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Site-Specific Proteolysis of Mini-F Plasmid Replication Protein RepE Destroys Initiator Function and Generates an Incompatibility Substance

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Plasmid F replication is controlled by a plasmid-specified Rep protein with both autorepressor and initiator functions. The mechanism by which these two functions of a Rep protein are balanced to achieve stable replication is unknown; however, we speculated in prior work that Rep protein modification could be involved. We report here that naturally proteolyzed F RepE protein has been detected and characterized. The processed molecule lost the first 17 N-terminal aminoacyl residues and initiator function but acquired increased specific DNA-binding affinity in the presence of *Escherichia coli* chromosomal DNA. When supplied in *trans*, the altered protein acts as an incompatibility substance and eliminates maintenance of F'*lac*. These findings indicate that protein processing has the potential to contribute to the overall control of DNA replication.

The F plasmid is a low-copy-number replicon whose concentration is maintained at parity with the host chromosome (2). Its maintenance depends on a plasmid-encoded Rep protein, designated RepE (14, 33, 37). RepE is a bifunctional protein which acts as both an autorepressor of its own transcription (29, 35) and an initiator of replication (5, 20). RepE binds to repeat sequences in the origin of replication, *oriS*, a secondary locus designated *copB*, and the *repE* operator in fulfilling these functions (Fig. 1) (16, 32). An increase in F plasmid copy number results from mutations in *repE* that reduce autorepressor but not initiator function (5, 10). The *copB* locus was thought to exert control by competing with *oriS* in binding RepE protein (11, 36), although this interpretation has been questioned recently (1, 22). While the major components of F replication control are known, the mechanism by which they interact to achieve equilibrium in plasmid copy number during cell growth is not clear.

In 1985, Trawick and Kline proposed a model for F plasmid replication control which is supported by computer simulation (38). According to this model, an irreversibly modified form of RepE binds to *oriS* and *copB*, while the unmodified RepE acts as the autorepressor. This speculation provided our impetus to search for modified RepE even though two previous reports on RepE purification had not indicated the existence of multiple forms (17, 32).

In this study, we have identified, purified, and characterized two truncated forms of RepE besides the full-length form of the protein. These truncations resulted from N-terminal, site-specific proteolysis. The smaller cleavage removed the first 11 aminoacyl residues and resulted in a protein that functions much like the unmodified RepE. The larger cleavage removed the first 17 aminoacyl residues and caused a loss of initiator activity. This loss was accompanied by an increased affinity for DNA binding at the operator and origin of replication in the presence of *Escherichia coli* chromosomal DNA, creating in effect a RepE molecule that is inhibitory to replication. While our observations do not conform to the previously proposed model, they are, however, consistent with the possibility of a novel role for protein processing in DNA replication control in the F plasmid.

MATERIALS AND METHODS

Bacteria and plasmids. The bacteria and plasmids used are listed in Table 1.

Cloning *repE*$^+$ and site-directed mutations. The full-length *repE* gene was amplified from pML31 by using oligonucleotide primers and the polymerase chain reaction (PCR). The 5′ primer contained an *Nde*I restriction site and the 3′ primer had an *Eco*RI site to facilitate cloning into pET-3b cut with these enzymes. All recombinants were sequenced to verify that mutations were not introduced by the PCR procedure. N-terminally truncated forms of RepE were produced by PCR and cloned into pET-8 as described above save that the 5′ oligonucleotides had homology to *repE* starting at codon 12 or 18; the RepE proteins produced from these mutants are designated Δ11 and Δ17, respectively. N-terminal sequencing of full-length RepE indicated that the *N*-formyl-methionine was removed from the mature protein. We presume this is true also for the Δ11 and Δ17 forms.

The *repE*::*lac*$^+$ translational fusion plasmid pBK859 (Table 1) was made by amplifying the F 45.173- to 45.345-kb region by PCR. This region contains the *repE* promoter operator and the first 35 codons of *repE*. The primer at 45.173 kb was modified to contain a *Hin*dIII restriction site, and the primer at 45.345 kb was modified to contain a *Bam*HI site. pACYC was cut with *Hin*dIII and *Bam*HI to delete the 5′ portion of its *tet* gene and replace it with the amplified F sequences. A pACYC::F recombinant was subsequently purified and restricted with *Bam*HI, and a *Bam*HI *lacZ*$^+$ cartridge was inserted to make pBK859.

