Cloned Bacteriophage ϕ X174 DNA Sequence Interferes with Synthesis of the Complementary Strand of Infecting Bacteriophage ϕ X174

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The insertion of a particular ϕ X DNA sequence in the plasmid pACYC177 strongly decreased the capacity of *Escherichia coli* cells containing such a plasmid to propagate bacteriophage ϕ X174. The smallest DNA sequence tested that showed the effect was the *Hind*II fragment R₄. This fragment does not code for a complete protein. It contains the sequence specifying the C-terminal part of the gene H protein and the N-terminal part of the gene A protein, as well as the noncoding region between these genes. Analysis of cells that contain plasmids with the "reduction sequence" showed that (i) the adsorption of the phages to the host cells is normal, (ii) in a single infection cycle much less phage is formed, (iii) only 10% of the infecting viral single-stranded DNA is converted to double-stranded replicative-form DNA, and (iv) less progeny replicative form DNA is synthesized. The reduction process is ϕ X174 specific, since the growth of the related G4 and St-1 phages was not affected in these cells. The effect of the recombinant plasmids on infecting phage DNA shows similarity to the process of superinfection exclusion.

The genome of the isometric bacteriophage ϕ X174 is a single-stranded ring of 5,386 nucleotides (17) and contains coding sequences for at least 10 proteins that are important in the propagation of the phage in the bacterial host (23). After the adsorption of the phage to the host cell and the injection of the viral DNA, this single-stranded DNA is converted to a double-stranded replicative-form (RF) DNA molecule. This process depends completely on host proteins. In a second step, some 20 progeny RF molecules are formed on the parental RF template. This step requires a functional A protein for the introduction of a nick in the viral strand of the parental RF DNA molecule at a specific site, called the origin of ϕ X DNA replication (7, 13). The 3'-OH end serves as a primer for viral strand synthesis. After one round of replication, the displaced viral strand is excised and circularized (8). On this viral strand the complementary strand synthesis takes place, resulting in a progeny RF molecule. Late in infection, when transcription and translation of the ϕ X genes have resulted in all gene products, displaced viral strands are packed into virus capsids, and this is followed by lysis of the cell. The ϕ X174 genome contains four intercistronic regions. Their function is unknown, but they may be important in gene expression or serve as signal sequences for

synthesis of complementary strand DNA, or both (18).

The insertion of fragments of the ϕ X174 genome in plasmids makes it possible to study the in vivo effects of single genes and combinations of genes on the host cell. Strains containing a recombinant plasmid with a complete and expressed ϕ X gene can be used for the propagation and analysis of mutant phages (11). The approach of cloning ϕ X sequences in plasmids can be especially useful for the study of those DNA regions that do not code for protein but do carry other genetic information, e.g., intercistronic regions and replication origins. The functions of these regions often cannot be analyzed by standard genetic methods.

During our present investigations, we observed that phage yields are significantly reduced after infection of cells that contain plasmids in which a particular segment of ϕ X DNA is inserted. The reduction process is expressed in an early step of the ϕ X infection life cycle, i.e., the conversion of the viral single-stranded DNA to the parental RF molecules.

MATERIALS AND METHODS

Bacterial strains and phages. The suppressorless strains *Escherichia coli* HF4704 (*thy*, *urvA*, ϕ X^s) and *E. coli* C *recA171* (*recA*, *rif*^r, ϕ X^s) were used as

transformation recipients. Phages *am3*, *amH57*, *am9*, and *amN1* are ϕ X174 amber mutants, respectively, in gene E, gene F, gene G, and gene H; *am86*, *am33*, and *amH90* are ϕ X174 amber mutants in gene A; and *ts27* is a temperature-sensitive mutant in gene F. The position of the mutations on the genetic and physical maps is shown in Fig. 1 (25).

Plasmids and plasmid isolation. pACYC177 (5) is an *E. coli* multicopy plasmid containing genes coding for resistance to ampicillin and kanamycin. It contains approximately 3,950 base pairs (bp) (P. J. Weisbeek, manuscript in preparation). The physical map of the plasmid and the location and the transcription direction of the antibiotic-resistance genes are shown in Fig. 1b (4). The single *Hind*II site within the ampicillin-resistance gene was used to insert ϕ X174 fragments.

All cells containing pACYC177 or a recombinant plasmid were grown in the presence of 35 μ g of kanamycin per ml. Plasmid DNA was isolated by alkaline sodium dodecyl sulfate lysis (2) and was purified by ultracentrifugation in CsCl gradients containing ethidium bromide (0.4 mg/ml).

