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Replication of mini-P1 plasmid DNA *in vitro* requires two initiation proteins, encoded by the *repA* gene of phage P1 and the *dnaA* gene of *Escherichia coli*

(*oriC* plasmid/*ori*λ plasmid)

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ABSTRACT We have developed an *in vitro* DNA-replication system that replicates exogenously added mini-P1 plasmid DNA. The system consists of purified P1 RepA protein and a partially purified mixture of *Escherichia coli* replication proteins. It is essentially the same as that described for the replication of *oriC* plasmid DNA [Fuller, R. S., Kaguni, J. M. & Kornberg, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7370–7374]. Mini-P1 DNA replication requires the *E. coli* DnaA initiation protein in addition to the P1 RepA initiation protein. The reaction is inhibited by rifampicin, novobiocin, and antibody to DnaB, suggesting the involvement of RNA polymerase, DNA gyrase, and DnaB protein. Replication is initiated in the region of the P1 origin of replication and proceeds unidirectionally as determined by electron microscopy. Thus, the *in vitro* system mimics the essential features of mini-P1 replication as suggested by genetic studies.

P1 prophage, like the *Escherichia coli* sex factor F, is a stringently controlled plasmid replicon maintained at a copy number approximately equal to the number of *E. coli* chromosomes (1). Mini-P1 plasmids containing only 1.5 kilobase pairs (kbp) of the 90-kbp P1 prophage retain the essential characteristics of the P1 replicon. They have been studied genetically and structurally because they are small and do not affect cell viability. Replication of mini-P1 requires the P1 initiation protein, RepA (2), the *E. coli* initiation protein, DnaA (3), and presumably many other *E. coli* proteins, although the *in vivo* requirements have not been explored extensively. Replication also requires a DNA site, the origin of replication, that is included within a 246-base-pair (bp) sequence of P1 DNA located to the left of the *repA* gene as diagramed in Fig. 1 (5). DNA sequence analysis has revealed that this region contains five direct repeats of a 19-bp sequence (2). The purified RepA protein binds to these repeats *in vitro* (6). Approximately 50 bp to the left of the RepA binding sites is a region of DNA of about 50 bp that contains 67% A+T. Further to the left are two 9-bp DnaA protein binding sites ("DnaA boxes") (7). To the right of the *repA* gene is a control region containing nine 19-bp repeats similar to those at the origin. These are required to maintain P1 plasmids at one copy per cell (8). Mini-P1 plasmids lacking this region, like the one used in these studies, are maintained at a copy number of about 8 (4).

The presence of multiple binding sites of a replicon-specific initiation protein and an A+T-rich region are characteristic of many replication origins. For example, the *E. coli* origin has four to five binding sites for DnaA protein (7), the phage λ origin has four binding sites for the λ initiation protein, O (9), and the origin regions of plasmids R6K (10), mini-F (11), pSC101 (12), and mini-P1 (6) have three to seven binding sites for their specific initiation proteins. The plasmid origins are more complex in that they have 9-bp DnaA boxes as well as binding sites for plasmid-encoded initiation proteins.

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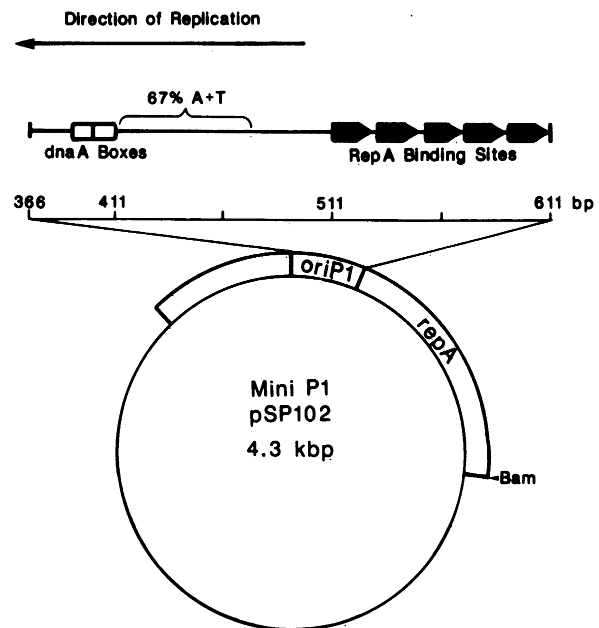

FIG. 1. Structural organization of the mini-P1 plasmid pSP102 (4). The 246-bp origin region (P1 coordinates 611–366; see ref. 2) is enlarged to show the position of the two DnaA boxes, the A+T-rich region, and the five RepA binding sites. The overall direction of replication, determined as described in *Results*, is indicated with an arrow.

