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Mini-F Plasmid Mutants Able To Replicate in the Absence of σ^{32}: Mutations in the *repE* Coding Region Producing Hyperactive Initiator Protein

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Mini-F plasmids cannot replicate in *Escherichia coli* strains (Δ*rpoH*) lacking σ^{32}, presumably because transcription of the *repE* gene encoding the replication initiator protein (RepE protein) depends mostly on RNA polymerase containing σ^{32}. We have isolated and characterized mini-F mutants able to replicate in Δ*rpoH* cells. Contrary to the initial expectation, five mutants with mutations in the *repE* coding region that produce altered RepE proteins were obtained. The mutations caused replacement of a single amino acid: the 92nd glutamic acid was replaced by lysine (*repE10*, *repE16*, and *repE25*) or glycine (*repE22*) or the 109th glutamic acid was replaced by lysine (*repE26*). These plasmids overproduced RepE protein and exhibited very high copy numbers. Two major activities of mutated RepE proteins have been determined in vivo; the autogenous repressor activity was significantly reduced, whereas the initiator activity was much enhanced in all mutants. These results indicate the importance of a small central region of RepE protein for both initiator and repressor activities. Thus the decreased *repE* transcription in Δ*rpoH* cells can be compensated for by an increased initiator activity and a decreased repressor activity of RepE, resulting in the increased synthesis of hyperactive RepE protein.

The replication initiator protein (RepE protein) of mini-F plasmid plays an essential and specific role in initiating replication from the origin, *ori2* (19, 30, 35). It has a molecular mass of 29 kDa and binds to the repeated sequence of 19 bp within the *ori2* region (21, 29). The RepE protein is also intimately involved in regulating plasmid copy number; the cellular concentration of RepE appears to determine the plasmid copy number, presumably by determining the frequency of replication initiation at *ori2*. The active role of RepE protein in the regulation of mini-F replication has been implied by several lines of evidence. First, structural alteration of RepE by a *cop* mutation affects plasmid copy number (3, 12, 26). Second, the *incC* region, containing 19-bp repeated sequences similar to those found at *ori2*, exhibits a modest affinity to RepE protein (29) and modulates plasmid copy number (26, 31). Third, the synthesis of RepE protein is autogenously regulated at the transcription level, with RepE serving as a repressor (14, 28, 34); a structural change in RepE protein (3) or the operator region that binds the repressor (25) can affect repression.

We have recently shown that transcription of *repE* encoding the RepE protein is mediated mostly by RNA polymerase containing σ^{32} (34), a minor σ factor involved in the heat shock response (10). The major promoter responsible for *repE* transcription actually has a sequence similar to that of the heat shock promoter for *rpoD* (34). In addition, *repE* transcription directed by RNA polymerase-σ^{32} was shown to be specifically repressed by RepE protein in the in vitro system employing purified RNA polymerase (34). In accordance with these results, mini-F cannot normally replicate in *rpoH* mutants and fails to transform *Escherichia coli* cells lacking σ^{32} (17).

As one approach to further examining the functional role and physiological significance of σ^{32}-mediated *repE* transcription in mini-F replication, we have isolated plasmid mutants able to replicate in the absence of σ^{32}. Contrary to our initial expectation, a number of mutations were found within the *repE* coding region rather than the *repE* promoter region. The mutations have been localized in the small central region of RepE protein, and the mutated proteins exhibit decreased activities in autogenous repression and increased activities in initiating replication at *ori2*. Consistent with these findings, a minor promoter apparently recognized by σ^{70} was found upstream of the major *repE* promoter (see Discussion).

MATERIALS AND METHODS

Bacterial strains and phage. All bacterial strains used are derivatives of *E. coli* K-12. Strains MC4100 [F⁻ *araD* Δ(*argF-lac*)*U169 rpsL relA flbB deoC ptsF rbsR*] (5), KY1603 [MC4100 Δ*rpoH30*::*kan zhf50*::Tn*10 suhX401* (λpF13-P*rpoD*$_{hs}$-*lacZ*)] (18), and KD1087 [F⁻ *mutD5* Δ(*tonB-trpAB*) *leu argE his spcA*] (7) have been described previously. λpF13-P*repE-lacZ* was constructed by inserting the *repE* promoter region (*AluI-SmaI* segment containing only the major promoter) into promoter-cloning vector pMS434 (13), and the resulting *repE-lacZ* operon fusion was transferred to λpF13 vector by in vivo recombination according to the procedure described previously (13).

Mini-F plasmids. Mini-F and related plasmids used are listed in Table 1. The parental mini-F plasmid pKV511 (33) used for isolation of mutants had been derived from pPB038, which was supposed to contain a Tn*3* insertion between *repE* and *incC* (2). This insertion was actually found to be within the *repE* coding region (therefore it was designated *repE317*; see Results). pKV5110 carrying the *repE* gene with the normal C-terminal sequence was constructed by inserting a synthetic double-strand DNA fragment (35 bp) into the *Eco*RV site of pKV511 (Fig. 1). Each of the *repE* mutations (*repE10*, *repE16*, *repE22*, *repE25*, and *repE26*) was then

