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Mini-F Plasmid Mutants Able To Replicate in *Escherichia coli* Deficient in the DnaJ Heat Shock Protein

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A subset of *Escherichia coli* heat shock proteins, DnaJ, DnaK, and GrpE, is required for mini-F plasmid replication, presumably at the step of functioning of the RepE initiator protein. We have isolated and characterized mini-F plasmid mutants that acquired the ability to replicate in the *Escherichia coli dnaJ259*. The mutant plasmids were found to replicate in any of *dnaJ*, *dnaK*, and *grpE* mutant hosts tested. In each case, the majority of the mutant plasmids carried a unique amino acid alteration in a localized region of *repE* coding sequence and showed an increased copy number, whereas the minority contained a common single base change (C to T) in the promoter/operator region and produced an increased amount of RepE. All RepE proteins with altered residues (between 92 and 134) exhibited increased initiator activities (hyperactive), and many showed reduced repressor activities as well, indicating that this region is important for the both major functions of RepE protein. These results together with evidence reported elsewhere indicate that the subset of heat shock proteins serves to activate RepE protein prior to or during its binding to the replication origin and that the mutant RepE proteins are active even in their absence. We also found that a C-terminal lesion (*repE602*) reduces the initiator activity particularly of some hyperactive mutant RepE proteins but does not affect the repressor activity. This finding suggests a functional interaction between the central and C-terminal regions of RepE in carrying out the initiator function.

The mini-F plasmid, derived from the F (fertility) factor, replicates as a low-copy-number plasmid (one to two copies per chromosome) in *Escherichia coli*. It contains a set of genes required for its characteristic mode of replication and partition, including the origin of replication (*ori2*), the *repE* gene encoding the initiator protein, the incompatibility gene *incC*, and the partition genes *sop* (*par*) (15). The minimum mini-F plasmid that consists of *ori2* and *repE* can stably replicate in *E. coli* but with high copy numbers (10 to 15 per chromosome) as a result of the lack of *incC*, which negatively regulates the copy number (13).

The *ori2* (*incB*) region contains four direct repeat sequences of 19 bp (21), the A+T-rich region, and binding sites for DnaA protein, which is required for replication of the mini-F plasmid (9, 16, 20) as well as the host chromosome (26). Similar sets of DNA sites have been found near the replication origins of several other plasmids and the bacterial chromosome (3) and appear to play fundamental roles in the initiation of DNA replication.

The RepE initiator protein binds to the *ori2* direct repeats and the *incC* repeated sequence that are highly homologous to each other. It also binds to the promoter/operator region of *repE* (19, 25), which contains an inverted repeat structure whose half sequence (10 bp) is similar to a part of the *ori2* and *incC* direct repeats (8-bp matches) (21). Binding of RepE protein to these DNA sites is essential for the initiation of replication, control of plasmid copy number, and autogenous repression of *repE* transcription (15). Thus, RepE protein is directly involved in initiation and control of DNA replication and in repression of its own synthesis at the transcriptional level.

We have previously shown that transcription of the *repE* gene is mediated primarily by a sigma factor (σ^{32}) which is specifically required for the heat shock response (27, 28). Furthermore, a subset of heat shock proteins (DnaK, DnaJ, and GrpE) was found to be required for replication of the mini-F plasmid (6, 12) like that of λ phage (7) or the mini-P1 plasmid (24). These heat shock proteins have been shown to play specific and synergistic roles in the initiation of replication for both λ and the mini-P1 plasmid. In mini-P1 plasmid replication, DnaK and DnaJ seem to activate the specific DNA binding function of the RepA initiator protein (31, 32). The DnaJ heat shock protein of *E. coli* is a basic protein of 41 kDa found mostly in the membrane fraction (7) and interacts directly with RepA by forming a stable binary complex (30). These results suggested that DnaJ might play an important, and possibly central, role in the control of mini-F plasmid replication by interacting with the RepE initiator protein.

To further examine the roles of heat shock proteins in mini-F plasmid replication, we have isolated and characterized mini-F plasmid mutants that acquired the ability to replicate in a *dnaJ*-defective host. It will be shown that these mutations affect either the central region of the *repE* coding sequence, resulting in the production of RepE with a hyperactive initiator activity, or the *repE* promoter/operator region, resulting in overproduction of RepE protein.