We have developed an *in vitro* system for the replication of mini-P1 plasmid DNA. Our goal is to understand the mechanism of initiation and regulation of a stringently controlled plasmid.

MATERIALS AND METHODS

***E. coli* Strains and Plasmids.** The mini-P1 plasmid used in these studies was pSP102 (ref. 4; Fig. 1); it was grown in *E. coli* strain N100 (13). The *oriC* plasmid was pCM960 grown in *E. coli* strain CM987 (14). The *ori*λ plasmid DNA was RF I (replicative form I; i.e., superhelical circular double-stranded) DNA prepared from *E. coli* strain JM101 (15) infected with M13 *ori*λ1 (16). Heat-induced cells of strain OR1265 (17), carrying the *repA* gene cloned under λ P_L (left promoter) control in plasmid pALA131 (6), were used to isolate the RepA protein. Heat-induced cells of strain N4830 (18), carrying the *dnaA* gene cloned under λ P_L control in plasmid pBF1509, were used for the purification of DnaA

protein. Plasmid pBF1509, derived from pBF110 (19) by the deletion of the λ *N* gene, was the gift of R. Fuller (Stanford University). *E. coli* strain SG4148 (Δ*lon*), derived from SG4144 (20), was used for the preparation of the host replication factors.

Proteins, Enzymes, DNA, and Chemicals. P1 RepA protein was purified from heat-induced cells carrying pAL131 by a procedure derived from that of Abeles (6). The procedure involved lysis by sonication, phosphocellulose column chromatography, low salt precipitation, and hydroxyapatite column chromatography. The final preparation was about 90% pure as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by staining with Coomassie blue (21) and was used in these experiments. The DnaA protein used was fraction III (19), the λ O protein used was fraction III (22), the λ P protein used was fraction III (22), and the DnaB protein used was fraction IV (23). DnaA, O, and P proteins were about 90% pure, and DnaB protein was about 80% pure as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis (21). The protein fraction containing host replication proteins was prepared by the method of Fuller *et al.* (24). Antibody to DnaA protein was the gift of A. Kornberg (Stanford University). Antibody to DnaB protein was raised in a rabbit injected with DnaB protein, fraction IV (23). Restriction endonuclease *Bam*HI was from Bethesda Research Laboratories.

pSP102 and pCM960 plasmid DNAs were purified as described (2). M13*ori*λ1 RF I DNA was purified by the method of Zinder and Boeke (25).

[*methyl*-^{3}H]dTTP was from New England Nuclear; unlabeled ribo-, deoxyribo-, and dideoxyribonucleoside triphosphates were from P.-L. Biochemicals. Polyvinyl alcohol type II was from Sigma.

Assay for P1 Plasmid DNA Replication. Reactions were similar to those described for *in vitro* replication of *oriC* plasmid DNA (24). Reaction mixtures (20 μl) contained 40 mM Hepes (pH 7.5); 20 mM KCl; 1 mM dithiothreitol; 2 mM ATP; 0.5 mM GTP, CTP, and UTP; 10 mM $Mg(OAc)_2$; 20 mM creatine phosphate; 2 μg of creatine kinase; 0.1 mM dATP, dCTP, and dGTP; 50 μM [*methyl*-^{3}H]dTTP (1000 cpm/pmol); 6% (wt/vol) polyvinyl alcohol; 300 pmol of pSP102 mini-P1 plasmid DNA (deoxynucleotide equivalent); 5 μl of a protein fraction from uninfected *E. coli* strain SG4148 (Δ*lon*) (containing about 50 mg of protein per ml); 100 ng of RepA; and 100 ng of DnaA. Reaction mixtures were incubated at 35°C for 20 min, and trichloroacetic acid-insoluble radioactivity was measured.

