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Location of a P1 Plasmid Replication Inhibitor Determinant within the Initiator Gene

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The P1 plasmid replication initiator protein, RepA, binds to its own promoter and represses transcription efficiently. There are only about 20 RepA dimers present per *repA* gene. A possible reason for this highly restrained expression became evident when *repA* expression was increased by using foreign promoters: with fivefold overexpression, the replication rate was diminished, and with 40-fold overexpression, replication was not detectable. The inhibition was P1 specific: growth of *Escherichia coli* and replication of pSC101, R6K, and mini-F plasmids were not affected. The activity is apparently not from RepA itself. Excess purified RepA did not inhibit replication in vitro. Mutations of the *repA* translation initiation codon reduced synthesis of the initiator but not the inhibitory activity. Deletion from either the N- or C-terminal ends of *repA* (28 and 69 codons, respectively, out of the 286-codon open reading frame) affected the initiator but not the inhibitory activity. Further deletions affected both the activities. These results demonstrate that the integrity of the initiator is not required for inhibition, but involvement of an unstable initiator fragment or of initiator mRNA cannot be ruled out.

Bacteriophage P1 is maintained in the prophage state as a plasmid whose copy number is comparable to that of the chromosome of the host, *Escherichia coli*. The basic 1.5-kilobase plasmid replicon consists of an origin and a control locus, *incA*, flanking an initiator gene, *repA*. Deletion of the control locus increases the plasmid copy number about eightfold (17). A salient feature of the replicon is the presence of nearly perfect repeats of a 19-base-pair (bp) sequence, five in the origin and nine more in *incA* (2). Purified RepA protein binds to all 14 repeats with nearly equal affinity (1). Within the origin repeats is the *repA* promoter, whose activity remains largely repressed, apparently due to RepA binding. This autorepression sets the basal level of RepA to only about 20 dimers per *repA* gene (22). Modest increases in RepA concentration by using foreign promoters help to increase plasmid copy number only in the absence of *incA*. Increasing RepA concentration fourfold beyond the basal level decreases copy number rather than increasing it any further (16). Replication stops when RepA concentration is increased 40-fold. Since this inhibitory activity was seen irrespective of the presence of the *incA* locus, we concluded that RepA has both positive and negative roles in P1 plasmid replication (6).

When mini-P1 replication was examined in vitro, even a 100-fold excess of purified RepA beyond what was required for maximal replication did not inhibit replication significantly (24). This suggests that the inhibitory activity in vivo was due not to RepA itself but to another product from the same region of DNA, one that presumably was eliminated during RepA purification for the in vitro experiments.

Here we show that several mutations in the *repA* gene that inactivate the initiator do not eliminate the inhibitory activity. These results support the view that RepA itself is not the inhibitor in vivo. The biochemical nature of the inhibitor remains to be determined.

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MATERIALS AND METHODS

Plasmid construction. Most of the plasmids used in this study to overexpress *repA* were derived from pALA162 (6). The plasmid was made from pBR322 by cloning between its *Hin*dIII and *Aat*II sites the *repA* gene and the adjoining 46 bp of *incA* (P1 coordinates 606 to 1569, Fig. 1). In this construct, the *repA* gene is devoid of its own promoter and is transcribed from the pBR322 *bla-p2* promoter. The transcription initiates at pBR322 coordinates 36 or 37 or both, just upstream of the *Hin*dIII site (4). In order to obtain similar plasmids without the residual *incA* sequences, a *Hin*dIII-*Bam*HI fragment of pSP102 (P1 coordinates 606 to 1529) carrying the *repA* gene only was cloned between identical sites of pRJM353 to generate the plasmid pRJM359. pRJM353 was obtained by destroying the original *Bam*HI site of pBR322 with S1 nuclease and by converting the *Eco*RI site to *Bam*HI with synthetic linkers. In pRJM359, *repA* is under the control of *bla-p2* promoter, as in pALA162. Deletions into *repA* were created by opening the plasmids with *Hin*dIII and digestion with either S1 nuclease (17) or with exonuclease III (exoIII) (12). pKM105 and pKM106 were obtained by the former method, and pKM107 was obtained by the latter. Mutations internal to *repA* were generated by oligonucleotide-directed mutagenesis (13). For point mutations, oligonucleotides 17 bases long with the desired change in the middle of the sequence were used. Deletions in pKM108, pKM109, pKM110, and pKM111 were generated by four oligonucleotides which were 30 bases long and which had 15 bases of homology on either side of the region to be deleted. Up to 246 bp was deleted by this method (pKM111; Fig. 1). Oligonucleotides have also been used to modify *repA* translation signals (see Table 5). Other details of the plasmids and other plasmids used in this work are described in Table 1 and Fig. 1.