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TABLE 1. Plasmids used

Plasmid	Relevant genotype	Reference or source
Mini-F and its derivatives		
pKV511	*ori2 repE317 bla*	33
pKV516	pKV511 *repE10*	This work
pKV518	pKV511 *repE22*	This work
pKV520	pKV511 *repE26*	This work
pKV5110	*ori2 repE*$^+$ *bla*	This work
pKV5111	pKV5110 *repE10*	This work
pKV5113	pKV5110 *repE22*	This work
pKV5115	pKV5110 *repE26*	This work
pKV718	*ori2 bla*	This work
pBK63	pMF45 *cop-44*	20
pBK80	pMF45 *cop-50*	20
ColE1-type plasmids		
pHY2-5	*ori*(pMB1) P*trp-repE*$^+$ *cat*	T. Tokino
pRPG16	*ori*(pMB1) *trpR*	11
pKV709	*ori*(pMB1) P*trp-repE317 cat*	This work
pKV7090	*ori*(pMB1) P*trp-repE*$^+$ *cat*	This work
pKV711	pKV7090 Δ*repE*	This work
pFO-B	*ori*(pMB1) *ori2 bla*	24
pKV719	*ori*(pMB1) P*trp-repE317 trpR cat*	This work
pKV7190	pKV719 P*trp-repE*$^+$	This work
pKV7191	pKV7190 P*trp-repE10*	This work
pKV7193	pKV7190 P*trp-repE22*	This work
pKV7195	pKV7190 P*trp-repE26*	This work
pMS434	*ori*(ColE1) *bla*	13
pBR322	*ori*(pMB1) *bla*	4
pACYC184	*ori*(P15A) *cat*	6

transferred to pKV5110 by replacing the *Sma*I-*Eco*RV fragment with that from each mutant plasmid. pBK63 and pBK80 carrying a *cop* (increased copy number) mutation were kindly supplied by B. Kline. A defective plasmid, pKV718, carrying mini-F *ori2* that can replicate only when RepE protein is supplied in trans, was constructed from pFO-B, a pBR322-based plasmid carrying the *Xho*I-*Hin*fI segment of mini-F (Fig. 1A) and the *bla* gene (encoding β-lactamase). pFO-B DNA was digested with *Apa*LI, and the 4.1-kb fragment containing *ori2* and *bla* was circularized to obtain pKV718.

Multicopy plasmids carrying the mini-F *repE* gene. The various ColE1-type multicopy plasmids used are listed in Table 1. pHY2-5 is a pBR322-based plasmid carrying the *repE* coding region fused to the *trp* promoter and the λ*cro* Shine-Dalgarno sequence (gift of T. Tokino); it was derived from pTA14 (29). To remove *incC* from this plasmid, the *Sma*I-*Pvu*I segment was replaced by that of pKV511, yielding pKV709 (Fig. 2). The *trpR* gene cut out from pRPG16 was then inserted into the *Bam*HI site of pKV709 to yield pKV719. In strains carrying pKV719 or any of its derivatives, *repE* transcription can be regulated by the *trp* repressor and intracellular concentration of tryptophan. The *Pst*I fragment of pKV719 was replaced by that from pKV5110 to yield pKV7190 carrying *repE* with the normal C-terminal sequence. To obtain plasmids carrying a *repE* mutation in similar constructs (pKV7191, pKV7193, and pKV7195), the *Sma*I-*Eco*RV segment of pKV7190 was replaced by the equivalent segment from the respective *repE* mutant plasmid.

Media and chemicals. The L broth (33) and medium E (32) used in most experiments have been described elsewhere. To manipulate the level of *repE* transcription from the *trp* promoter, medium E supplemented with 0.5% Casamino Acids (Difco), 0.5% glucose, and various concentrations of L-tryptophan was used. Solid media contained 1.2% agar, and ampicillin (50 μg/ml) or chloramphenicol (20 μg/ml) was used for selection of drug-resistant transformants.

Determination of β-galactosidase activity. Samples of a culture (0.5 ml) were mixed with 0.5 ml of Z buffer in an ice bath; cells were disrupted with chloroform and sodium dodecyl sulfate (SDS) and assayed for β-galactosidase essentially as described previously (22).

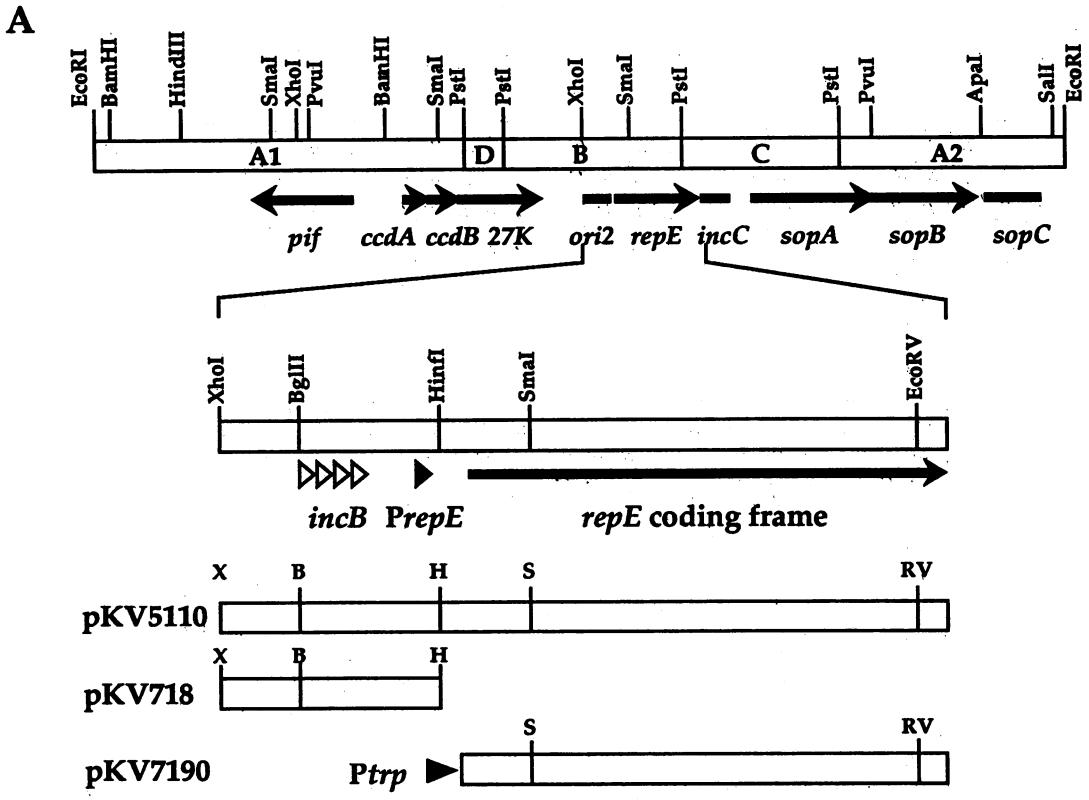
Immunoblotting of RepE protein. Cells were treated with 5% trichloroacetic acid, washed in acetone, and heated in SDS-sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto Immobilon PVDF transfer membrane (Millipore) as described previously (27). RepE protein was detected by treatment with specific rabbit antiserum (gift of K. Matsubara), anti-rabbit immunoglobulin biotinylated antibody, and horseradish peroxidase-streptavidin (Amersham, Buckinghamshire, United Kingdom). Staining was done by a Konica Immunostaining HRP-kit (Konica Co., Tokyo, Japan).

