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Genetic Assay for Small Fragments of Bacteriophage φX174 Deoxyribonucleic Acid

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The double-stranded replicative form deoxyribonucleic acid (RF-DNA) of bacteriophage φX174 was fragmented by pancreatic deoxyribonuclease, and the complementary strand fragments were then annealed to intact viral single strands. When such complexes infected *Escherichia coli* spheroplasts, some of the progeny virus bore genetic markers derived from the RF-DNA fragments. In this way, genetic markers have been salvaged from DNA fragments less than 50 nucleotides in length. This method is potentially useful as a specific assay to aid in the purification of genetically defined DNA fragments and also as a mechanism for the incorporation of small chemically synthesized DNA sequences into viral genomes.

The deoxyribonucleic acid (DNA) of the single-stranded bacteriophage φX174 is one of the most promising for detailed chemical studies, including base sequence determinations (7, 10, 13). Although its genome length of 5,500 nucleotides (15) places it among the smallest characterized DNA species, the task of a complete structure determination by present methods is overwhelming. For this reason, we are interested in developing methods for the isolation of specific small fragments of φX174 which will be suitable materials for such chemistry. The approach we are taking is based upon the canonical assumption that it is possible to purify anything for which we have a specific assay. We describe here a method by which small fragments of the φX genome may be assayed to determine which genetic markers they contain. In combination with the many available φX174 mutations (2, 5, 16, 17), this assay should provide a useful tool for the identification of almost any desired fragment of the genome.

The procedure which we use to assay DNA fragments is outlined in Fig. 1. The double-stranded replicative form of φX DNA (RF) is purified from infected cells (11) and then fragmented by pancreatic deoxyribonuclease. The resulting pool of fragments is mixed, under annealing conditions, with intact viral single-stranded DNA ("+" strands) bearing a conditional lethal mutation. Some of the annealed structures formed in this way should be genetically heterozygous, being composed of a wild-type (*wt*) fragment of the complementary strand ("−" strand) and a complete mutant "+" strand. The mixture will also contain, of course, annealed structures composed of the mutant "+" strand paired with "−" strand fragments which do not cover the site of the mutation, as well as unannealed "+" strand fragments from the RF digest. These annealed complexes are infective to *Escherichia coli* spheroplasts, using the standard φX DNA assay (6). Some of the progeny from such an infection bear the *wt* marker from the RF fragment. Particles bearing such "salvaged" markers can be selectively counted by performing a plaque assay under conditions which are restrictive for the conditional lethal mutation in the "+" strand. We use the word "salvage" to describe the recovery of phage genetic markers from DNA fragments. We do not like to use "rescue" because this word has generally been used to describe the recovery of markers from complete (but dead) genomes.

Our reason for attempting this method of assay was a hope that the annealed "−" strand fragment would be enzymatically completed, to produce a complete complementary "−" strand, within the infected spheroplast. A possible mechanism is illustrated in Fig. 2. Since the 3′ end of the pancreatic deoxyribonuclease fragment bears a hydroxyl group, it would be expected to function as a primer for a DNA polymerase which could add nucleotides directly to the end of this fragment. Ligase could then seal the end of the completed "−" strand to the 5′-phosphorylated end of the deoxyribonuclease fragment. The resulting RF molecule would be genetically heterozygous. Replication of this RF would yield two homozygous molecules, one bearing the *wt*

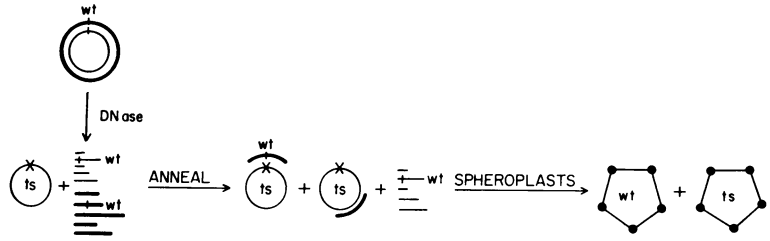

FIG. 1. *Assay of genetically defined φX DNA fragments. The "+" strands are indicated by thin and "−" strands by thick lines. The conditional lethal mutation carried by the "+" strand is represented here as a temperature-sensitive (ts) marker. The RF carries the wild-type (wt) allele at this site.*

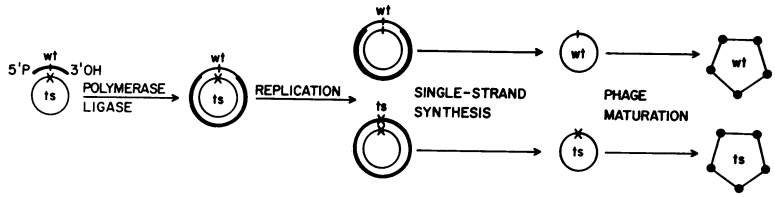

FIG. 2. *Proposed salvage mechanism for fragment markers. The "+" strands are indicated by thin and "−" strands by thick lines. Material derived from the original RF fragment is represented as a thinner segment of the "−" strand.*

marker derived from the fragment and one bearing the conditional lethal mutation from the intact "+" strand. We would then expect these progeny RF molecules to produce "+" strands and, hence, phage particles of the corresponding genotypes.