Purification of RepE protein. The protocol of Masson and Ray (17) was used with the culture containing plasmid pREPE5 except that induction times with arabinose were 8 h. Cells were induced at an optical density at 595 nm (OD_{595}) of 0.2 by addition of arabinose (1% final concentration). At harvest, the culture had an OD of 0.85, which was equivalent to approximately 600 mg of protein. From this, we purified about 2 to 3 mg of homogeneous RepE protein.

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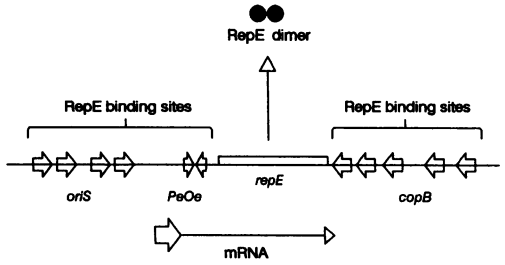

FIG. 1. Arrangement of the *repE* gene and known RepE protein-binding sites in the F plasmid. The product of *repE* is a 29,000-Da protein that acts as a dimer. Transcription of *repE* begins at promoter *Pe*. RepE binds at sites indicated by arrows. Control of *repE* transcription is autogenous and achieved by RepE binding to the operator *Oe* (small inverted arrows). Each repeat in *Oe* is 8 bp and identical in sequence to the first 8 bp of the nine 19-bp repeats that constitute the origin of replication, *oriS*, and *copB* (larger arrows).

RepE was purified from cultures harboring recombinant pET-*rep* expression vectors by the following protocol. A 3-ml culture in Luria (L) broth (18) supplemented with ampicillin (25 μg/ml) and chloramphenicol (25 μg/ml) was inoculated into 200 ml of L broth containing the same antibiotics and grown at 37°C to an OD_{600} of 0.5. RepE production was induced for 3 h with 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested in 20 ml of TEMG (20 mM Tris-HCl, 450 mM KCl, 1 mM sodium EDTA, 10 mM β-mercaptoethanol, 10% glycerol [pH 7.5]) and sonicated for a total of 4 min in 30-s pulses with the cell vessel contained in an ice-salt bath. The insoluble RepE was removed from the sonicate by centrifugation at 35,000 × *g* for 15 min. The pelleted RepE was overlaid with 20 ml of TNT (50 mM Tris-HCl, 200 mM NaCl, 0.5% Triton X-100 [pH 7.5]) and sonicated for 5 to 10 s to dissolve most of the pellet except for RepE. RepE was separated from other dissolved pellet proteins by centrifugation and then dissolved in TEDNG (50 mM Tris-HCl, 1 mM disodium EDTA, 1 mM dithioerythreitol, 250 mM NaCl, 5 M guanidine-HCl [pH 8.0]) with a 5- to 10-s pulse of sonication. The RepE was left in TEDNG overnight at 4°C, clarified by centrifugation, and then dialyzed at room temperature for a total of 3 h against two changes of buffer MESEKMG (20 mM morpholineethanesulfonic acid, 0.1 mM disodium EDTA, 300 mM KCl, 10 mM β-mercaptoethanol, 10% glycerol [pH 6.0]). After clarification by centrifugation, the RepE was purified by passage over a Mono S HR5/5 (Pharmacia LKB) anion-exchange resin, followed by removal of low-molecular-weight contaminants by gel exclusion chromatography through a Superose 12 HR10/30 (Pharmacia LKB) column. All three forms of RepE chromatographed as dimers in this last step. RepE-containing fractions were then pooled and concentrated to approximately 0.5 to 1.0 mg/ml, and MESEKMG buffer was adjusted to contain 50% glycerol. RepE is stable for longer than 12 months at −20°C in this condition.

Detection of RepE forms in crude cell lysates. Bacteria were cultured in L broth for a minimum of eight generations in the exponential phase, sampled, and then allowed to grow overnight to obtain a stationary-phase sample. Samples were mixed immediately with an equal volume of SDS lysis buffer (2% sodium dodecyl sulfate [SDS], 20% glycerol, 0.05% bromphenol blue, 20% β-mercaptoethanol, 250 mM Tris-HCl [pH 6.8]) and boiled. Boiled samples were frozen at −20°C until gel electrophoresis in 15% polyacrylamide (29.2% acrylamide–0.8% bisacrylamide)–0.1% SDS. After electrophoresis, proteins were transferred to polyvinylidene difluoride paper by the procedure of Towbin et al. (34). Proteins were detected by application of either rabbit polyclonal anti-RepE or mouse monoclonal anti-RepE followed by anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated to alkaline phosphatase. Alkaline phosphatase activity was detected by using a chromogenic substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)-Nitro Blue Tetrazolium (NBT), or it was detected photometrically by using Lumi-Phos 530 (Lumigen, Inc.) and X-ray film (24).

