Protein-DNA Interactions in Regulation of P1 Plasmid Replication

ANN L. ABELES,* LUCRETIA D. REAVES, AND STUART J. AUSTIN

Laboratory of Chromosome Biology, BRI-Basic Research Program, National Cancer Institute-Frederick Cancer Research Facility, Frederick, Maryland 21701

Received 11 April 1988/Accepted 10 October 1988

The P1 RepA protein appears to play three roles in P1 plasmid replication: acting at the origin both as a specific initiator and as a repressor of transcription, and interacting with the copy-control locus *incA* to bring about a negative control of initiation. We have used the DNase I footprinting technique to show that RepA binds specifically to repeat units of a 19-base-pair consensus sequence present in both the origin and *incA* control regions. RNA polymerase was shown to bind to two specific regions within the origin repeats. One of these constitutes the known promoter sequence for the *repA* gene. We show evidence that the polymerase can be efficiently displaced from the promoter by subsequent RepA binding, thus providing a direct mechanism for RepA autoregulation. Under the conditions used, there were no obvious differences in the affinities of individual repeat sequences for the purified protein.

The prophage form of bacteriophage P1 is maintained in its host, *Escherichia coli*, as a low-copy-number plasmid. Despite the fact that cells may only contain one or two copies of the plasmid, generation of plasmid-free segregants occurs very rarely (31). This fact implies that the plasmid makes use of a highly accurate system for the regulation of its DNA synthesis and the subsequent segregation of the copies to daughter cells.

All necessary plasmid-borne information for controlled replication is present in a 2-kilobase region of the 100-kilobase P1 genome (3). This region contains a functional origin of some 250 base pairs (bp), followed by an open reading frame for the synthesis of the RepA protein and a copy-control locus, *incA* (3, 10). The origin is the only part of the sequence that is essential in *cis* to the plasmid for P1-specific replication. The RepA protein is also required but can be supplied in *trans* to the plasmid (10). Plasmids lacking the *incA* locus are able to replicate but have an eightfold-higher copy number than does the wild type (8, 28).

P1-specific replication can be observed in vitro by using templates that contain only the minimal origin (2). Highly purified RepA protein is the only P1-specified product required for in vitro synthesis and is absolutely required for replication (2, 36). Thus, RepA is presumably an initiator protein that directs the host DNA-synthetic machinery to initiate replication at the P1 origin. Binding of RepA to the origin DNA was detected by a gel band-retardation method (1). The origin region that bound RepA contained five imperfect repeats of a 19-bp sequence with the consensus 5'-GATGTGTGCTGGAGGGAAA-3', which suggested that this sequence constitutes a specific binding site for the protein. A further nine repeats with the same consensus sequence are present in the *incA* copy-control locus. DNA fragments containing subsets of these *incA* repeats also bind purified RepA in gel-band retardation assays (1).

In addition to acting as a specific initiator, RepA binding at the origin must serve an additional purpose. The promoter for expression of the *repA* gene is located within the origin region, corresponding to a sequence spanning the rightmost three 19-bp repeats (10). RepA synthesis is autoregulated by repression of transcription of the *repA* gene (10). The sim-

plest explanation of this effect would be that RepA binding to the repeat region blocks *repA* promoter function.

In summary, RepA appears to play three roles in P1 replication: it acts at the origin both as a specific initiator and as a repressor of transcription, and it interacts with the copy-control locus to bring about a negative control of initiation. To further investigate the action of RepA with the relevant DNA sites, we have examined these interactions by DNase I footprinting.

MATERIALS AND METHODS

Bacterial strains and plasmids. The RepA protein was purified from *E. coli* K-12 OR1265 (F⁻ Str^r *his* *ilv* *galp308::IS2* [λ N7 N53 *ci857* *cro27* *cry*]) (30) carrying plasmid pALA131 (1). Strain N100 (F⁻ *galK2* Str^r *recA*) (14) was the host for preparing plasmid DNA for footprinting. The plasmids used were pBR322 (6), pALA12, pALA18, and pALA145 (3), and pALA148 and pALA335 (1).