MATERIALS AND METHODS

Phage strains. All of the phage used in this paper were derived from wild-type φX174 (*wt*). The cistrons in which various conditional lethal mutations lie are indicated in the text. The letter names for the φX174 cistrons were agreed upon at the small DNA phage conference held at the California Institute of Technology on 7 November 1970. The Roman numeral names previously used by the Sinsheimer group are also indicated.

Each strain used in these experiments bears an amber mutation (*am3*) in the lysis cistron [cistron E(I)], in addition to any other mutations which may be present, to facilitate production of large quantities of phage and RF-DNA (4, 9, 11). Since all strains are identical in this respect, the *am3* mutation will not always be indicated in the text.

E. coli strains. HF4714 is φX174-sensitive and su^+ (amber). This is the usual indicator strain for plaque assays. CR/φX is su^+ (amber) and is resistant to infection by *wt* phage. It is sensitive to infection by phage bearing the host range mutation HaHb.

W6 is the host normally used for spheroplast assays of infective DNA. Whole cells of this strain are resistant to infection by φX174. P3478, which is deficient in the production of DNA polymerase, and its parent W3110 were the gift of J. Cairns. Both are resistant to infection by φX174 phage particles.

Spheroplast assays for infective DNA. Assay of infective DNA was performed essentially as described by Guthrie and Sinsheimer (6). Infected spheroplasts were incubated at 30 C or, in a few experiments (*see* figure legends), at 37 C for 3 to 5 hr. Progeny phage released by treatment in a Vortex mixer with a few drops of chloroform were then plaque-assayed.

Selective plaque assays. In most experiments, the progeny phage from infected spheroplasts were selectively assayed for particles bearing the wild-type allele of a temperature-sensitive or a cold-sensitive mutation (designated ts^+ or cs^+). Phage bearing a ts^+ allele were selectively assayed at 40 C on HF4714 indicator. Phage bearing a cs^+ allele were selectively assayed at 25 C on HF4714. Total phage (phage bearing either the mutant or the wild-type allele) were assayed at 30 C for experiments involving *ts* mutations and at 37 or 40 C for experiments involving *cs* mutations. Phage bearing the host range mutation HaHb were selectively assayed by using an indicator composed of 90% CR/φX and 10% HF4714.

DNA preparations. The "+" strands were prepared by phenol extraction of purified virus (4).

RFI (supercoiled RF) was purified from infected cells essentially as described by Komano and Sinsheimer (11).

RF fragments. Purified RF was treated with bovine pancreatic deoxyribonuclease (Sigma Chemical Co., 1× crystallized and lyophilized, or Worthington Biochemical Corp., 1× crystallized) at various concentrations ranging from 0.0004 to 0.04 μg/ml to control the size of the resulting fragments (*see* figure legends for exact conditions in each case). Digestion was carried out in the presence of 0.001 to 0.002 M Mg^{2+} at 30 C. The reaction was stopped by adding an excess of ethylenediaminetetraacetic acid (EDTA), and the product was converted to single-stranded fragments by heating to 100 C for 3 to 10 min followed by quench-