Restriction enzyme analysis. The restriction endonucleases *Hae*III, *Hind*II, and *Hha*I were purchased from New England Biolabs Inc. The digestion mixtures (45 μ l) contained, in all cases, 10 mM Tris-hydrochloride, pH 7.5, 0.1 mM EDTA, 10 mM MgCl₂, 10 mM β -mercaptoethanol, and 100 μ g of bovine serum albumin per ml. Complete digestions were carried out at 37°C for 2 h. Partial digestions were carried out at low enzyme-to-DNA ratios and were stopped by phenol extraction and alcohol precipitation of the reaction mixture.

Ligation procedure. T4 DNA ligase was isolated from an induced λ T4 *lig* lysogen (15). Complete and partial *Hae*III or *Hind*II digests of wild-type ϕ X RF DNA were ligated with *Hind*II-linearized pACYC177 DNA in a ϕ X-DNA-ends to vector-DNA-ends ratio of 4. The ligation mixture contained 10 μ g of DNA, 15 U of enzyme, 30 mM Tris-hydrochloride, pH 7.2, 0.5 mM ATP, 10 mM dithiothreitol, and 8 mM MgCl₂, and the procedure was carried out at room temperature for 3 h.

Characterization of transformants. After transformation of Ca²⁺-treated cells, transformants that were resistant to 35 μ g of kanamycin per ml and sensitive to 50 μ g of ampicillin per ml were screened for the presence of recombinant plasmids by infection with phage amber and thermosensitive mutants. Genetic recombination between the wild-type ϕ X sequence inserted in the plasmid and the mutant DNA of the infecting phage will result in wild-type phages if the mutant used maps within the inserted ϕ X region. When the inserted ϕ X sequence contains a complete gene that is expressed, complementation may occur and mutant phages will be formed. The phages resulting from either recombination or complementation will give rise to an increase in the number of plaques on the suppressorless strains, compared with the background level that results from the presence of wild-type revertants in the mutant phage lysate. Only mutants with a reversion frequency equal to or lower than 10^{-4} can be used in the test. An increase in the number of plaques of at least 10 times the background level was used as a positive indication for the presence of the corresponding DNA fragment (or at least a portion of it).

Gel electrophoresis. Electrophoresis of plasmids and

ϕ X DNA was performed on horizontal slab gels of 1.4% low-melting-point agarose (Bethesda Research Laboratories Inc.) in the presence of 2 μ g of ethidium bromide per ml. The electrophoresis buffer was 0.04 mM Tris-0.01 M sodium acetate-0.001 M EDTA, pH 7.6. When the gel had to be scanned for radioactivity, it was cut into 1.5-mm slices which were dissolved in 0.5 ml of water at 65°C. The solution was mixed with 10 ml of a mixture consisting of 23 parts of toluene solution [5 g of 2,5-diphenyloxazole (PPO) per liter and 0.05 g of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP) per liter] and 7 parts of Triton X-100 and was analyzed for radioactivity in a liquid scintillation counter.

Restriction enzyme digests were analyzed on horizontal 1% agarose gels or on vertical polyacrylamide gels. In the latter case, a gel containing a 3 to 10% gradient in acrylamide was used.

Labeling and purification of *am3* phages. *E. coli* HF4704 cells (100 ml) treated with 50 μ g of mitomycin C per ml (Sigma Chemical Co.) at a density of 5×10^8 cells per ml were infected with *am3* phages at a multiplicity of infection (MOI) of 5. Each culture contained 100 μ Ci of [¹⁴C]thymine (Radiochemical Centre, Amersham, England) (14). Two hours after infection, the cells were harvested, washed, and lysed with lysozyme at 4°C for 16 h. The solution was then cooled to -70°C and thawed to room temperature again twice. The lysate was freed from cell debris by centrifugation and was purified on a 10 to 30% sucrose gradient, resulting in a phage preparation of 2.2×10^{11} phage per ml and 1.5×10^5 cpm/ml.

Adsorption of phage. Ten minutes after infection of a mid-log-phase culture grown in the presence of 35 μ g of kanamycin and 0.3 mM CaCl₂, a 50- μ l portion was taken, added to 5 ml of fresh medium containing 100 μ l of HCCl₃, and mixed thoroughly for 30 s. After 10 min at 0°C, the mixture was titrated for phage, and the amount of nonadsorbed phage was calculated.