In vivo assays of RepE autorepressor function. Heteroplasmid cells were constructed as shown in Table 2. A pACYC plasmid (pBK859) was modified to contain a *lac* reporter

TABLE 1. Bacteria and plasmids

Strain or plasmid	Genotype	Reference
E. coli		
CSH50	*ara* Δ(*lac pro*) *thi* Str^r, a K-12 strain	18
BK342	CSH50 *recA1* (F'*lac*)	25
BL21	(λDE3) *ompT* r_B^- m_B^-, a B strain; λDE3 is λ imm^{21} *lacI*$^+$ *lacUV5*:T7 RNA polymerase; T7 RNA polymerase induced by IPTG (pLysS)	30
Plasmids		
pML31	Mini-F Km^r (Fig. 1)	15
pMF21	pML31 with *Bam*HI deletion 40.5 to 42.85 kb[a] F sequences	13
pREPE5	*repE* fused to *araB* promoter, Tc^r; *repE* expression induced by arabinose	16
pBK207	44.1- to 45.85-kb *Pst*I fragment of F cloned into *Pst*I site of pBR322; Ap^s Tc^r	25
pBK633	pUC8:F *Oe* 45.161 to 45.348 kb of F as *Alu*I-*Sma*I fragment cloned into *Eco*RI/*Sma*I-digested pUC8; Ap^r	This work
pBK634	pUC8:F *oriS* 45.000 to 45.161 kb of F as *Bgl*II-*Alu*I fragment cloned into *Eco*RI/*Sma*I-digested pUC8; Ap^r	This work
pBK819	*repE* Δ11 cloned into pET-8; used to produce Δ11-RepE from phage T7 promoter	This work
pBK820	*repE* Δ17 cloned into pET-8; used to produce Δ17-RepE from phage T7 promoter	This work
pBK857	*repE* cloned into pET-3b; used to produce Δ1-RepE from phage T7 promoter	This work
pBK859	pACYC containing F 45.143-45.345 kb:*lacZ*$^+$ translational fusion; Cm^r (see text)	This work
pLysS	pACYC plasmid producing phage T7 lysozyme; Cm^r	30
pET-3b	pBR322 containing phage T7 promoter (ϕ10) and translation signal (S10); Ap^r	30
pET-8	Same as pET-3b but has *Nco*I site instead of *Nde*I; Ap^r	30

[a] Kilobase coordinates are based on the data of Murotsu et al. (21), in which the reference *Pst*I site of F is at coordinate 44.100 kb.

TABLE 2. Autorepression by different RepE forms

Heteroplasmid construct[a]	β-Galactosidase sp act[b]	% Repression
pET-3b	594	0
pET-8	606	0
pBK857 (Δ1)	259	56
pBK819 (Δ11)	221	64
pBK820 (Δ17)	257	57

[a] Each host cell contained pBK859 (*lac*$^+$ *cat*$^+$) in addition to the pET vector or recombinant listed. Assays were done in triplicate.
[b] Miller units (18).

gene translationally fused to the F *repE* operator promoter. To preclude misinterpretation of β-galactosidase results due to fluctuations in reporter plasmid concentration, plasmid concentration was monitored by measuring plasmid-determined chloramphenicol acetyltransferase activity (3). In all cases, the pBK859 levels were equal (data not shown).

DNA-protein binding assays. pUC plasmids containing cloned mini-F sequences 45.0 to 45.161 kb (*oriS*) or 45.161 to 45.348 kb (*repE* operator, designated *Oe*) were the source of appropriate restriction fragment target sites. After restriction, plasmid vector and F fragments were end labeled with a radiolabeled deoxyribonucleotide by using the Klenow form of DNA polymerase. Before RepE binding constants were measured, the portion of active RepE was determined by reacting a small, known amount of protein with a known excess amount of *repE* operator DNA. The fractional amount of operator bound was used to calculate the fractional amount of active RepE. RepE produced from pREPE5 cultures was about 70% active; in the pET expression system, activity ranged from 95 to 100%. Binding and assay conditions were as described previously (17) except that in some cases *E. coli* chromosomal DNA (0.1 mg/ml final concentration) was present in the assay tube. While not shown here, control experiments were done with slightly larger F sequences that produced more than one labeled fragment upon restriction. Only the fragments containing the *Oe* or *oriS* target were observed to shift in response to RepE exposure. Also, the binding could be competed appropriately with unlabeled DNA containing *Oe* or *oriS* sites.