RESULTS

Inhibitory activity from *repA* mutated in the initiation codon ATG. The inhibitory activity associated with overexpression of *repA* is most conveniently assayed by measuring lysogeny

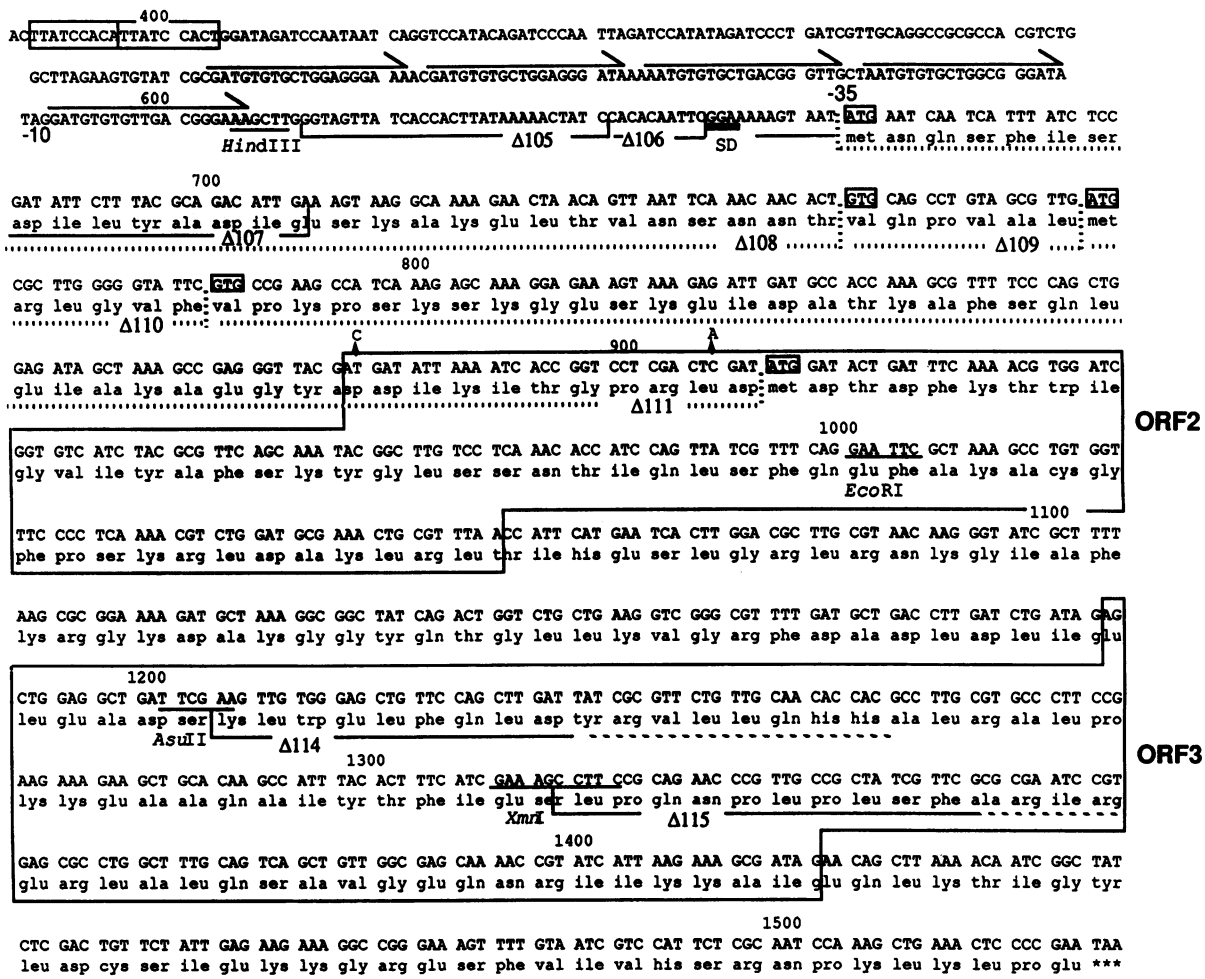

FIG. 1. The primary structure of the mini-P1 replicon origin and the *repA* gene (reproduced with modification from references 2 and 6). The minimal origin as shown here starts with two tandem DnaA boxes (TTATCCACA/T) and ends with five arrows, which represent binding sites of the RepA protein. The −10 and −35 regions mark the *repA* promoter region, and SD marks the putative ribosome-binding site. *repA* ORF starts with an ATG (boxed), and the amino acid sequence of the entire ORF is shown below the sequence. Four other internal start codons in the same reading frame as *repA* are also boxed. ORF2 and ORF3 show the next two largest ORFs, and they are both in the +1 frame. The positions of various deletion and point mutations and also some of the major restriction sites that have been used in this work are also indicated.

of cells infected with integration-deficient λ–mini-P1 phages (Table 2). Since the prophages are unable to integrate, their continued presence in a growing colony requires functioning of the plasmid replicon. Any activity that blocks plasmid replication also prevents colony formation when the presence of the prophage is selected (19). Table 3 shows that when RepA was supplied in 40-fold excess over that produced by the wild-type plasmid, lysogeny of the infecting λ–mini-P1 phage, λDKC235, decreased by 4 orders of magnitude (lines 1 and 2).