Mutagenesis of mini-F plasmid. *E. coli mutD* strains produce an altered epsilon subunit of DNA polymerase III and are defective in the proofreading step of DNA synthesis, resulting in mutations at a very high frequency (7). Such a *mutD* strain (KD1087) carrying pKV511 was used as a source of plasmid DNA in the isolation of mini-F mutants.

RESULTS

Isolation of mini-F *repE* mutants able to replicate in strain lacking σ^{32}. Mini-F plasmid cannot replicate in *rpoH* mutants, specifically in the Δ*rpoH* mutant completely lacking σ^{32}. Since the latter mutant grows only at or below 20°C (36), a temperature-resistant revertant (KY1603) of the Δ*rpoH* mutant that can grow at temperatures up to 40°C by virtue of excess GroE proteins (18) was used as host bacterium in the isolation of plasmid mutants. pKV511 was used as the parental mini-F plasmid because of its simple structure, consisting essentially of *ori2*, *repE*, and *bla* (Fig. 1A). It was first shown that pKV511 DNA cannot transform KY1603 cells to ampicillin resistance at 30°C. However, transformants appeared at a low frequency (about 10^{-7} of that for the *rpoH*$^+$ host) with mutagenized plasmid DNA. Plasmids recovered from these colonies could transform the Δ*rpoH* strain (KY1603) at high frequencies. Thus, about 20 independent mutant plasmids able to replicate in the absence of σ^{32} were isolated. Each mutation has been localized to a specific mini-F segment by exchanging segments between mutant and parental plasmids and then testing the resulting plasmids for their abilities to replicate on KY1603 cells. These experiments permitted us to classify the mutant plasmids into two groups; one group contained a mutation in the *Sma*I-*Eco*RV segment (Fig. 1A), thus within the *repE* coding region, and the other group contained a mutation in the *Eco*RV-*Pvu*I segment (Fig. 2). The former group was analyzed in this study.

Nucleotide sequence analysis of *repE* mutations. The *Sma*I-*Eco*RV fragment from each mutant and the parental plasmid (pKV511) was ligated to pUC118, and the nucleotide sequences in both directions were determined by the standard chain termination method. All five mutations were found to contain single nucleotide substitutions: three mutations (*repE10*, *repE16*, and *repE25*) altered the 92nd glutamic acid (GAA) to lysine (AAA), one mutation (*repE22*) altered the



B

pKV5110 and pKV7190 (*repE*)

EcoRV HpaI

CGCGATATCACTTCCATGACGACAGGATAGTCGTTAAC

ArgAspIleThrSerMetThrThrGly***

pKV511 and pKV719 (*repE317*)

EcoRV Tn3

CGCGATATCACTTCCATGAGGGGTCTGACGCTCAGTGGA-

ArgAspIleThrSerMetArgGlyLeuThrLeuSerGly-

ACGAAAACTCACGTTAAGGGATTTTGGTCATGA

ThrLysThrHisValLysGlyPheTrpSer***

FIG. 1. (A) Physical map of mini-F and mini-F portions of composite plasmids used. The mini-F f5 segment (40.3 to 49.4 kb of the F coordinate map) is shown at the top. Arrows indicate the locations and orientations of known genes, and filled bars represent sites or regions. Filled arrowheads indicate promoters, while open arrowheads show the 19-bp repeated sequence at the *ori2* (*incB*) region. In addition to the DNA segments shown, pKV5110 and pKV718 carry an ampicillin resistance gene (*bla*). See Fig. 2 for the structure of pKV719. X, *Xho*I; B, *Bgl*II; H, *Hin*fI; S, *Sma*I; RV, *Eco*RV. (B) Nucleotide and predicted amino acid sequences of C-terminal portions of wild-type *repE* and *repE317*. *repE317* represents a Tn*3* insertion as indicated, resulting in the production of a RepE protein with altered C-terminal structure. See Materials and Methods for the construction of pKV5110 and pKV7190.

