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Biochemistry

Cloning of bacteriophage fd gene 2 and construction of a plasmid dependent on fd gene 2 protein

(gene expression and overproduction/origin of replication/phage miniplasmid/safe cloning vector)

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ABSTRACT **Bacteriophage fd gene 2 was cloned in plasmid pBR325. Cells carrying the hybrid plasmid produce about 200 times more enzymatically active fd gene 2 protein than did cells infected with phage fd wild type, as measured by replication of phage fd replicative form I *in vitro*. Cloned gene 2 supports replication of an artificial phage fd miniplasmid consisting of the origin of bacteriophage fd replication and a gene coding for kanamycin resistance. This plasmid occurs in high copy numbers and is viable only in cells carrying the cloned fd gene 2 or in cells infected with phage fd. Because the miniplasmid is not propagated in natural hosts, it can be considered a safe cloning vector. Its fusion with the gene 2 hybrid plasmid provides an autonomous replicon independent of the *polA* function of the host cell. fd gene 2 is the only phage-encoded *trans*-acting function required for replication of double-stranded fd DNA *in vivo*.**

Gene 2 protein of bacteriophage fd is a key enzyme for DNA replication of the phage. It is a specific endonuclease that cleaves supercoiled phage fd DNA at a unique site in the viral strand (1), thus creating the origin of viral strand replication (2). Furthermore, gene 2 protein provides a topoisomerase activity for specific relaxation of fd replicative form I DNA (RFI; covalently closed, circular, and superhelical) (3). It is involved in initiation of unwinding of the fd double strands and cleavage and circularization of the replicating viral strands (4). Gene 2 protein could be isolated in amounts of only 10 or 100 μg from 1 kg of cells infected with wild-type or *am*51 fd phage, respectively. In order to facilitate purification of gene 2 protein and to make it available for physical and chemical studies, it was desirable to amplify the amount of the enzyme by cloning fd gene 2 on a multicopy plasmid.

It was also of interest whether cloned gene 2 protein would support replication of a plasmid containing only the phage fd origin for its initiation. Replication of such a plasmid should be dependent on the gene 2 function provided in *trans*. An analogous experiment has been carried out on the broad host range plasmid RK2: a *trans*-acting function of RK2 was shown to be required for replication of a plasmid whose origin was derived from RK2 (5).

MATERIALS AND METHODS

Bacterial Strains, Bacteriophages, and Plasmids. *Escherichia coli* H403 is H570 (*polA1, endA1, rnsA100, tsx*) with the F episome and a reversion of *polA*. GM48 (*dam-3 dcm-6 thr-1 leu-6 thi-1 lacY galK2 galT22 ara-14 tonA31 tsx-78 supE44*) (6) was obtained from H. L. Heyneker via D. Blohm. An F episome was transferred into this strain. *E. coli* strains H517 (F^+, *rep-3*), 1101 (F^+, *endA1, supE*), HfrC6 (HfrC, *met, sup*$^-$), and the RNA phage fr were from the collection of H. Hoffmann-Berling. Phages fd *am*11 (gene 2 amber), fd *am*51 (gene 5 amber) (2), and phages fd 11 and fd 106 (7) have been described. Phage fd 11 carries an *Eco*RI restriction endonuclease site between gene 2 and the origin of replication at position 5830/1 in the fd genome; phage fd 106 is a cloning vector bearing an insertion of the genes for kanamycin (Km) and chloramphenicol (Cm) resistance at position 5572/3. Plasmid pBR325 has been described (6) and its physical structure has been analyzed (8).

Isolation of DNA. The isolation of plasmids (9) was modified as follows: After lysozyme treatment urea instead of Triton X-100 was added to a final concentration of 2 M to achieve complete lysis. No additional salt was required for polyethylene glycol precipitation of the nucleic acids. Phage fd RFI to be cleaved by *Atu* I restriction endonuclease was isolated as described (2) from strain GM48 F^+ (6, 10).