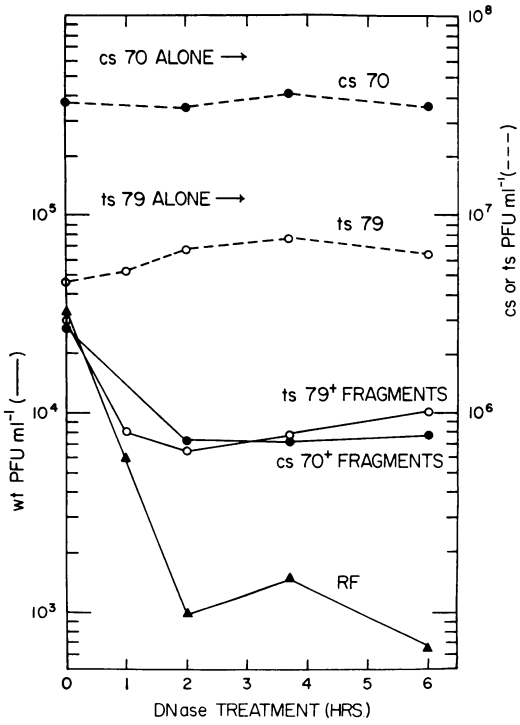

FIG. 3. *Recovery of genetic markers from φX RF fragments. Purified φX am3 RFI (absorbancy at 260 nm = 0.30) was treated with pancreatic deoxyribonuclease at a concentration of 4 × 10⁻⁴ μg/ml in the presence of 1 μg of bovine serum albumin per ml, 0.001 M $MgSO_4$, and 0.01 M Na_2HPO_4:NaH_2PO_4, pH 8. Samples were taken immediately before the addition of deoxyribonuclease (zero-time sample) and after various periods of incubation with deoxyribonuclease at 30 C. Each sample was immediately made 0.002 M in EDTA, heated to 100 C for 10 min, and quickly frozen. After all of the samples had been collected, the RF fragments were annealed with ts 79 and with cs 70 single-stranded DNA. Purified single-stranded DNA was diluted to 10 μg/ml in 2X SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 0.002 M EDTA. A 100-μliter amount of diluted single-strands was mixed with 10 μliters of deoxyribonuclease-treated RF, and the mixture was incubated at 67 C for 2 hr. The treated RF samples were also subjected to the annealing conditions in the absence of single-stranded DNA as a control. Annealed samples were next diluted for spheroplast infectivity assay. Symbols: ○—○, ts 79⁺ wild-type markers salvaged from RF fragments annealed to ts 79 plus strands (assayed at 40 C); ○----○, total phage (which are almost all ts 79) in the same spheroplast assays as ○—○; assayed at 30 C; ●—●, cs 70⁺ wild-type markers salvaged from RF fragments annealed to cs 70 plus strands (assayed at 25 C); ●----●, total phage (which are almost all cs 70) in the same spheroplast assays as ●—●; assayed at 37 C; ▲—▲, phage in the assays of treated, annealed RF in the absence of "+" strands. The levels of wt revertants in the "+" strand preparations were measured by subjecting a sample to annealing conditions in the absence of RF and then assaying selectively for wt as above and for total phage. The wt revertant levels are 4.6 × 10² ts 79⁺ per ml and less than 10² cs 70⁺ per ml (not indicated since they are off scale). The total phage yields from unannealed "+" strands were somewhat higher than following annealing to RF (indicated by arrows).*

ing in an ice bath. Fragment preparations were stored at −20 C.

Sephadex fractionation of DNA fragments. DNA fragments were fractionated on the basis of size by chromatography on Sephadex G-100. The elution buffer contained 0.01 M tris(hydroxmethyl)aminomethane (Tris; *p*H 8.1), 0.002 M EDTA, and either 0.01 or 1.0 M NaCl (*see* figure legends). Columns were run at room temperature, the flow rate was controlled by a peristaltic pump at a rate of 20 ml/hr, and 4.0-min fractions were collected. The absorbance of the effluent was continuously monitored at 260 nm (Beckman DB) or at 254 nm (Isco UA-2).

Hohn and Schaller (8) showed a simple logarithmic relation between K_d on Sephadex columns and chain length for oligonucleotides up to about 15 nucleotides in length. [$K_d = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the oligonucleotide, V_o is the elution volume of a molecule much larger than the pore size of the gel, and V_t is the elution volume of a molecule much smaller than the pore size of the gel.] They also reported experiments with Sephadex chromatography of transfer ribonucleic acid (tRNA). To estimate the sizes of DNA fragments eluted from Sephadex, we have used *E. coli* tRNA as a marker, assumed that the logarithmic relation between K_d and chain length holds for chains up to 200 in length and also that K_d approaches 1 for very short chains. We find that this method gives an accurate estimate of the chain length of 5*S* RNA (120 nucleotides), indicating that our extrapolation of the results of Hohn and Schaller above a chain length of 15 is valid. Since tRNA has a great deal of secondary structure, it may well be somewhat more compact than the average DNA oligonucleotide of equal chain length. If this is true, then we have probably slightly overestimated the chain lengths of the RF fragments.

RESULTS

Salvaging genes from fragments. Evidence for the recovery of the wild-type alleles of two different φX genetic loci from DNA fragments is presented in Fig. 3. RFI which is *wt* for both markers was subjected to a low concentration of pancreatic deoxyribonuclease for various time periods. The fragments resulting from melting and quickly cooling the treated RF were then annealed to circular "+" strands bearing either the *ts* 79 [cistron G(III)] or the *cs* 70 mutation [probably cistron H(II), *see* reference 2], and the resulting complexes were used to infect spheroplasts of *E. coli*. The progeny phage resulting from these infections were selectively assayed for

wt markers derived from the fragmented RF. The level of residual infectivity in the fragment preparation was also measured after subjecting the treated RF to the whole procedure in the absence of "+" strands. Such residual infectivity could result from circular strands, either "+" or "−" (12, 14), unbroken by the deoxyribonuclease treatment or conceivably from annealed complexes containing only fragmented strands.

The annealing procedure clearly results in salvaging the *wt* allele in the case of both markers. The residual infectivity in the fragment preparation and the levels of *wt* revertants in the "+" strands were small compared to the level of *wt* phage obtained from the complete annealing mixture. The observed recovery of fragment markers was, perhaps, surprisingly high. It appears that these *wt* alleles are approximately one-fourth as infective in fragments as in intact RF molecules (Fig. 3). The interpretation of this result is complicated, however, by the fact that the specific infectivity of RF is about 20 times lower than for "+" strands (3). The specific infectivity of the annealed complexes composed of one mutant "+" strand and a *wt* fragment is unknown.

It was important to show that this recovery of *wt* alleles exhibits the expected genetic specificity. This may be done essentially by showing that fragments of an RF bearing a conditional lethal mutation do not produce any increase in the number of *wt* progeny when annealed to "+" strands carrying the same mutation. To demonstrate this, we produced fragments of *cs* 70 RF which were then tested by annealing with *cs* 70 plus strands and also with *ts* 79 plus strands. The results, presented in Table 1, show that the *cs* 70 RF fragments can contribute *ts* 79^+ alleles but not *cs* 70^+ alleles to progeny phage. A simultaneous control experiment in which *wt* RF (both *ts* 79^+ and *cs* 70^+) was fragmented and tested under identical conditions confirmed that both the *cs* 70^+ and the *ts* 79^+ alleles can be salvaged from fragments.