When the translational initiation codon ATG was mutated to ATA, RepA protein could not be detected by Western immunoblotting (columns 2 and 3 in Fig. 2). However, the mutant plasmid still inhibited lysogeny significantly (pKM102; Table 3, line 3). The inhibitory activity was still dependent upon a high rate of *repA* transcription; the same base change in a low RepA producer plasmid did not have any effect on the frequency of lysogeny (pKM101; Table 3, line 5). Use of λDKC251, a *repA* missense phage whose replication, and hence stable lysogeny, depends upon a source of RepA in *trans*, confirmed that the mutant plasmid was defective in initiator synthesis (Table 3, lines 4 and 5).

An in vivo measure of RepA synthesis, albeit indirect, was obtained by assaying the ability of the protein to repress an authentic *repA* promoter in *trans* (autorepression). The *repA* promoter activity was followed by use of gene fusion to *lacZ* (22). We have shown previously that RepA produced from a wild-type mini-P1 plasmid represses another *repA* promoter in *trans* so that its activity is reduced from about 250 Miller units to 1 Miller unit. When the RepA concentration was increased 40-fold compared with the wild-type level, the repression did not improve detectably: the basal level still remained 1 U (see pALA162, Table 3, last column). With a mutation in the ATG initiation codon, the repression was less complete: the basal level increased from 1 to 36 U (see pKM102, Table 3, last column). Since the repression was less efficient than was achieved with the wild-type plasmid, the RepA level from pKM102 must have been below the wild-type level, as was indicated by the Western analysis (Fig. 2). From these studies we conclude that excess *repA* transcription inhibits replication of mini-P1 plasmids in *trans* in the absence of excess RepA protein.

Inhibitory activity from *repA* deletion mutants. If RepA itself is not the inhibitory element, can parts of the *repA* gene