Media and buffers. The cells were grown in L broth (14) with 25 μ g of ampicillin per ml, and plasmids were amplified by the addition of chloramphenicol to 0.2 mg/ml. Plasmid DNA and DNA fragments were suspended in TE buffer (10 mM Tris chloride, 1 mM EDTA) (23). The footprinting reactions were performed in buffer A (20 mM Tris chloride [pH 8], 40 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 50 μ g of bovine serum albumin per ml, 25 to 50 mM NaCl). Footprinting reactions with RNA polymerase were performed in buffer B, in which 20 mM Tris acetate (pH 8) and 40 mM potassium acetate were used in place of the Tris chloride and KCl in buffer A (21). ATP (500 μ M) and 50 μ M each CTP and UTP were added to buffer B to allow formation of initiation complexes. The buffer used for the polynucleotide kinase reactions was described by Maxam and Gilbert (25). Electrophoresis was performed in Peacock buffer (29). Sephadex G25-SF (Pharmacia Fine Chemicals, Piscataway, N.J.) was equilibrated in TE buffer. Salmon sperm DNA, (Pharmacia) used as a carrier in the footprinting reactions, was suspended in TE buffer at 4 mg/ml and sonicated until the average fragment size was less than 1,000 bp. RepA buffer was 50 mM Tris chloride (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 1 mM β -mercaptoethanol, and 10% glycerol.

Enzymes. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.),

* Corresponding author.

FIG. 1. Map of the P1 replicon and fragments used for DNase I footprinting. (A) Map of the minimal P1 replicon. The origin region is marked by an open box above the map. The open reading frame for the essential replication protein, *repA*, is marked with a stippled arrow below the map. Note that the map is discontinuous in order to emphasize the regulatory regions at the ends of the replicon. The numbers above the map give the coordinates in base pairs. The repeat sequences present in the origin and in the copy-control element *incA* are numbered and shown by small solid arrows below the map. The location of the predicted DnaA binding site is labeled. The substrate sites for the *dam* methylase are shown by the short vertical lines marked GATC. The location of the *repA* promoter is marked with a short arrow and labeled *PrepA*. Locations of several restriction sites are shown above the map. (B) Maps of the P1 regions used for footprinting. The plasmids containing cloned regions of P1 used to prepare the DNA for footprinting are shown. The regions excised from these plasmids and the strands whose footprints were determined are shown below the P1 map. For clarity, some of the restriction sites used to obtain the fragments are indicated. The solid box in the map of pALA335 indicates the location of the *rep-11* deletion. In some cases, the sites used to excise the fragments were in the adjoining vector, such as the *ClaI* and *EcoRI* in pALA145, the *HindIII* in pALA148, and the *BamHI* in pALA335. The fragments are more fully described in Materials and Methods. The P1 DNA is indicated by the striped line. The small arrows below the line show the direction in which the sequence was read, and asterisks indicate the location of the 5' ^{32}P label.

Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or New England BioLabs, Inc. (Beverly, Mass.) and were used as recommended by the suppliers. T4 polynucleotide kinase was from Pharmacia, lysozyme and bovine serum albumin were from Sigma Chemical Co. (St. Louis, Mo.), calf intestine alkaline phosphatase was from Boehringer Mannheim, and *E. coli* RNA polymerase was from Promega Tec. P1 RepA protein was purified as previously described (1) and was stored at 0°C in RepA buffer containing 0.5 M NaCl. DNase I (Boehringer Mannheim) was suspended in 2.5 mM HCl at 3 mg/ml and stored at 4°C.

Gel electrophoresis. Restriction digests were monitored in and DNA fragments were isolated from 5% polyacrylamide gels run in Peacock buffer (29). Footprint gels were 6% polyacrylamide gels with 8 M urea and were run in 1.1× Peacock buffer. The dye for the reactions was 7 M urea, 0.05% bromphenol blue, and 0.1% xylene cyanol FF.

DNA preparation. Plasmid DNA was extracted from cells and purified as previously described (3). The plasmids were digested by restriction enzymes, and the desired fragments were isolated and extracted from polyacrylamide gels according to the method of Maxam and Gilbert (25). Fragments to be labeled with ^{32}P were treated with calf intestine alkaline phosphatase, phenol extracted, and alcohol precipitated and then suspended in 50 μl of TE buffer and purified over a 1-ml column of Sephadex G25-SF essentially as described by Maniatis et al. (23) except that the buffer was TE. Labeling and purification of DNA fragments were done as previously described (1) except that the unincorporated ATP was removed by passage over a 1-ml Sephadex G25-SF