MATERIALS AND METHODS

Bacterial strains and phages. All bacterial strains used are *E. coli* K-12 and are listed in Table 1. MC4100 and derivatives carrying a mutation in a heat shock protein gene were used in most experiments. Strains KY1453, KY1454, KY1455, and KY1456 were constructed by introducing *dnaJ259*, *grpE280*, *dnaK204*, or *dnaJ*::Tn*10*-42 from KY1457, DA16, CAG13350, or CG992, respectively, by transduction with phage P1*vir*, selecting for tetracycline resistance (Tn*10*), and screening for temperature-sensitive

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strains		
MC4100	F⁻ *araD* Δ(*argF-lac*)*U169 rpsL relA flbB deoC ptsF rbsR*	4
MT852	RB85T *dnaJ259 thr*⁺ *thy*⁺	29
KY1453	MC4100 *dnaJ259 car-96*::Tn*10*	This work
KY1454	MC4100 *grpE280 pheA*::Tn*10*	This work
KY1455	MC4100 *dnaK204 thr*::Tn*10*	This work
KY1456	MC4100 *dnaJ*::Tn*10*-42	This work
KY1457	MT852 *car-96*::Tn*10*	This work
KY1603	MC4100 Δ*rpoH30*::*kan zhf-50*::Tn*10 suhX401* (λpF13-P*rpoD-lacZ*)	17
NRK156	MC4100 *dnaK756 thr-34*::Tn*10*	12
DA16	DA15 *grpE280*	2
CAG13350	MC1061 *dnaK204 thr*::Tn*10*	33
KD1087	F⁻ *mutD5* Δ(*tonB-trpA,B*) *leu argE his spcA*	5
CG992	B178 *dnaJ*::Tn*10*-42	23
MA194	AT2358 *mutT1*	1
Plasmids		
pKV5110	*ori2 repE*⁺ *bla*	13
pKV531	pKV5110 *repE602*	This work
pKV711	pKV7090 P*trp*-Δ*repE*	13
pKV718	*ori2 bla*	13
pKV7190	*ori*(pMB1) *trpR cat* P*trp-repE*⁺	13
pKV739	pKV7190 *repE602*	This work
pJE3021	40.3–49.3 F *copA1*	11
pKV740	pKV511 *ori*(pMB1) *repE*Δ*C57*	This work

growth. A temperature-resistant revertant (KY1603) of the Δ*rpoH* mutant lacking σ^{32} (17) and phage λP*repE-lacZ*, used for monitoring *repE* transcription (13), were described previously.

Mini-F plasmids. Mini-F and related plasmids used are listed in Table 1. Construction of pKV5110 was described previously (13). pKV531 was identical with pKV5110 except that it contained a frameshift mutation (*repE602*) at the C-terminal end of *repE* (Fig. 1B). Mini-F plasmid mutations were isolated from pKV531 (see below) and were separated from *repE602* by removing the *Sma*I-*Eco*RV (635 bp) or *Sty*I (647 bp) fragment from the original mutant plasmids and introducing them into pKV5110 by replacing the equivalent segment of the latter plasmid. The *copA1* mutation of pJE3021 (11) was also introduced into pKV5110 by the same procedure. pKV740 was a mini-F–*ori*(pMB1) composite plasmid that produced RepE protein lacking the C-terminal 57 amino acids (an amber codon was inserted to terminate translation). Structures of all *repE* mutant plasmids constructed were confirmed by nucleotide sequencing.

Plasmids used for *repE* expression controlled by the *trp* promoter. The ColE1-type multicopy plasmid pKV7190 used for production of RepE protein has been described previously (13). pKV739 was identical with pKV7190 except for a frameshift mutation (*repE602*) at the C-terminal end of *repE* (Fig. 1B). They both carried *repE* under the control of the *trp* promoter/operator and repressor, and the amount of RepE protein produced was manipulated by changing the tryptophan concentration in the medium. Plasmids that produce the mutant forms of RepE were constructed by replacing the *Sma*I-*Eco*RV fragment of pKV7190 or pKV739 by that from each of the *repE* mutant plasmids. The structures of plasmids constructed were confirmed by using appropriate restriction enzymes and by nucleotide sequencing.

Media. L broth and medium E have been described elsewhere (13). Medium E supplemented with 0.5% Casamino Acids, 0.5% glucose, and various concentrations of L-tryptophan was used to adjust the level of *repE* transcription from the *trp* promoter. Agar plates contained 1.2% agar. Ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), and tetracycline (12.5 μg/ml) were used for maintenance as well as for selection of drug-resistant transformants or transductants.

Mutagenesis of mini-F plasmids. The parental mini-F plasmid was introduced into strain KD1087 or MA194 carrying the mutator *mutD5* or *mutT1*, respectively, and plasmid DNA was prepared from a number of independent cultures to ensure the independence of mutants obtained. Alternatively, the parental plasmid DNA was treated with 0.4 M hydroxylamine at 37°C for 26 h under conditions described previously (10).