Incompatibility assays. BK342 (F'*lac*$^+$) was transformed with pET Ap^r plasmids expressing the N-terminally truncated RepE described in the Results section. Aliquots of transformed cells were plated on MacConkey lactose agar (Difco) supplemented with 25 μg of ampicillin per ml, and incompatibility was scored as elimination of the Lac$^+$ phenotype.

RESULTS

RepE purification: identification of three forms. Initially, we produced RepE protein from the pREPE5 clone by the procedures of Masson and Ray (16). From 8.0 liters of culture, we recovered about 1 to 2 mg of purified soluble protein. One-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the pREPE5-encoded RepE displayed a broad band when stained with Coomassie blue. Subsequent nonequilibrium, two-dimensional gel electrophoresis indicated that this RepE preparation contained primarily two species in roughly equivalent amounts (data not shown). This latter analysis was scaled up, and the two forms were extracted from the gel for N-terminal analysis. The results indicated that the larger species was deleted for the first 11 N-terminal residues and the smaller species was deleted for the first 17 residues (Fig. 2). These altered forms of RepE must represent posttranslational processing, because subsequent DNA sequence analysis of the pREPE5 clone showed that the full *repE* gene was present (data not shown).

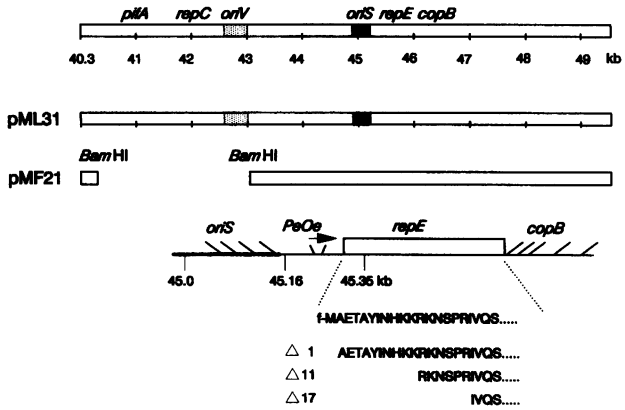

FIG. 2. Maps of mini-F plasmids and RepE protein sequences. The f5 *Eco*RI restriction fragment of plasmid F is 9.5 kb starting from coordinate 40.3 kb. Plasmid pML31 and its *Bam*HI deletion derivative pMF21 also contain a 7.5-kb *Eco*RI fragment with *Tn*903 (Km^r). The iterons in *oriS* and *copB* (diagonal lines) are 19 to 22 bp in length. The *repE* operator, *Oe*, is an inverted repeat whose sequence is the first 8 bp of the *ori*/*cop* iterons. *Pe* represents the *repE* promoter. The *repE* (45.25 to 46.0 kb) gene encodes a 251-aminoacyl protein. References for these facts have been summarized (8, 9, 12).

In a successful attempt to produce full-length RepE, we cloned *repE*$^+$ next to a phage T7 promoter in the pET expression plasmid described by Studier et al. (30). The recombinant was designated pBK857 (Table 1). RepE made from the T7 system was largely insoluble. We used this insolubility to advantage for an initial purification by low-speed centrifugation, then denatured the granules with guanidine-HCl, and reconstituted 95 to 100% of the protein to an active form which was purified to homogeneity as described in Materials and Methods. We routinely recovered 1 to 2 mg of RepE from a 250-ml culture. Purified RepE was analyzed by two-dimensional gel electrophoresis and revealed only one spot (data not shown). Aminoacyl sequencing showed that this RepE started with residue number 2 of the known sequence. In other words, just the *N*-formyl methionine of RepE was removed. The different forms are designated Δ1-RepE, Δ11-RepE, and Δ17-RepE to conform with the observed processing.

Detection of processed RepE produced in F$^+$ bacteria. *E. coli* CSH50 containing mini-F pML31 was cultured in L broth and sampled in the log and stationary phases of growth. Harvested cells were immediately lysed in boiling detergent to prevent proteolysis. Subsequently, the lysates were frozen and later analyzed by Western immunoblotting with a polyclonal anti-RepE. The protein load per lane was the maximum permissible within the limits of the technique. The results in Fig. 3 show that no protein the size of Δ17-RepE is detectable in log-phase cells, whereas roughly equal amounts of protein of the sizes of Δ1- and Δ17-RepE are present in stationary-phase cells. The smaller, putative RepE is identified conclusively as the Δ17 form first by its size and reactivity with polyclonal anti-RepE (Fig. 3), second by its weak and identical reactivity with a monoclonal