Enzymes. Restriction endonucleases (except *Atu* I) were from commercial sources (BRL; Boehringer Mannheim). Phage T4 DNA ligase was isolated from the overproducing strain 1100(λ_{228} dT4 lig) (11). *E. coli* DNA polymerase I was purified as described (2).

To purify *Atu* I restriction enzyme (12) cells of *Agrobacterium tumefaciens* strain B6 were mechanically opened and the homogenate was fractionated with polyethyleneimine and ammonium sulfate, and subsequent chromatography on Bio-Rex70, DEAE-cellulose, Ultrogel AcA 34, and hydroxyapatite. The preparation contained residual amounts of a single-strand specific endonuclease (12).

Plasmid Construction. In general, DNA (25 μg/ml) was cleaved by restriction endonucleases in 20 μl of buffer containing 20 mM Tris·HCl at pH 7.5/10 mM $MgCl_2$/1 mM 2-mercaptoethanol. Reaction mixtures with *Atu* I endonuclease additionally contained bovine serum albumin at 500 μg/ml. Incubation was carried out for 1 hr at 35°C and the reaction was terminated by heating to 70°C for 10 min.

Heat-stable enzymes were removed by dilution of the reaction mixture with H_2O to 100 μl and subsequent phenol treatment. The aqueous phase was applied to a Ultrogel AcA 54 column equilibrated with H_2O. The exclusion volume was collected and lyophilized.

Gap filling was carried out with 20 ng of *E. coli* DNA polymerase I for 500 ng of DNA in 100 μl of buffer under conditions described elsewhere (13). The DNA was ligated with 100 ng of T4 ligase and 5–25 μg of DNA per ml in 100 μl of buffer containing 50 mM NaCl, 60 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM ATP. Incubation was performed at 4°C for 2 hr for staggered-end or 6 hr for blunt-end ligation.

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Abbreviations: RF, replicative form DNA; RFI, covalently closed circular superhelical (replicative form) DNA; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; kb, kilobase(s).

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Cloning. The DNA was cleaved and ligated as indicated in the text. Ca^{2+}-treated cells were transformed according to Mandel and Higa (14), and plated on rich medium containing antibiotics in a concentration of 20 μg [Km, tetracycline (Tc)], 50 μg (Cm), or 100 μg [ampicillin (Ap)] per ml of agar. H403 cells were preinfected with phage fd at a multiplicity of infection of 5 at a cell density of 3×10^8 cells per ml. Single colonies from the infection experiment were then rendered competent by Ca^{2+} treatment (14).

RESULTS

Cloning of phage fd gene 2: Overproduction of gene 2 protein

Construction of a Gene 2 Hybrid Plasmid. This experiment was designed for overproduction of gene 2 protein in *E. coli* cells after construction of a hybrid plasmid genome *in vitro*. Previous studies on the killing effect of gene 2 protein (for a review see ref. 15) indicate that the expression of this protein on a multicopy plasmid might possibly interfere with the cellular metabolism. We have therefore chosen a cloning strategy that should result in a high yield of hybrid genomes containing the information of fd gene 2 in a defined arrangement (Fig. 1). An *Eco*RI/*Atu* I restriction fragment bearing the entire nucleotide sequence for only gene 2 was derived from fd 11 RFI DNA (7). Its insertion into the *Eco*RI/*Hin*dIII sites of plasmid pBR325 (6, 8) resulted in the deletion of a section of the structural gene encoding Cm resistance. It also placed the gene encoding Tc resistance under the control of strong fd promotors located proximal to gene 2, and within gene 2 proximal to genes *X* and 5 (16). The hybrid construction is outlined in Fig. 2. Cells transformed by this hybrid DNA were selected for growth on Ap and Tc and checked for Cm sensitivity and for gene 2 function by complementation of gene 2 amber fd phages on the *sup*$^-$ host H403. Small colonies contained hybrid plasmids with an intact gene 2. One of these clones, pTM11, was further analyzed.