TABLE 1. Description of plasmids

Name	Replicon	Drug resistance phenotype	Relevant content of P1 DNA (coordinates)[a] and comments	Source or reference
pALA69	pBR322	Ap	*repA* (606-1569); provides nearly wild-type amounts of RepA from unknown promoter	6
pALA162	pBR322	Ap	Similar to pALA69 but provides 42 times the wild-type amount from the vector *bla-p2* promoter	6
pBH20	pBR322	Ap	*lac-po* at the *Eco*RI site of pBR322	16
pDL19	pUC19	Ap	Extra *Bgl*II and *Xmn*I sites, otherwise same as pUC19	8
pGM10	pBR322	Ap	Same as pSP120 except *repA* contains Δ108	Fig. 1
pGM11	pBR322	Ap	Same as pSP120 except *repA* contains Δ115	Fig. 1
pGM12	pBR322	Ap	Same as pRJM359 except *repA* contains Δ108 and Δ115	Fig. 1
pGM13	pUC19	Ap, Cm	Same as pKM123 except *repA* contains Δ107 and Δ115	Fig. 1
pGM14	pUC19	Ap, Cm	Same as pGM13 except *repA* contains Δ108 and Δ115	Fig. 1
pGM15	pBR322	Ap	Same as pKM131 except for GGAG to CCTC change	Table 5
pGM16	pBR322	Ap	Same as pKM131 except for 16-bp deletion around SD region	Table 5
pIP3	R6K	Km	No P1 DNA	18
pJD2	pUC19	Ap, Cm	Same as pDL19 except for *cat* gene at *Bam*HI site	J. Devito and A. Das
pKM101	pBR322	Ap	Same as pALA69 except for G to A change at 666	Table 3
pKM102	pBR322	Ap	Same as pALA162 except for G to A change at 666	Table 3
pKM105	pBR322	Ap	Same as pALA162 except contains Δ105	Fig. 1 and Table 4
pKM106	pBR322	Ap	Same as pALA162 except contains Δ106	Fig. 1 and Table 4
pKM107	pBR322	Ap	Same as pALA162 except contains Δ107	Fig. 1 and Table 4
pKM108	pBR322	Ap	Same as pALA162 except contains Δ108	Fig. 1 and Table 4
pKM109	pBR322	Ap	Same as pALA162 except contains Δ109	Fig. 1 and Table 4
pKM110	pBR322	Ap	Same as pALA162 except contains Δ110	Fig. 1
pKM111	pBR322	Ap	Same as pALA162 except contains Δ111	Fig. 1
pKM114	pBR322	Ap	Same as pRJM359 except contains Δ114	Fig. 1 and Table 4
pKM115	pBR322	Ap	Same as pRJM359 except contains Δ115	Fig. 1 and Table 4
pKM123	pUC19	Ap, Cm	*repA* contains Δ106 and Δ115 and is cloned between *Hin*dIII-*Xba*I sites of pJD2	This work
pKM126	pUC19	Ap, Cm	Same as above except *repA* contains Δ109 and Δ115	This work
pKM127	pUC19	Ap, Cm	Same as above except *repA* contains Δ114	This work
pKM130	pBR322	Ap	Same as pALA162 except for deletion of ATG initiation codon (664–666)	This work
pKM131	pBR322	Ap	Same as pKM108 except *repA* SD region (612–647) substituted with synthetic DNA	Table 5
pKM132	pBR322	Ap	Same as pKM131 except for deletion of GTG codon	Table 5
pLG339	pSC101	Km, Tc	No P1 DNA	20
pMF21	F	Km	No P1 DNA	14
pRJM353	pBR322	Ap	*Bam*HI site of pBR322 destroyed and a 10-bp *Bam*HI linker inserted at the *Eco*RI site	This work
pRJM359	pBR322	Ap	*repA* (606–1529) cloned between *Hin*dIII and *Bam*HI sites of pRJM353	This work
pSP120	pBR322	Ap	pBH20 carrying *repA* (606–1529) between *Hin*dIII and *Bam*HI sites	16

[a] See reference 2 and Fig. 1 for coordinates.

be deleted without affecting the inhibitory activity? A deletion from the 5′ end suggested that the Shine-Dalgarno (SD) region was required. In pKM105, the inhibitory activity was normal, but in pKM106, where the deletion just touched the SD region, the RepA level was reduced fivefold and the inhibitory activity was lost, suggesting that the poor translatability of the *repA* message caused a loss of significant inhibitory activity (Fig. 1; Table 4). Similarly, a larger deletion covering the translation control region and beginning of the *repA* gene was also completely inactive for the inhibitory activity (pKM107; Table 4). It was not obvious why the SD region would be required for activity in the absence of efficient translation initiation (i.e., from the ATA start codon). We shall show later that some inhibitory activity is present even when the initiation codon is deleted.

