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Biochemistry

Construction and characterization of an *Escherichia coli* plasmid bearing a functional gene *G* of bacteriophage φX174*

(*in vitro* recombination/transformation/gene-specific suppressor/spike protein)

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ABSTRACT **In order to study the mutagenic effects of site-specific, covalent modifications of biologically active DNA, we need host cells that are permissive for any type of mutation that might be produced *in vivo* from the modified DNA. Specifically, we require a general, *in vivo* complementation system for the bacteriophage φX174 gene *G*, an essential gene that we have chosen for our initial studies of chemical mutagenesis. Toward this end, we have constructed a plasmid (pφXG) that carries a functional copy of φX174 gene *G*. Three different bacterial strains that are nonpermissive for *am9*, a gene *G* amber mutant, have been transformed with pφXG. The transformants are now permissive for this gene *G* mutant, but not for the gene *A* or *E* mutants that have been tested. This paper describes the construction and the biochemical characterization of this plasmid, pφXG, and describes some of the biological properties exhibited by the pφXG-bearing strains.**

A large class of mutagens, including most ultimate carcinogens, are reactive electrophiles that produce many different kinds of covalent modifications when they react with DNA (1). At present, there is no direct method for determining which of these modifications produce mutations, nor is there any direct way of showing what type of mutation (base substitution, frameshift, or large deletion) is produced by different kinds of premutational lesions in DNA. Our laboratory has begun an intensive effort to develop methodology that will enable us to explore this problem directly.

The availability of extensive information regarding the structure and biology of bacteriophage φX174 (2–4) makes this virus an especially attractive model for our initial studies. Briefly, our approach is as follows: First, a well-characterized modification is introduced into DNA at a single, preselected site by a combination of chemical and enzymatic synthesis. Second, the biological response of this site-altered DNA is examined by isolating mutant phage produced by transfection of permissive cells. Third, the precise change produced by the site-specific modification is determined by determining the sequence of the region of interest in DNA isolated from the mutant phage. In principle, covalent modifications of the DNA, including those produced by carcinogens, can be examined by this procedure, and the molecular expression of any mutagenic activity displayed by the premutational lesion can be defined.

Towards this goal, we need to develop bacterial strains capable of suppressing not only base substitutions, but also frameshifts and large deletions in gene *G*, an essential gene of φX174 that we have chosen for our initial studies. In principle, complementation provides a general solution to this problem, but there are serious technical difficulties in using a helper DNA in the transfection process. Therefore, we have investigated the possibility of using a plasmid carrying φX174 gene *G* to produce the gene *G* product, a virus spike protein, constitutively in the host cell. In this communication, we describe the construction and characterization of a ColE1 type plasmid containing a segment of φX174 replicative form (RF) DNA carrying gene *G*. We show that this plasmid, pφXG, renders three previously nonpermissive *Escherichia coli* strains (H514, HF4704, and HF4740) permissive for φX174 *am9*, a phage mutant defective in gene *G* (5).

METHODS AND MATERIALS

φX174 Strains. Wild type was from R. C. Warner; *am9* (a gene *G* mutant), *am3* (a gene *E* mutant), and *am86* (a gene *A* mutant) were from R. L. Sinsheimer (5).

***E. coli* Strains.** *E. coli* C (wild type) was from R. C. Warner. The C/K_{12} hybrids, H514 Su^-, ϕX^s, *end, uvrA, thyA, arg,* r_K, m_K (6), HF4704 F^-, ϕX^s, Su^-, *thy, uvrA* (7), and HF4714 F^-, Su^+, ϕX^s, *arg, his, leu, thr, pro* (5, 7) were obtained from J. Hurwitz. HF4740 F^-, Su^-, ϕX^s, *recA13,uvrA103, tlr* was obtained from P. Howard Flanders. HB129 $S^r m$, r_B, m_B, *gal, lac, leu, pro,* B_1, *end* carrying pMB9 (8, 9) was from M. Ptashne's laboratory.

Growth of Bacteria and Virus. Luria broth and L plates were used as rich media (10). L-tet medium and plates were Luria broth (or L plates) containing 15 mg of tetracycline per liter. Virus was prepared from single plaques grown on HF4714 Su^+ using the defined medium, modified 3XD (11). Titers were measured by the soft agar techniques on R plates (10).