Analysis of Hybrid Clones. Digestion of purified plasmid pTM11 with *Alu* I, *Hpa* II, and *Eco*RI/*Hpa* I nucleases indicated that the insert in the hybrid plasmid yields restriction fragments corresponding to the *Alu* I C, *Hpa* II D and F, and *Eco*RI/*Hpa* I fragments of bacteriophage fd 11 (data not shown). Thus, gene 2 is contained within these restriction fragments (7, 17).

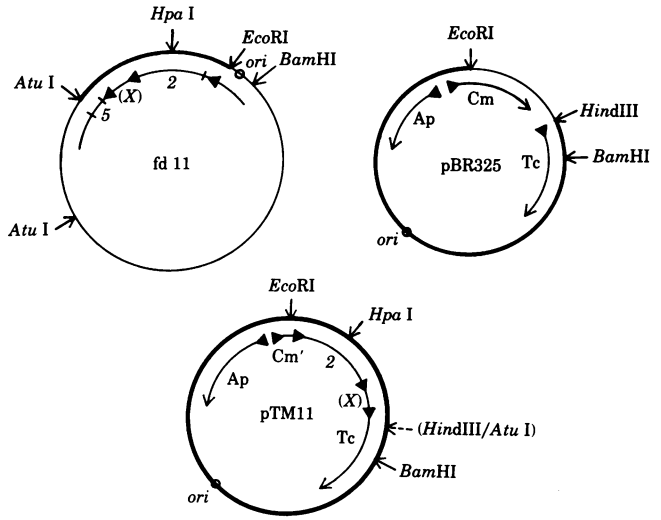

FIG. 1. Restriction maps of phage fd 11 [6.4 kilobases (kb)] (7), pBR325 (6.0 kb) (8), and pTM11 (6.2 kb). ➤, Promoters and direction of transcription. Peripheral arrows indicate cleavage sites of restriction endonucleases. Figures inside designate phage genes.

The gene 2 protein encoded by plasmid pTM11 supports growth of fd gene 2 amber phages: Because strain H403 carrying pTM11 does not indicate single plaques upon infection with F-specific phages, for a quick procedure drops of phage solution were applied to plated cells, which led to a weak response of phage growth (see legend to Fig. 2). Growth of phage could be demonstrated for fd wild type and fd gene 2 amber (fd *am*11), but not for fd gene 5 amber (fd *am*51). The *sup*$^-$ strain H403 without plasmid or transformed with pBR325 did not propagate fd *am*11 or fd *am*51 (data not shown). To prove that growth of fd *am*11 on H403(pTM11)—and on H403(pTM12), which is a derivative of H403(pTM11) (see below)—is due to complementation and not to recombination, the mutant phage was grown on this strain in liquid rich medium. Under these conditions phage propagation is improved compared to plating. Fig. 3 shows an increase in fd *am*11 titer and also in amounts of wild-type phage after infection. This result demonstrates complementation of the gene 2 amber mutation, although recombination events or reversions occur to a minor extent in the mutated gene 2 of the phage.

Overproduction of fd Gene 2 Protein. Biochemical evidence for synthesis of functional gene 2 protein was obtained by studies on replication of phage fd RF in soluble extracts of H403(pTM11) cells. Table 1 shows the high replication capacity of extracts from this strain. When H403(pTM12) cells were transformed with the fd miniplasmid pfdA1 (see below), extracts from these cells also showed high gene 2 protein activity. Overproduction of gene 2 protein in strain H403(pTM11) and strain H403(pTM12, pfdA1) exceeds that of wild-type fd-infected cells by about 200-fold and that of fd *am*51-infected cells by about 10-fold (Table 1).

Gene 2 protein purified from strain H403(pTM11) by a method described previously (2) showed the properties of gene 2 protein purified from phage fd *am*51-infected cells, including its specific endonuclease and topoisomerase activities (3). Furthermore, plasmid pTM11 in *E. coli* H517 (*rep-3*) allowed preparation of a gene 2 protein that was completely free of contaminating *rep* protein.