The necessity to retain the SD region prompted us to study internal deletions of *repA* that left the SD region intact. The inhibitory activity was still present in pKM108, in which the first 28 codons of *repA* were absent. This result supports the idea that the significant defect in pKM107 was the lack of the SD region and not the deletion of the first 15 codons. The mutant plasmid, pKM109, with the first 34 codons of *repA* deleted was defective for the inhibitory activity. Since this plasmid retains the SD region and the initiation codon intact, the sequences between the codons 28 to 34 must be important for the activity. As expected, further deletions into *repA*

TABLE 2. Description of phage strains

Name	Relevant characteristics	Source, comments, and/or reference
λDKC201	λ-P1:5R*c*I857	19
λDKC235	λKAN-P1:5R	16
λDKC236	λ-P1*prepA-lacZ*	Measures *repA* promoter activity (17)
λDKC251	λKAN-P1:5R*rep*-6	Recessive and presumed missence mutation in *repA* (3)
λDKC257	λCAT-P1:5R*rep*-6	This work
λDKC259	λCAT-P1:5R	Egon Hansen

TABLE 3. Inhibitory activity from *repA* mutated in the initiation codon

Resident plasmids			Lysogenization frequency of infecting phage		Activity of *prepA-lacZ* gene fusion[a] (Miller units)
Name	*repA* allele present	RepA amount relative to wild-type plasmid λDKC201	λDKC235 (wild type)	λDKC251 (*repA* missense)	
pBR322			1	10^{-4}	250
pALA162	Wild type	42	10^{-4}	10^{-4}	1
pKM102	Mutant	<1	10^{-3}	10^{-4}	36
pALA69	Wild type	2	1	1	1
pKM101	Mutant	≪1	1	10^{-4}	250

[a] A monolysogen of λDKC236 phage carrying the *prepA-lacZ* gene fusion was used to measure *repA* promoter activity (17).

(codons 1 to 40 and 1 to 82) that brought the next two initiation codons adjacent to SD were also inactive (pKM110 and pKM111; Fig. 1). We conclude that the first 28 codons of *repA* that inactivate the initiator (see below) are dispensable for the inhibitory activity.

The last 69 codons, at least, could be deleted from the 3′ end without affecting the inhibitory activity (pKM115; Fig. 1 and Table 4). The deletion, however, eliminated initiation activity (see below). Deletion of the next 37 codons (pKM114) inactivated both activities. These deletion mutants establish that the integrity of the initiator gene was not required to see strong inhibitory activity.

Initiator activity from *repA* deletion mutants. In order to measure initiator activity of *repA* deletion mutants that retain the inhibitory activity, the mutant genes were recloned under *lac-po* in the vector pBH20 so that the expression level could be controlled (16). When the mutant *repA* genes from pKM108 and pKM115 were transferred to pBH20, the resultant plasmids pGM10 and pGM11 permitted lysogeny of wild-type λ–mini-P1 phage but failed to support lysogeny of an isogenic phage with a missense mutation in *repA* (λDKC257 and λDKC259, respectively). The presence of control plasmid pSP120 (pBH20 carrying the wild-type *repA* gene [16]) allowed lysogeny of both phages. As expected, when *repA* expression was increased by inducing expression from the *lac* promoter, lysogeny of the wild-type phage was also affected, indicating that the inhibitory activity could be expressed from the new vector. Plasmids pKM109 and pKM114 (Fig. 1), which have larger deletions into *repA* and thereby have lost the inhibitory activity, were also unable to support initiation. Thus, certain mutations that affect the integrity of the *repA* gene and inactivate its initiation function can retain the inhibitory activity.