Enzymes. Restriction endonuclease *Eco*RI was from Miles. *Hae* III and *Hpa* II were gifts from R. Roberts; *Hha* I was from A. Jeffrey. Lambda exonuclease was a gift from C. Radding's laboratory. Deoxynucleotidyltransferase was from PL Biochemicals. RNase A was from Worthington Biochemicals.

DNA Preparations. φX174 RF DNA was prepared by a modification of Godson and Boyer's method (12). Plasmid DNA was amplified in the presence of chloramphenicol (200 μg/ml) and was isolated by minor modifications of the "cleared lysate" method Clewell (13). For the electrophoretic identification of recombinant clones, plasmid DNA was partially purified as follows: Lysates were prepared from 20 ml of chloramphenicol-treated cultures essentially according to Clewell except that 20 μg of RNase was added together with the detergent solution. After the chromosomal material was pelleted, the clear lysates were extracted twice with phenol and then three times with ether. The DNA was recovered by ethanol precipitation. Some detergent coprecipitated with the DNA, but caused no problems with the electrophoretic assay.

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Abbreviations: RF, replicative form; TE buffer, 10 mM Tris·HCl, pH 8.0/1 mM EDTA; bp, base pairs; restriction fragments are named according to the number of base pairs they contain.

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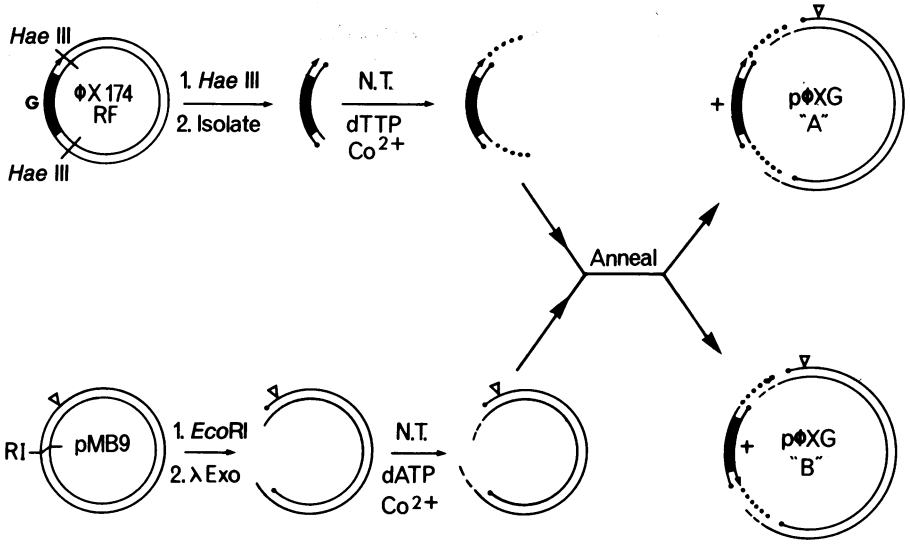

FIG. 1. Experimental protocol for the biochemical construction of hybrid molecules from pMB9 DNA and a segment of ϕX174 RF DNA. λ Exo is λ exonuclease; N.T. is terminal deoxynucleotidyltransferase (20). Heavy dots symbolize 5′ ends. "A" and "B" are the two possible orientations for the inserted viral DNA fragment relative to the *Hin*dIII restriction site shown as an open triangle (9). The 5′ to 3′ direction of the viral plus strand (+) is indicated by the arrowhead.

Gel Electrophoresis and Electron Microscopy. Gel electrophoretic techniques used have been described (14). Electron microscopy was by the formamide (50%) and the aqueous techniques described by Davis *et al.* (15). The restriction fragment *Hae* 1342† from ϕX RF DNA was used as an internal size marker.