Properties of Gene 2 Hybrid Clones. The slow growth of strain H403(pTM11), presumably due to the overproduction of gene 2 protein, favors the accumulation of spontaneous faster growing mutants. After approximately 15 generations of growth in antibiotic-free medium, 1% of the cells carried plasmids with

Table 1. Properties of gene 2 hybrid cell clones

Strain	Activity of gene 2 protein*	Infection by phages fd and fr†	Transformation efficiency of pfdA1‡
H403	—	Normal	9×10^{-7}§; $<10^{-9}$¶
H403(pTM11)	7.0	Very low‖	—
H403(pTM12)	2.1	Normal‖	1×10^{-4}
H403 fd-infected	0.03	—	3×10^{-5}
H403 fd *am*51-infected	0.6	—	—
H403(pTM12, pfdA1)	8.0	Not observed	—
H517	—	—	$<10^{-9}$
H517(pTM12)	—	—	$<10^{-9}$

* DNA synthesis on fd RFI in a soluble cell extract was measured (2); the activity of gene 2 protein is given in units/mg of protein (3).
† Plaque formation was measured as described in legend to Fig. 2.
‡ Transformation with a mixture of pTM12 and pfdA1 [transformants per cell per μg of DNA (14)].
§ Contransformation with pTM12 and pfdA1.
¶ pTM12 DNA previously linearized with *Pst* I endonuclease, which leaves pfdA1 intact.
‖ See Fig. 3.