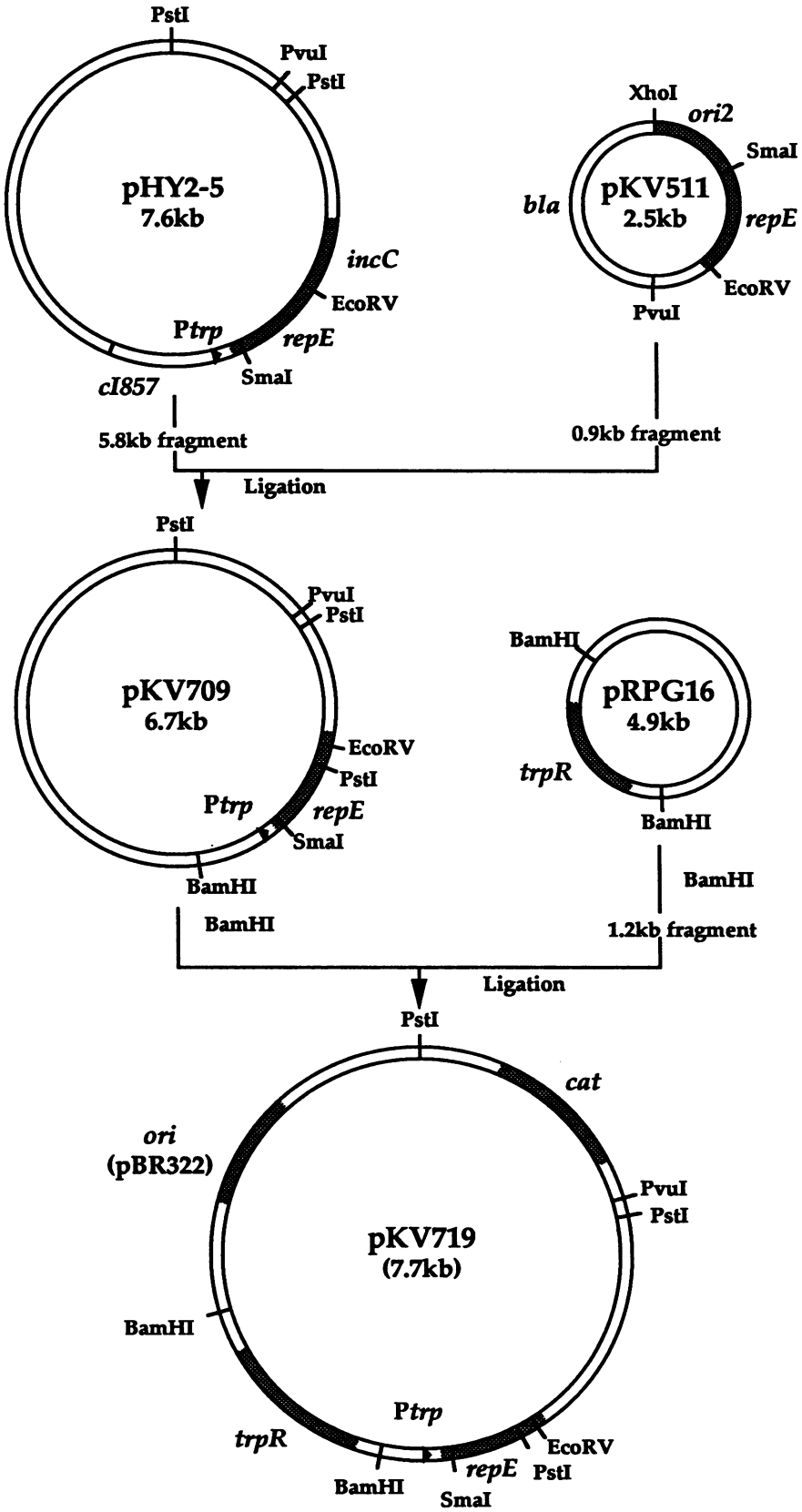


FIG. 2. Construction of multicopy plasmid pKV719 carrying *repE*, whose expression is regulated by the *trp* promoter-operator and *trpR* repressor. See Materials and Methods for details of procedures, including those for construction of derivative plasmids.

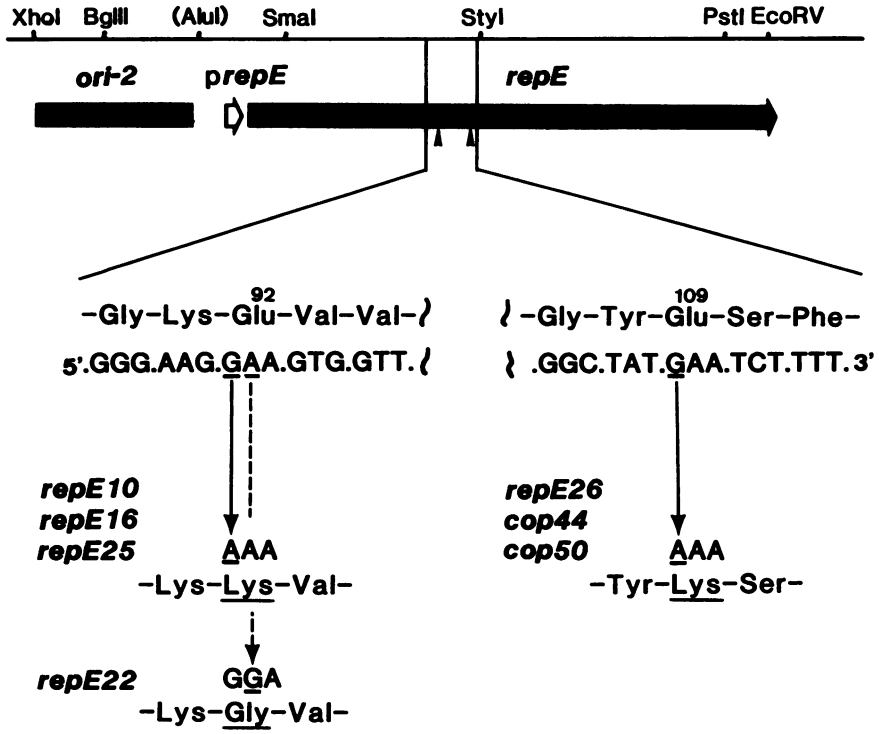


FIG. 3. Alterations of nucleotide and predicted amino acid sequences in the mini-F *repE* mutants. Amino acid numbers are based on the finding that the N-terminal methionine is processed and is not found in the mature RepE protein (29).