The fragment assay exhibits an approximately linear relationship between the number of *wt* phage produced and the concentration of DNA fragments in the annealing mixture (Fig. 4). It should therefore be practical to use the assay to quantitate the concentration of fragments bearing a particular *wt* allele during purification of such a fragment.

Size of genetically active fragments. We can make a rough estimate of the number of deoxyribonuclease hits per RF molecule by measuring inactivation of infectivity to spheroplasts, following melting of the treated RF. We assume that this gives a measure of the number of surviving circular strands ("+" and "−") and that deoxyribonuclease hits are randomly distributed. Such an analysis is complicated by the differences in specific infectivity between RF and "+" strands (3) and possibly between "+" and "−" strands (12, 14). A calculation based on these considerations results in an estimate of four to eight hits per strand or an average fragment size of 700 to 1,400 nucleotides for the longest deoxyribonuclease treatment presented in Fig. 3. Since ϕX contains eight known cistrons (5, 16, 17), these fragments are about one or two genes in length.

To estimate the size of genetically active fragments more accurately and to investigate the possibility of using smaller fragments, we fractionated RF digests on Sephadex columns. Figure 5 displays the result of Sephadex G-100 fractionation of a *cs* 70 RF digest. These chromatographic conditions separate oligonucleotides with chain lengths ranging from about 10 to 200 (Fig. 5a). Our estimates of chain length are based on the results of Hohn and Schaller (8; *see above*). The absorbance profile of the RF digest shows a broad distribution of fragment sizes over the effective range of the column plus a peak of very small material (Fig. 5b). Individual fractions from this column were assayed for *ts*γ^+ activity (Fig. 5c). [*ts*γ is a mutation in cistron

TABLE 1. *Genetic specificity of the assay*[a]

RF fragments	"+" Strands	cs^+ PFU/ml (25 C)	ts^+ PFU/ml (40 C)
cs 70	None	$<10^1$	$<10^1$
cs 70	*cs* 70	$<10^1$	
cs 70	*ts* 79		1.9×10^3
cs^+	None	$<10^1$	$<10^1$
cs^+	*cs* 70	3.7×10^3	
cs^+	*ts* 79		5.5×10^3
None	*cs* 70	1.0×10^1	
None	*ts* 79		7×10^1

[a] RF bearing the *cs* 70 mutation (*am*3 *cs* 70) and RF bearing the *wt* allele at this site (indicated by cs^+ in the table, the full genotype being *am*3 HaHb), were each treated with 0.004 μg of pancreatic deoxyribonuclease per ml, 0.002 M $MgSO_4$ at 30 C for 2 hr. The reaction was terminated by the addition of 0.005 M EDTA, and the fragments were melted for 10 min at 100 C and then chilled in ice. Fragments (either *cs* 70 or cs^+) were annealed with "+" strands (either *cs* 70 or *ts* 79 at 10 μg/ml) in 2× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 0.002 M EDTA at 65 C for 2 hr. Samples were then diluted in 0.05 M Tris (*p*H 8.1) and assayed in spheroplasts at 37 C. The results of selective assays for ts^+ progeny phage (assayed at 40 C) or cs^+ progeny phage (assayed at 25 C) are tabulated. PFU, plaque-forming units.

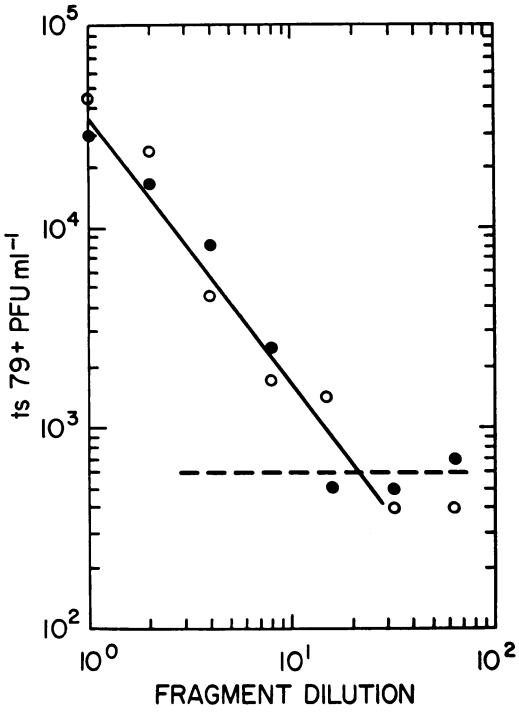

FIG. 4. *Assay of diluted RF fragments. This experiment used the Sephadex-fractionated RF fragments described in Fig. 5b. Fractions from the column were diluted in 1.0 M NaCl, 0.002 M EDTA, 0.01 M Tris, pH 8.1 (the column elution buffer). A series of six twofold dilutions was performed. A 20-μliter amount of diluted RF fragments was mixed with an equal volume of ts 79 plus strands at a concentration of 2 μg/ml in 0.05 M Tris(pH 8.1) at 0 C for spheroplast assay (37 C). The concentration of ts$^+$ phage (assayed at 40 C) in the spheroplast tube is plotted versus fragment dilution. Symbols: ○, dilution data for fraction 30, Fig. 5b (fragments are about 150 nucleotides long); ●, dilution data for fraction 40, Fig. 5b (fragments are about 75 nucleotides long). The solid line arbitrarily drawn through the data has a negative slope of 1.3. The horizontal dashed line indicates the background level of revertants present in the "+" strand preparation.*