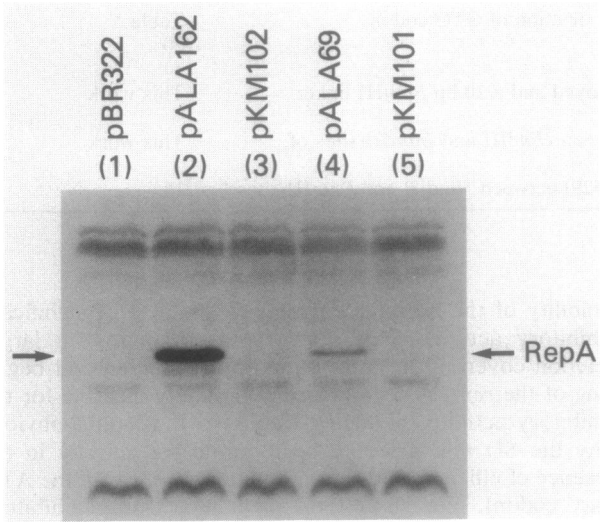

FIG. 2. Western blot of RepA. Total protein extract (300 μg) was added to each lane from N100 cells carrying different plasmids, as identified at the top of the gel. Protein extracts were made as described previously (22). In lane 1 the cells do not carry a source of RepA. The next two lanes show the amounts of RepA from pALA162 and an isogenic plasmid, pKM102, carrying the ATG to ATA mutation in the *repA* initiation codon. In lane 4 the source of protein is from pALA69, which produces about 1/20 of the protein produced by pALA162 (22). Since no RepA band is visible in lane 3, RepA synthesis must have been reduced at least 20-fold due to the G to A mutation. pKM101 is identical to pALA69 except for the G to A mutation and, as expected, no RepA band is visible in lane 5 also. The arrow identifies the RepA band.

Role of translation initiation signals. In order to distinguish between a requirement for the sequences of the SD region per se and a requirement for translation initiation, 51 bp of untranslated *repA* mRNA (coordinates 612 to 662) was replaced with a 19-bp synthetic SD sequence essentially as described by Crowl (7). The results obtained with the plasmid that is thus modified (pKM131) show that a different but functional SD sequence can substitute for the authentic SD region of *repA* without loss of the inhibitory activity (Table 5). In other words, the authentic SD sequence is not a requirement for the activity. We also determined the effect of reducing the complementarity of the SD region to 16S rRNA, as shown for plasmid pGM15 (Table 5). When the core sequence of the SD region GGAG was changed to CCTC, no inhibitory activity could be detected by our standard assay. As expected, complete deletion of the SD region was also not tolerated (pGM16, Table 5). On the other hand, some inhibitory activity was still present when we left the SD region intact but deleted the GTG initiation codon (pKM132; Table 5) or the ATG initiation codon from pALA162 (pKM130; Table 1). It appears that although there is an absolute requirement for an efficient SD region, the translation initiation codon need not be efficient.

Determination of minimal sequence requirement for inhibitory activity. The above results indicated that the sequences around the SD region and between coordinates 748 and 1313 (not eliminated by deletions 108 and 115) are important for the inhibitory activity. To determine whether the activity is totally absent in *repA* fragments carrying the larger deletions 107, 109, and 114, the fragments were cloned under the *lac* promoter in the pUC19-derived vector pJD2 (pGM13, pKM126, and pKM127; Table 1). The idea was to increase *repA* expression by the combined action of increased promotion (the *lac* promoter is stronger than *bla-p2*) and increased copy number (pJD2 has a higher copy number than pBR322). Cloning in pJD2 was designed in such a way that a

TABLE 4. Inhibitory activity from *repA* deletion mutants

Plasmid name	Deletion present	Deletion size (P1 coordinates)	Relative levels of RepA	Inhibitory activity[a]	Initiation activity
pKM105	Δ105	28 bp (614–641)	1.3	+	Not tested
pKM106	Δ106	38 bp (614–651)	0.2	−	+
pKM107	Δ107	94 bp including codons 1–15 (614–707)	Not done	−	−
pKM108	Δ108	Codons 1–28 (664–747)	<0.1	+	−[b]
pKM109	Δ109	Codons 1–34 (664–765)	0.3	−	Not tested
pKM114	Δ114	Codons 181 to end (1204–1529)	0.3	−	Not tested
pKM115	Δ115	Codons 217 to end (1314–1529)	0.6	+	−[b]

[a] Inhibitory activity was measured by lysogeny by using λDKC235, as in Table 3. Reduction of lysogenization frequency by 4 orders of magnitude indicates presence of activity (+); normal lysogeny indicates lack of the activity (−).

[b] *repA* fragments were recloned into pSP120 to reduce expression (pGM10 and pGM11; Table 1), and initiation activity was measured by lysogeny as in Table 3. See text for further details.

translation termination codon was created immediately after the *repA* sequences to avoid gene fusion to the vector sequences. In any event, increasing the expression level did not cause the activity to be regained. (The exception was deletion 106, which, although inactive in the pBR322 background [in pKM106], did show the inhibitory activity from the pJD2 vector [in pKM123; Table 1]. A closer inspection of the sequence revealed that the cloning in pJD2 resulted in the addition of 12 codons from the vector to the *repA* open reading frame [ORF] and that the fusion gene was under the control of the *lac* SD region.) These results thus support our earlier conclusion (stated in the beginning of this paragraph) regarding the sequence requirement for the inhibitory activity.