same glutamic acid (GAA) to glycine (GGA), and another mutation (*repE26*) altered the 109th glutamic acid (GAA) to lysine (AAA) (Fig. 3). Thus, three representative mutant plasmids, pKV516, pKV518, and pKV520, carrying *repE10*, *repE22*, and *repE26*, respectively, were used for subsequent analyses.

In the course of sequencing the mutant plasmids, it was noted that the C (carboxy)-terminal region of *repE* in all plasmids used differed from the published sequence (23) of wild-type *repE*. Evidently, Tn*3* had been inserted into the C-terminal region of *repE* during the isolation of pPB038 plasmid (2). Three C-terminal amino acids (threonine, threonine, and glycine) had been replaced by an unnatural stretch of 17 amino acids (Fig. 1B); therefore the alteration was designated *repE317*. In view of this unexpected finding, we have constructed a set of wild-type and mutant mini-F plasmids carrying *repE* with the normal C-terminal structure by the procedure described in Materials and Methods. The latter series of plasmids are designated pKV5110, pKV5111, pKV5113, and pKV5115, carrying *repE*$^+$, *repE10*, *repE22*, and *repE26*, respectively.

Stability and copy number of *repE* mutants. As expected from the selection procedure employed, all the mutant plasmids originally isolated (thus carrying *repE317* as well) showed high stability in the Δ*rpoH* strain. In contrast, no stable transformant was obtained when the *repE10*, *repE16*, or *repE25* mutant plasmid was introduced into *rpoH*$^+$ cells. In the set of mutants in which the C-terminal lesion (*repE317*) of RepE has been repaired, stability varied depending on the mutation: *repE10* and *repE26* were relatively stable in the Δ*rpoH* strain but much less stable in the *rpoH*$^+$ strain, whereas *repE22* was stable in both *rpoH*$^+$ and Δ*rpoH* strains (Table 2).

To determine the copy numbers of the mutant plasmids, they were transformed into the *rpoH*$^+$ strain harboring a compatible multicopy plasmid, pACYC184, as an internal reference (the copy number of pKV5110 was not affected by pACYC184). Unexpectedly, the *repE10* plasmid (pKV5111) that failed to transform MC4100 (*rpoH*$^+$) was able to transform, at a normal frequency, the same strain carrying pACYC184. This *repE10* plasmid apparently exhibited an extremely high copy number in MC4100 (*rpoH*$^+$) carrying pACYC184, although frequent loss of plasmid precluded an accurate estimation (Table 3). pKV516 carrying both *repE10* and *repE317* mutations similarly exhibited a high copy number in the *rpoH*$^+$ strain. The *repE22* plasmid (pKV5113)

TABLE 2. Stability of plasmids at 30°C[a]

Plasmid	% of cells harboring plasmid	
	MC4100 (*rpoH*$^+$)	KY1603 (Δ*rpoH*)
pKV5110 *repE*$^+$	>99.8	NT
pKV5111 *repE10*	<0.1	87
pKV5113 *repE22*	>99.5	80
pKV5115 *repE26*	5.0	88
pKV511 *repE317*	>99.8	NT
pKV516 *repE10 repE317*	<0.2	>99.8
pKV518 *repE22 repE317*	99	86
pKV520 *repE26 repE317*	>99.5	76
pBR322	>99.8	>99.8

[a] Cultures grown in a selective medium (with ampicillin) were used to inoculate L broth at 10^3 cells per ml and shaken overnight at 30°C. Samples were diluted and plated on L agar, and colonies that appeared after 1 to 2 days were replica plated onto L agar containing ampicillin to score plasmid-harboring cells. NT, Not tested.

TABLE 3. Copy numbers of mini-F plasmids relative to that of pACYC184

Plasmid	Copy number in [a]:	
	MC4100(*rpoH*$^+$)	KY1603(Δ*rpoH*)
pKV5110 *repE*$^+$	0.36 (1.0)	NT
pKV5111 *repE10*	2.80[b] (7.8)	1.48 (4.1)
pKV5113 *repE22*	1.43 (4.0)	0.86 (2.4)
pKV5115 *repE26*	2.74[b] (7.6)	1.78 (4.9)
pKV511 *repE317*	0.50 (1.4)	NT
pKV516 *repE10 repE317*	2.73 (7.6)	1.72 (4.8)
pKV518 *repE22 repE317*	1.73 (4.8)	0.29 (0.8)
pKV520 *repE26 repE317*	1.59 (4.4)	0.40 (1.1)

[a] Cells harboring both mini-F and pACYC184 plasmids were grown overnight in L broth containing ampicillin and chloramphenicol. Plasmid DNAs were prepared, digested with *Eco*RI and *Bgl*II, and examined by agarose (1%) gel electrophoresis. DNA bands were scanned by a densitometer (Biomed Instruments Inc.; model SLR 2D/D), and the density of mini-F DNA was normalized to that of pACYC184 DNA and corrected for the size difference. Averages of at least five experiments are presented. Values in parentheses are those relative to the *repE*$^+$ plasmid (pKV5110), 5 to 10 copies of which are found per chromosome in MC4100. NT, Not tested.