G(III).] This activity also shows a broad distribution with a maximum at fragments of about 100 nucleotides in length; however, the very small fragments (less than 20 nucleotides) appeared inactive. We rechromatographed two fractions from a position slightly smaller than tRNA (Fig. 5d). The absorbance of this material was too small to measure in the column effluent, but the *ts*γ$^+$ genetic activity was easily detectable. This activity emerged from the column as a reasonably sharp peak at the position expected (Fig. 5d). We conclude from this experiment that genetic markers may be recovered from DNA oligonucleotides the size of tRNA.

The genetic activity of even smaller fragments is demonstrated by the experiment shown in Fig. 6. Again RF fragments were chromatographed on Sephadex G-100, but this time in the presence of low salt to improve the resolution of smaller nucleotides (8). The fragments were annealed to "+" strands at low temperature (25 C; references 18, 19) to facilitate annealing of small fragments. Initial fractionation of this fragment preparation showed a peak of biologically active fragments slightly smaller than tRNA. Rechromatography of fragments with an estimated chain length of 30 to 40 nucleotides demonstrated that these fragments are genetically active in our assay. These pieces are small enough to be extremely useful in sequence studies, once a specific fragment of this size has been purified. Further refinements in procedure may allow the detection of even smaller fragments.

In performing fragment assays, we have successfully employed a variety of annealing conditions (*see* figure and table legends). We now routinely use the conditions described in the legend to Fig. 6, particularly when assaying small fragments.

Necessity of annealing. We have done several experiments which are designed to test the model suggested in Fig. 2 for the incorporation of genetic markers carried on DNA fragments into infective phage particles.

First, we have shown that actual annealing of the fragments to the mutant "+" strand is necessary for efficient salvaging of *wt* markers. Table 2 compares the numbers of *wt* markers recovered following annealing at 67 C for 2 hr with an identical mixture of fragments and "+" strands prepared at room temperature immediately before spheroplast infection. Annealing increased the yield of *wt* progeny phage 20 times but had no effect on the total number of progeny (which are predominantly *ts*).

Once the RF fragments have been annealed to "+" strands, melting of the annealed complexes destroys the ability of the fragments to contribute genetic markers upon infection of spheroplasts. Figure 7 shows melting profiles of fragment markers. "Gene size" fragments bearing the *ts* 79$^+$ alleles melt at 80 to 85 C (Fig. 7a), which is similar to the melting temperature of intact RF under the same conditions. "tRNA size" fragments bearing the *ts*γ$^+$ allele appear to melt at a lower temperature and over a broader range (approximately 60 to 80 C). These small fragments clearly melt at a lower temperature than RF (Fig. 7b). The melting of RF is easily measured since it results in an increase in infectivity to spheroplasts (Fig. 7b; reference 3).