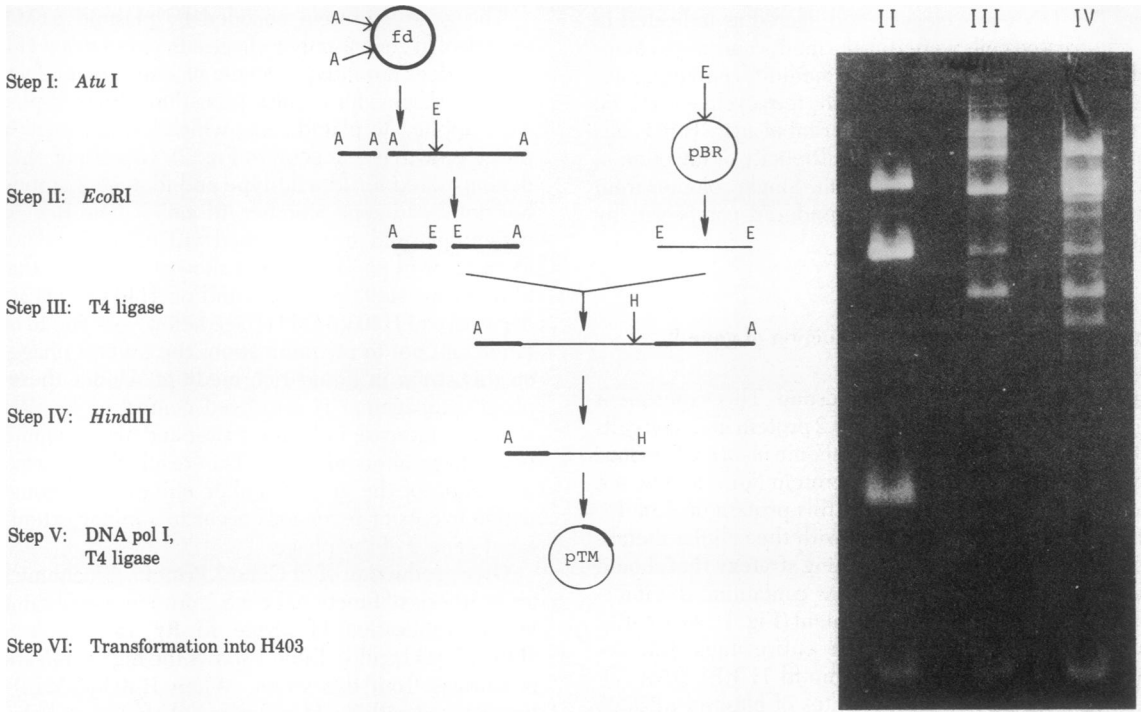

FIG. 2. Scheme for inserting fd gene 2 into pBR325 and selective cloning. Step I: Phage fd 11 RFI (1 μg) was cleaved with *Atu* I endonuclease and the reaction mixture was extracted with phenol and lyophilized. Step II: The cleavage product of step I and plasmid pBR325 (0.2 μg) were cleaved with *Eco*RI endonuclease. Step III: The cleavage products of step II were ligated with phage T4 ligase only at the *Eco*RI cuts. Ligation did not occur for *Atu* I sites before treatment with DNA polymerase I. As shown in lane III of the gel, fragments bearing a *Eco*RI end were efficiently ligated, whereas the 1-kb *Atu* I fragment, which can be seen at the bottom of the gel, was not a substrate for ligation. This phenomenon can be accounted for by the trace nuclease contamination in *Atu* I. Step IV: Products of ligation to the right arm of linear pBR325 (Fig. 2) were eliminated by treatment with *Hin*dIII endonuclease. Step V: Sticky ends of the DNA were filled by synthesis with DNA polymerase I and ligated with T4 DNA ligase. Step VI: Ap-resistant transformants were selected and then scored for Tc resistance and Cm sensitivity. Cultures of positive colonies were plated and droplets of the phages (10^5 plaque-forming units/ml) fd, fd *am*11, and fd *am*51 were applied to the surface. After incubation at 37°C about 25% of the clones were found to complement fd *am*11. Capital letters indicate cleavage sites for restriction endonucleases: A, *Atu* I; E, *Eco*RI; and H, *Hin*dIII. On the right, cleavage and ligation pattern in a 1.2% agarose gel after individual steps of the hybrid construction.

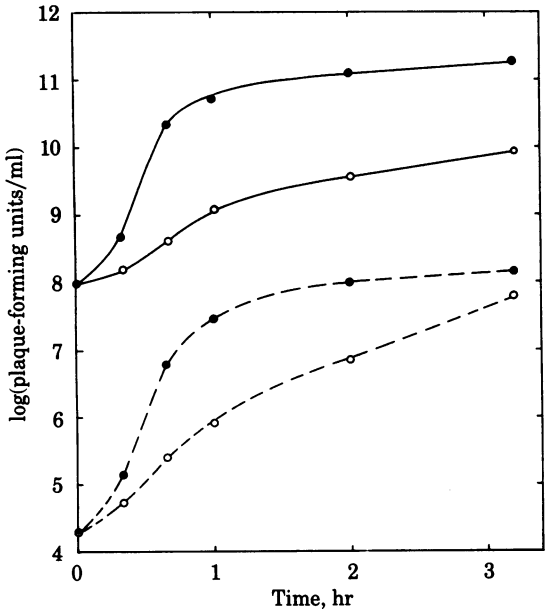

FIG. 3. Complementation of phage fd *am*11 by H403(pTM11) (○) and H403(pTM12) (●). The cells were grown at 37°C to 1×10^8 cells per ml and infected with one fd *am*11 phage per cell, and the phages were titered on strain 1101 (——) or, for revertants, on HfrC6 (-----). The frequency of revertants in the infecting phage mutant was 2×10^{-5}.

inactive gene 2. This could be remedied by growing cells in the presence of Tc, because many of the spontaneous mutants in gene 2 showed polar effects on the expression of the Tc resistance gene.

The hybrid cell clone H403(pTM11) shows a low capacity for propagation of F-specific phages (Table 1, Fig. 3) and does not survive Ca^{2+} treatment used for the preparation of competent cells (data not shown). We therefore isolated a spontaneous mutant of plasmid pTM11 by retransformation of plasmid DNA into H403 cells. This derivative plasmid, termed pTM12, showed a restriction pattern identical to that of pTM11 for the endonucleases *Eco*RI/*Hpa* I/*Bam*HI and *Hpa* II (data not shown) and still complemented fd gene 2 amber phages (Fig. 3). The plasmid pTM12 expresses gene 2 protein in low quantities, but it does not interfere with growth of F-specific phages (Table 1) and allows the cell to survive Ca^{2+} treatment to a normal extent. For this reason the plasmid pTM12 was used as the basis for development of a vector system described below.