Biochemical Construction of Plasmid–Viral DNA Hybrid Molecules. The general procedure of Jackson *et al.* (16) was used except that the DNA ligase step was omitted (17). The pMB9 DNA was digested with *Eco*RI in buffer (80 mM Tris·HCl, pH 8.0/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol) under conditions in which more than 95% of the circles were converted into linear forms as determined by electron microscopy. The linear DNA (20 μg) was taken up in 200 μl of buffer (67 mM glycine-KOH, pH 9.4/4 mM $MgCl_2$) and was digested with 65 units of λ exonuclease (18) for 1 hr at 0°. DNA was recovered by the following steps in sequence: one phenol extraction, three ether extractions, and ethanol precipitation. Exonuclease-treated linear pMB9 DNA (10 μg) was dissolved in 100 μl of transferase buffer (140 mM Tris-cacodylate adjusted to pH 6.9 with KOH/1 mM $CoCl_2$/1 mM 2-mercaptoethanol) (19) containing 10 nmol of [^{3}H]dATP (1.1 Ci/mmol) and was incubated with 66 units of deoxynucleotidyltransferase (20) for 160 min at 37°. The reaction was terminated by one phenol and three ether extractions, and the DNA solution was dialyzed overnight against two changes (2 liters each) of TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA). Deoxyadenylate incorporation was estimated from trichloroacetic acid-precipitable radioactivity to be about 100 residues per 3′ end of pMB9. The viral fragment, *Hae* 1342, was prepared by digesting ϕX174 RF DNA with *Hae* III and fractionating the products on a 35% preparative polyacrylamide slab gel by procedures similar to those described elsewhere (14). Four picomoles of *Hae* 1342 were incubated (no λ exonuclease) with 55 units of transferase in 100 μl of transferase buffer containing 10 nmol of [^{3}H]dTTP (1.8 Ci/mmol) at 37° for 60 min. Poly(dT)-tailed DNA was isolated as above. Incorporation of thymidylate was estimated to be about 80 residues per 3′ end of *Hae* 1342.

One microgram of poly(dA)-tailed, linear pMB9 DNA (~0.28 pmol) and 0.25 μg of poly(dT)-tailed *Hae* 1342 (~0.3 pmol) were annealed by incubation in 200 μl of buffer (100 mM $CaCl_2$ in TE buffer) at 50° for 30 min followed by slow cooling to 22° over several hours. In two control experiments, the tailed pMB9 DNA alone and the tailed viral DNA fragment alone were put through identical annealing procedures. Electron microscopy revealed that when both types of DNA were present during annealing, at least 10% of the linear DNA was converted into circular form. No circles were detectable in control experiments.

Transformation. Calcium-shocked cells were prepared from exponentially growing cultures according to Cohen *et al.* (21), except that $CaCl_2$ concentration was increased to 50 mM. Annealed DNA (0.15 pmol) in 200 μl of buffer (50 mM $CaCl_2$ and 10 mM $MgCl_2$ in TE buffer) at 0° was mixed with 400 μl of the calcium-shocked cell suspension and put through the following sequential steps (22): incubation at 0° for 15 min, incubation at 42° for 2 min, incubation at 22° for 10 min, addition of 0.3 ml of 3 × concentrated L broth, incubation at 37° for 30 min, and plating of 0.1 ml on L-tet plates. Tetracycline-resistant colonies became visible after 12 hr of incubation of 37°.

The recombinant DNA experiments were done under P 1 containment as outlined in the "National Institutes of Health Recombinant DNA Research Guidelines, Part II."

RESULTS

Cloning of Segment of φX174 DNA Bearing Gene *G*. *Hae* 1342† is the largest fragment produced by *Hae* III digestion of ϕX174 RF DNA and contains the entire structural gene (gene *G*) for a spike protein as well as parts of genes *F* and *H* (23, 24). By the experimental procedures schematized in Fig. 1, we have cloned this viral fragment into the *E. coli* plasmid, pMB9, a convenient vehicle carrying a ColE1 linear DNA with *Eco*RI. Then poly(dA) tails were added. Poly(dT) tails were added to the viral fragment. Equimolar amounts of the two molecular species were annealed, and the resulting mixture was used to transform the *E. coli* host, H514. Transformants were selected by plating in the presence of tetracycline. DNA from 11 of the 346 tetracycline-resistant colonies obtained was isolated and examined by agarose gel electrophoresis. Out of 11 transformants examined, two had a plasmid DNA with a mobility identical to the parental plasmid, pMB9; nine had more slowly

† This is fragment Z1 according to the widely used, but less descriptive, nomenclature (2).