Electron Microscopy of P1 DNA Replication Intermediates. *In vitro* P1 replication reactions were as described above except that 4 mM 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP) was added to terminate DNA synthesis and the reaction volume was 75 μl. Incubations were for 10 min at 35°C. Reactions were stopped by the addition of 1% $NaDodSO_4$ and 50 mM EDTA. DNA was purified essentially as described (26). Samples were heated at 70°C for 3 min and then incubated for 60 min at 37°C with proteinase K (0.5 mg/ml). The mixtures were phenol-extracted and ethanol-precipitated, and the pellets were resuspended in 25 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA. The samples were then incubated with pancreatic RNase (5 μg/ml) at 37°C for 45 min and again phenol-extracted and ethanol-precipitated. To ensure complete removal of $NaDodSO_4$, the pellets were resuspended in 100 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA and reprecipitated with ethanol. The pellets were resuspended in 50 μl of 0.02 M NaCl/5 mM EDTA. One-microliter (about 10 pmol of pSP102 DNA) samples were spread for electron microscopy (27). The spread films were transferred to carbon-coated grids, shadowed at a low angle with platinum/palladium vapor, and viewed in a Phillips EM400 electron microscope. A Tektronix 4956 digitizer was used for length measurements of DNA molecules.

RESULTS

Replication of Mini-P1 Plasmid DNA *in Vitro*. The plasmid DNA used in our studies is diagramed in Fig. 1. It contains the origin of replication of mini-P1, the *repA* gene, the chloramphenicol acetyltransferase gene, and about 650 bp of pBR322 DNA that does not contain the pBR322 origin of replication. *In vivo* the plasmid is maintained at a copy number of about 8. The requirements for the *in vitro* replication of this mini-P1 DNA template are shown in Table 1. DNA synthesis depended on purified RepA protein and a crude protein fraction prepared from *E. coli* cells not carrying the P1 plasmid. Fig. 2 shows that the RepA protein, as determined by the appearance of a polypeptide of 32 kDa (6), was coeluted on phosphocellulose column chromatography with the activity required for mini-P1 replication, suggesting that the RepA protein and not a minor contaminating species in the preparation was required. Table 1 shows that synthesis was greatly stimulated by the addition of purified DnaA protein even though the host protein fraction was prepared from *dnaA*$^+$ cells. This result suggests that DnaA protein was a limiting component in the host protein fraction. DNA synthesis also required exogenously added mini-P1 DNA, ATP and an ATP regenerating system, and polyvinyl alcohol. The reaction was inhibited by rifampicin and novobiocin, suggesting a role of RNA polymerase and DNA gyrase, respectively. DNA synthesis was also inhibited by antibodies to DnaA and DnaB proteins.

Fig. 3 shows the kinetics of DNA synthesis and the temperature dependence of the reaction. At 36°C there was a 5-min lag before dTMP incorporation began. At 24°C very little synthesis occurred even after 30 min. Similar effects have been seen with *oriC* (26, 28) and *ori*λ (unpublished observation) plasmid templates under similar reaction conditions.

The DNA specificity of the *in vitro* replication system is shown in Table 2. Reaction mixtures supplemented with DnaA alone, RepA alone, or λ O and P proteins together were insufficient for DNA synthesis with mini-P1 plasmid DNA. Unlike mini-P1 DNA, *oriC* plasmid DNA was replicated when DnaA protein alone was added. RepA alone was

Table 1. Requirements for *in vitro* replication of mini-P1 DNA

Reaction components	Mini-P1 DNA synthesis, pmol of dTMP incorporated
Complete	14.8
− P1 RepA protein	0.9
− DnaA protein	1.7
− *E. coli* enzyme fraction	<0.2
− Mini-P1 plasmid DNA	<0.2
− ATP and creatine phosphate	<0.2
− Polyvinyl alcohol	<0.2
+ Rifampicin (25 μg/ml)	0.6
+ Novobiocin (25 μg/ml)	0.4
+ Antibody to DnaA protein	0.6
+ Antibody to DnaA protein + DnaA protein	6.8
+ Antibody to DnaB protein	0.3
+ Antibody to DnaB protein + DnaB protein	7.1

Reactions were carried out as described in *Materials and Methods*. Antibody fractions were incubated with the *E. coli* enzyme fraction, DnaA protein, and RepA protein, either with or without additional DnaA protein (0.1 μg) or DnaB protein (0.6 μg), for 10 min at 0°C, prior to the addition of the rest of the reaction components.