To define the minimal sequence requirement more precisely, we combined the deletions 108 and 115 in a single fragment to generate the plasmid pGM12. Surprisingly, the inhibitory activity could not be detected, although individually neither deletion prevented the activity. The results were same when the fragment was transferred to pJD2 (pGM14; Table 1). In other words, increased expression of the doubly deleted *repA* gene did not cause the inhibitory activity to reappear. Since no protein band from pGM14 hybridizable to RepA antibody was detected, the presumptive inhibitor, be it RNA or protein, must have been unstable. Alternatively, the internal *repA* fragment bounded by deletions 108 and 115 cannot encode the inhibitor.

ORF2 and ORF3 are probably irrelevant for inhibitory activity. Within the *repA* coding sequences there are two other ORFs which are 59 and 76 amino acids long (in frame +1 with respect to *repA*), marked ORF2 and ORF3 in Fig. 1. ORF3 most likely does not encode a protein since it lacks authentic translation initiation signals. Moreover, the possibility that ORF3 is relevant to the inhibitory activity is made unlikely by the observation that deletion of 32 of the 76 ORF3 codons (as in pKM115) did not cause loss of the inhibitory activity. Translation initiation signals of ORF2 appeared to be more authentic but were not considered significant when analyzed by the Perceptron algorithm (21). The translation signals of *repA* were the only ones recognized when the entire 1-kilobase region containing the *repA* gene was scanned in both directions. Nevertheless, we undertook to alter, by site-directed mutagenesis, the putative initiation codon of ORF2 from ATG to ACG at coordinate 879 and created an amber codon at coordinate 906 by a C to A change of the codon TCG. Neither of the two mutations affected the inhibitory activity (Fig. 1). From these results, we conclude that ORF2 and ORF3 are irrelevant to the inhibitory activity.

TABLE 5. Role of translation initiation signals

Plasmid name	Synthetic ribosome-binding site sequence[a]	Inhibitory activity[b]
pKM131	AGCTTAAAAATTAAGGAGGATATCGTGCAG	+
pGM15	----------CCTC----------	−
pGM16	-----[ ]----------	−
pKM132	----------[ ]---	±

[a] The SD sequence and the initiation codon are overlined. The first five bases are part of *Hin*dIII site. The hyphens indicate identity and the boxed spaces indicate the extent of the deletions.

[b] Inhibitory activity was measured by lysogeny of λDKC235 as in Table 3. Lysogens were rare in the case of pKM131, indicating the presence of strong inhibitory activity. Although lysogeny was normal in the cases of pGM15, pGM16, and pKM132, the prophage was unstable in the case of pKM132 in the absence of selection (66% loss in 20 generations).

DISCUSSION

We reported earlier that overproduction of RepA inhibited mini-P1 replication in *trans* (6). In this paper we report that the inhibition does not require either excess *repA* translation or the integrity of the *repA* gene. The inhibitory activity was maintained even when *repA* translation was reduced below the physiological rate by mutating the translation initiation codon ATG. Although the initiation codon could be deleted, there was a requirement for a functional SD sequence. Also required was the middle two-thirds of the *repA* ORF (codons 29 to 216 of the 286-codon-long ORF), with extension to either the N- or C-terminal end. The exact nature of the inhibitor remains to be determined.

The requirement of the SD region suggests that the inhibitor is a protein and, most simply, a part of the RepA protein itself. This is supported by the following results. An in-frame deletion of the first 28 codons of *repA* left the inhibitory activity intact (pKM108; Table 4). The activity was lost by introduction of an amber mutation at coordinate 997 (*rep*-103; see reference 3) or of a 10-bp linker at the *Eco*RI site at coordinate 1000. In the case of the amber mutation, the activity was regained in the presence of efficient amber suppressors (unpublished data). The observation that *repA* deleted for either the N- or the C-terminal ends retains the inhibitory activity whereas the double deletion (pGM14; Table 2) does not is also accommodated in this model by assuming instability of the truncated protein. Indeed, no RepA fragment could be detected from pGM14 by immunoblotting. However, the existence of plasmids that despite a mutation in the initiation codon do synthesize the inhibitor, but do not reveal any RepA fragment by immunoblotting (e.g., pKM102; Table 3), is not easily explained by assuming the inhibitor to be part of RepA. The fragments could be unstable and yet be active or could be only weakly antigenic.