column, followed by addition of salt and ethanol precipitation. The fragments used are shown in Fig. 1 (except for the pBR322 fragment, which was the *EcoRI*-to-*BamHI* fragment of pBR322, end labeled with [^{32}P]ATP and cut with *ClaI*). The others were prepared as follows: the *BamHI*-to-*EcoRI* fragment of pALA145, which encompasses the *incC* region (bp 473 to 606, with a *BamHI* linker added at bp 473) was end labeled and cut with either *ClaI* or *DdeI* to enable reading of each strand. The *DraI* (*AhaIII*)-to-*HindIII* fragment of pALA148, which carries the origin region (bp 87 to 598 [*HincII*]) was cut with *TaqI*, labeled, and cut again with *DdeI*. *DdeI* cuts at bp 488 between the last methylation site and repeat 14. The *DraI*-to-*BamHI* fragment of pALA335, encompassing the *rep-11* mutant origin, was cut with *HinfI*, end labeled, and cut again with *DdeI*. The *BamHI* fragment of pALA18, which carries the *incA* region (bp 1507 to 1812, with *BamHI* linkers attached at the *AluI* sites), was end labeled and cut with either *HhaI* or *Sau3AI*. The *PvuII*-to-*HindIII* fragment of pALA12 (bp 1379 to 1851, the *incA* region) was cut with *HgiAI*, end labeled, and cut again with *TaqI*.

Footprinting. The footprinting reactions were adapted from Galas and Schmitz (13) and were performed in 100 μl of buffer A with 0 to 120 pmol (0 to 10 μg) of RepA and 0.02 to 0.8 pmol (0.005 to 0.04 μg) of ^{32}P -labeled DNA fragment per reaction. RepA (0.2 to 0.8 $\mu\text{g}/\mu\text{l}$) was diluted 1/5 into RepA buffer just before addition to the reactions. The reactions were assembled, except for the DNase I (and heparin when used), and allowed to equilibrate at 30 or 37°C for 20 to 30 min. The DNase I (3 mg/ml) was diluted 1/10 in 10 mM Tris

chloride (pH 8) just before use. A 1- μ l amount of the dilute DNase I was used for each reaction, giving a final concentration of 3 μ g/ml. The reactions were stopped after 1 to 3 min by the addition of 100 μ l of phenol-chloroform-isoamyl alcohol (24:24:1) and 11 μ l of 3 M sodium acetate containing 0.36 mg of sonicated salmon sperm DNA per ml. The samples were mixed by vortexing and centrifuged for 5 min, and the phenol layer was removed. The samples were reextracted with phenol, alcohol precipitated, and dried. When RNA polymerase was used, the reactions were performed in buffer B. Buffer B contained ATP, CTP, and UTP (the complement of the first one or two bases of the *repA* message) to allow the RNA polymerase to form an initiation complex. (RNA polymerase footprints were not detected with only ATP and CTP present.) Each reaction mixture contained 0.04 to 0.9 U of RNA polymerase, and the preincubation time was 30 min at 37°C. Most of the experiments were performed with 0.9 U of RNA polymerase. In some cases, the reactions were treated with 5 μ g of heparin per ml for 10 s before addition of DNase I. The heparin treatment was used to reduce the nonspecific binding of the RNA polymerase to the DNA. However, it was not used when RepA was present since it disrupted the RepA-DNA interactions. The Cherenkov counts of the dried samples were determined, and the samples were resuspended in the urea dye to give 2,000 to 4,000 cpm/ μ l. A 2- μ l amount of each sample was applied per lane of the 6% urea-polyacrylamide gel, and the gels were electrophoresed at 1.5 to 2 kV. Equal portions of the end-labeled DNA were also used for the A+G and C+T sequencing reactions described by Maxam and Gilbert (25). These reactions were electrophoresed simultaneously to allow identification of the sequences in the footprinting reactions. The gels were dried and exposed to X-AR5 film (Eastman Kodak Co., Rochester, N.Y.) for 1 to 5 days at -70°C.