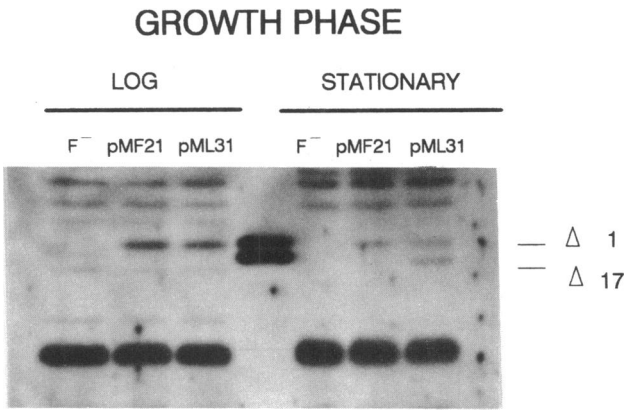

FIG. 3. Characterization of RepE protein forms in crude cell lysates produced from mini-F$^+$ bacteria. Bacteria containing pML31 or pMF21 were cultured in L broth and lysed with boiling SDS detergent as described in the Materials and Methods section. An amount of culture equal to 0.8 OD_{600} units was loaded per lane for log-phase cultures, and half of this amount was loaded for stationary-phase cultures. Lane 4, purified Δ1- and Δ17-RepE standards, 15 ng of each. Since the level of RepE from plasmids with a wild-type copy number is low, Lumi-Phos 530 (Lumigen, Inc.), a chemiluminescent substrate, was used to detect mouse anti-rabbit IgG antibody conjugated to alkaline phosphatase.

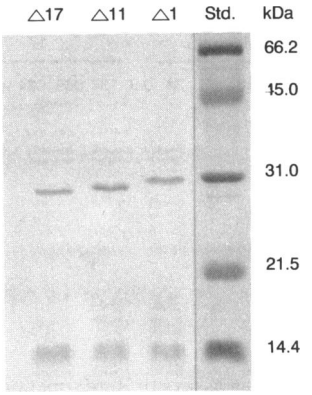

FIG. 4. Leaky expression of RepE forms in CSH50. The pET::*repE* plasmids producing the indicated truncated form of RepE were transferred into CSH50 by transformation and expression of the RepE forms monitored by Western analysis (34) as described in the text. RepE was stained with mouse monoclonal anti-RepE antibody and detected by using goat anti-mouse IgG conjugated with alkaline phosphatase and the BCIP-NBT chromogenic substrate. The positions of molecular mass standards (Std.) are indicated.

anti-RepE that binds very strongly to Δ1-RepE but weakly to authentic Δ17, and finally by its complete lack of reactivity with a monoclonal that binds Δ1-RepE somewhere between residues 2 and 11 (data not shown). These results indicate that processing occurs naturally and is not just a consequence of excessive RepE overproduction with the pREPE5 expression vector.

Plasmid pMF21 (Fig. 2), a deletion derivative of pML31 lacking most of *pifA* and *oriV* and lacking all of *repC*, was also examined for production of different RepE forms. The results in Fig. 3 show that pMF21 did not produce detectable amounts of Δ17-RepE in either log- or stationary-phase cells. However, the question of whether Δ17-RepE is present in log-phase cells is not definitively settled by these results because of technical limitations in immunoblotting, as related in the Discussion.

In vivo functional analysis of RepE forms. The T7 expression system is a tripartite one, containing a lambda lysogen with phage 21 immunity and a cloned T7 RNA polymerase gene under control of the *lacUV5* promoter. The second component is a recombinant plasmid with a T7 promoter next to a cloning site (pET). The third component is a compatible plasmid with the T7 lysozyme gene (pLysS). The T7 lysozyme neutralizes the leaky expression of T7 RNA polymerase. To test the three different forms of RepE for initiator function, we first cloned wild-type *repE* or *repE* sequences deleted for codons 2 through 11 or 2 through 17 into a pET plasmid next to the T7 promoter. In strain CSH50 (pLysS$^-$), which does not make T7 RNA polymerase, these recombinant plasmids produced significant levels of RepE due to readthrough transcription from a vector promoter (Fig. 4). After we established the existence of leaky expression of the *repE* genes encoding different forms, we transformed bacterial hosts containing one of these recombinants with circular F *oriS*-Cm^r DNA. This DNA cannot be replicated unless functional RepE is present in *trans*. Hosts producing Δ1- or Δ11-RepE could be transformed with equal and high efficiency (10^5 transformants per μg of *oriS* DNA), but the host producing Δ17-RepE could not be transformed at all.