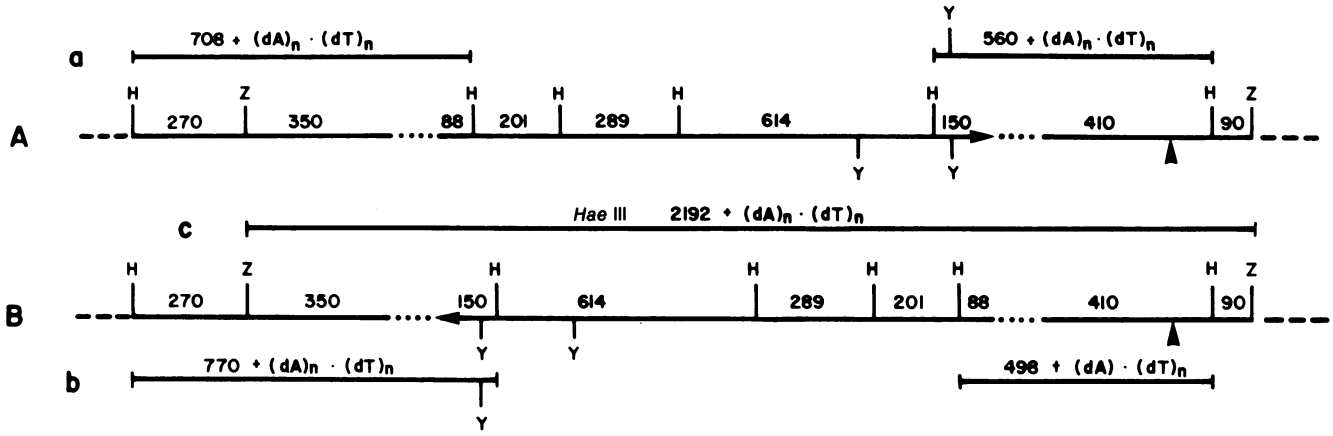

FIG. 2. A partial restriction cleavage map of pφXG105. (*A*) pφXG105 in the "A" orientation (see Fig. 1). (*B*) pφXG105 in the "B" orientation. (*a* and *b*) Predicted *Hha* I bridge fragments for orientations "A" and "B," respectively. (*c*) Predicted *Hae* III fragment from pφXG. H, Y, and Z refer to cleavage sites for the restriction endonucleases *Hha* I, *Hpa* II, and *Hae* III, respectively; ▲ marks the *Hin*dIII site that is used to orient pMB9 relative to its *Eco*RI site (9). The arrowhead gives the orientation (5′ → 3′) of the φX174 (+) strand; the dots represent $(dA)_n$·$(dT)_n$ bridges.

moving DNA, suggesting a larger molecule. All the more slowly moving plasmids had similar mobilities, indicating similar size.

In order to see if gene *G* was functional in any of these transformants, we screened them for plaque formation with φX174 *am9*, a gene *G* mutant incapable of growth on the parent bacterial strain (H514) from which these transformants were derived. The results showed that the two tetracycline-resistant transformants whose plasmid DNA had the same mobility as the pMB9 marker did not form any plaques. The remaining nine transformants (harboring larger, apparently hybrid, plasmids) gave plaques, although not all of them had the same plating efficiency. Two of these nine transformants (named H514/pφXG105 and H514/pφXG109) were selected for further analysis.

Characterization of pφXG105 and pφXG109. In addition to the electrophoretic mobilities mentioned above, pφXG105 and pφXG109 were characterized by electron microscopy and restriction mapping. Electron microscopy gave average contour lengths of: (*a*) poly(dA)-tailed, linear, pMB9 DNA, 1.68 μm; (*b*) poly(dT)-tailed φX *Hae* 1342, 0.35 μm; (*c*) circular molecules produced by annealing (*a*) and (*b*), 1.98 μm; and (*d*) pφXG105 and pφXG109, 2.07 μm.

Fig. 2 shows the partial restriction map for pφXG constructed from available information (2, 9). Fig. 3*a* shows the *Hae* III cleavage pattern for purified pφXG105 DNA as well as for φX RF DNA and pMB9 DNA. The map predicts that insertion of φX *Hae* 1342 at the single *Eco*RI site of pMB9 should cause pMB9 *Hae* 850 (9) to be replaced by a new fragment, pφXG *Hae* 2192 + $(dA)_n$·$(dT)_n$. Lanes 2 and 3 of Fig. 3*a* shows this. The new fragment is estimated to be about 2400 base pairs (bp) from its mobility.

