agarose gel electrophoresis. After removal of HaeIII by phenol extraction (Materials and Methods), the sample was mixed with 80 μg of pBR322 DNA partially digested with AluI to give a total volume of 0.9 ml in standard buffer. To this was added 100 μl ligase buffer and T4 DNA ligase (0.4 units in 5 μl). The mixture
The determination of these infectivities was performed since: (a) the success of the general experimental approach (Fig. 1) depends on the fact that linear DNA has a lower infectivity than circular and, (b) this is the first report that describes the linearization of φX viral DNA in an intercistronic region.

(f) Insertion of AluI fragments of pBR322 into the J-F intercistronic region

The partially duplex molecule which was linearized specifically at the HaeIII site in the J-F intercistronic region was reacted with T4 DNA ligase along with an excess of an AluI digest of pBR322 DNA. Controls for this reaction are shown in lanes (a) to (d) of Figure 6. Circular φX (+) strand DNA and linearized partially double-stranded φX (+) strand were treated with T4 DNA ligase and samples, before and after the ligase reaction, were analyzed. As expected, the distribution of φX (+) strand in the circular form (lane (a), upper band) as compared to the randomly linearized form (lane (a), lower band) remained unchanged after the ligase reaction (lane (b)). The partially double-stranded φX (+) strand, previously linearized by cleavage at the duplex HaeIII site (lane (c)), was circularized by the action of T4 DNA ligase (lane (d)). This confirmed that the linearized molecule in lane (c) had double-stranded ends.

Lane (e) shows the ligation products of the partially duplex φX (+) strand DNA which was linearized by HaeIII when reacted in the presence of AluI fragments of pBR322. Most of the DNA is shifted into the position characteristic for its circular form. A novel band also appears which migrates between the linear and circular forms. This band may be linear partially double-stranded φX (+) strand extended in length by one or more AluI fragments at either one or both double-stranded ends. The smear of DNA throughout the lower part of this lane is the bulk of the AluI-digested pBR322 DNA and its various ligation products.

As outlined in Figure 1, the formation of circular parental φX (+) strand molecules in this ligation reaction is inevitable. To reduce the level of infective parental DNA, a final treatment with HaeIII was performed to cleave all regenerated double-stranded HaeIII sites. Figure 7 shows the various forms of circular and linear φX (+) strand DNA in lanes (a) to (d). Lane (e) shows the ligation products presented in Figure 6(e) after extensive digestion with HaeIII. Most of the φX (+) strand was converted to the linear form.

(g) Isolation of phage mutants

The mutant DNA (as in Fig. 7 lane (e)) was used to transfect CaCl2-shocked E. coli cells as described in Materials and Methods. In two such experiments (using approximately 0.2 μg DNA/transfection), a total of 13 plaques were found. Phage were isolated from these plaques and preliminarily designated I to XIII. Since neither the size nor the morphology of their plaques identified these strains as mutants, small cultures of E. coli were infected and RF DNA was isolated by a rapid phage screening procedure

was incubated for 20 h at 15°C, after which an additional 10 μl ligase (0.8 unit) was added, and the reaction time was extended by an additional 20 h. 20 μl of this reaction mixture were analyzed on 1.4% agarose gels in TAE buffer at 200 V. A portion of the reaction mixture analyzed after 40 h ligation is shown in lane (e). Markers for the gel and controls for the ligation reaction are as follows: φX (+) strand DNA before (lane (a)) and after ligation (lane (b)); linear partially double-stranded φX (+) strand DNA before (lane (c)) and after ligation (lane (d)).
Fig. 7. *Hae*III treatment of ligation products. The ligation products shown in Fig. 6 (e) were digested with *Hae*III at 65°C for 4 h as in Fig. 5. A portion was analyzed as in Fig. 6 by agarose slab gel electrophoresis (lane (e)). Markers for the gel were: φX (+) strand DNA before (lane (a)) and after treatment with *Hae*III for 5 min at 65°C (lane (b)); partially double-stranded φX (+) strand DNA before (lane (c)) and after treatment with *Hae*III (lane (d)).
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(Materials and Methods). A \( HaeIII \) digest of these DNAs clearly identified all 13 as mutants of the desired form. Hence, RF DNA was then prepared by the standard procedure and was used for further restriction analysis.