[b] These values have been corrected for plasmid-free segregants (40 to 60%) that appeared in the cultures. Such an instability means that the actual copy numbers may be much higher than indicated.

showed a moderately high copy number (three- to fourfold of *repE*$^+$ plasmid copy number), whereas the *repE26* plasmid (pKV5115) showed a very high copy number in the *rpoH*$^+$ strain, as did the *repE10* plasmid. The *repE317* lesion itself caused a small but significant increase (ca. 1.5-fold) in the copy number.

Hyperproduction of altered RepE proteins. The increased copy numbers observed may be due to an increased amount or activity (or both) of RepE protein due to mutations. Accordingly, the levels of RepE protein produced in cells carrying the wild-type or mutant plasmid were determined semiquantitatively by immunoblotting with specific antiserum against RepE protein (Fig. 4). It is evident that all *repE* mutants tested (except *repE317*) produced appreciably higher amounts of RepE protein than did control plasmid pKV5110 (lane 2). The RepE protein levels for *repE10* and *repE26* plasmids in Δ*rpoH* cells (lanes 7 and 9) were particularly high (at least four- to fivefold that of *repE*$^+$ plasmid in *rpoH*$^+$ cells). The apparent difference in RepE26 levels between *rpoH*$^+$ and Δ*rpoH* cells (lanes 5 and 9) probably reflects the difference in plasmid stability (Table 2). The electrophoretic mobility of RepE317 protein was significantly slower than that of RepE$^+$, in agreement with the altered molecular size as predicted from the nucleotide sequence (lanes 2 and 3).

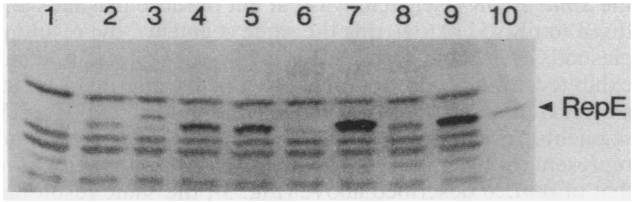
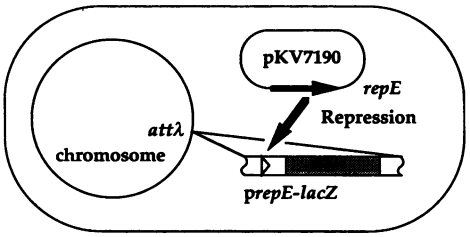


FIG. 4. Immunoblotting of RepE protein. Log-phase cultures (0.5 ml; A_{600} = 0.5) of MC4100 (lanes 1 through 5) or KY1603 (lanes 6 through 9) carrying each plasmid were treated with acid, washed in acetone, and heated in 40 μl of buffer. Samples (15 μl) were subjected to 12% SDS-gel electrophoresis and transferred to a membrane for Western blotting (immunoblotting). Lanes: 1, no plasmid; 2, pKV5110 (*repE*$^+$); 3, pKV511 (*repE317*); 4, pKV5113 (*repE22*); 5, pKV5115 (*repE26*); 6, no plasmid; 7, pKV5111 (*repE10*); 8, pKV5113 (*repE22*); 9, pKV5115 (*repE26*); 10, 0.4 μg of purified wild-type RepE protein.

A. Repressor Activity



B. Initiator Activity

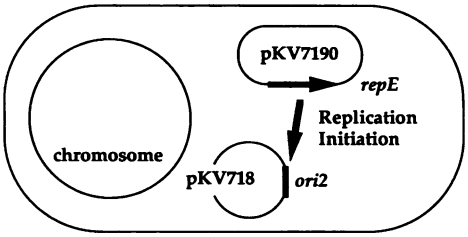


FIG. 5. Strategies for assaying repressor and initiator activities of RepE protein in vivo. (A) Repressor activity was determined by measuring *lacZ* expression mediated by the *repE* promoter (P*repE*) in the presence of various amounts of RepE protein produced by pKV7190 (or its derivative). (B) Initiator activity was determined by measuring the copy number of the *ori2* plasmid (pKV718) in the presence of various amounts of RepE protein produced by pKV7190 (or its derivative).

Repressor activity of altered RepE proteins. To determine the repressor activity of the mutant RepE proteins in vivo, a set of Δ*lac* strains was prepared that were lysogenic for λpF13-P*repE-lacZ* and carrying pKV7190 (or a derivative thereof) in which the *repE* gene (wild type or mutant) was transcribed from the *trp* promoter (Fig. 5A). In these strains, *repE* transcription from the λ prophage genome can be monitored by measuring β-galactosidase activity in the presence of specific RepE protein, whose amounts can be varied by varying the tryptophan concentration in the medium. The dependence of the RepE protein level (produced from pKV7190) on tryptophan concentration was verified by immunoblotting experiments; as tryptophan concentration increased, RepE synthesis decreased (data not shown). The levels for all mutated RepE proteins have been determined and shown not to differ significantly from one another or from that for the wild type (within ±20%), indicating that none of the *repE* mutations affected the stability of RepE protein appreciably.