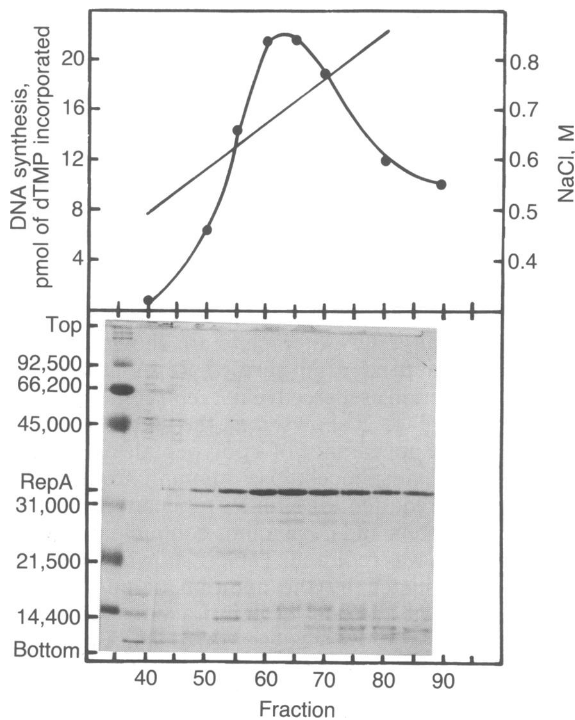

FIG. 2. Coelution of RepA protein with mini-P1 replication activity. A crude lysate (390 mg of protein) was prepared from 15 g of cells induced to overproduce RepA (6). It was adjusted to 0.25 M NaCl and applied to a P11 column (2.5 × 15 cm) equilibrated with 10% (vol/vol) glycerol/1 mM EDTA/1 mM dithiothreitol/50 mM Tris·HCl, pH 7.5, containing 0.25 M NaCl. The column was developed with a 700-ml gradient from 0.25 M to 1.25 M NaCl in the same buffer. Seven-milliliter fractions were collected. (*Upper*) Aliquots (1 μl) of fractions were assayed for P1 DNA-synthesis activity in mixtures as described in *Materials and Methods* but lacking RepA. (*Lower*) Aliquots (10-μl) of fractions were boiled for 10 min in Laemmli sample buffer, electrophoresed in a $NaDodSO_4$/10% polyacrylamide gel, and stained with Coomassie blue (21). Bio-Rad $NaDodSO_4$/PAGE low molecular weight protein standards were applied to the first well (M_r values shown at left).

insufficient, but its presence with DnaA neither stimulated nor inhibited *oriC*-dependent DNA synthesis. λ O and P proteins were also insufficient for *oriC* replication. In contrast to mini-P1 and *oriC* DNA, *ori*λ DNA was not replicated by RepA or DnaA protein. Its replication required the addition of λ O and P proteins.

Effect of RepA Concentration. Mini-P1 DNA synthesis increased with increasing concentrations of RepA (Fig. 4).

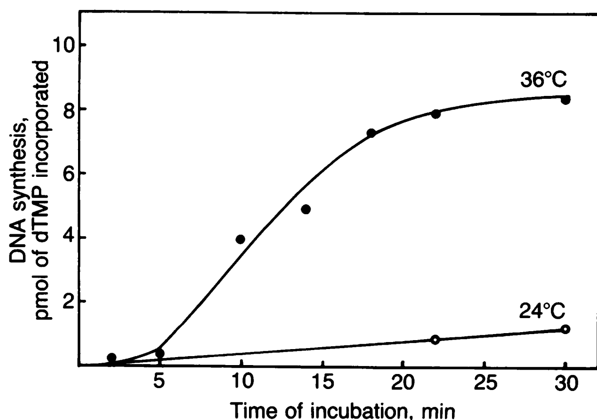

FIG. 3. Kinetics of mini-P1 DNA synthesis. Reaction mixtures were incubated for the indicated times at either 24°C or 36°C.