Figure 8 shows a \( HaeIII \) digest of RF DNA from the parental \( \Phi X174 \) am 3 as control and all mutant strains containing an insertion (isolation of strain 30 is described below). Whereas the parental RF DNA shows a typical \( HaeIII \) pattern (Sanger et al., 1977), fragments 5 and 8 are missing in each mutant digest. Instead, an additional band of variable size appears, which is larger than 504 bp in each case. Since the \( HaeIII \) site in the \( J-F \) intercistronic region lies between the \( HaeIII \) fragment 5 (310 bp) and the \( HaeIII \) fragment 8 (194 bp) (see Fig. 12), this indicates an insertion into this region. The sizes of these inserts range between 50 and 170 bp as judged by their migration in polyacrylamide gels. Further characterization of these insertions by restriction mapping is presented in the accompanying paper (Müller & Wells, 1980).

Figure 9(a) shows a \( HaeIII \) digest of RF DNA from strains VII, XI and XII as compared to the parental strain. In these cases, the \( HaeIII \) site in the \( J-F \) intercistronic region has also been destroyed and fragments 5 and 8 are fused for these mutants. However, the fusion fragments are slightly smaller than 504 bp, suggesting the loss of some DNA. For a more accurate estimate of the size of the deletions, RF DNAs were cleaved with \( HhaI \) (Fig. 9(b)). The \( HhaI \) fragment 12 of parental \( \Phi X174 \) is 93 bp long (Sanger et al., 1977) and contains all of the \( J-F \) intercistronic region. Figure 9(b) shows that the \( HhaI \) fragment 12 for each mutant has been shortened, while all other \( HhaI \) fragments remained unaltered. The \( HhaI \) fragment 12 of strain VII is not easily detectable as it comigrates with fragment 13. From their migration behavior (of fragment 12), the size of the deletions was estimated to be 7 bp for strain VII, 4 bp for strain XI, and 26 bp for strain XII. Sequence analysis of strain XII in this region showed the absence of 27 bp in the \( J-F \) intercistronic region (Müller & Wells, 1980).

(h) Maximum size of permissible inserts into the \( J-F \) intercistronic region

In an attempt to determine the maximum length of DNA that can be inserted without impairment of lytic growth, mutants were isolated by a slightly different procedure. As outlined in Figure 10, this approach theoretically allows for the isolation of \( \Phi X174 \) mutants with an insertion of an \( AluI \) fragment of pBR322 which carries a \( HaeIII \) site. When \( HaeIII \)-linearized partially double-stranded \( \Phi X \) (+) strand DNA is treated with T4 DNA ligase in the presence of \( AluI \) fragments of pBR322, three possible types of products are formed, namely partially double-stranded circular parental strands, and those carrying either insertions or deletions (Fig. 1). The mixture of these molecules was used for transfection of \( E. coli \) HF4714, a mixture of phage were isolated, and \( \Phi X \) (+) strand DNA was prepared by phenol extraction. The (–) strand of the 404 bp \( Taq \) I fragment was hybridized to this DNA mixture and the DNAs were then treated with \( HaeIII \) at 65°C. Assuming that only the parental \( \Phi X \) (+) strands can form a perfectly base-paired region at the \( HaeIII \) site, only parental DNA should be linearized, while mutant DNAs (even if they contain a \( HaeIII \) site in the inserted sequence) should remain circular.

Transfection with DNA products was carried out and 48 plaques were picked randomly. Phage isolated from these plaques were analyzed by the rapid screening
FIG. 8. Restriction analyses of insertion mutants. RF DNAs (approx. 3 μg) were digested with HaeIII and products were separated on a 4% polyacrylamide slab gel. The fragments obtained in the control (c) digest are marked by arrows and labelled according to Sanger et al. (1977). The position where a fragment of the combined length of fragment 5 and 8 would migrate is indicated as 5–8. The wt designation indicates the digest of φX174 am3 DNA.
Fig. 9(a).
Fig. 9. Restriction analyses of deletion mutants. (a) *Hae*III digest of RF DNA from *φX174* am3 (control) and mutant strains VII, XI and XII. 1 to 3 µg of RF DNAs were digested with *Hae*III and electrophoresed on a 4% polyacrylamide slab gel. Fragments of the control digest were marked as in Fig. 8. (b) *Hha*I digest of RF DNA from *φX174* am3 and mutant strains VII, XI and XII. 4 to 5 µg RF DNA were digested with *Hha*I and electrophoresed on an 8% polyacrylamide slab gel. Fragments of the control digest were designated as described above. Fragment 12 contains the J-F intercistronic region. The wt designation indicates the digest of *φX174* am3 DNA. Other details are in Materials and Methods.
Deletions (HaeIII site destroyed) + wt (HaeIII site intact) + Insertions (HaeIII site destroyed)

Transfect E.coli

Isolate (+) strand DNA and hybridize with the (−) strand of the 404 bp TaqI fragment