When the wild-type RepE protein was provided by pKV7190 in the maximum amount (without the addition of tryptophan), β-galactosidase activity decreased to about 8% of that of the control cell carrying the vector alone (no RepE protein produced) (Fig. 6). When RepE protein synthesis was repressed by excess L-tryptophan (50 μg/ml), β-galactosidase activity increased (ca. threefold) to 27% of the

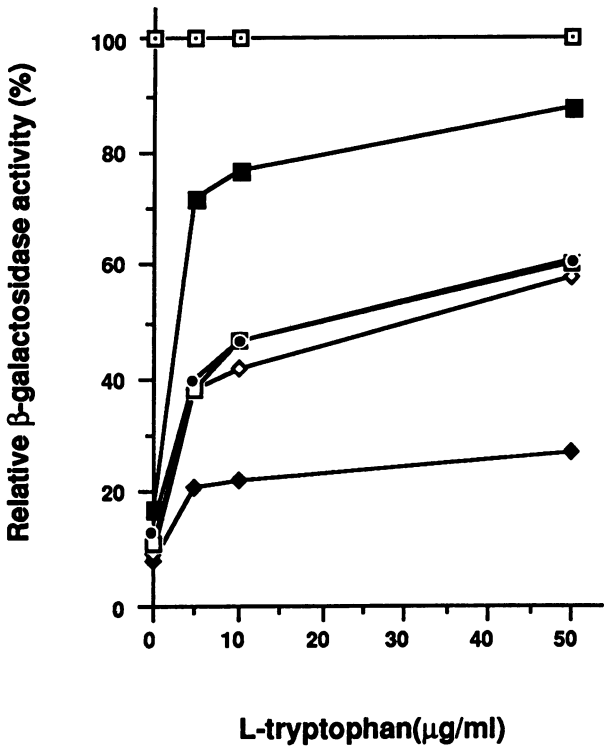


FIG. 6. Relative repressor activities of RepE proteins. Cells of MC4100 lysogenic for λpF13-P*repE-lacZ* and harboring pKV7190 (or its derivative) were grown to log phase in medium E-Casamino Acids containing glucose, chloramphenicol (20 μg/ml), and L-tryptophan at the concentrations indicated and were assayed for β-galactosidase activity. The values obtained were normalized to that for cells harboring a control plasmid [pKV711 (Δ*repE*)] grown without tryptophan. The 100% value was 220 Miller units (22). Symbols: ⊡, no RepE protein; ◆, RepE$^+$; ■, RepE317; ◇, RepE10; ●, RepE22; □, RepE26.

control activity. When the mutant RepE protein was similarly provided by the pKV7190-derivative plasmid carrying *repE10*, *repE22*, or *repE26*, β-galactosidase activities were significantly higher than those with the wild-type RepE protein for any of the protein levels (tryptophan concentrations) examined. These results clearly indicate that all the *repE* mutations tested impaired the RepE repressor function significantly. In addition, the RepE protein with the C-terminal lesion (*repE317*) was found to be much less active as a repressor than the wild-type or other mutant proteins examined.

Initiator activity of mutant RepE proteins. To determine the initiator activity of mutant RepE proteins as a function independent of repressor activity, we measured the copy number of a defective mini-F *ori2* plasmid (pKV718) whose replication depends quantitatively on the supply of RepE protein from another plasmid (pKV7190 or its derivative) in the wild-type cell (Fig. 5B). Again, the amount of RepE protein was manipulated by varying the tryptophan concentration in the medium.

When wild-type RepE protein was supplied by pKV7190, the copy number of *ori2* plasmid increased in parallel with the amount of RepE protein produced; the lower the tryptophan concentration, the higher the amount of RepE protein and thus the higher the copy number (Table 4). The RepE317 protein showed a slightly reduced initiator activity

TABLE 4. Relative initiator activity deduced from the copy number of the *ori2* plasmid (pKV718)[a]

RepE protein produced	Copy no. with L-tryptophan at (μg/ml):			
	0	5	10	50
Wild type	5.3	4.1	2.1	0.54
RepE317	3.5	3.0	1.5	0.25
RepE10	—[b]	—	12.3[c]	1.8
RepE22	9.0	6.7	3.9	0.85
RepE26	—	16.1[c]	11.6	1.7

[a] MC4100 cells harboring both pKV718 and pKV7190 (or its derivative that produces the RepE protein as indicated) were grown in medium E-Casamino Acids containing glucose, ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), and L-tryptophan. Log-phase cells were harvested, and plasmid DNAs were prepared, digested with *Eco*RI, and subjected to agarose gel electrophoresis. pKV718 DNA bands were quantitated by densitometer tracing as described in Table 3, footnote *a*, and copy numbers relative to that of pKV7190 (internal reference) are presented. The copy number of pKV7190 (or its derivatives) was unaffected by the *repE* mutant alleles carried therein.

[b] —, No stable transformants were obtained because of severe inhibition of host cell growth.

[c] These values have been corrected for plasmid-free segregants in the cultures used.

as judged by the present assay system. In contrast, much smaller amounts of RepE10 protein proved quite active in supporting *ori2* plasmid replication, indicating that this protein has a very high initiator activity. Cell growth was normal when excess tryptophan was present, but it was severely inhibited when tryptophan was low or absent, indicating that large amounts of RepE10 protein are deleterious to the cell. It should be noted that such a growth inhibition was observed only when cells contained the *ori2* plasmid. These results probably explain our failure to obtain stable transformants by the *repE10* plasmid in *rpoH*$^+$ cells (Table 2). In contrast, the RepE22 protein containing a different amino acid at the same position (residue 92) exhibited a moderately high initiator activity with no inhibitory effect on cell growth. The RepE26 protein with lysine instead of glutamic acid at residue 109 exhibited a very high initiator activity; large amounts of RepE26 protein were also inhibitory to the cell, and the cultures tended to accumulate plasmid-free cells, though the inhibition was much less severe than with the RepE10 protein.