RESULTS

RepA protein recognizes the 19-bp repeat sequences in the origin. We studied the interaction of purified RepA protein with the origin DNA by using the DNase I protection (footprinting) method (13). Examples of the footprints with RepA are shown in Fig. 2 and summarized in Fig. 5A. Figures 2A and B compare the protection patterns observed for a fragment of pBR322 and the P1 *incC* region. For roughly the same low molar ratios of RepA to DNA (Fig. 2, lanes c), RepA specifically protected the P1 DNA and showed little binding to pBR322. At higher molar ratios (lanes b), some nonspecific binding to pBR322 was observed, whereas there was little change in the P1 footprint. At the highest ratios used in this figure, there was some nonspecific binding on the P1 DNA. Figures 2C and D compare the protection patterns for the strands across the *incC* region. For comparable molar ratios (Fig. 2C, lane c, and 2D, lane b), specific protection was observed for the repeats. In Fig. 2C, the third or fourth bases from the 3' end of the repeat showed enhanced digestion for repeats 14, 13, 12, and 10. This enhancement was not observed for repeat 11. However, the protection pattern was about the same for all of the repeats. For the other strand (Fig. 2D), protection was observed for all repeats at all concentrations of RepA shown. On this strand, bases more toward the middle of the repeats showed enhanced cutting for repeats 12, 13, and 14 and a weaker effect for repeats 11 and 10. As the RepA concentration was increased, there was some evidence of increasing protection of bases in repeats 13 and 14 (Fig. 2C)

and in all repeats in Fig. 2D. It appears that the individual repeats had similar affinities for the protein and that the loading order was more or less random. It is not possible to say whether the subtle differences in protection of the individual repeats reflect differences in the sequences or in protein binding to the repeats. Regions of the origin outside the repeats showed some nonspecific binding, but no pattern of protection and enhancement was observed.

Protection of the repeat sequences of both DNA strands was detected, although the strand distribution of the protected regions appeared somewhat asymmetric. The spacing of the origin repeats was such that essentially the same region of sequence was exposed every two turns of the helix. This is more easily visualized in the helical drawing in Fig. 3. This projection also reveals how the protein protects the DNA and leaves an opposing region of each strand exposed to enhanced attack by DNase I.

We carried out analogous binding studies to DNA containing the mutant origin *rep-11*. This mutant has lost two of the five origin repeats by a recombination event that leaves repeats 13 and 14 intact and creates a hybrid repeat from portions of repeats 10 and 12. The protection pattern was minimally disturbed by this deletion. Protection of repeats 13 and 14 was unaltered from the wild-type case, and the hybrid repeat gave a pattern like that for the wild-type repeat 11, whose sequence it closely resembled (data not shown).

RepA displaces RNA polymerase from the origin repeats. Binding studies using *E. coli* RNA polymerase were carried out under conditions in which abortive initiation of transcription can occur but further synthesis is blocked. Under these conditions, the polymerase forms a relatively stable open complex at the promoter (15). The RNA polymerase protection pattern observed was different from that given by RepA alone (Fig. 4). The interaction of RNA polymerase as well as RepA plus RNA polymerase with the upstream origin region is shown in Fig. 4A. RepA alone showed little protection of this region (data not shown). Likewise, RNA polymerase did not specifically protect the DNA of this region except perhaps near the end of the fragment (top of Fig. 4A, lane b). However, RNA polymerase did show some interaction with this fragment, as can be seen by the changed band intensities over the whole fragment. When RepA was present along with RNA polymerase, this protection and most of the other interactions were lost and the pattern was similar to that with RepA alone or without any added protein. There were a few bases showing enhanced digestion in the presence of RNA polymerase, and many of these enhancements were lost when RepA was added. The protection pattern for the region of the *incC* repeats is shown in Fig. 4B through D. When RepA was added after the RNA polymerase, the protection pattern was similar to that with RepA alone (Fig. 4B, compare lanes a through c). After addition of RepA, the patterns of protection and enhanced bands in the region of the repeats were typical of the RepA pattern. RNA polymerase protection is located across the region of the known *repA* promoter (10) (Fig. 4C and D, lanes d through f). The protection patterns with RepA alone or with RepA and RNA polymerase as well as with three concentrations of RNA polymerase are shown in Fig. 4C and D. RNA polymerase protection was predominantly over the *repA* promoter, located between repeats 12 and 11 and across repeat 10. RNA polymerase protection was more pronounced on the upper strand (Fig. 4D).