A vector system functionally dependent on fd gene 2

Construction of an fd Miniplasmid. If gene 2 is the only phage function involved in the propagation of fd RF, the plasmid pTM12 should support replication in *trans* of a minimized plasmid containing the fd origin. In order to construct such a miniplasmid an *Eco*RI/*Bam*HI fragment of fd 11 containing the origin (Fig. 1) (7) was fused to an *Eco*RI/*Bam*HI fragment of fd 106 (map not shown) (7) encoding Km resistance and the 3′ end of the Cm resistance gene. H403(pTM12) cells were transformed with this hybrid DNA and selected for Km resistance.

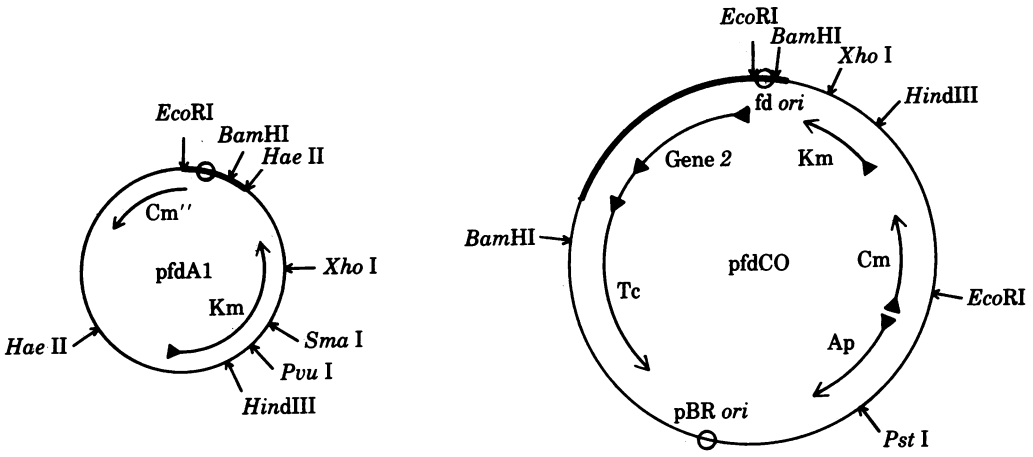

FIG. 4. Restriction maps of plasmids pfdA1 (2.4 kb) and pfdCO (8.6 kb). The thickened line indicates phage fd sequences. For additional explanations see legend to Fig. 1.

Cm-sensitive cells harbored the miniplasmid, designated pfdA1 (Figs. 4 and 5).

Properties of the Miniplasmid. Plasmid pfdA1 bears the fd origin as the only functional origin of replication and no fd structural genes. Consequently it is viable only in strains synthesizing gene 2 protein—i.e., H403(pTM12) or fd-infected cells (Table 1). H517(pTM12, *rep*$^-$) cells do not support replication of pfdA1 in accordance with the requirements of phage fd RF replication (ref. 18, unpublished data).

Plasmid DNA isolated from H403(pTM12, pfdA1) cells contains both species of plasmids, as expected (Fig. 5). The high copy nature of pfdA1 is reflected in the increased intensity of the plasmid band in the gel. It can also be seen that plasmids pTM12 and pfdA1 do not extensively form cointegrates *in vivo*. The absence of *in vivo* cointegrate formation is confirmed by the failure of plasmid DNA from H403(pTM12, pfdA1) to transform H517 (*rep*$^-$) cells to Km resistance (Table 1), because pfdA1 cannot be maintained under *rep*$^-$ conditions other than as a fusion to the *rep*-independent plasmid pTM12.