Progeny RF DNA isolation. Mid-log-phase cultures (100 ml) of the plasmid-containing cells were infected with wild-type phage at an MOI of 5, 1 min after addition of 5 mCi of [¹⁴C]thymine to the cultures. Ten minutes after infection, chloramphenicol was added to a final concentration of 35 μ g/ml. After 1 h at 37°C, the cells were harvested, and all double-stranded DNA was isolated by the alkaline lysis method (2). The resulting DNA preparation was analyzed by gel electrophoresis on horizontal 1.4% low-melting-point agarose gels in the presence of ethidium bromide.

Parental RF DNA isolation. Plasmid-containing cultures were grown to a density of 3×10^8 cells per ml in the presence of 35 μ g of kanamycin per ml. After 10 min of incubation in 150 μ g of chloramphenicol per ml, the cells were infected with [¹⁴C]-labeled *am3* phages at an MOI of 1. After 30 min at 37°C, the cells were harvested and washed three times with 0.05 M sodium tetraborate and 0.0025 M EDTA. The gentle lysis method with lysozyme, sodium dodecyl sulfate, and pronase allows isolation of the parental RF (1). The DNA preparation was analyzed on horizontal 1.4% low-melting-point agarose gels in the presence of ethidium bromide.

RESULTS

Selection of recombinant plasmids. A mixture of DNA fragments obtained by complete or partial digestion of ϕ X174 RF DNA with the

FIG. 1. (a) Genetic and physical map of bacteriophage ϕ X174. Z and R refer to the restriction fragments produced by *Hae*III and *Hind*II digestion, respectively. The map positions of the mutants are indicated by dots. (b) Restriction map of pACYC177 (4, 5). IR = inverted repeat.

restriction enzymes *Hae*III and *Hind*II was ligated with *Hind*II-linearized pACYC177. The ligation mixture was used to transform competent cells of the strains *E. coli* HF4704 and *E. coli* C *recA171*. Kanamycin-resistant, ampicillin-susceptible transformants were expected to carry a recombinant plasmid with a ϕ X DNA fragment inserted in the *Hind*II site of pACYC177. The genetic content of the inserted ϕ X sequences was determined by titrating ϕ X

amber and thermosensitive mutants on the cells containing the recombinant plasmids (see Materials and Methods). The results of the analysis for a number of recombinant plasmids are given in Table 1. On the basis of these data and the known map position of the mutants used (Fig. 1a), a minimal estimate of the length and the contents of the inserted phage DNA was made. It follows that pPR111 contains at least a portion of fragment Z_{6b} , pPR216 of fragment Z_{6a} ,

TABLE 1. Titration of phage mutants on suppressorless HF4704 cells containing different recombinant plasmids

Plasmid ^a	Mutant (gene) and reversion frequency							
	<i>am3</i> (E), 2.5 × 10 ⁻⁵	<i>ts27</i> (F), ^b 5 × 10 ⁻⁴	<i>amH57</i> (F), 2.5 × 10 ⁻⁵	<i>am9</i> (G), 10 ⁻⁵	<i>amN1</i> (H), 4 × 10 ⁻⁵	<i>am86</i> (A), 10 ⁻⁶	<i>am33</i> (A), 10 ⁻⁴	<i>amH90</i> (A), 2.5 × 10 ⁻⁵
Group A								
pACYC177	1.0 ^c	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pPR111	1.0	1.0	1.0	1.3	1.0	0.9	100	0.9
pPR216	1.3	1.5	1.3	1.7	1.1	0.8	1.0	39
pPR256	12	350	4,000	50,000	110	0.9	3.0	0.6
Group B								
pPR141	0.1	0.1	0.1	0.3	0.1	55	0.1	0.04
pPR213	0.1	0.15	21	96	20	31	16	0.15
pPR224	38	15	200	350	100	95	0.05	0.03
pPR241	0.05	0.03	0.06	42	16	23	0.1	0.04
pPR253	0.04	0.01	0.03	0.03	83	28	190	10
pPR266	0.1	0.03	29	850	200	31	270	0.1

^a All but one of the recombinant plasmids were constructed from ϕ X *Hae*III fragments; the exception is pPR141, which contains ϕ X *Hind*III fragments.

^b The number of plaques at the nonpermissive temperature of 41°C was measured.