The map also predicts that the *Hha* I digest of pφXG should show three φX fragments (201, 289, and 614 bp) as well as two new bridge fragments that join pMB9 and φX *Hae* 1342 DNA. The exact size of the bridge fragments will depend upon the orientation of φX *Hae* 1342 relative to pMB9 and the length of the $(dA)_n$·$(dT)_n$ tails. The map also predicts that pMB9 *Hha* I 1030, containing the *Eco*RI site (9), should disappear. Fig. 3*b* verifies these predictions. From its mobility, the larger bridge fragment is estimated to be 830 bp in both pφXG105 and pφXG109; no such fragment appears in either the pMB9 or φX RF DNA digests by themselves. The smaller bridge fragment is estimated to be about 600 bp for pφXG105 and 625 for pφXG109.

The data in Fig. 3 *a* and *b* show that φX *Hae* 1342 bearing gene *G* has been inserted into the *Eco*RI site of pMB9. Two different orientations (see Fig. 1) are possible, each giving slightly different bridge fragments (left: 708 + tail or 770 + tail; right: 560 + tail or 498 + tail; see Fig. 2). Since these values are rather similar for the different orientations and since the length of the tails is not known with precision, it is difficult to determine the orientation from the estimated size of the bridge fragments in the *Hha* I digest. However, the map predicts that the left *Hha* I bridge fragment will not change in a double digest of pφXG with *Hha* I and *Hpa* II if φX *Hae* 1342 has the "A" orientation shown in Fig. 1, while the right *Hha* I bridge fragment will be shortened by 40 bp. The reverse will be true for the opposite orientation ("B", Fig. 1). The cleavage pattern in Fig. 3*c* shows that the left *Hha* I bridge fragment remains constant at 830 bp. The right bridge (600 bp) disappears and a new fragment of 540 bp appears. *Hha* I 613 disappears, as it must (in either orientation) since it contains a *Hpa* II site (2). These data suggest strongly that φX *Hae* 1342 DNA has the orientation shown as "A" in Fig. 1. Similar results (not shown) were obtained with pφXG109.

Biological Properties of Bacterial Hosts Carrying pφXG105 or pφXG109. Table 1 summarizes the results of experiments in which we tested the ability of the nonpermissive host, H514, to form plaques with φX174 amber mutants defective in genes *A*, *E*, and *G* before and after transformation with pφXG105 and pφXG109. Within the limits of these data, the suppression is specific for gene *G* mutants.

When the gene *G* nonsense mutant, *am9*, was grown on the plasmid-bearing strain, H514/pφXG105, in soft agar, very small turbid plaques were barely visible in 3 hr at 37° compared to large (~2 mm), clear plaques produced by wild-type phage. Several individual plaques were picked and examined on lawns of permissive and nonpermissive test strains (HF4714 Su^+ and C Su^-), neither of which carry the plasmid. In a typical experiment, ⅓ of the virus in a single plaque from H514/pφXG105 was wild type. Plaques picked after longer incubation had an even greater wild-type content. This was peculiar to H514/pφXG105 lawns since the *am9* stock used in these experiments gives plaques containing $<10^{-5}$ wild-type virus on the permissive host, HF4714. Results similar to those for H514/pφXG105 were obtained with H514/pφXG109.

To investigate the production of wild-type phage in plasmid-bearing cells infected with *am9*, recombination was measured by an infective center assay. H514/pφXG105 cells