F^+ cells containing pTM12 together with pfdA1 cannot be infected with phage fd (Table 1). The reason for this is not known, but it might rely on the insufficient formation of F pili. On the other hand, plasmid pfdA1 can be transferred to fd-infected cells, where it is maintained as a separate replicon. It is, however, not packaged into infectious particles, even though efficient packaging of the helper phage itself takes place (Fig. 6). Thus, the 261-base pair segment in pfdA1 that originates from the intergenic region of phage fd does not supply a sufficient packaging function. By inserting an additional segment harboring the packaging signal of phage fd this property can be conferred to the miniplasmid (unpublished data).

The characteristics of pfdA1 suggest that it could be used as a safe cloning vector, because it cannot be packaged and can be propagated only in cells containing a source of fd gene 2 protein.

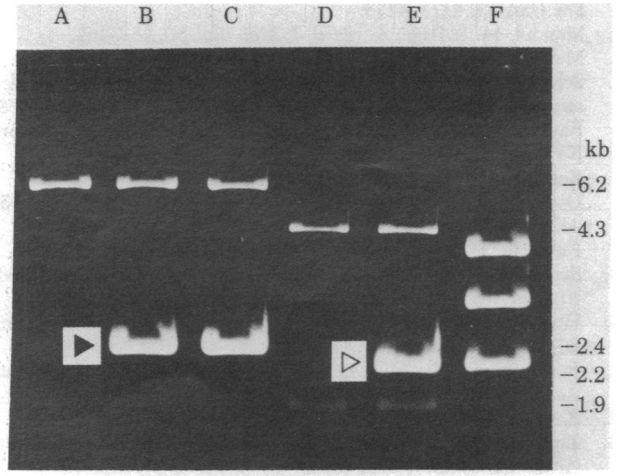

FIG. 5. Agarose gels of the constructed hybrids. Lane A, pTM11 after *Eco*RI cleavage; lane B, pTM12 and pfdA1 after *Eco*RI cleavage; lane C, pTM12 and pfdA1 after *Bam*HI cleavage; lane D, pTM11 after *Bam*HI/*Eco*RI double digestion; lane E, pTM12 and pfdA1 after *Bam*HI/*Eco*RI double digestion; lane F, fd 106 after *Bam*HI/*Eco*RI double digestion. Linear plasmid pfdA1 is marked by ▶; the fragment bearing the Km resistance gene (lane E, pfdA1; lane F, fd 106) is marked by ▷. The difference in migration of these two bands results from the removal of the fragment containing the origin of replication of fd 11. The weak bands represent pTM DNA.

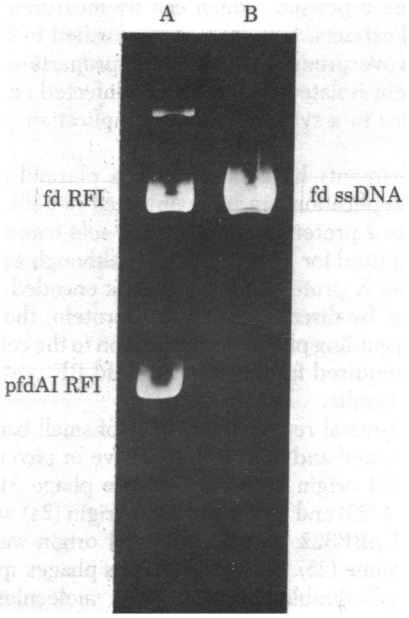

FIG. 6. Maintenance of plasmid pfdA1 in fd-infected cells. Superhelical DNA (lane A) and phage particles (lane B) were isolated from cultured cells, which had been infected with bacteriophage fd and transformed by plasmid pfdA1. Aliquots of the covalently closed circular DNA and the deproteinized phage particles were electrophoresed through a 1.2% agarose gel. The positions of circular fd single-stranded DNA (ssDNA) and of RFI of fd and pfdA1, respectively, are indicated. Single strands of pfdA1 are not visible on the gel, indicating a packaging efficiency of less than 0.5%.

One further advantage is that cells transformed by a gene 2-dependent replicon are easily cured of this plasmid by growing the cells at 42°C for a few generations (data not shown), because gene 2 protein is a heat-sensitive enzyme *in vivo* and *in vitro* (2).