Interestingly, there was a second region in the origin protected by RNA polymerase. The lower strand was protected in a region extending from repeat 14 leftward toward

FIG. 3. Regions of the origin-repeat sequences protected from DNase I digestion by RepA. The sequence of the region is displayed on a cylindrical projection of a B-DNA helix. Symbols: ■, indicate regions strongly protected by RepA; ---, indicate less well protected areas. Points showing enhanced digestion are marked with lines through the sequences. The -35 and -10 sequences in the promoter region and the +1 position for the message are marked. (Note the symmetry in the regions marked by the bow-shaped brackets.)

the core of the origin (Fig. 4B, lane b). This binding also appeared to be reversed by subsequent addition of RepA (Fig. 4B, lane c). Figures 5B and C summarize the protection observed with RNA polymerase and both proteins together.

There was evidence of interactions of the polymerase with some additional sequences outside the repeat regions of the origin near the DnaA recognition sequences. Some of these interactions were not inhibited by RepA and may have been

FIG. 4. Footprints of the P1 *ori* region in the presence of RepA and RNA polymerase. The two left lanes of each panel contain the A+G and C+T sequencing reactions. Lanes marked o contain no RepA or RNA polymerase. (A) 0.04 pmol of the pALA148 *TaqI-DdeI ori* region; (B) 0.05 pmol of the pALA148 *HindIII-DdeI incC* region. Protein amounts: a, 10 pmol of RepA; b, 2.6 pmol of RNA polymerase; c, 2.6 pmol of RNA polymerase added first, mixture incubated for 3 min at 37°C, and then 10 pmol of RepA added. Lanes marked o contain no RepA or RNA polymerase. (C) 0.2 pmol of the pALA145 *EcoRI-DdeI incC* fragment; (D) 0.2 pmol of the pALA145 *BamHI-ClaI incC* fragment. Protein amounts: a, 10 pmol of RepA; b, 10 pmol of RepA added, mixture incubated for 3 min at 37°C, and then 2.6 pmol of RNA polymerase added; c, 2.6 pmol of RNA polymerase added, mixture incubated for 3 min at 37°C, and then 10 pmol of RepA added. Lanes d through f: 2.6, 5.2, and 7.8 pmol, respectively, of RNA polymerase added. The reactions were carried out as described in Materials and Methods.

FIG. 5. Summary of the DNase I footprinting for the P1 origin region from several footprinting experiments. (A) Regions of the P1 origin protected by RepA from DNase I digestion; (B) regions protected by RNA polymerase; (C) regions protected by both RepA and RNA polymerase when present together. Symbols: ■, regions of strong protection; ---, regions of weaker protection; ↓, places showing enhanced digestion; ↗, positions of strongly enhanced digestion; →, beginning of the minimal origin. The proposed DnaA-binding site (12), *dam* methylase sites, and repeat sequences are boxed. The -35, and -10 regions of the promoter and the +1 position showing the start of the RepA message are indicated (10). The small numbers above each line of sequence give the base pair coordinates (the terminal 0s are located above the numbered base pairs). In some regions, DNase I does not cleave between some of the bases. If these regions were surrounded by regions showing good protection, the solid bars were continued across the regions.

nonspecific interactions, since RNA polymerase will bind DNA nonspecifically.

RepA protein interacts with the repeat sequences of the copy-control region, *incA*. As we have previously shown by a gel band-retardation assay, RepA also interacts with the DNA region containing the *incA* repeats. An example of DNase I footprinting of this region is shown in Fig. 6, and the data are summarized in Fig. 7. The *incA* repeats differed from those in the origin in that they were separated by longer spacer regions, generally of 10 or 20 bp. RepA gave considerable protection of the DNA throughout the repeat region, sometimes extending into the spacer regions. The spacers between repeats 9 and 8, 8 and 7, 7 and 6, 6 and 5, and 3 and

2 showed symmetrical patterns of enhanced cutting on each strand, which indicated that part of these sequences remained unprotected and were left in a configuration that was DNase I sensitive. RNA polymerase showed very little interaction with the *incA* region, and addition of both RepA and RNA polymerase gave a pattern very similar to that obtained with RepA alone (Fig. 6).