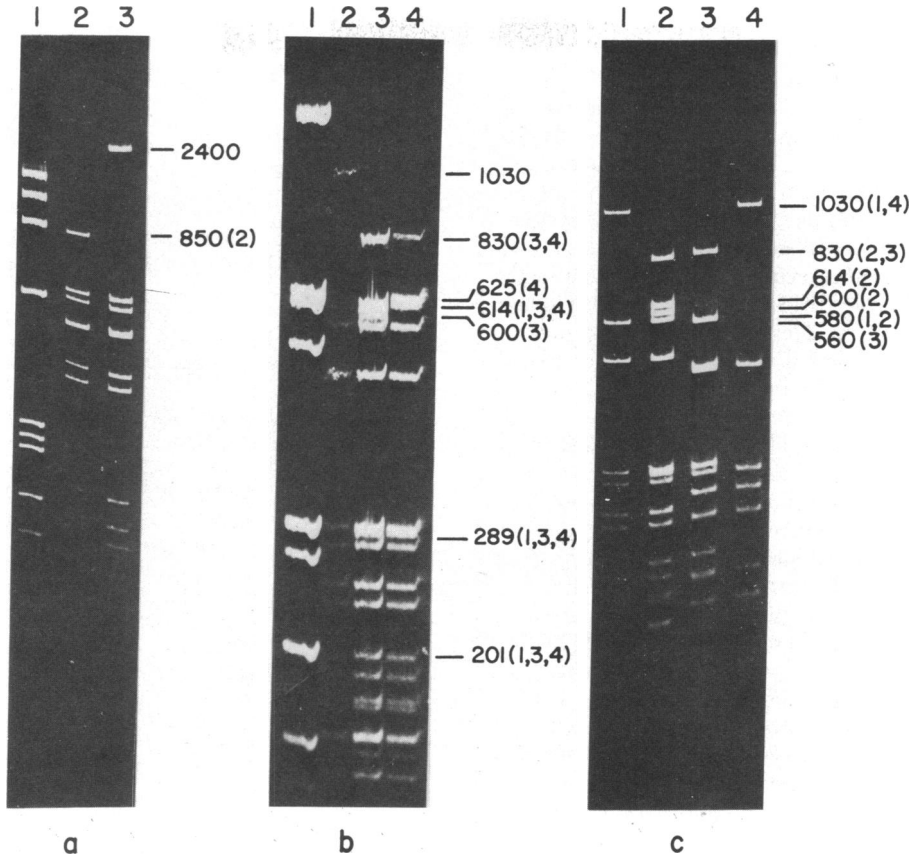

FIG. 3. Fractionation of restriction endonuclease cleavage products from pφXG105 and pφXG109. DNA (2–10 μg) was digested with the appropriate enzyme(s) in 100 μl of buffer (60 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$/6 mM 2-mercaptoethanol) for 3 hr at 37°. Reaction was terminated by one phenol and three ether extractions. Ten microliters of a dye mix (0.5% bromophenol blue and 0.5% xylene cyanol in 50% glycerol) were added. Electrophoresis was on vertical 5% polyacrylamide slab gels (20 × 40 × 0.15 cm) in 90 mM Tris-borate/2.5 mM EDTA (pH 8.3) at 400 V until the bromophenol blue marker reached or just ran off the bottom (10–12 hr). Gels were stained for 30 min in 0.5–1 μg of ethidium bromide per ml and photographed under UV light. Sizes of certain relevant restriction fragments are indicated. For easier identification of certain bands, the slots in which they occur are given in parentheses. (*a*) *Hae* III cleavage patterns of φX174 RF DNA (slot 1), pMB9 DNA (slot 2), and pφXG105 DNA (slot 3). (*b*) *Hha* I cleavage patterns of φX174 RF DNA (slot 1), pMB9 DNA (slot 2), pφXG105 (slot 3), and pφXG109 (slot 4). (*c*) *Hha* I and *Hpa* II double-digestion patterns of pMB9 and pφXG105 DNA: pMB9 cleaved with *Hha* I (slot 1), pφXG105 cleaved with *Hha* I (slot 2), pφXG105 cleaved with *Hha* I and *Hpa* II (slot 3), and pMB9 cleaved with *Hha* I and *Hpa* II (slot 4).

infected with *am9* were diluted and plated on lawns of the permissive and nonpermissive test strains. Wild-type phage was found in 0.03% of the infected cells. Clearly recombination by itself cannot explain why *all* of the plaques examined on lawns of HF514/pφXG105 were mixtures of mutant and wild-type phage.

An important clue to the plaque purity puzzle came from single cell, single burst experiments (25) carried out according to Benbow *et al.* (7). In a 200-tube experiment, an average burst size of 10 was obtained when H514/pφXG105 was infected with *am9* compared to about 300 when the same plasmid-bearing host was infected with wild-type phage. Thus, both recombination and a small burst size seem to be involved in the production of mixed plaques when *am9* is grown on H514/pφXG105.