^c The number of plaques resulting from the titration of mutants on cells containing pACYC177 is set to 1.0.

pPR256 of the fragments *Z*₇, *Z*₈, *Z*₁, and *Z*₂, etc. (see Fig. 1a). Fragment *Z*₂ is not completely integrated into plasmid pPR256 because only one (*amN1*) of the two mutants that map on fragment *Z*₂ (*amN1* and *am86*) gave a positive result in the test. The same holds for fragment *Z*₁ in pPR241, because titration of *am9* phage gave a positive result, whereas the titration of *amH57* phage scored as negative.

Titration of *amH57* and *am9* phages on the strain containing pPR256 resulted in a large increase in the number of plaques. In this plasmid the complete F and G genes may be present, so that the products of these two genes can complement the amber mutation of the infecting phages. The lower value in the test when the F mutant *ts27* was plated on the strain containing pPR256 is the consequence of the higher reversion frequency of this mutant. In a *recA* background an increase in the number of plaques can only be the result of complementation. As can be seen in Table 2, the F and G genes in pPR256 were indeed expressed, as they complemented the mutations of the infecting *amH57* and *am9* phages. Titration with *am3* and *amN1* phages had no effect in the *recA* cells, so the positive

score for these mutants in the *recA*⁺ titration was apparently the result of recombination. Such a titration assay, testing for recombination between recombinant plasmids and phage genomes, can thus be used as an easy method to determine whether or not a bacterial strain is recombination deficient. A surprising observation was that several plasmid-containing strains (Table 1, group B) showed a strong decrease in the number of plaques formed, in comparison with the number of plaques formed on the pACYC177-containing control strain. Moreover, all the plaques formed were much smaller than normal. No differences in growth rate between the different plasmid-containing strains were observed. The effect apparently does not interfere with the identification of the inserted sequences, although the actual increases might otherwise have been larger. This reduction in plaque formation was illustrated more clearly by the titration of wild-type phages on the different plasmid-containing cells. As shown in Table 3, the efficiency of plating on the HF4704 strains containing the plasmids belonging to group B was 6 to 100 times lower than that on the strains containing the plasmids belonging to group A. If

TABLE 2. Titration of phage mutants on *E. coli* C *recA* cells containing recombinant plasmids

Plasmid	Mutant (gene)				
	<i>am3</i> (E)	<i>amH57</i> (F)	<i>am9</i> (G)	<i>amN1</i> (H)	<i>am86</i> (A)
pACYC177	1.0 ^a	1.0	1.0	1.0	1.0
pPR256	1.2	5,500	2,700	1.0	1.0

^a The number of plaques resulting from the titration of mutants on cells containing pACYC177 is set to 1.0.

TABLE 3. Effect of ϕ X DNA insertions on wild-type phage production

Plasmid	Efficiency of plating ^a in:		Phage yield in a single infection (HF4704) ^b
	<i>E. coli</i> HF4704 (<i>recA</i> ⁺)	<i>E. coli</i> C <i>recA171</i>	
Group A			
pACYC177	1.0	1.0	1.0
pPR111	1.0	1.0	1.0
pPR216	1.0	1.0	0.9
pPR256	1.0	1.0	1.0
Group B			
pPR141	0.13	0.01	0.12
pPR213	0.16	0.005	0.17
pPR224	0.035	0.005	0.024
pPR241	0.008	0.003	0.010
pPR253	0.010	0.003	0.010
pPR266	0.010	0.004	0.012

^a Wild-type ϕ X174 phage was titrated at 37°C on the plasmid-containing cells. The plaque number on the pACYC177-containing strain is set to 1.0.

^b Cultures of the plasmid-containing cells were infected at an MOI of 5 with *am3* phage. The yield after 30 min at 37°C was measured and related to the yield of the pACYC177-containing cells.

the host was recombination deficient, the differences were even more pronounced. A lower efficiency of plating was always accompanied by a smaller plaque size. The reason for the decrease in plaque number became clear after the phage yield of a single infection in the plasmid-containing cells was measured. The decrease in plaque number was in parallel with the decrease in progeny phage production (Table 3).

Restriction enzyme analysis of the recombinant plasmid. The results of the recombination and complementation experiments provide only a rough estimate of the ϕ X DNA sequences present in the recombinant plasmids. The exact nature and length of the insertions were determined by restriction enzyme analysis. All but one of the recombinants were constructed from ϕ XHaeIII fragments; the exception, pPR141, contains ϕ XHindII fragments. As the ϕ X sequence were cloned into the single HindII site of pACYC177, the HindII enzyme was used to characterize the ϕ X DNA inserts in the recombinant plasmids. Comparison of the digests with the HindII digest of ϕ X174 RF DNA allows identification of most of the inserted ϕ X sequences. The results are shown in Fig. 2 and Table 4.