DISCUSSION

Our studies indicate that some initial events at the origin can now be defined. RepA appears to bind to individual repeat units in the tightly packed origin array in a rather

FIG. 6. Examples of the *incA* footprint gels. Footprints of both strands of the *incA* region encompassing repeats 6 through 9 are shown. The two left lanes of each panel show the sequence as determined by the Maxam and Gilbert A+G and C+T sequencing reactions (25). Lanes a, c, d, and g show the DNA treated with DNase I. In lanes b, the DNA was preincubated with 9 pmol of RepA for 30 min before DNase I treatment. In lanes e, the DNA was preincubated with 2.6 pmol of RNA polymerase for 30 min before DNase I treatment. In lanes f, the DNA was preincubated with 2.6 pmol of RNA polymerase for 2 min, 9 pmol of RepA was added, and the incubation was continued for 30 min before DNase I treatment. Details of the reactions are given in Materials and Methods. Lanes in panel A contained ca. 8 ng (0.04 pmol) of the *Bam*HI-to-*Sau*3AI fragment of pALA18; lanes in panel B contained ca. 8 ng (0.03 pmol) of the (5') *Taq*I-to-*Hgi*AI fragment of pALA12 (see Fig. 1). The sequence of each fragment, beginning at the bottom and reading left to right, is shown.

FIG. 7. Summary of the regions of *incA* protected by RepA from DNase I digestion. The fragments used for footprinting are shown in Fig. 1, and an example of an *incA* footprint gel is shown in Fig. 6. The technique is described in Materials and Methods. Symbols: ■, strongly protected regions; — — —, areas of weaker protection; ↓, points of enhanced digestion. The repeated sequences are boxed and numbered. The small numbers over the sequences give the base pair coordinates. The 0s are over the numbered base pairs.

uniform fashion. The RepA protein used in these assays is competent for initiation *in vitro*, using isolated origin DNA as a template (2). As no significant binding to any other region of the origin was seen under the conditions used, it seems likely that RepA binding to the repeat sequences is the primary event that gives rise to a RepA-specific signal to trigger initiation. Binding to multiple repeats seems to be essential for this signal, since the *rep-11* mutant that lacks two repeat units from within the array has no origin function (1, 5) but RepA still binds efficiently to the remaining repeats. This binding of the replication protein to the repeats associated with the plasmid replicon has been observed for several other plasmids, such as F, Rts1, R6K, and pSC101, and has been documented by footprinting studies (11, 17, 18, 20, 22, 24, 26, 33, 35). The replication proteins for plasmids R6K and pSC101 are able to displace RNA polymerase from their promoters (11, 20, 35). This is probably the mechanism by which they autoregulate their production. However, the mechanism by which this binding participates in regulating replication has not been elucidated.

Our results suggest that for P1, origin binding for initiation and *repA* transcription control are two aspects of the same event. Specific binding of RNA polymerase to the promoter sequence encompassing the three rightmost origin repeats was detectable in the absence of RepA. RepA appears to displace RNA polymerase from the transcription-initiation complex. This displacement is likely to be the basis for P1 RepA autoregulation, as has been shown for pSC101 and R6K (11, 20, 35).

We found some evidence for a second possible RNA polymerase-binding site in the leftmost part of the origin-

repeat region (Fig. 4B) and are currently investigating whether this region represents a genuine promoter. The fact that this site is also competed for by RepA binding suggests that it might play some role in origin-region control. Inspection of the sequence suggests that there is a possible promoter here (−35 at bp 522 through 517 and −10 at bp 499 through 494) that reads to the left, i.e., in the direction of the replication fork (36). Host RNA polymerase is required for mini-P1 replication *in vitro* (36), and it is conceivable that this leftward polymerase-binding site is involved positively in primer formation or negatively in transcriptional activation of the origin. The ability of RepA protein to compete with polymerase for binding to this site could then be the basis for the ability of RepA to switch off replication when present in the cell at abnormally high concentrations (10, 28, 32). We note that this putative binding site is near the end of the fragment (20 to 30 bp from the end) and could therefore represent artifactual end binding. This binding was also not observed for the reactions using about four times more DNA and the same amount of RNA polymerase (Fig. 4C and D, lanes d).

The F, Rts1, and P1 plasmids each have two sets of repeat sequences that bind their respective replication proteins (17, 18, 24, 26, 33). These sequences have been shown to negatively regulate plasmid replication (3, 8, 9, 17, 19, 26). The negative role for the P1 *incA* locus in replication control can be demonstrated in two ways: deletion of *incA* repeats causes an increase in copy number (8, 28), and addition of an excess of these sequences to the cell, either in the host chromosome or on a plasmid vector, causes loss of a resident P1 plasmid, presumably by blocking its replication

(4, 8, 27). Binding of RepA protein to the *incA* region initially suggested that the negative effect exerted by *incA* worked by titrating RepA. It was thought that the RepA concentration might normally be limiting for initiation and that *incA* might exert its effect by binding sufficient RepA to further limit availability of the protein to the origin (8, 10, 28). However, as RepA synthesis is autoregulated, it would be expected that the free RepA concentration would tend to be kept constant irrespective of withdrawal of protein from the pool by binding to the *incA* sites, making the titration model less attractive. Recent work by Chatteraj et al. has shown that the inhibition due to the *incA* repeats cannot be overcome completely by additional RepA and therefore must be exerting its inhibition by some mechanism other than sequestering of RepA (9, 27).