Table 2. DNA specificity of the *in vitro* DNA replication system

Plasmid DNA template	Protein(s) added to *E. coli* enzyme fraction: RepA	DnaA	λ O and P	DNA synthesis, pmol of dTMP incorporated
Mini-P1	+	+	–	27.0
	+	–	–	2.6
	–	+	–	0.9
	–	+	+	<0.2
oriC	+	+	–	13.1
	+	–	–	0.6
	–	+	–	14.6
	–	–	+	<0.2
*ori*λ	+	+	–	<0.2
	+	–	–	<0.2
	–	+	–	<0.2
	–	–	+	26.1

Reaction conditions for measuring mini-P1 DNA synthesis were as described in *Materials and Methods*. Mixtures for measuring *oriC* and *ori*λ DNA synthesis contained 300 pmol of pCM960 *oriC* plasmid DNA or M13*ori*λ1 plasmid DNA, respectively, instead of mini-P1 DNA. Where indicated, 100 ng of RepA, 100 ng of DnaA, or 1 μg of λ O protein and 50 ng of λ P protein were added.

Synthesis reached a plateau at about 2 μg of RepA per ml, at which the molar ratio of RepA dimers to binding sites on the P1 DNA was about 1. A 100-fold excess of RepA inhibited the reaction about 25%.

With high concentrations of RepA, 100-fold higher than the K_m, mini-P1 DNA synthesis did not require additional DnaA protein above the amount present in the *E. coli* enzyme fraction (Table 3). The addition of this amount of RepA to reaction mixtures measuring *oriC* DNA synthesis did not satisfy the DnaA requirement, suggesting that the RepA was not contaminated with DnaA. Furthermore, with these conditions, mini-P1 DNA synthesis was still sensitive to antibody to DnaA protein, suggesting that the absolute requirement for DnaA had not been bypassed. These results suggest that the role of DnaA protein in mini-P1 replication may be different than its role in *oriC* replication.

Identification of the Origin of Replication and the Direction of Replication. The DNA products synthesized *in vitro* were isolated from reaction mixtures by phenol extraction, treated with RNase, and analyzed by electron microscopy as described in *Materials and Methods*. The spreading condition for electron microscopy (27) was such that the DNA, rather than appearing as supercoiled molecules, appeared as relaxed closed circular molecules with occasional small denaturation

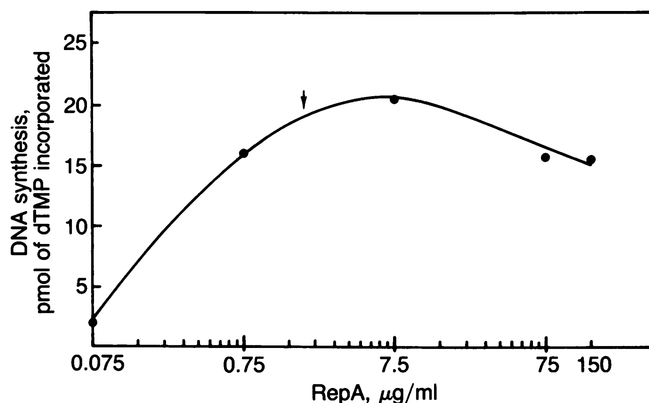

FIG. 4. Effect of RepA concentration on mini-P1 replication. DNA synthesis was measured in replication reaction mixtures as described in *Materials and Methods* but with the indicated amounts of RepA. Arrow indicates the condition where RepA dimers are equimolar with RepA DNA binding sites.