The plasmids pACYC177 and pPR111 were linearized by the HindII enzyme. The HindII site of pPR111 is situated within fragment Z₉. This fragment, as well as fragment Z₁₀, was shown to be present in pPR111 by electron microscopy of heteroduplexes of the plasmid

with single-stranded ϕ X DNA (A. van der Ende, personal communication). Plasmid pPR141 resulting from the ligation of ϕ XHindII fragments in the HindII site of pACYC177 was not cut at all. Apparently the ligation did not restore the HindII sites at the ends of the inserted DNA, probably because of the action of a small exonuclease contamination in the enzyme preparation. The plasmid pPR216 does contain Z_{6a} (Table 1). The adjacent fragments Z₉ and Z_{6b} were not present; Z₉ is absent because the plasmid has no HindII site, and Z_{6b} is absent because of the negative result in the recombination test with *am33* phages (Table 1).

Comparison of the HindII digestion patterns of the plasmids that were cleaved more than once by the enzyme with the complete ϕ XHindII digest identified some of the ϕ X DNA sequences in these plasmids. The inserted ϕ X DNA was arranged in the same order as in the complete ϕ X genome, as might be expected from the ligation of fragments of a partial HaeIII digestion into the vector DNA. Table 4 shows the fragments whose presence is indicated by the HindII digestion.

The length of the fragment containing the kanamycin-resistance gene in pPR241 is almost identical to that of the linearized pACYC177 molecules. As the pPR241 DNA can recombine with the *am9* mutant DNA (and not with the *amH57* DNA) to form wild-type phages, the ϕ X DNA insertion includes a sequence running from just upstream of the R₁-R₉ HindII site up to the Z₂-Z_{6b} junction, thus resulting in a plasmid with a total length of approximately 5,700 bp.

In the case of pPR256, the size of the ϕ X DNA insertion (with an incomplete Z₂ fragment) was determined by comparison of its HhaI digest with the HhaI digests of pACYC177 and ϕ X174 (Fig. 3). The HhaI map of ϕ X174 is shown in Fig. 4. The HhaI map of pACYC177 is not known; however, the sequence of the gene coding for ampicillin resistance can be deduced from the completely sequenced plasmid pBR322 (19, 20). The single HindII site of pACYC177 is situated within a HhaI fragment with a length of 337 bp. All but one of the bands on the gel from the HhaI digest of pACYC177 were also seen in the pPR256 digest. The missing fragment indeed has a length of approximately 340 bp. Two fragments in the pPR256 HhaI digest did not correspond to one of the fragments in the pACYC177 digest or in the ϕ X174 digest. These fragments have lengths of 560 and 162 bp. Because the position of the HindII site in the 337 fragment of pACYC177 is known exactly, it can be inferred that the insert starts at nucleotide 435 and ends at approximately nucleotide 3,904, situated just within the H gene (17). The orienta-

FIG. 2. Determination of the length and the nature of the ϕ X DNA insertions in the recombinant plasmids by electrophoresis on 1.4% agarose gels before and after *Hind*II digestion. (a) Lane 1, pPR216; lane 2, pPR141; lane 3, pPR111; lane 4, pACYC177; lane 5, ϕ X174 RF DNA; lane 6, pACYC177 + *Hind*II; lane 7, pPR111 + *Hind*II; lane 8, pPR141 + *Hind*II; lane 9, pPR216 + *Hind*II. (b) Lane 1, ϕ X174 RF DNA; lane 2, pPR213; lane 3, pPR224; lane 4, pPR241; lane 5, pPR253; lane 6, pPR256; lane 7, pPR266; lane 8, pACYC177. (c) Lane 1, ϕ X174 RF DNA + *Hind*II; lane 2, pPR213 + *Hind*II; lane 3, pPR224 + *Hind*II; lane 4, pPR241 + *Hind*II; lane 5, pPR253 + *Hind*II; lane 6, pPR256 + *Hind*II; lane 7, pPR266 + *Hind*II; lane 8, pACYC177 + *Hind*II. The intensity of the bands representing the small fragments is too low to be visualized on the gel.

tion of the ϕ X insertion of pPR256 is such that the direction of transcription of the ϕ X DNA is opposite to that of the ampicillin- and kanamycin-resistance genes in pACYC177. As shown in Table 4, there is a fair agreement between the sizes of the plasmids estimated after gel electrophoresis and the size calculated from the results of the restriction enzyme analysis. Figure 4 summarizes all the results of the characterization of the recombinant plasmids.