Construction of a Hybrid Plasmid Bearing the Phage fd Origin and fd Gene 2. Because plasmid pTM12 supplies a function for replication of pfdA1 we fused together the two replicons. This was done by cleavage of both plasmids with *Eco*RI endonuclease and subsequent ligation of the fragments with T4 ligase. In one orientation Cm resistance should be restored because the two plasmids carry complementary sections of the Cm gene (Figs. 1 and 4). Thus cells were transformed with the ligated DNA and selected for Cm resistance. The fused plasmids formed a new replicon, termed pfdCO, which has the expected size and bears four antibiotic resistances (Km, Cm, Ap, Tc). It can be propagated in H517 (*rep*$^-$) and in H570 (*polA*$^-$) cells, indicating two functional origins of replication in pfdCO: that of bacteriophage fd and the ColEI origin of pBR325. Its reduction to a smaller size should increase its desirability as a convenient cloning vector bearing an autonomous replicative unit of bacteriophage fd.

DISCUSSION

Cloning of DNA segments of the *E. coli* phages on plasmids often interferes with the metabolism of the *E. coli* cell. Maintenance of fragments with strong *E. coli* phage T7 promoters has been achieved in the Gram-positive *Staphylococcus aureus* (19). Successful cloning of intact genes of small bacteriophages has been shown for gene G of phage ϕX174, although complementation experiments have shown that interference of the gene product with phage growth results in a small burst size (20).

We have succeeded in the molecular cloning of phage fd gene 2. Cells containing fd gene 2 cloned in pBR325 produce high levels of gene 2 protein, which can be measured in partially purified cell extracts. Gene 2 protein purified to homogeneity from such an overproducer has the same properties as functional gene 2 protein isolated from phage fd-infected cells. It is also active *in vitro* in a system for fd RF replication (unpublished data).

Our experiments have shown that a plasmid carrying the origin of fd replication can be maintained in cells with cloned gene 2. Gene 2 protein therefore is the sole *trans*-acting function of fd required for RF propagation, although a possible role played by the X protein, whose gene is encoded within gene 2 (16), cannot be discounted. Gene 3 protein, thought to be a pilot protein guiding phage RF replication to the cell membrane (21), is not required for cellular phage fd RF synthesis on the basis of our results.

Recently several replication origins of small bacteriophages have been cloned and shown to be active *in vivo* and *in vitro*. The phage G4 origin was inserted into phage M13 (22), the phage ϕX174 (23) and the phage M13 origin (24) were inserted into plasmid pBR322, and the phage f1 origin was duplicated in the f1 genome (25). Also, filamentous phages spontaneously produce small double-stranded DNA molecules and miniphages *in vivo*, which bear the origin of replication, but no functional genes (26, 27). In this paper we have demonstrated that the cloned fd origin can be made functional by the cloned gene 2 of bacteriophage fd *in vivo*.

We believe that the miniplasmid is a desirable candidate on which to base development of an improved cloning vector. (*i*) The vector is safe by virtue of the inability of pfdA1 to be packaged into infectious particles. Its dependence on the presence of the foreign gene 2 limits its existence to its intended host. (*ii*) The pfdCO plasmid consists of an autonomous replicon, which is independent of the host *polA* function. (*iii*) pfd plasmids maintain a high copy number in the host cell and allow easy purification of their DNA. (*iv*) the pfd replicon does not share homology with other frequently used cloning vectors, making it useful in cross-hybridization experiments. (*v*) Cells are easily cured of pfd plasmids by growth at elevated temperature. (*vi*) The option of packaging single-stranded plasmid DNA into phage particles is present and can be supplied to pfd plasmids by insertion of an additional nucleotide sequence that bears the packaging signal of bacteriophage fd (refs. 23 and 28; unpublished data). The ease with which single-stranded plasmid DNA is obtained facilitates sequence analysis by the method of Sanger *et al.* (29), hybridization experiments, and any other manipulation requiring large quantities of single-stranded genome.

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