Isolation of mini-F mutants that can replicate in a *dnaJ*-defective host. When MT852 (*dnaJ259*) was transformed by mini-F plasmid pKV5110 DNA with selection for ampicillin resistance at 30°C, no stable transformants but numerous pseudotransformants that prohibited isolation of potential mutants appeared. This problem was overcome by using a similar plasmid (pKV531) with, in addition, a frameshift mutation (*repE602*) at the C-terminal end of *repE*; pKV531 replicated in the wild-type (MC4100) but not in the *dnaJ*-defective host, with no pseudotransformants being found (presumably because of reduced initiator activity; see below). The plasmid copy number was 8 to 10 per host chromosome, which is slightly lower than in pKV5110. Mutagenized plasmid DNAs were introduced into MT852 (*dnaJ259*), selecting for ampicillin-resistant transformants at 30°C. Plasmid DNAs were extracted from the transformants, and those that could transform MT852 at high efficiencies were kept for further study.

Initiator and repressor activities of RepE protein. The initiator and autogenous repressor activities of RepE protein were determined as previously described (13).

Other methods. Recombinant DNA experiments and nucleotide sequencing were carried out by standard procedures (18). The methods for determination of cellular levels of

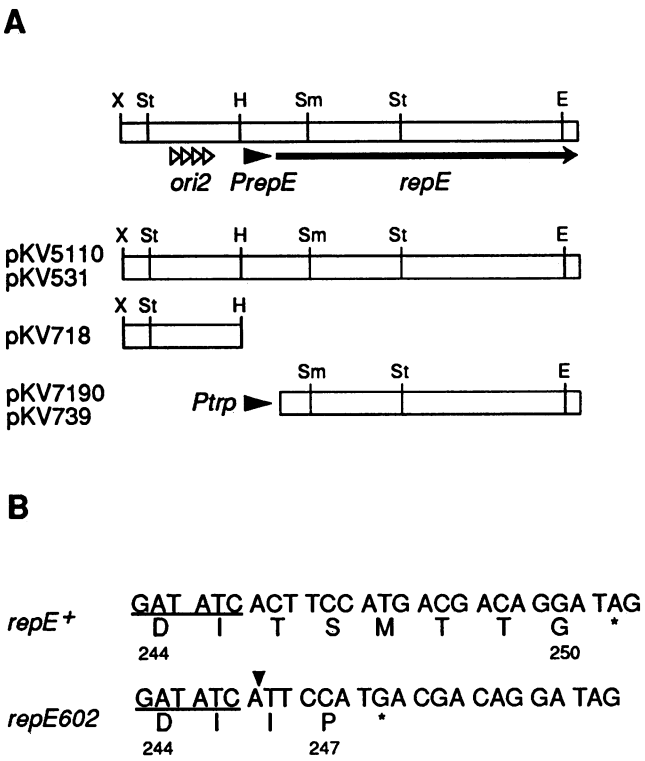

FIG. 1. Physical map of mini-F plasmids and the mini-F plasmid portion carried by the chimera plasmids. (A) The mini-F plasmid *ori2* and the *repE* regions are indicated at the top. Filled arrowheads indicate promoters, and open arrowheads indicate the 19-bp repeated sequences. In addition to the DNA segments shown, all mini-F plasmids used (pKV5110, pKV531, and pKV718) carried the ampicillin resistance *bla* gene. pKV7190 and pKV739 are derivatives of pBR322 carrying the mini-F *repE* gene controlled by the *trp* promoter and repressor and the chloramphenicol resistance gene (13). Restriction sites: X, *Xho*I; St, *Sty*I; H, *Hin*fI; Sm, *Sma*I; E, *Eco*RV. (B) Nucleotide and predicted amino acid sequences of the C-terminal end of wild-type *repE* and of the frameshift mutant *repE602*, caused by a deletion of one base as indicated by the arrowhead. The *Eco*RV site is underlined.