Non-paired loop in fragment strand (HaeIII site destroyed) + Complete hybridization (HaeIII site intact) + Non-paired loop in (+) strand (HaeIII site destroyed)

Circular molecules Linear molecules

Circular molecules

Transfect E.coli

**Fig. 10.** Scheme for the isolation of ϕX174 mutants with maximum size inserts. The scheme is a continuation of that described in Fig. 1 and starts with the products of the ligation reaction. A portion of this reaction mixture was used to transfect E. coli, and a plate lysate containing a mixture of all viable phage was used for propagation of phage for the purification of (+) strand DNA. Hybridization of the (−) strand of the 404 bp TaqI fragment to the (+) strand DNA, HaeIII restriction at 65°C and transfection of E. coli with the reaction products were carried out as described in Materials and Methods. The wt designation indicates the digest of ϕX174 am3 DNA.

A typical HaeIII digest of RF DNAs obtained by this procedure is shown in Figure 11. Though the digests in some cases are incomplete and recoveries vary from sample to sample, the typical HaeIII digest pattern is visible in all cases. The absence of bands 5 and 8 and the appearance of a new band migrating close to HaeIII fragment 4 identifies a strain as an insertion mutant. As judged by this technique, 18 mutants were isolated. None of these contained an insert with an internal HaeIII site. The largest insert of approximately 160 to 170 bp was found in strain 30 (shown in Fig. 8); this insert is shown in the following paper (Müller & Wells, 1980) to be 163 bp long. The other mutants were not characterized further.

### 4. Discussion

A series of viable mutants with sequence changes in the intercistronic region between genes J and F, for which no biological function is known, were constructed using a new technique for site-specific mutagenesis. Three important factors contributed to the success of these experiments. (a) Cloning of the 404 bp TaqI fragment permitted the
Fig. 11. HaeIII digest of RF DNAs obtained by the "miniprep" procedure. Phage from 48 plaques were isolated and RF DNA prepared by the rapid phage screening procedure (Materials and Methods). A typical HaeIII digest of such minipreps is shown for 12 strains. The characteristic HaeIII fragments of wt φX174 RF DNA are marked by arrows. Digests in which fragments 5 and 8 were missing and a new band of appropriate intensity appeared migrating close to fragment 4 were indicative of a mutant strain. This is the case for strains 34, 38, 41, and 44.
use of relatively large quantities of material throughout so that all reactions could be monitored by gel electrophoresis. Thus, conditions could be established for driving key in vitro reactions to virtual completion. (b) Strand separation and use of the Taq I fragment (−) strand facilitated a total conversion of φX (+) strands to the partially double-stranded form. This is a necessary prerequisite for successful elimination of parental genomes since unhybridized viral DNA will not be linearized, and thus rendered inactive, in the final Hae III reaction. (c) The final Hae III cleavage step linearized parental genomes, thus rendering them inactive for transfection. The final yield of mutant phage compared to the amount of (+) strand DNA at the beginning of the reaction was very low (approx. 0.001 %) and was due, in part, to the elimination of all mutant genomes containing an Alu I fragment with an internal Hae III site and all parental φX genomes. However, this final step was of great importance since all 13 phages isolated were mutants.

Other workers (Humayun & Chambers, 1979) have shown that mutants of φX174 may be constructed with limiting amounts of fragment DNA and without strand separation of the fragment used for hybridization. However, these investigators had a powerful screening procedure for identifying mutant phages. Since the biological function of the J-F intercistronic region is unknown, we did not have a similar screening technique. Hence, it was intolerable in our study to have a large population of parental DNA (unhybridized with the complementary fragment and thus not cleaved by Hae III) in the final transfection step.