Alternatively, the inhibitor could be the product of a small ORF activated by the SD region at a distance by translational coupling. Although the normal spacing between the SD region and the initiation codon is 5 to 12 nucleotides, reinitiation far downstream from a termination codon is possible by translational coupling (11).

There are examples in other systems where internal fragments control functioning of the primary protein. For example, transposable element Tn*5* encodes a transposase and an inhibitor protein in the same reading frame. The inhibitor is shorter than the transposase by 55 N-terminal amino acids (25). Similarly, in the case of certain of the single-stranded DNA phages, truncated versions of the initiator proteins are separately translated from an initiation codon within the initiator gene. In the case of f1 phage, the smaller protein is a powerful inhibitor of phage-specific DNA synthesis (10).

A second possibility is that the inhibitory element is *repA* mRNA. In that case, the requirement for the translation initiation signals could be for proper folding of the RNA or for protection of the RNA from nuclease degradation. These sequences per se might also be important.

The RNA might function by binding to RepA or some other host initiation factor so as to reduce its initiation potential. Interaction with a host factor seems unlikely in view of the fact that the activity is P1 specific. We have determined that under the condition of 40-fold overexpression of RepA, the growth rate of *E. coli* is not affected within experimental error. The stability of three other replicons, mini-F (pMF21), mini-R6K (pIP3), and mini-pSC101 (pLG339), was also unaffected under conditions that caused loss of the mini-P1 from more than 97% of cells by 20 generations of growth in the absence of selection (unpublished data). Another possibility could be that the RNA directly interferes with the origin function by hybridizing with homologous sequences.

In order to gain independent evidence for the location of the inhibitor, we have isolated RepA mutants that function as initiators even when overproduced (S. K. Pal and D. K. Chattoraj, unpublished data). The DNAs of three of these mutants have been sequenced, and the mutations map at three widely separated locations in the *repA* gene at codons 4, 198, and 270. It is noteworthy that codons 4 and 270 fall into regions that can be deleted without losing the inhibitory activity (see pKM108 and pKM115, respectively; Table 4). Since the regions cannot be deleted simultaneously without losing activity, the regions may contribute indirectly to inhibitor synthesis. It is also possible that mutations in these codons affect the target of the inhibitor rather than its synthesis. Finally, the mutant initiators may bypass the inhibitory effect by making some other stage of the initiation process more proficient. We also find that the autorepression of RepA is unaffected in the presence of the inhibitor (unpublished data). Therefore, if RepA is the target of the inhibitor, it does not affect RepA synthesis or its DNA-binding activity; it very likely acts on RepA posttranslationally. Such a possibility is raised by the observation that autorepression but not the synthesis of RepA was significantly reduced in cells defective in the heat shock genes *dnaJ*, *dnaK*, and *grpE* (K. Tilly, D. K. Chattoraj, and M. Yarmolinsky, unpublished data). The implication is that these gene products are involved in posttranslational modification of RepA for its DNA-binding activity.

Replication of two other plasmids, R6K (9) and Rts1 (23), is also inhibited when their initiator genes are overexpressed. It has been assumed in both systems that the inhibitory element is the initiator itself. It appears that unlike the case in mini-P1, overproduction of the amino terminal third of the R6K initiator suffices for this inhibition (A. Greener, M. Filutowicz, and D. R. Helinski, unpublished data, cited in reference 15). The Rts1 situation is similar to that of mini-P1 in that the mutations that destroy the initiator still retain the inhibitory activity in some cases. In all three systems, initiator mutants exist that promote replication when the initiator is overproduced. These studies establish that in addition to control by repeated RepA-binding sequences, several plasmids have evolved a second control element that is initiator-associated and that might function similarly. The widespread occurrence of this kind of inhibitory activity justifies considering the function to be an element of replication control.

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