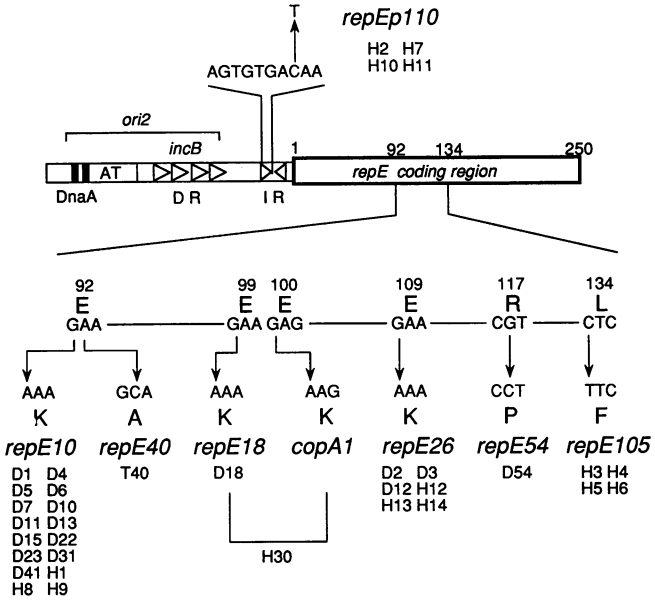

FIG. 2. Alterations in nucleotide and predicted amino acid sequences in *repE* mutants. The mini-F plasmids used (pKV531 and pKV5110) are shown with mutations in the promoter/operator (above) or in the coding region (below). All mutants obtained (unique or recurrent) are indicated below the representative ones referred to in the text. Mutants obtained by using *mutD* or *mutT* bacteria or by treatment with hydroxylamine are indicated as D, T, or H, respectively, followed by numbers. DnaA, DnaA binding region; AT, A+T-rich region; DR, direct repeat; IR, inverted repeat.

RepE protein and for assaying β-galactosidase were essentially as described previously (13).

RESULTS

Isolation of mini-F mutants capable of replicating in a *dnaJ*-defective host. Mini-F plasmids cannot replicate in a bacterial host carrying either the *dnaK*, *dnaJ*, or *grpE* mutation at 30°C, which permits normal cell growth (12). A number of independent mutant plasmids that can replicate in the *dnaJ259* strain MT852 were obtained. Mini-F plasmid pKV531, carrying a *repE602* frameshift mutation (Fig. 1B), was used as the parental plasmid for technical reasons (see Materials and Methods). To localize the mutations on the plasmid DNA, each of the mutant plasmids was digested with appropriate restriction enzymes. The resulting fragments were then substituted for the equivalent fragment of the parental plasmid, and the resulting recombinants were tested for the ability to replicate in the *dnaJ*-defective host. Thirty mutations were found to be localized within the *Sma*I-*Eco*RV segment (635 bp), thus affecting the *repE* coding region (Fig. 1A). Four mutations were found in the *Sty*I-*Sma*I segment (407 bp) containing the replication origin (*ori2*) and the 5′ portion of *repE*.

The alteration(s) in nucleotide sequence was then determined. Among the 30 mutations in the *Sma*I-*Eco*RV segment, 22 were found to carry a base substitution causing a replacement of glutamic acid at residue 92 or 109. They are identical to the *repE10* or *repE26* mutation, respectively, both of which were previously isolated as mutants capable of replicating in a σ^{32}-deficient host (13) (Fig. 2). Seven other mutants contained single base substitutions that had not been reported: mutations affecting residue 92 (*repE40*), 99 (*repE18*), or 117 (*repE54*) and four recurrent mutations (*repE105*) affecting residue 134. Finally, there was a mutant containing two base changes, one identical with *repE18* and the other identical with *copA1*, a high-copy-number mutation characterized previously (11) (Fig. 2). Since a plasmid with the original *copA1* mutation alone, when introduced into pKV531, was found to replicate in the *dnaJ*-defective host, this plasmid instead of the double mutant was used for the further study. In addition to these *repE* mutations with amino acid substitutions, the four mutations localized to the *Sty*I-*Sma*I fragment were all shown to carry the same base change (C to T; designated *repEp110*) at the −10 region of the *repE* promoter/operator, identical with that reported previously as deficient in autogenous repression (22).

Replication of mutant plasmids in other heat shock-related mutant bacteria. We tested whether the mutant plasmids obtained can replicate in bacterial hosts defective in the other heat shock proteins (DnaK and GrpE) or the heat shock sigma factor σ^{32}. For this and subsequent experiments, each plasmid mutation was separated from the *repE602* lesion present in the parental plasmid by replacing

TABLE 2. Efficiencies of transformation by mini-F mutants[a]

Strain	Mini-F plasmids carrying *repE* allele:									
	+	*10*	*18*	*26*	*105*	*copA1*	*p110*	*602*	*40–602*	*54–602*
MC4100 wild type	1.0	0.6	0.8	0.3	0.9	0.8	1.0	0.9	0.9	1.0
KY1453 *dnaJ259*	(0.1)	0.2	0.5	0.6	0.7	0.4	0.4	10^{-5}	0.6	0.4
KY1456 *dnaJ*::Tn*10*	(0.2)	0.5	0.5	0.4	0.9	0.6	0.8	10^{-4}	0.4	0.5
KY1455 *dnaK204*	10^{-4}	0.5	0.4	0.2	0.4	0.3	0.3	10^{-4}	0.3	1.0
NRK156 *dnaK756*	(0.1)	0.4	0.7	0.4	0.6	0.6	0.3	10^{-5}	0.8	1.1
KY1454 *grpE280*	(10^{-2})	0.1	0.3	0.8	0.7	0.7	0.3	10^{-4}	0.3	0.6
KY1603 Δ*rpoH30*	10^{-3}	0.3	10^{-4}	0.3	0.5	10^{-2}[b]	0.1[b]	10^{-5}	10^{-4}	0.8