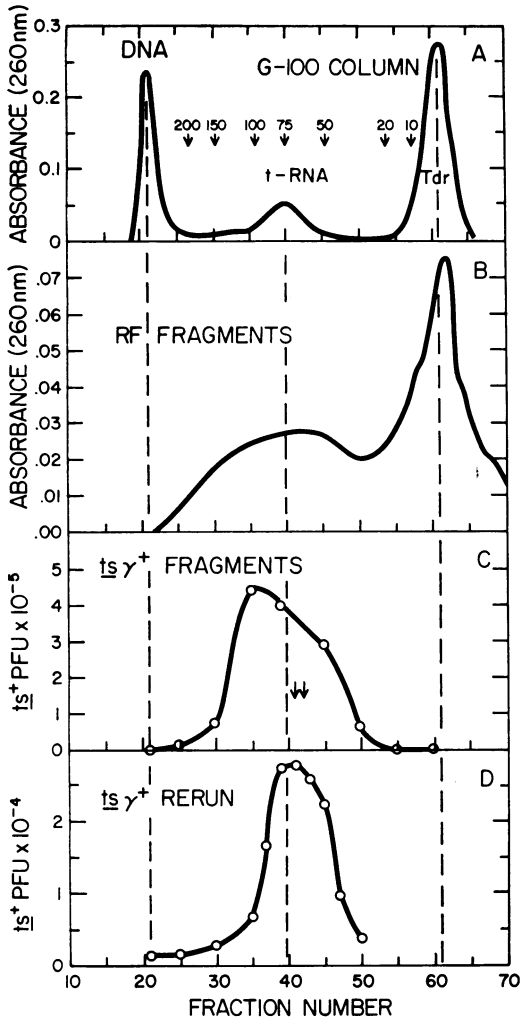

FIG. 5. *Sephadex column chromatography of RF fragments. Fragments were run on a column (1 by 90 cm) of Sephadex G-100 in 1.0 M NaCl buffer. (A) Column calibration: the sample contained 50 μg each of calf thymus DNA (void volume marker, V_o), E. coli tRNA, and thymidine (total volume marker, V_t). Elution volumes for polynucleotides of various chain lengths estimated from the position of the tRNA peak (reference 8; see Materials and Methods) are indicated by arrows. (B) Chromatography of RF fragments: φX RFI bearing the cs 70 mutation was dialyzed into 0.03 M Tris buffer (pH 7.2). To 4.0 ml of this RFI, having an absorbance of 0.37 at 260 nm, was added 0.001 M $MgSO_4$ and 0.02 μg of pancreatic deoxyribonuclease per ml. This mixture was incubated at 30 C for 1 hr, EDTA was added to a concentration of 0.003 M, and the mixture was heated 3 min at 100 C, cooled quickly to 0 C, and applied to the column. (C) Biological assay of RF fragments: 20 μliters of each fraction from the column described in (B) above was mixed with an equal volume of φX tsγ single-stranded DNA at a concentration of 2 μg/ml in 0.05 M Tris buffer (pH 8.1) to give the annealing mixture (with an NaCl concentration of 0.5 M). This mixture was then incubated at 65 C for about 100 min, diluted 20× into 0.05 M Tris(pH 8.1), and used to infect spheroplasts. The resulting progeny phage were assayed selectively for ts+ markers from the fragments. The concentration of infective ts+ particles in the spheroplast incubation tube is plotted here. Control infections with fragments subjected to the whole annealing procedure in the absence of tsγ single strands showed no detectable infectivity in any fraction. The number of ts+ revertant progeny produced by tsγ single strands subjected to the procedure in the absence of fragments was insignificant. (D) Assay of rerun RF fragments: fractions 41 and 42 from the column run described in (B) above were rerun on the same column. Fractions were assayed in spheroplasts as described in (C) above, along with the same control infections.*

Salvaged markers are recombinant. To test one prediction of the salvage mechanism proposed in Fig. 2, we have performed fragment assays by using RF and "+" strands which are genetically distinguishable at two different sites. On the basis of the model, any salvaged marker would be derived from the deoxyribonuclease-treated RF molecule, whereas any marker lying outside the region covered by the fragment would be derived, by copying, from the "+" strand template. Phage bearing salvaged markers should, therefore, be genetically recombinant with respect to the genotypes of the RF and "+" strand preparations from which they are derived, provided that the markers employed are separated by a distance larger than the size of the RF fragments.

In the experiment described in Table 3, the RF carried a host range marker, HaHb, which can be selectively assayed as well as the wild-type allele of the *tsγ* mutation. When assayed by annealing with *tsγ* plus strands, both these markers were salvaged from fragments of this RF. However, the two markers were independently salvaged to produce the recombinant types *tsγ* HaHb and *wt* (ts^+ $HaHb^+$). The independent salvaging of HaHb and $ts\gamma^+$ markers shows that they are separated by a distance larger than the size of the RF fragments employed (tRNA-size pieces) and supports the model presented in Fig. 2.

Fragment assays in mutant spheroplasts. It seemed conceivable that some mutant strain of *E. coli* might be unable to incorporate RF fragments into infective phage. In fact, all of the strains we have tested do salvage RF fragments. Spheroplasts of a DNA polymerase-deficient (*pol* A^-) strain of *E. coli* salvaged $ts\gamma^+$ fragments with a frequency which was not significantly different from the parental *pol* A^+ strain. The fractions of ts^+ progeny were 0.48% (*pol* A^-, P3478), 0.97% (*pol* A^+, W3110), and 2.1%