Table 3. Requirement for DnaA protein is different for replication of mini-P1 and *oriC*

Proteins added to *E. coli* enzyme fraction			DNA synthesis, pmol of dTMP incorporated	
RepA	DnaA	Antibody to DnaA	*oriC*	Mini-P1
+	+	−	17.1	19.0
−	+	−	17.5	0.4
+	−	−	0.2	14.2
+	−	+	ND	<0.2
+	+	+	ND	8.8

Reaction mixtures contained 300 pmol of either mini-P1 DNA or *oriC* plasmid DNA. Where indicated, 1 μg of RepA (10-fold more than described in *Materials and Methods*) and 0.2 μg of DnaA protein were added. Incubations with antibody to DnaA were as described in the legend to Table 1. ND, not done.

bubbles. Apparently the DNA was at the threshold of denaturation. The frequency of branched molecules (θ structures) indicative of replication intermediates was less than 1% of all circular molecules.

To increase the number of replication intermediates, ddTTP was included in the *in vitro* reaction mixtures. In the presence of this chain terminator, about 10% of the DNA molecules observed were θ structures. Some of these θ molecules were three-stranded in the replicated region (D loops). Another 10% of the structures were interlocked circles, figure-eight forms, and aggregates of branched molecules. All of these structures were at least an order of magnitude less prevalent in DNA preparations from reaction mixtures without RepA. To map the position of the branch points in θ structures, the DNA was linearized by digestion with *Bam*HI endonuclease prior to electron microscopic examination. As expected, linear structures were seen with replicated regions instead of θ structures (Fig. 5*A*). They were presumed to be early replication intermediates whose forks had not passed the *Bam*HI site. DNA molecules were also seen that had a Y at each end (Fig. 5*B*). These were presumably late replication intermediates in which the restriction site fell within the replication bubble. The distance from each end of the molecule to the first fork and the distance between forks were measured for 69 molecules. These data indicated that one fork was preferentially positioned at about 26% from one end, independent of the size of the replication bubble. This position coincides with the position of the P1 origin relative to the *Bam*HI site as defined genetically (ref. 5; Fig. 1). The data were further analyzed by plotting the distance of each of the branch points (Y_1 and Y_2) from the end of the shorter of the two unreplicated regions,

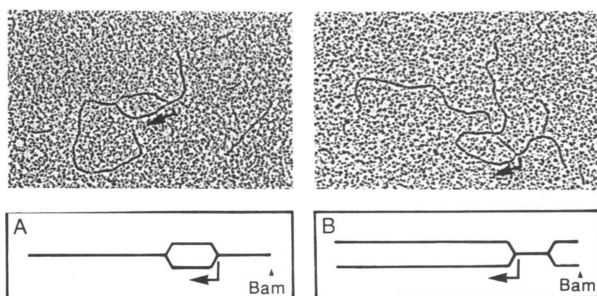

FIG. 5. Electron micrographs of replication intermediates of mini-P1 plasmids linearized with *Bam*HI endonuclease. An example of an early replication intermediate is shown in *A*; a later replication intermediate is shown in *B*. For each, the tail of the arrow marks the origin and the head shows the direction of replication as deduced from the position of the branch points relative to the *Bam*HI site (see Fig. 6). For clarity the molecules are also shown schematically below the micrographs.

except when the replicated region (X) exceeded 50% of the molecule (Fig. 6). The slope of the line representing the second branch point is about 1, and the two lines can be extrapolated to the same point at 0% replication. These results are expected when replication starts from a unique origin and proceeds unidirectionally. Thus, in our *in vitro* reaction mixtures, mini-P1 DNA replication is initiated in the region of the P1 origin and proceeds unidirectionally leftward (summarized in Fig. 1). The electron micrographic data also show that about 30% of the molecules initiated replication outside of the P1 origin, but these starts appeared to be random.