[a] Mini-F plasmids used were pKV5110 with *repE*$^+$ or its derivatives, each carrying a mutant *repE* allele as indicated. Frequencies of transformants with mini-F plasmids at 30°C, relative to those with pBR322, were normalized to that with the wild-type host. Values in parentheses refer to pseudotransformants.
[b] Small colonies appeared at reduced frequencies, indicating that these mutant plasmids can replicate in the Δ*rpoH* host but with reduced efficiencies.

appropriate DNA fragments; where this separation failed (*repE40* and *repE54*), however, the original mutants containing *repE602* were used. The resulting plasmids containing single *repE* mutations were introduced into the isogenic set of mutant bacteria, and ampicillin-resistant transformants were selected at 30°C. In contrast to the parental plasmid pKV531 (*repE602*) or pKV5110 (*repE*$^+$), the mutant plasmids were all capable of transforming the *dnaK*- or *grpE*-defective host, though the apparent efficiencies varied according to the specific plasmid or bacterial host used (Table 2). Furthermore, strain KY1456 carrying a Tn*10* insertion at the proximal region of *dnaJ* supported replication of these mutant plasmids, suggesting that the complete lack of DnaJ function can be tolerated by these mutant plasmids.

On the contrary, a bacterial strain lacking σ^{32} (Δ*rpoH*) permitted replication of only some mutant plasmids (*repE54-repE602* and *repE105*) besides *repE10* and *repE26*, as shown previously (13), and barely supported replication of other mutants (*copA1* or *repEp110*) (Table 2). In addition, mutant plasmids carrying *repE18* or *repE40-repE602* were virtually unable to replicate in the Δ*rpoH* host. The reason for the latter finding is perhaps that transcription of *repE* or supply of heat shock proteins other than DnaK, DnaJ, or GrpE, or both, are reduced to the levels that are insufficient for replication.

The copy number of mutant plasmids. To examine the effects of mutations on the regulation of plasmid replication, the plasmid copy number was determined both in the wild type and in the *dnaJ259* strain (KY1453) at 30°C. The mutant plasmids used above were introduced into a strain harboring the compatible plasmid pACYC184, which served as an internal reference in estimating the copy number (Table 3). Thus, the mutations *repE10* and *repE26* were found to give very high copy numbers in the *dnaJ259* strain as well as in the wild-type host. The *copA1* plasmid also exhibited a high copy number, particularly in the *dnaJ*-defective host. Most other mutants gave slightly lower copy numbers in the *dnaJ* host than in the wild-type host, presumably because of some indirect effects of the *dnaJ* mutation. Paradoxically, the plasmids with high copy numbers showed marked instability, probably because of strong inhibitory effects exerted on growth of the host cell (13). In contrast, the copy number of the *repE105* plasmid was only slightly higher, and that of the *repE18* plasmid was essentially the same as that of the parental mini-F plasmid. The *repE54-repE602* double mutant plasmid, but not the *repE40-repE602* plasmid, showed a high copy number, primarily as a result of the lack of autogenous repressor activity (see below). Attempts to isolate plasmids containing *repE54* or *repE40* alone were unsuccessful. The likely explanation for this failure is that the *repE40* and *repE54* mutants, having potentially high initiator activities (see below), cause extremely high copy numbers and strongly inhibit cell growth. The copy numbers of mutant plasmids *repE10*, *repE18*, and *repE26* in the *dnaK756*, *grpE280*, and Δ*rpoH* mutant bacteria did not differ appreciably from those observed in the wild-type host (data not shown).