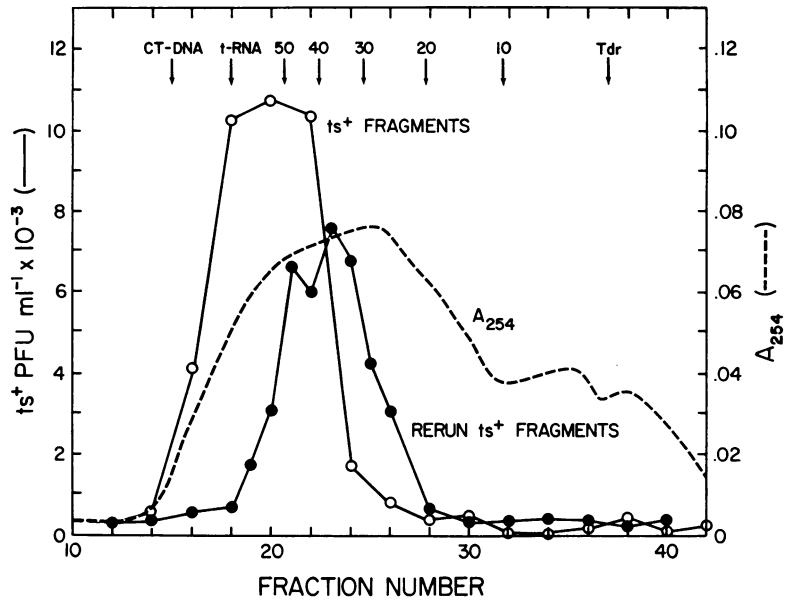

FIG. 6. *Sephadex fractionation of very small RF fragments. A Sephadex G-100 column (12 mm by 79 cm) was eluted with 0.01 M NaCl buffer. CT-DNA indicates the elution position for calf thymus DNA, tRNA indicates the position of E. coli transfer RNA, Tdr indicates the position of thymidine, and the numbered arrows indicate the elution positions estimated for oligonucleotides of various chain lengths. am3 RF at a concentration of 0.43 absorbancy at 260 nm units/ml was treated with pancreatic deoxyribonuclease at a concentration of 0.04 μg/ml in 0.05 M Tris (pH 8.1), 0.001 M $MgSO_4$ for 1 hr at 30 C. The reaction was terminated by adding 0.003 M EDTA and heating the mixture to 100 C for 3 min. The sample was quickly cooled in ice and them immediately applied to the column. The dashed line represents the absorbance of the eluate at 254 nm. Fractions were assayed for tsγ^+ activity (indicated by open circles) as follows. A 20-μliter amount of a fraction was mixed with 20 μliters of tsγ plus strands at a concentration of 2 μg/ml in 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 8.1. This annealing mixture was heated for 15 min at 57 C and then incubated at 25 C for about 20 hr. Samples were next diluted 20× into cold 0.05 M Tris (pH 8.1) for spheroplast assay. The concentrations of ts$^+$ phage (assayed at 40 C) in the spheroplast tubes are plotted (open circles). Fractions assayed in the absence of added "+" strands showed no significant infectivity [less than 5 × 10^1 plaque-forming units (PFU)/ml], and the level of ts$^+$ revertants in a control assay of the "+" strand preparation was also very low (about 6 × 10^1 PFU/ml). Fractions 23 and 24 from the run described were heated to 100 C for 2 min, quick-cooled, and applied to the same column under identical conditions. Fractions from this rerun column were assayed as described above. The tsγ^+ activity of the rerun fragments is indicated by solid circles.*

(W6, our standard spheroplast strain). Similar results have been obtained for strains carrying *uvr*A, *uvr*B, *uvr*C, *rec*$^{-13}$, *rec*$^{-21}$, and *rec*$^{-22}$ mutations. These results are consistent with the idea that RF fragments are completed to form intact genomes by the action of the usual RF-synthesizing mechanism and not by some system which is specific for the repair of broken DNA.

DISCUSSION

The assay described here permits the detection of genetically defined fragments of the complementary "−" strand of φX174 RF-DNA. The assay works for fragments 30 to 40 nucleotides in length or larger. We are now attempting to use this assay in conjunction with techniques for separating DNA fragments to isolate specific fragments of the φX174 genome.

The model shown in Fig. 2 accounts for what we now know of the mechanism by which genetic markers are salvaged from DNA fragments in our assay. The DNA polymerase which extends the fragment to form a complete "−" strand appears not to be Kornberg's polymerase. It seems quite possible that the actual mechanism may be complicated by factors such as (i) repair of heterozygous RF molecules to produce homozygous ones and (ii) the action of exonucleases (such as those associated with DNA polymerase) on the annealed fragment.

The assay procedure may also be regarded as a method for incorporating small DNA fragments into an intact viral genome. Biologically active

TABLE 2. *Annealing is necessary for marker recovery*[a]

ts 79 plus strands	RF fragments	Annealing	ts 79 PFU/ml	ts 79+ PFU/ml
+	+	Yes	1.3×10^6	6.0×10^3
+	+	No	1.7×10^6	3.0×10^2
+	−	Yes	1.2×10^6	3×10^1
+	−	No	1.7×10^6	$<10^1$
−	+	Yes	$<10^1$	$<10^1$
−	+	No	7×10^1	$<10^1$

[a] "Gene-size" fragments of *cs* 70 RF, prepared as described in the legend to Fig. 7a, were used as the source of *ts* 79+ markers. Fragment, "+" strand mixtures (composed as in Fig. 7a), as well as separated fragments and "+" strands, were either annealed for 2 hr at 67 C or else simply mixed at room temperature immediately before spheroplast assay. All samples were diluted $10^3\times$ into 0.05 M Tris (*p*H 8.1) for spheroplast assay. The titers of progeny phage bearing the *ts* 79+ allele (assayed at 40 C) or the *ts* 79 allele (assayed at 30 C) are tabulated. PFU, plaque-forming units.