DISCUSSION

We have described an *in vitro* system that replicates mini-P1 DNA. This study establishes that mini-P1 replication *in vitro* requires two initiation proteins, RepA and DnaA, in agreement with the results of genetic experiments (2, 3). Genetic studies could not determine whether DnaA acted directly at the P1 origin or acted indirectly through other host proteins (3). This second possibility could not be excluded because of the observations that DnaA regulates gene expression (29) and DnaA boxes occur in places other than the origins of replication (7). Our results show that DnaA protein is directly involved in the initiation of P1 replication, although its binding to the DnaA boxes in the origin region remains to be shown. It should be noted that plasmids like mini-R1 and

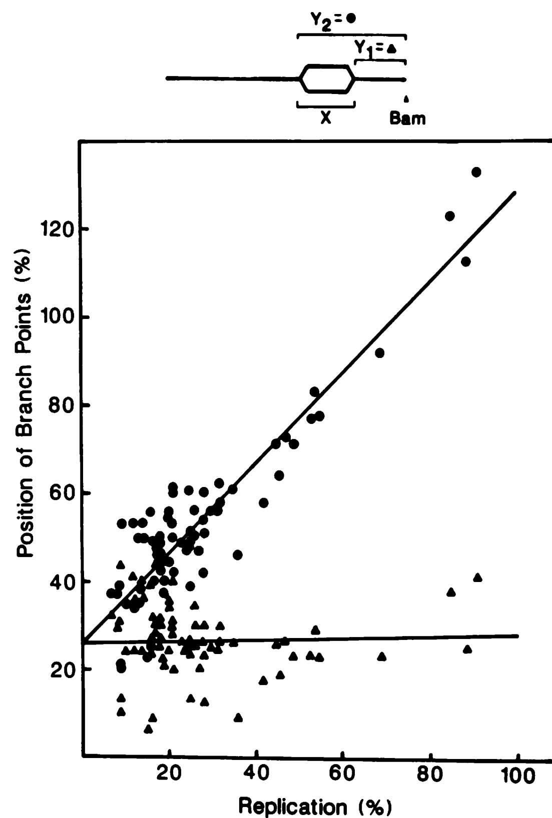

FIG. 6. Distribution of the position of branch points in mini-P1 replication intermediates. Replication intermediates were analyzed after linearization with *Bam*HI. As shown in the schematic at the top of the figure, X represents the length of the replicated DNA between the two branch points. Y_1 represents the shorter of the two unreplicated arms when $X < 50$ (Y_1 represents the longer arm when $X > 50$). Y_2 represents $X + Y_1$. For highly replicated molecules, X can approach 100; Y_2 in that case can approach 126, since the average value of Y_1 is about 26, the position of the origin. Two lines were drawn by linear regression analysis of the points X,Y_1 (▲) and X,Y_2 (●).

pBR322 possess DnaA boxes within 100 bp of their origins and may also utilize DnaA protein (34, 35). Although mini-P1 and mini-F do not replicate in *dnaA*-null mutants, both plasmids can integratively suppress *dnaA* temperature-sensitive mutants (3, 30). These observations, as well as our results (Table 3), suggest that the role of DnaA protein in P1 and F replication may be different from its role in *E. coli* replication.

Our *in vitro* results are surprising in one respect: replication was not appreciably inhibited by high concentrations of RepA, even with molar ratios of RepA dimers to binding sites of greater than 100. In contrast, *in vivo* mini-P1 replication, as measured by plasmid stability in the absence of selection, was decreased slightly when the molar ratio of RepA to binding sites was 10 and was undetectable when the ratio was 60 (ref. 5 and unpublished data). Excess RepA might render the origin less active for initiation due to aggregation of the protein with itself, DNA, or other host proteins. Alternatively, it is possible that overproduction of RepA causes increased expression of another plasmid-encoded factor that inhibits replication *in vivo*. There are other open reading frames within the *repA* genes of both mini-P1 and mini-F, but their significance has not been established (2, 31). The *in vitro* results might then be explained by assuming that the second factor was removed during the purification of RepA. Further biochemical studies should reveal the mechanism of replication inhibition.

Our *in vitro* results establish the position of the origin and the direction of mini-P1 replication. Since the direction of replication of mini-P1 DNA has not been determined *in vivo*, the physiological significance of the *in vitro* results remains to be seen. Two other replicons, mini-F and pSC101, have origin regions organized similarly to P1 in that each contains initiation protein binding sites, DnaA boxes, and an A+T-rich region. Both of these plasmids replicate *in vivo* unidirectionally from the origin away from the initiation protein gene (32, 33) as does mini-P1 *in vitro*.

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