Cellular levels of mutant RepE proteins. The amounts of RepE protein produced by the mutant plasmids, except for those (*repE10* and *repE26*) exhibiting a particularly high instability (13), were determined by immunoblotting using a specific antiserum (Table 3). In general, a good correlation was found between the plasmid copy numbers and the levels

TABLE 3. Copy numbers and RepE protein levels of the mutant plasmids

Plasmid	Copy number in[a]:		RepE protein level in[b]:	
	MC4100 (*dnaJ*$^+$)	KY1453 (*dnaJ259*)	MC4100 (*dnaJ*$^+$)	KY1453 (*dnaJ259*)
repE$^+$	1.0		1.0	
repE10	22[c] (89)	10[c] (25)		
repE26	19[c] (60)	7.2[c] (29)		
copA1	5.6	20[c] (91)	1.6	1.0
repE18	1.2	0.8	1.0	0.7
repE105	1.9	1.0	2.7	1.4
repEp110	2.7	2.2	6.5	2.7
repE602	0.7		1.0	
repE40-repE602	1.8	0.8	1.9	1.0
repE54-repE602	22[c] (90)	6.3[c] (65)	4.1	3.0

[a] Strains harboring both mini-F and pACYC184 plasmids were grown overnight in L broth containing ampicillin and chloramphenicol. Plasmid DNAs were prepared, treated with *Eco*RI and *Bgl*II, and subjected to agarose gel electrophoresis. DNA bands were scanned by a densitometer, and the density of mini-F plasmid DNA was normalized to that of pACYC184 DNA and corrected for the size difference. Averages of three experiments are shown. Values represent those relative to the *repE*$^+$ plasmid (pKV5110) in MC4100, which was 10 to 15 per host chromosome (the copy number ratio of pKV5110 to pACYC184 was 0.39.
[b] Cells carrying each plasmid were grown to log phase; samples were taken and mixed with a fixed amount of cells producing truncated RepE (RepEΔC57), which served as an internal reference. Following treatment with trichloroacetic acid, proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The RepE protein levels were determined by immunoblotting with specific antiserum essentially as described previously (13). The quantitation was carried out within a linear range by using appropriate controls, and the results were normalized to that for the *repE*$^+$ plasmid (pKV5110) in the wild-type host (MC4100).
[c] Value corrected for plasmid-free segregants (25 to 90%, as shown in parentheses) that appeared in the cultures. Such an instability implies that the actual copy number may be much higher than indicated.

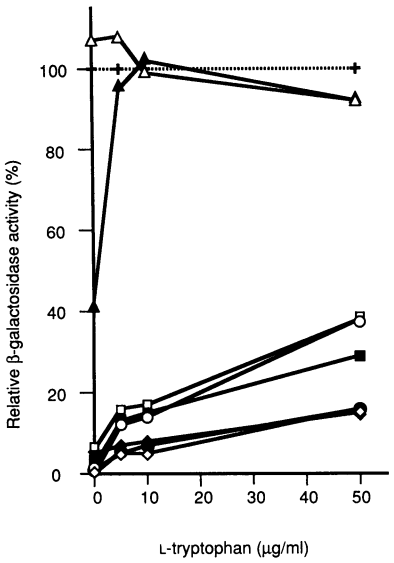

FIG. 3. Relative repressor activities of RepE proteins with a normal C-terminal end. MC4100 cells lysogenic for λpF13-P*repE-lacZ* and harboring pKV7190 (P*trp-repE*$^+$) or its derivative carrying a mutant *repE* allele were grown to log phase in medium E containing chloramphenicol (20 μg/ml) and L-tryptophan as indicated. Portions of the culture were assayed for β-galactosidase activities, which were normalized to the activity for cells with a control plasmid lacking *repE* (pKV711). As the L-tryptophan concentration increased, RepE synthesis decreased and P*repE*-directed synthesis of β-galactosidase increased. Symbols: +, no RepE protein; ◇, RepE$^+$; ○, RepE10; ●, RepE18; □, RepE26; ■, RepE40; △, RepE54; ▲, RepE105; ◆, CopA1.

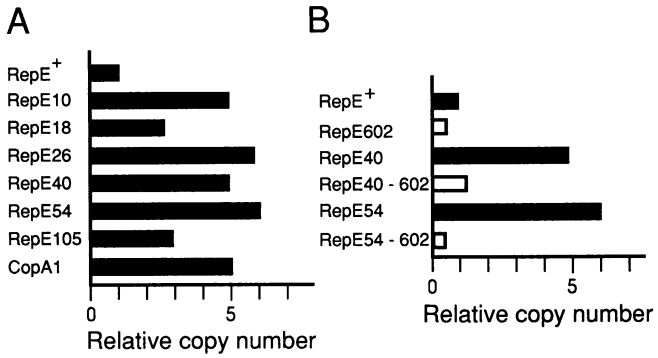

FIG. 4. (A) Initiator activities of RepE proteins with a normal C-terminal end, as determined by copy numbers of *ori2* plasmid (pKV718). MC4100 cells harboring both pKV718 and pKV7190 (or its derivative) were grown to log phase in medium E containing ampicillin (20 μg/ml), chloramphenicol (20 μg/ml), and L-tryptophan 50 μg/ml. Plasmid DNAs were prepared, linearized by *Eco*RI digestion, and subjected to agarose gel electrophoresis. DNA bands were scanned by a densitometer, and the density of the pKV718 band was normalized to that of pKV7190 (or its derivative) to obtain relative copy numbers. The value obtained for each mutant RepE is presented relative to that of the wild-type RepE (which varied to some extent). (B) Effects of the *repE602* C-terminal lesion on initiator activities. Closed bars, RepE proteins with a normal C-terminal end, as in panel A; open bars, RepE proteins with an altered C-terminal end.