Effect of the recombinant plasmids on the ϕ X DNA replication. Comparison of the results in Fig. 4 with those in Table 3 shows that all plasmids that cause reduction of phage growth

have in common the insertion of approximately the nucleotides 3,904 to 4,202 or, in fact, the larger part of the *Hind*II fragment R₄. This sequence includes the C-terminal part of the gene H protein, the N-terminal part of the gene A protein, and the noncoding region of 63 bp between these two genes. The reduction effect is completely ϕ X174 specific, as the related phages G4 and St-1 (3, 9) are not affected by the plasmids containing the reduction sequence (data not shown).

We have determined at which moment in the ϕ X174 life cycle the interference of the plasmids occurs. First, it was found that the adsorption of

TABLE 4. Size and content of the ϕ X DNA in the recombinant plasmids

Plasmid	Size on gel ^a	Size calculated ^b	ϕ X fragments used in ligations	ϕ X fragments found after <i>Hind</i> III digestion of the recombinant plasmids	Content of the insertions in the recombinant plasmids
pPR111	4,400	4,421	<i>Hae</i> III, complete digest	—	Z _{6b} , Z ₉ , Z ₁₀
pPR141	4,450	4,445	<i>Hind</i> II, complete digest	—	R ₄
pPR213	6,700	6,662	<i>Hae</i> III, partial digest	R ₂ , R ₄ , R ₆	Z ₁ , Z ₂ , Z _{6b}
pPR216	4,250	4,221	<i>Hae</i> III, partial digest	—	Z _{6a}
pPR224	7,800	7,722	<i>Hae</i> III, partial digest	R ₁ , R ₂ , R ₄ , R ₆ (2 $\times$), R ₇	Z ₇ , Z ₅ , Z ₈ , Z ₄ , Z ₁ , Z ₂
pPR241	5,700	5,730	<i>Hae</i> III, partial digest	R ₂ , R ₄ , R ₆	Z ₁ (partial), Z ₂
pPR253	5,600	5,580	<i>Hae</i> III, partial digest	R ₄ , R ₆	Z ₂ , Z _{6b} , Z _{6a}
pPR256	7,300	7,412	<i>Hae</i> III, partial digest	R ₁ , R ₂ , R ₆ (2 $\times$), R ₇	Z ₇ , Z ₅ , Z ₈ , Z ₄ , Z ₁ , Z ₂ (partial)
pPR266	7,200	7,265	<i>Hae</i> III, partial digest	R ₁ , R ₂ , R ₄ , R ₆	Z ₄ , Z ₁ , Z ₂ , Z _{6b}

^a Estimated values in bp, as determined after gel electrophoresis.

^b Calculated values in bp, determined by adding the lengths of the ϕ X fragments whose presence is suggested to the length of the pACYC177 plasmid (3,950 bp; P. J. Weisbeek, manuscript in preparation).

the phages to the bacteria was the same whether or not a plasmid with the reduction sequence was present in the cells. Further, the effect of the plasmids on ϕ X RF DNA synthesis was measured. Several strains containing plasmid were infected with ϕ X174 wild-type phage in a [¹⁴C]thymine-containing medium in the presence of 35 μ g of chloramphenicol per ml. Under these conditions the RF-to-RF DNA replication continues, whereas the RF-to-single-stranded DNA stage of replication is blocked. The replicating DNA was labeled, and the label was quantified after separation of the DNA by electrophoresis on agarose gels (see Materials and Methods). Figure 5 shows an analysis of the different DNA preparations. In each lane ϕ X174 RF DNA as well as plasmid DNA can be seen. In lanes b and h (from cells containing the nonreducing plasmids pPR216 and pPR256) more ϕ X RF DNA is present than in the lanes a, c, d, g, and i (from cells containing the plasmids in which the reducing sequence is inserted). To quantitate the differences, we scanned each lane for radioactivity. The activity in lanes b and g containing pPR216 and pPR253 is plotted in Fig. 6. The profiles in these two lanes are characteristic of cells with a nonreducing plasmid and a reducing plasmid, respectively. The latter cells have a two- to threefold reduced amount of ϕ X RF_I + RF_{II} DNA. This result shows that not only was phage formation affected but also the level of ϕ X RF replication was lowered by the presence of these plasmids.