To exclude the possibility that Δ17-RepE is a superinitiator causing such a large replication of F *oriS*-Cm^r DNA that it is toxic, we transformed cells with a pBR322 recombinant containing the F *oriS* locus (pBK207) (Table 1). The F sequences in pBK207 are identical to those in the F *oriS*-Cm^r DNA circles. We found greater than 10^5 transformants per μg of pBK207 DNA with CSH50 recipients which were producing the Δ17-RepE. This result would not be expected if Δ17 caused overinitiation of *oriS* in pBK207.

A heteroplasmid system was also constructed to see whether RepE autorepressor function was lost by site-specific proteolytic processing. The heteroplasmid constructs are shown in Table 2 along with typical results. These data show that all three forms reduce expression from a *repE* promoter/operator-*lacZ* translational fusion by approximately 60%. It is known from published work (29) that the Δ1 form is capable of about 90% repression in a similar (but not identical) type of heteroplasmid system. Clearly, our system is only qualitative and not optimal; hence, we can only conclude that processing to the Δ17 form does not destroy autorepressor function.

DNA-binding properties of RepE forms. The binding properties of RepE forms were examined in a gel retardation assay as described in Materials and Methods and the legend to Fig. 5. RepE protein is cationic and binds to eucaryotic and procaryotic carrier DNA when one of these is present in the binding buffer (data not shown). When no carrier was present, we measured a dissociation constant (K_d) for the Δ1 form of 3.0 (±0.6) × 10^{-10} M (Table 3), in agreement with previous findings (17). For the Δ17 form, we measured a value of 2.0 (±0.75) × 10^{-10} M. We repeated these measurements in the presence of *E. coli* chromosomal DNA, since this is more reflective of RepE's natural environment. To our surprise, we found that the K_d for the Δ1 form was increased over 50-fold but the K_d for the Δ17 form increased only 7.5-fold. The net result is that in the presence of a chromosome, the Δ17 form is 10-fold more effective in binding operator than is the Δ1 form.

The Δ17 form is also more effective in binding to *oriS*

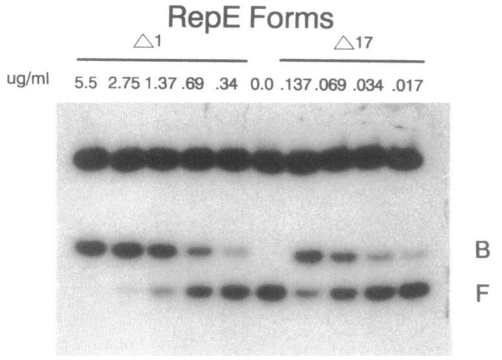

FIG. 5. Gel retardation assays of RepE operator bound to different RepE forms. RepE protein produced from pBK857 (Δ1) or pBK820 (Δ17) was mixed at the indicated concentrations with restricted, ^{32}P-end-labeled pBK633 (operator) DNA (5×10^{-10} M) and analyzed by electrophoresis in a 5% polyacrylamide gel as described in the text. *E. coli* chromosomal DNA (0.1 mg/ml) was present in the binding reaction mix. The slowest band is the plasmid vector; B and F, bound and free DNAs, respectively. Band intensities in assays of this type were determined with an electronic scanner (Ambis) and not from the autoradiogram. One microgram of RepE per ml is 1.7×10^{-8} M for the dimer form.

target sequences as well (Fig. 6). A mathematical analysis like that used for *repE* operator is not applicable to *oriS* because the latter forms multiple species of complexes with RepE. Therefore, we chose to simply depict the differences in the protein affinities by plotting total *oriS* DNA bound as a function of RepE concentration (Fig. 6B). This representation shows that at comparable concentrations, the Δ17 binds significantly more DNA than does the Δ1 form. Given the results in Fig. 5 and 6, we predicted that when supplied in *trans* to normal F, the Δ17 form should inhibit plasmid replication, that is, should cause plasmid incompatibility.

Incompatibility analysis of different RepE forms. Plasmid incompatibility is the inability of two plasmids to coexist in a host in the absence of selective pressure. RepE-mediated incompatibility was tested by transforming plasmid recombinants of pET (Ap^r) encoding the different forms of *repE* into CSH50 (F'*lac*) *recA* and selecting for ampicillin-resistant colonies on MacConkey lactose agar. The results in Table 4 show that only the Δ17 form of RepE caused loss of F'*lac*. The Δ1 and Δ11 forms were not expected to eliminate F'*lac* because they are replication-competent forms of RepE. The failure of the various mutant forms to cause incompatibility is not due to poor protein expression, because each of the various recombinants has a comparable leaky expression of the cloned *repE* gene (Fig. 4; other data not shown).