For insertion into the J-F intercistronic region, we chose a partial Alu I digest of the sequenced pBR322 DNA (Sutcliffe, 1978). The largest fragment without an internal Hae III site is Alu I fragment 6, 281 bp long. On the other hand, the shortest Alu I fragment containing at least one Hae III site is fragment 8, 226 bp in length. In an attempt to determine the maximum amount of additional DNA that may be packaged into the φX capsid without abolishing infectivity, we tried to isolate all possible mutants which were constructed in the ligation reaction. Essentially all circular DNA molecules obtained in this step were used for transfection without discrimination by Hae III restriction (Fig. 19). Thus even genomes containing larger inserts with internal Hae III sites were permitted to establish lytic growth. To avoid screening hundreds of plaques, however, (+) strand DNA was isolated from a mixture of all progeny phage, hybridized with the 404 bp Taq I fragment (−) strand and restricted with Hae III. Subsequent transfection then resulted in the isolation of 18 mutants out of 48 plaques screened. This relatively high yield (40 %) of mutant phage indicates that a successful reduction of parental phage could be achieved with the final Hae III restriction. With the exception of strain 30, all mutants obtained had inserts ranging in size from 20 to 70 bp, as judged by polyacrylamide electrophoresis. Strain 30 had an insert of 163 bp (Müller & Wells, 1980), the largest insert found to date. That larger inserts were not found may indicate that addition of more than ~160 bp to the φX174 genome would prevent packaging, or otherwise present a negative survival advantage by which these mutants were outgrown by parental phage or mutants with smaller inserts. Alternatively, it is possible that hybridization of the 404 bp Taq I (−) strand to the mutant (+) strand DNA does, in fact, provide a suitable (albeit weak) Hae III site, thereby rendering these mutant genomes inactive. Complete control studies on the reaction of Hae III with “non-paired loop” substrates such as those shown in Figure 10 have
not yet been performed; also other features of the substrate requirements for HaeIII remain to be elucidated (Blakesley et al., 1977).

Our successful isolation of several viable φX174 mutants with a modified or partially deleted J-F intercistronic region has several biological implications. Sequence comparisons between the φX and G4 genomes revealed that extensive sequence homology in the coding region of these two closely related phages has been maintained; however, this is not the case for the intercistronic regions, especially between genes J and F. Yet the secondary structures which have been proposed for the three larger intercistronic regions have been well maintained in size and geometry in both phage genomes (Fiddes & Godson, 1978; Godson et al., 1978a,b). The primary sequence of the J-F intercistronic region of both φX and G4 may fold into a stable hairpin structure, which has been implicated as a terminator of transcription in vitro as well as in vivo (Godson et al., 1978b; Fiddes & Godson, 1978). Since the HaeIII site which we have used in the construction of the described mutants is located in this proposed hairpin structure (Fig. 12), comprising part of the stem and part of the loop, insertions of 50 and more bp into this region should greatly diminish the stability of this particular secondary structure. This is also to be expected for the deletion mutants, at least for strain VII (7 bp deleted) (Müller & Wells, 1980) and strain XII (27 bp deleted), where the HaeIII site has been destroyed (Fig. 9(a)). Consequently, part or all of the potential hairpin sequence has been removed in these cases. Since all of these mutants grow in the absence of helper functions, we conclude that part of the J-F intercistronic region and formation of a hairpin structure in this sequence are not essential for lytic growth under laboratory conditions.

Three genes (B, K and E) are known at present in φX and G4 to overlap with the coding regions of the other genes (Godson et al., 1978b). It has been tempting, there-

\[
\begin{align*}
7001 & & 3 \\
HaeIII & & 5 \\
HaeI & & 8 \\
\text{Gene order} & & 12 \\
\text{D} & & \text{J} & & \text{F} \\
\text{Intercistronic region J-F} & & & & & & & & & & & & & & & & & & & & & & & & & & & & \text{End of J} & & \text{HaeIII site} & & \text{Start of F} \\
965 & & 1000
\end{align*}
\]

**Fig. 12.** A portion of the φX174 genome has been redrawn from Sanger et al. (1977) to illustrate the correlation between the various restriction fragments used in this work and the genetic map. The sequence of the J-F intercistronic region is shown to indicate the potential secondary structure (Godson et al., 1978b), the termination codon of gene J (T-A-A), the ribosome-binding site (A-G-G-A), and the start codon of gene F (A-T-G).
fore, to speculate on the evolutionary driving forces that have created complicated genomes of this type. Formation of overlapping genes, rather than extension of the genome by additional DNA sequences, has been argued to be possibly favored because of the physical constraints imposed on these genomes by the limited size of the phage capsids. Alternatively, it has been suggested that at least genes K and E were later evolutionary developments, using pre-existing sequences in a different reading frame (Godson et al., 1978b). Other aspects, such as energetic considerations, the existence of intercistronic regions, and the positioning of genes as a consequence of regulatory mechanisms, obscure the answer to this problem even more and it becomes clear that much more information is necessary before these genomes and their gene order can be fully understood. Our data contribute to this understanding since we showed that the addition of at least 163 bp to the genome of φX is feasible. Thus, it seems improbable that physical constraints on the size of the DNA were the evolutionary reasons for overlapping of some sequences since, for example, the coding region of gene K is only 170 bp long.

Finally, we note that because of the nature of the J-F intercistronic region, containing a ribosome-binding site as well as a potential terminator of transcription, these φX mutants provide interesting new tools for the study of transcription termination and translation in φX174.

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