It was not yet clear whether RF replication itself was inhibited or whether the reduction had its effect on an earlier step in the phage life cycle. This was tested by infecting the strains containing pACYC177, pPR141, and pPR241 with [¹⁴C]thymine-labeled *am3* phages in the presence of 150 μ g of chloramphenicol per ml. At this chloramphenicol concentration all pro-

tein synthesis is inhibited, and therefore only the formation of the parental RF DNA is allowed (21). Figure 7 shows the distribution of the label in the agarose gels after electrophoresis of the DNA preparations. In cells with the control

FIG. 3. Electrophoresis of the *Hha*I digests of pPR256 (lane 1), pACYC177 (lane 2), and ϕ X174 RF DNA (lane 3) on a 3 to 10% acrylamide gradient gel. (a) and (b) indicate the new fragments in pPR256; (c) indicates the pACYC177 fragment missing in pPR256.

Fig. 4. Contents of the ϕ X DNA insertions of the recombinant plasmids.

plasmid pACYC177 without any ϕ X DNA insertion, 90% of the ^{14}C label recovered from the infected cells was found in the RF_I and RF_II fractions, whereas 10% of the label was recovered as single-stranded phage DNA. The single-stranded DNA peak probably represents eclipsed but not properly penetrated single-stranded DNA molecules (16). In the cells with the reducing plasmids pPR141 and pPR241, the amounts of RF_I and RF_II DNA decreased to less than 10% of the amounts found in the pACYC177 plasmid, but the amount of single-stranded DNA was similar in all cases. Since the adsorption of the phages was normal, much label must be present as small, nondiscrete DNA fragments that were partly lost during the DNA isolation procedure, but were partly found at the end of the gel (slice numbers 60–70). Our interpretation is that, in cells with a reducing plasmid, part of the infecting single-stranded DNA was converted to parental RF DNA and the nonconverted single-stranded DNA was gradually broken down to smaller fragments. In those cells in which a parental RF DNA molecule has been formed, about normal amounts of progeny RF DNA can be made (at lower or no chloramphenicol concentration). This may account for the much less pronounced reduction in progeny RF DNA, as shown in Fig. 6. A pretreatment of the cells with rifampin (100 $\mu\text{g}/\text{ml}$) for 10 min did not influence the experimental data. The same amount of the infecting single-stranded DNA was permitted to be converted to RF DNA, whether or not an incubation with rifampin preceded phage infection. The conclusion is that the intracellular presence of the ϕ X DNA sequence around the intercistronic region between the genes H and A strongly influences the propagation of ϕ X174 phages by inhibiting the conversion of the infecting single-stranded DNA to parental RF molecules.

DISCUSSION

Different parts of the ϕ X174 DNA sequence have been cloned into the single *Hind*II site of pACYC177. The recombinant plasmids have been characterized by recombination, complementation, and restriction enzyme analysis. The titration assay described, testing for recombination between recombinant plasmids and phage genomes, can be used as an easy method for determining whether or not a phage-sensitive bacterial strain is recombination deficient.

Infection with ϕ X174 of some of the HF4704 strains containing plasmid results in a lower efficiency of plating, caused by the lower phage yield of each infection cycle, than infection of a pACYC177-containing strain. Moreover, the plaques formed are much smaller and have fuzzy edges. In the recombination-deficient strain *E*.

FIG. 5. Presence of phage RF DNA in plasmid-containing cells. Electrophoresis on 1.4% low-melting-point agarose of lysates of plasmid-containing cells after a 1-h infection with ϕ X wild-type phages (MOI, 5) in a medium containing 35 μ g of chloramphenicol per ml and [14 C]thymine. The cells contained pPR213 (lane a), pPR216 (lane b), pPR224 (lane c), pPR241 (lane d), pPR253 (lane g), pPR256 (lane h), pPR266 (lane i), or no plasmid (lane j). Lanes e and f show ϕ X RF DNA and pACYC177 as markers, respectively. Minor bands represent dimer molecules of both the plasmids and the ϕ X RF molecules.

coli C recA171, the effect is even more pronounced. Comparison of the content of the insertions (Fig. 4) and the differences in efficiency of plating (Table 3) show that the plasmids with the reduction effect have the ϕ X *Hind*II fragment R_4 in common. Plasmid pPR256, which contains only the left part of the R_4 fragment, shows no reduction, however. Thus, the presence of the sequence between the nucleotides 3,904 and 4,202 of the complete ϕ X DNA sequence is responsible for the reduction effect. This part of the sequence encloses the end of the H gene, the proximal part of the A gene, and the noncoding region between these two genes (nucleotides 3,917 to 3,981). The intergenic region contains the major transcription termination site and the gene A promoter.