TABLE 3. Dissociation constants for RepE forms[a]

RepE form	K_d (M) with carrier DNA:	
	None or poly(dI-dC) · poly(dI-dC)	*E. coli*
Δ1	$3.0 (\pm 0.6) \times 10^{-10}$ (6)	$1.76 (\pm 0.23) \times 10^{-8}$ (4)
Δ17	$2.0 (\pm 0.75) \times 10^{-10}$ (4)	$1.5 (\pm 0.22) \times 10^{-9}$ (3)

[a] Defined as molarity of RepE dimers that gave 50% binding of operator DNA fragments. Values are averages ± standard deviations; the number in parentheses represents the number of assays.

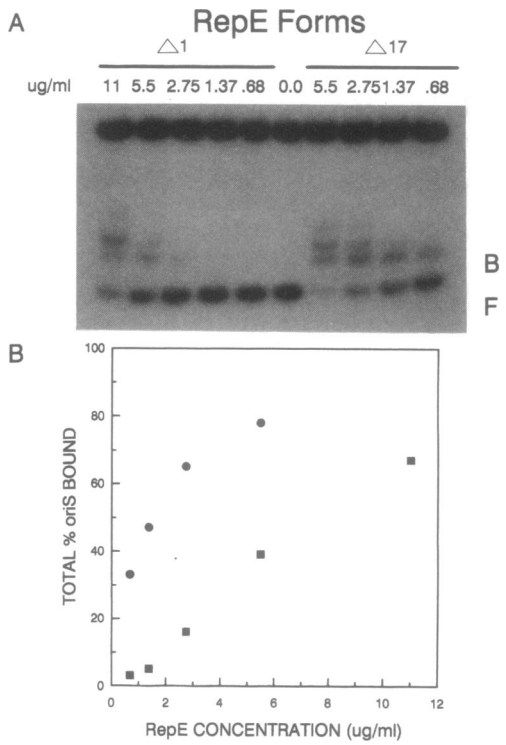

FIG. 6. Affinity of Δ1 and Δ17 RepE forms for *oriS* DNA. Binding assays were done as described in the text and the legend to Fig. 5. (A) Autoradiographic results. (B) Plot of the results in panel A. Total % *oriS* bound is calculated as bound/(bound + free DNA). ●, Δ17-RepE; ■, Δ1-RepE.

DISCUSSION

F has long been considered a model system for control of replication because its copy number is on a parity with that of host chromosome. Its stature as a model has been enhanced by the recent clarification that timing of F replication in the cell cycle is unique (7), a property that previously had only been observed with the host chromosome. Analysis of the natural history of the F RepE protein should provide insight into the control of DNA replication for this model system.

In this study, three forms of F RepE protein have been identified, purified to homogeneity, and characterized. Two of the forms, Δ1- and Δ17-RepE, can be found in crude cell extracts after SDS-PAGE analysis and can therefore be considered natural states of the protein. The third form, Δ11-RepE, has only been found after extensive purification

TABLE 4. Incompatibility between F'*lac* and truncated forms of RepE

Incoming plasmid *repE* gene form[a]	Phenotype distribution[b]
Δ1	100% Lac$^+$
Δ11	100% Lac$^+$
Δ17	5% Lac$^+$, 15% Lac$^\pm$, 80% Lac$^-$

[a] The incoming plasmid was a derivative of pET containing the *repE* gene genetically altered by the indicated N-terminal deletion.

[b] For each transformation, over 1,000 colonies were examined. In the Δ17 group, the Lac$^\pm$ designation refers to sectored colonies. Sectoring likely represents incompatibility events occurring during colony development.

by column chromatographic procedures and likely represents a product of in vitro proteolysis.

The functional and biochemical changes caused by processing RepE to the Δ17 form indicate that the modified protein is suited as a negative regulatory element. In the presence of *E. coli* DNA, Δ17-RepE binds with greater affinity to both *oriS* and *repE* operator than does Δ1-RepE. In vivo, the Δ17 form is functionally inactive as an initiator. These results implied that Δ17-RepE could potentially function as an inhibitor in competition with the Δ1 form. To test this prediction, we set up an incompatibility assay in which Δ17 protein was supplied in *trans* and showed that an F'*lac* plasmid could not be maintained. Incompatibility could have occurred because of Δ17-RepE competing with Δ1-RepE for *oriS* or by the Δ17 form more actively repressing the F'*lac* *repE*$^+$ gene. Since we observed that Δ17-RepE forms dimers, incompatibility could also have occurred through formation of heterodimers with the Δ1 form. Our results do not allow us to choose or exclude any of these possibilities.