This suggested that because of the small burst size, many cycles of infection and lysis must occur before a plaque becomes visible. During this period, the probability of recombination between the plasmid DNA and the mutant viral DNA to give wild-type viral DNA is high. Once this occurs, wild-type phage is produced rapidly because its burst size is 30 times that of *am9* in this plasmid-bearing cell. To test this hypothesis, we lowered the recombination frequency of pφXG and *am9* DNA by transforming a recombination-defective, nonpermissive host (HF4740 *recA13*) with pφXG105. A tetracycline-resistant transformant, permissive for *am9*, was obtained without difficulty. Two types of plaques were observed after infection of this recA⁻, pφXG-bearing host with *am9*. Most of the plaques were small with a regular edge. These plaques had less than 0.1% wild-type phage. About 2% of the plaques were large with an irregular edge. These plaques contained a large amount of wild-type phage. Thus, reduction of the recombination frequency by the recA⁻ mutation makes it possible to isolate plaques that are essentially pure *am9*. Why 2% of the plaques are mixed is still not clear to us.

These biological effects are not peculiar to H514/pφXG since very similar effects have been observed with another bacterial strain, HF4704, transformed with pφXG105 or pφXG109.

Table 1. Plaque formation by φX174 mutants on *E. coli* containing pφXG plasmids

φX174	Location of mutation	Plaque formation H514	H514/ pφXG105	H514/ pφXG109
Wild type	—	+	+	+
am9	Gene *G*	−	+	+
am3	Gene *E*	−	−	−
am86	Gene *A*	−	−	−

DISCUSSION

Gene *G*, an essential gene in bacteriophage φX174, is required for synthesis of single-stranded viral DNA from RF. Its gene

product is a spike protein in the virion (3, 4). If one wishes to isolate and propagate both conditional and unconditional lethal mutants that might arise from a covalent modification of the DNA in a virulant phage such as ϕX174, one must have a system that is permissive not only for base substitutions, but also for frameshifts and large deletions. Gene-specific suppression by complementation using a plasmid vehicle such as the one described here would appear to provide such a general system.

We anticipated a number of potential difficulties in constructing a functional strain carrying pϕXG. For example, the restriction fragment ϕX *Hae* III 1342 does not contain a known promoter (2, 26, 27). Also, production of viral spike protein in an uninfected cell might be lethal. None of these materialized, but one problem did arise. The burst size of *am9*, a gene *G* nonsense mutant, on the pϕXG-bearing host, H514, is only 5–10% that of wild-type phage on H514/pϕXG. The small burst size is apparently due to the known polar effect of the *am9* mutation on gene *H* (28). Our most recent experiments (unpublished) have shown that minicells carrying pϕXG produce a large amount of gene *G* spike protein. The gene *H* product, another spike protein, must be furnished by the infecting mutant phage. Because of the severe polarity of the *am9* mutation, only a small amount of H protein seems to be formed (28), and this limits the burst size. Further support for this comes from experiments (unpublished) in which we have transformed an amber suppressor strain that is *non*permissive for *am9* with pϕXG. The transformant not only becomes permissive for *am9*, but the burst size increases 7-fold compared to the transformants described in this paper that do not carry a suppressor tRNA.

The small burst size combined with recombination leads to plaques containing a mixture of wild-type and *am9* phage. Pure *am9* can be isolated from plaques grown on a recombination-deficient, pϕXG-bearing host such as HF4740/pϕXG even though the burst size is still small. This is not generally useful for our proposed system since the *recA* function seems to be required for many interesting, carcinogen-induced mutations (29, 30). This does not appear to be serious since we propose to produce mutants by transfection of *spheroplasts* with a site-specific, covalently modified DNA. Transfection of spheroplasts derived from cells that are resistant to infection by the intact virus produces a single burst of virus from DNA. Since we find only 0.03% wild-type virus when single cells of H514/pϕXG infected with *am9* are plated, we expect no more than this from a single burst of transfected spheroplasts.

In terms of developing a general system for detecting and propagating any type of mutation in gene *G*, polar effects produced by nonsense codons may present a problem with certain frameshift mutations. For example, a frameshift that obliterates the normal termination codon of gene *G* without generating a new termination codon in the short (eight nucleotides) nontranslated region between *G* and *H* may show *translational* polarity due to readthrough even if *transcriptional* polarity is overcome by using a pϕXG-bearing strain carrying an appropriate suppressor tRNA. For these reasons, it appears that a host carrying a plasmid with functional copies of *both* genes *G* and *H* may be required before the system will be completely permissive for base substitution, frameshift, and large deletion mutations in gene *G*.

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