It has been reported recently (6) that the filamentous phage M13 also contains a DNA region that, when inserted in a plasmid, reduces the growth of infecting phages to 2 to 3%. This region comprises the nucleotides 5,867 to 5,995 of the M13 sequence (24) and is part of the intergenic region in front of gene II. This gene codes for a protein that has a function comparable to that of the gene A protein of ϕ X174.

Insertion of the ϕ X174 R_4 fragment in one of the *Hind*II sites of pBR322 also results in a plasmid that interferes with ϕ X phage growth (R. Versteeg, personal communication). The reduction effect is also highly specific, since ϕ X174 is affected and the related phages G4 and

St-1 are not. The general structure of the genomes of these phages is comparable to that of the ϕ X174 genome, although in the case of G4 there is only a little sequence similarity with

Fig. 6. Distribution of radioactivity in the lanes containing the plasmids pPR216 (●) and pPR253 (○) in Fig. 5. Each lane was cut into 1.5-mm slices which were dissolved and counted. Direction of migration is from left to right.

FIG. 7. Fate of viral phage DNA after infection of plasmid-containing cells in the presence of 150 μ g of chloramphenicol per ml with [14 C]thymine-labeled phages (MOI, 1). Sixty minutes after infection, DNA was isolated and electrophoresed on 1.4% low-melting-point agarose gels. The gels were cut into 1.5-mm slices, and the amount of radioactivity was determined. The cells contained pACYC177 (●), pPR141 (○), or pPR241 (▲). Direction of migration is from left to right.

ϕ X174 in the intercistronic region between genes H and A (10). The St-1 sequence is not yet known. Thus, probably it is not a host cell process that is the main cause of the reduction effect.

Our experiments show that the interfering ϕ X DNA sequence decreases the parental phage formation through an inhibition of the conversion of the infecting single-stranded DNA into parental RF DNA. As the amount of progeny RF DNA formed was in good relation to the amount of parental RF formed (at lower chloramphenicol concentration and with correction for differences in MOI between the experiments), we assume that the process of RF DNA replication itself is not affected. There is a similarity between the phenomenon described here and the process of superinfection exclusion, described several years ago for ϕ X174 and S13 (12, 22). The conversion of superinfecting viral DNA to parental RF DNA was shown to be blocked, about 7 min after the first infection. Superinfection exclusion was eliminated under conditions in which the replication of the first infecting phage was inhibited or delayed (e.g., gene A mutants, chloramphenicol) (12, 22). The reduction effect we observed is, however, unaffected by chloramphenicol and rifampin (Fig. 5-7). Therefore, protein synthesis, as well as RNA synthesis, in the infected cell is not necessary. Moreover, the restriction fragment R_4 does not

code for a complete protein. Single-stranded DNA that is not converted to RF molecules will ultimately be broken down, the efficiency of this process being dependent on the particular conditions of the experiment and on the strain used.

How can a double-stranded ϕ X DNA sequence present in the cell interfere with the conversion of infecting viral single-stranded DNA? No phage protein is involved, as the viral single-stranded DNA has not yet been transcribed. We suggest that the ϕ X DNA sequence in the plasmid interacts in some way with an intracellular component, e.g., a membrane or a protein, that is present in limiting amounts. When this component is also required for the conversion of the infecting single-stranded DNA to RF molecules, then both types of DNA molecules will compete for the same site, resulting in exclusion of the single-stranded DNA. This intracellular component might well be the "essential bacterial site" at which the parental RF DNA is thought to be replicated (26). We conclude from our results that the process of conversion of the infecting viral DNA to RF molecules is spatially connected with the sites at which the intracellular double-stranded phage DNA is located during replication. This relationship between the two types of phage DNA suggests a strong coupling of the different stages of viral DNA replication.

As a concluding remark, we can say that,

besides the regions specifying the plus- and minus-strand origins of replication, a third DNA sequence plays an important role in the replication of bacteriophage ϕ X174.

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