Our incompatibility results represent the second report of plasmid elimination by using a "piece" of a Rep protein. The first example is with the plasmid R6K system (4), as discussed below. These examples mimic the dominant negative effects with antisense RNA currently in vogue as a form of intracellular immunity except that protein is involved. As a practical example, one might use this approach to prevent or reduce transformation by a tumor virus whose replication is dependent on an autorepressor/initiator protein, for example, simian virus 40 (6, 23).

Similar observations of negative initiation control not related to autorepression associated with Rep proteins or *rep* genes have been made with other plasmid systems. Overexpression of replicon-specific *rep* genes causes inhibition of P1 (19), R6K (4), and Rts1 (31) replication. For R6K, the amino-terminal third of the molecule acts as the inhibitor. With Rts1, the Rep protein is assumed to be the inhibitor. For P1, the nature of the inhibitor is uncertain but seems to be either a portion of mRNA or the Rep protein.

Multiple forms of an autorepressor/initiator protein are not unprecedented. The DnaA protein used for initiation at the *E. coli* chromosomal origin, *oriC*, binds ATP, ADP, and acidic phospholipids but is only active as an initiator when bound to ATP (26, 27). Little is known about the distribution of the various DnaA forms or their functions.

We note that our original model for control of replication involved irreversible processing of RepE. One processed form bound to *oriS* and *copB*, while the other bound to just the *repE* operator. The model was so constructed that processing and binding to the first two loci did not upset the RepE equilibrium needed to establish autoregulation of *repE* expression. In the present work, we see that all forms bind to all target sites examined. It is not immediately clear whether the model can be modified to handle these observations or should be discarded.

Any future model involving Δ17-RepE must account for its relative binding to F DNA target sites compared with the Δ1 form. The results in this article show that (i) there is little, if any, difference between the two proteins in the absence of competitor DNA (Table 3) and (ii) there is potentially a very significant difference in the presence of *E. coli* chromosome (Table 3; Fig. 5 and 6). At present, the true magnitude of the difference is not known, but it must be at least 10-fold with respect to the *repE* operator (Table 3). Further binding studies are in progress to clarify this issue.

The unexpected results seen in Table 3 with *E. coli* chromosomal DNA on binding of Δ1- and Δ17-RepE to the operator and *oriS* deserve further comment. If the chromosomal DNA contained cryptic F, it should have affected the binding of both RepE forms equally. Southern analysis indicated that the *E. coli* chromosomal DNA we used did not react with an F *repE* probe (data not shown). Both the Δ1- and Δ17-RepE forms had about the same affinity for *repE* operator in the presence of herring sperm DNA (data not shown), suggesting that both forms have the same nonspecific DNA-binding constant. Given these observations, a simple interpretation is that *E. coli* DNA contains a cryptic Δ1-RepE binding site not recognized by the Δ17 form. Even so, we cannot eliminate the possibility that nonspecific protein-DNA interactions are at the root of our observations in Table 3. Consistent with this possibility is the fact that the 16 amino acids lost from Δ17 are very basic. Clearly, further work is needed to understand these results.

An unresolved issue is whether Δ17-RepE exists at significant levels in log-phase cultures. The fact that pMF21 and pML31 have the same copy number (28) and are stably inherited seems at odds with the possibility that Δ17-RepE is regulatory, since pMF21 apparently does not make truncated protein (Fig. 3). Conceivably, though, both plasmids make this form in small but effective amounts in log-phase cells, while only pML31 makes a large amount in stationary-phase cells. The protein loaded in Fig. 3 represents the maximum amounts allowable without destroying resolution and was taken from unfractionated, detergent-lysed cells. This protocol was used to preclude unwanted proteolysis. Unfortunately, the total RepE in log-phase F$^+$ cells is so low that immunoblotting will not detect Δ17-RepE if it represents only 5% or so of the total.

When RepE was purified by our chromatographic technique from 4-liter log-phase cultures containing either pMF21 or pML31 mini-F plasmid, 9 and 11%, respectively, of the total RepE was found in the Δ17 form. A reconstruction experiment in which purified Δ1-RepE was seeded into a lysate made from an F$^-$ culture showed that during purification, proteolysis converted 5% of it to the Δ17 form. This conversion occurred despite the presence of several protease inhibitors. These results are consistent with the existence of Δ17-RepE in log-phase cultures, but they are not definitive. Toward this end, construction of a genetically engineered mini-F with a RepE protein that cannot be proteolysed should be informative.

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