of RepE produced by the most plasmid mutants. The RepE levels for high-copy-number mutants (*copA1* and *repE54-repE602*) were clearly underestimated as a result of the plasmid instability. It is interesting to note that the promoter mutation *repEp110*, which caused only a slightly higher copy number in the wild-type host, produced a large excess (six- to sevenfold of the wild-type level) of RepE protein. The latter observation suggests that the increased RepE levels are not sufficient to explain the marked increase in copy numbers of many of the *repE* mutants examined above; the hyperactive mutant RepE proteins are mainly responsible for the extremely high copy numbers observed.

Repressor activity of the mutant RepE proteins. The autogenous repressor activities of the mutant RepE in *repE* transcription were determined in vivo by monitoring β-galactosidase synthesis from the P*repE-lacZ* operon fusion on λ prophage in cells harboring pKV7190 (or its derivative) that produces RepE under *trp* promoter control. In some cases, pKV739 carrying the *repE602* lesion was used instead of pKV7190 to produce RepE containing two mutations (see Materials and Methods). The cellular amounts of RepE produced were adjusted by various concentrations of L-tryptophan in the media. Among the mutant RepE proteins tested, RepE54 was found to have almost completely lost the repressor activity, and RepE105 showed a markedly reduced activity (Fig. 3). The repressor activity was slightly but significantly reduced with RepE40, as observed previously with RepE10 and RepE26 (13), and hardly affected with RepE18 or CopA1. The *repE602* lesion at the C terminus of RepE exerted little effect on the repressor activities of wild-type RepE, RepE54, or RepE40 (data not shown). The amounts of mutant RepE produced (determined by immunoblotting) did not vary significantly from that of the wild-type RepE except that the level of RepE105 was reduced by about 50%, suggesting that RepE105 may be slightly unstable. Together, these results suggest that the RepE segment of around 117 to 134 residues is particularly important for the autogenous repressor function.

Initiator activity of the mutant RepE proteins. The initiator activities of the mutant RepE were determined by measuring the copy number of the *ori2* plasmid (pKV718) in the presence of limited amounts of RepE supplied by derivatives of pKV7190 (P*trp-repE*) carrying a *repE* mutation. All of the mutant RepE proteins examined showed significantly higher initiator activities (two- to sixfold) than did the wild-type RepE (Fig. 4A). In particular, high initiator activities were observed with RepE40, RepE54, and CopA1, as well as RepE10 and RepE26 reported previously (13). RepE18 exhibited a slightly increased activity. Interestingly, the *repE602* lesion drastically reduced the initiator activity of RepE40 or RepE54 when combined with each of the latter mutations (Fig. 4B). Consistent with this finding was the observation that transformation of the *ori2* plasmid into cells harboring the *repE40-repE602* or *repE54-repE602* plasmid was much less frequent (by 1 or 2 orders of magnitude) than with cells harboring the *repE*$^+$ plasmid (data not shown). These results suggest that the central region around residues 92 to 117 and the C-terminal region of RepE may functionally interact with each other in the initiation of plasmid DNA replication. Table 4 summarizes the major properties of *repE* mutant plasmids presented above to facilitate comparison.

DISCUSSION

We have previously shown that the inability of the mini-F plasmid to replicate in the *dnaK*, *dnaJ*, and *grpE* mutant bacteria can be rescued by excess RepE protein supplied in

TABLE 4. Summary of properties of *repE* mutant plasmids[a]

repE allele	Transformation		Plasmid stability[b]	Plasmid copy no.[b]	RepE level[b]	RepE repressor activity	RepE initiator activity
	dnaJ259	*ΔrpoH30*					
+	−	−	+	+	+	+	+
10	+	+	−	+++	ND[c]	±	+++
18	+	−	+	+	+	+	++
26	+	+	−	+++	ND	±	+++
40	ND	ND	ND	ND	ND	±	+++
54	ND	ND	ND	ND	ND	−	+++
105	+	+	+	+	+	−	++
copA1	+	±	+	++	+	+	+++
p110	+	±	+	+	++	ND	+
602	−	−	+	+	+	+	±
40-602	+	−	+	+	+	±	+
54-602	+	+	−	+++	++	−	±