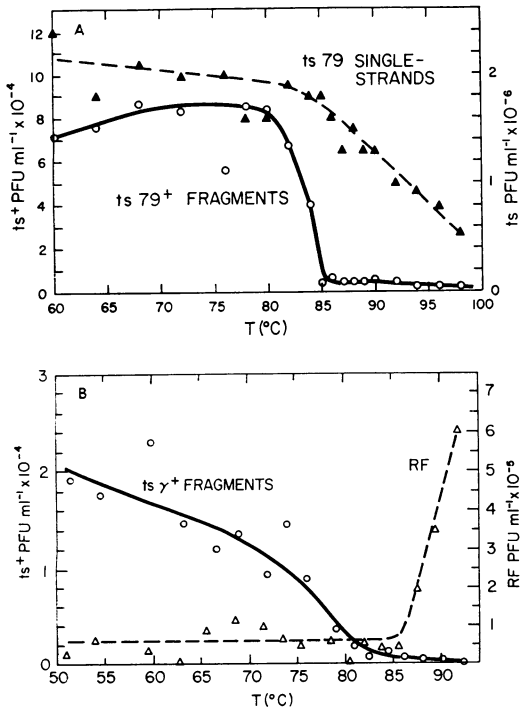

FIG. 7. *Melting of annealed RF fragments. (A) This experiment employed "gene size" pieces prepared as follows. cs 70 RF at a concentration of 0.53 absorbancy at 260 nm units/ml in 0.01 M Na_2HPO_4:NaH_2PO_4 (pH 8), 0.001 M $MgSO_4$ was treated with pancreatic deoxyribonuclease at a concentration of 0.004 μg/ml plus 1 μg of bovine serum albumin per ml at 30 C. At various times, the reaction was stopped by adding 0.003 M EDTA to samples of the mixture, and the treated RF was melted at 100 C for 10 min and then chilled in ice. Surviving infectivity of the treated RF, measured after annealing, reached a limiting value of about 10^{-3} of the original level after 2 hr of deoxyribonuclease treatment. The 22-hr sample was annealed to ts 79 plus strands: 10 μliters of RF fragments was mixed with 100 μliters of ts 79 plus strands at a concentration of 10 μg/ml in 2X SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.001 M EDTA and incubated at 67 C for 2 hr. For melting, the annealed complexes were diluted into 0.1 M KH_2PO_4 adjusted to pH 7 with KOH. The sample was placed in a jacketed cell connected to a circulating water bath and heated at a constant rate of about 1 C/min. At various temperatures, samples were withdrawn and diluted into chilled 0.05 M Tris (pH 8.1) for spheroplast assay. The open circles indicate the results of selective assay for ts 79+ markers (salvaged from the RF fragments) at 40 C. Triangles indicate total phage assayed at 30 C. (This is essentially a measure of the ts 79 progeny of the "+" strands and, therefore, measures heat inactivation of "+" strands during the experiment.) (B) This melting experiment employed RF fragments slightly smaller than tRNA. Fractions 43 and 44 from the G-100 fractionation described in Fig. 5b were pooled and mixed with an equal volume of tsγ plus strands (2 μg/ml in 0.05 M Tris, pH 8.1) and annealed at 64 C for 110 min. For melting, the annealed complexes were diluted 10× into 0.1 M KH_2PO_4 : K_2HPO_4, pH 7.0. Melting and spheroplast assays were performed as described above. As a control, a sample of untreated cs 70 RF was melted and assayed in the same manner. Open circles indicate the melting of tsγ+ markers from the annealed complexes, measured by plaque assay at 40 C. Triangles indicate the melting profile of the intact RF.*

fragments of the φX genome could be chemically synthesized by presently available techniques (1) if the necessary sequences were known, since fragments only half the size of Khorana's synthetic tRNA gene are active in our assay. The necessary information to permit such a synthesis may have to await an actual sequence analysis of some portion of the φX genome. Alternatively, it might soon be possible to guess a functional sequence, which would anneal adequately, directly from amino acid sequence information.

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TABLE 3. *Salvaged markers are recombinant*[a]

Annealing mixture		Total HF 4714 (30 C)	ts^+ HF 4714 (40 C)	HaHb CR/φX (30 C)	ts^+ HaHb CR/φX (40 C)
"+" Strands	RF fragments				
ts	None	7.9×10^6	$\sim 2 \times 10^2$	8×10^2	$<10^1$
ts	HaHb	5.9×10^6	5.7×10^4[b]	1.8×10^4[c]	4.0×10^2
None	HaHb	$\sim 5 \times 10^1$	$\sim 1 \times 10^2$	$<10^2$	$<10^1$

[a] RF (absorbancy at 260 nm = 0.12) bearing the host range marker HaHb was treated with pancreatic deoxyribonuclease [0.02 μg/ml in 0.05 M Tris (*p*H 8.1), 0.001 M $MgSO_4$ at 30 C for 1 hr) and then melted (100 C, 3 min) to produce fragments. A 50-μliter amount of the fragment preparation was mixed with 50 μliters of $ts\gamma$ plus strands [2 μg/ml in 10× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 0.005 M EDTA]. The mixture was annealed at 55 C for 3 hr and then diluted 20 times in 0.05 M Tris (*p*H 8.1) for spheroplast infection. Control samples containing either "+" strands or RF fragments alone were subjected to annealing conditions and assayed for infectivity. The numbers tabulated are progeny phage per milliliter in the spheroplast tubes, when assayed under the various conditions listed. Plaque formation at 40 C selectively assays ts^+ phage, whereas plaques on CR/φX demonstrate the presence of HaHb. Spot tests were performed by stabbing plaques with sterile pins. These pins were then stabbed into plates which had been preseeded with appropriate indicator bacteria.

[b] Spot tests on CR/φX (30 C) showed 1 of 50 tested was HaHb.

[c] Spot tests on CR/φX (40 C) showed 4 of 50 tested were ts^+.

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