Examination of previously isolated *cop* mutants. Among the several known *cop* mutations affecting *repE*, two plasmids that exhibit copy numbers about 10 times as high as that of the wild type (pBK63 and pBK80 carrying the *cop-44* and *cop-50* mutations, respectively) (20) were tested and found to be able to replicate in the Δ*rpoH* strain (KY1603). When the *Sma*I-*Eco*RV segment was cut out of these plasmids and fused to pKV5110 (deleting the same segment), the resulting plasmids were able to replicate in the Δ*rpoH* strain. They exhibited a high copy number, as did the original mutant plasmids (data not shown). Sequencing of the *Sma*I-*Eco*RV segments revealed that both *cop-44* and *cop-50* mutations represent a single base change identical to each other and to that of *repE26* described above (Fig. 3); the same result has been obtained by B. Kline (personal communication). The same nucleotide alteration has also been reported for one of the mutations associated with the *cop3* mutant (3).

DISCUSSION

All the mini-F *repE* mutants characterized in this study showed increased copy numbers and RepE protein levels due to a single amino acid change within the narrow region of

the RepE initiator protein. The altered RepE proteins exhibited decreased autogenous repressor activities, explaining in part the increased RepE protein level and plasmid copy number. They also showed markedly increased initiator activities for *ori2* replication, which should contribute to the high plasmid copy numbers. These results indicate that the marked decrease in *repE* transcription caused by the lack of σ^{32} allowed us to select for mutant plasmids that can constitutively produce hyperactive RepE proteins. The results also imply that *repE* transcription occurs at a fair efficiency even in the absence of σ^{32}. In fact, a new promoter transcribed by RNA polymerase-σ^{70} has been identified upstream of the major *repE* promoter recognized by σ^{32} (32a). Transcription from this minor promoter, which can also be repressed by RepE protein, becomes more pronounced in the absence of transcription from the downstream *repE* promoter.

Previous studies on the *repE* mutants affected in autogenous repression suggested that the C-terminal region of RepE protein is involved in repression of *repE* transcription (3). The present result with *repE317* is in good agreement with this proposition. However, the RepE protein level and copy number observed are not as high as those which might have been expected from the marked decrease in repressor activity (Fig. 4 and Table 3), perhaps because the initiator activity of RepE317 protein is also decreased (Table 4). All the mutations (*repE10*, *repE22*, and *repE26*) affecting the initiator activity simultaneously affect the repressor activity significantly. This suggests that the region of RepE protein (residues 92 to 109) defined by these mutations interacts with the carboxy-terminal region (affected by *repE317*) in exerting its repressor function.

Among the mutations affecting the DNA initiator activity, *repE26* represents a typical *cop* mutation; three *cop* mutations (*cop-3*, *cop-44*, and *cop-50*) previously isolated in high-copy-number mutants actually contained a mutation identical to *repE26* (3; Fig. 3). This clearly indicates the importance of this and adjacent residues in RepE protein for initiator activity in mini-F replication. In contrast, mutants bearing the three recurrent mutations (*repE10*, *repE16*, and *repE25*) have been isolated presumably because of the selection procedure employed, i.e., on the basis of their abilities to replicate in Δ*rpoH* cells. The inability of the latter group of plasmids to yield stable transformants with *rpoH*$^+$ bacteria is probably related to the extremely high initiator activity of RepE protein produced (Table 4). The highly excessive DNA initiation at *ori2* may titrate out one or more essential host proteins, such as those required for chromosomal-DNA replication, and consequently inhibit cell growth. The question of why the *repE10* plasmid can transform the same *rpoH*$^+$ strain when it carries pACYC184 remains to be investigated.

Two other *repE* mutants with an increased copy number have previously been reported to contain a single amino acid replacement in RepE protein at a region very close to that affected by the mutations we are discussing: glutamic acid to lysine (*copA1*) or histidine to tyrosine (*copA2*) at the 100th or 129th residue, respectively (12). It has not been reported whether these mutations affect the repressor or initiator activity of RepE protein. Most, if not all, of the high-copy-number *repE* mutations thus far reported therefore affect a nearby region (between the 92nd and 129th residues) of the RepE protein, emphasizing the importance of this region in the control of plasmid copy number. Furthermore, the same amino acid change (glutamic acid to lysine) in three neighboring residues (92, 100, and 109) appear to have similar drastic effects on plasmid copy number, suggesting that this region is directly involved by its interaction with DNA (*ori2*) or other proteins in exerting the initiator and/or repressor activity. High-copy-number mutations affecting the replication initiator protein have been isolated from several other plasmids that are similar to mini-F in structure and function of replication origin regions (1, 9, 15, 16). The sites of these *cop* mutations also tend to form a cluster at the central region of the respective protein, suggesting that a basically similar mode of Rep protein function is operating in these plasmids.

It has recently been shown that mini-F replication requires certain heat shock proteins (DnaK, DnaJ, and GrpE) whose syntheses are markedly enhanced upon temperature upshift. Mutants deficient in any one of these proteins are unable to be transformed by mini-F DNA (8, 17). Unlike wild-type mini-F plasmids such as pKV5110, the *repE* mutants isolated in this study (*repE10*, *repE22*, and *repE26*) are able to transform the heat shock protein mutants at normal efficiencies (17; unpublished result). Moreover, wild-type mini-F plasmids can transform heat shock mutants if the mutants already carry another compatible plasmid that can supply excess RepE protein in trans. These results taken together indicate that the set of heat shock proteins somehow assists the functioning of RepE protein in the initiation of mini-F replication (17). Analysis of the interplay between the RepE protein and host cell proteins including heat shock proteins should prove important for further understanding of mini-F replication and its regulation.

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