[a] The data presented in Fig. 3 and 4 and Tables 2 and 3 are summarized in a semiquantitative way.
[b] Determined in the wild-type host (MC4100).
[c] ND, not determined.

trans by a compatible plasmid (12). Similarly, the *repE* promoter/operator mutant (*repEp110*) that overproduced RepE was able to replicate in the same set of mutant bacteria, though the plasmid copy number in the wild-type host was relatively little enhanced (two- to threefold); it also replicated poorly in the Δ*rpoH* host (Table 2). In contrast, the majority of other mutant plasmids carried a mutation within the *repE* coding region and produced RepE proteins with higher, though varying, initiator activities and some with very low repressor activities (Fig. 3 and 4). All of these plasmids replicated in any of the *dnaJ*, *dnaK*, and *grpE* mutants tested, including *dnaJ* and *dnaK* null mutants, albeit at reduced efficiencies. Apparently, these heat shock proteins work synergistically in mini-F plasmid DNA replication, as has been shown for replication of λ phage (7) and the mini-P1 plasmid (31). Only the plasmid mutants that produce extremely hyperactive RepE were obtained in the previous selection with the Δ*rpoH* strain (13), whereas some mutant plasmids obtained in this study (*repE18* and *repE602-repE40*) produced RepE with moderately high initiator activities and failed to replicate in the Δ*rpoH* strain (Table 2). Thus, the greater capacity of replication initiation appeared to be required for the mini-F plasmid to replicate in the absence of σ^{32} than in the absence of any of the heat shock proteins. This conclusion is reasonable because σ^{32} plays a major role both in production of the set of heat shock proteins (8) and in transcription of the *repE* gene (28).

Kawasaki et al. (14) recently studied the DNA binding properties of RepE protein purified from the wild type and several hyperactive *repE* mutants. The RepE proteins obtained were dimers for all the plasmids examined. The binding affinity of the wild-type RepE was very low but was strikingly enhanced by DnaJ (but not DnaK) protein. Moreover, the hyperactive RepEs from the *repE10*, *repE26*, and *copA1* mutants showed much higher binding affinities to the *ori2* repeated sequence than did the wild-type RepE but somewhat lower affinities to the *repE* operator. These results agreed well with the properties of the mutant RepE studied in vivo (13), namely, the higher initiator activities and plasmid copy numbers and slightly lower repressor activities. It thus appears that the DnaJ heat shock protein plays a major role in activating RepE at a stage prior to or during its binding to DNA and that the hyperactive RepE proteins are in the partially active form without the aid of DnaJ protein. In the case of the mini-P1 plasmid, whose replication mechanism is similar to that of the mini-F plasmid, the RepA initiator protein dimers are known to form complexes with DnaJ dimers (30). Such complexes, when incubated with DnaK protein plus ATP, can be converted to active RepA monomers that bind efficiently to the origin DNA (31, 32).

The higher initiator activities of the mutant RepE proteins examined so far are the result of single amino acid changes at the restricted region between residues 92 and 134 (Fig. 2), and some are simultaneously affected in the repressor function. This is in good accord with the previous proposition that the RepE region around residues from 92 to 129 may be important in the control of plasmid copy number (13). Among the mutant RepE proteins examined in this study, RepE54, which is altered at residue 117 (proline instead of arginine), may represent a unique case because it has lost the autogenous repressor activity almost completely while exhibiting a high initiator activity (Fig. 3 and 4). Another mutant protein, RepE105, with an alteration at residue 134 (phenylalanine replacing leucine) showed much reduced repressor activity possibly accompanied by protein instability. In contrast, all of the neighboring mutations affecting the residues between 92 and 109 (*repE10*, *repE40*, *repE18*, *copA1*, and *repE26*) affected the repressor activity only slightly if at all (Fig. 3) (13). Thus, the narrow region spanning residues 117 to 134 appears to be particularly important in the autogenous repressor function of RepE protein.

Another striking observation related to RepE function is the intragenic suppression of high initiator activities associated with the *repE40* or *repE54* mutation (affecting residue 92 or 117, respectively) by a C-terminal frameshift mutation (*repE602*); the *repE602* lesion by itself also affected the initiator activity significantly (Fig. 4). The repressor activity of *repE40* or *repE54* was not significantly affected by *repE602*. These results indicate the involvement of a functional interaction between the region defined by these mutations (the residues spanning 92 through 117) and the C-terminal region of RepE protein in exerting the initiator activity at some stage(s) of replication initiation. Direct examination of DNA binding properties of RepE from several mutants described here, as well as studies on the mechanism of DnaJ-mediated RepE activation, will be important for further elucidation of the structure and function of RepE and the role of heat shock proteins in mini-F plasmid replication.

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