Trawick and Kline (34) proposed a model in which two forms of the Rep protein exist, a repressor and an initiator. We have seen no evidence of two forms of the protein in our preparations. The same preparations that show binding to these DNA sequences also support replication *in vitro* at the same molar ratios of RepA to DNA (2). Another suggestion for the mechanism of control requires that a strict hierarchy of RepA-binding affinities exists, differentiating between autoregulatory, initiation, and control sites (7). Our results do not favor this idea, since the affinities for the regions responsible for these activities seem to be more or less equal.

Pal and Chatteraj present a model in which the compensatory effect of autoregulation is prevented by assuming that protein bound to the control repeats can still act as a repressor by causing DNA folding (27). They propose that replication control of P1 occurs by steric hindrance when the *incA*-RepA complexes fold back and bind the *incC* repeats, thus blocking both RepA production and initiation of replication.

The data from our experiments would tend to support this model. Our footprinting experiments indicated that RepA bound to both the *incC* and *incA* repeats and did not show strong differences in affinity between the individual repeats. However, the protection pattern observed for the *incC* repeats was different from the pattern for *incA*, which suggests that the protein-DNA interactions may be somewhat different for the two sets of repeats. The possibility that the *incA* and *incC* repeats may interact differently with RepA is also suggested by the fact that RepA appears to be a dimer in solution (32), and yet the binding sites do not show the symmetry usually associated with the binding of dimers such as is seen with the λ repressor (16). In addition, the mobility shift patterns observed with RepA binding to intact *incA* fragments suggested that the interactions with *incA* might be more complex than the binding shown with *incC* (1). Although Pal and Chatteraj suggest that the main block to replication is by the *incA*-RepA-*incC* complexes, other combinations are possible. Plasmids deleted for *incA*, although they exist at a slightly higher copy number, also exhibit controlled replication (8, 27). In these cases, inhibition could be via *incC*-RepA-*incC* interactions. These interactions may not be quite as effective as those involving *incA* repeats because the copy number increases to about 10 for *incA*-deleted plasmids (8, 27). This role for inhibition by the *incC* repeats is also supported by our early observations that the *incC* repeats, when present on the high-copy-number pBR322 plasmids, blocked P1 replication but had no obvious effect on P1 stability when present in *trans* on a low-copy-number vector (3).

ACKNOWLEDGMENTS

We thank our colleagues Kathy Martin, Therese Brendler, and Michael Davis for helpful advice and careful reading of the manuscript, Janet Newlands and Richard Gourse for advice for RNA polymerase footprinting, and Julie Ratliff for final preparation of the manuscript.

This research was sponsored by the National Cancer Institute under contract NO1-CO-74101 with Bionetics Research, Inc.

LITERATURE CITED

1. Abeles, A. 1986. P1 plasmid replication: purification and DNA-binding activity of the replication protein RepA. *J. Biol. Chem.* **261**:3548-3555.
2. Abeles, A. L., and S. J. Austin. 1987. P1 plasmid replication requires methylated DNA. *EMBO J.* **6**:3185-3189.
3. Abeles, A., K. Snyder, and D. Chatteraj. 1984. P1 plasmid replication: replicon structure. *J. Mol. Biol.* **173**:307-324.
4. Austin, S., F. Hart, A. Abeles, and N. Sternberg. 1982. Genetic and physical map of a P1 miniplasmid. *J. Bacteriol.* **152**:63-71.
5. Austin, S. J., R. J. Mural, D. K. Chatteraj, and A. L. Abeles. 1985. Trans- and cis-acting elements for the replication of P1 miniplasmids. *J. Mol. Biol.* **183**:195-202.
6. Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:95-113.
7. Chatteraj, D. K., A. L. Abeles, and M. B. Yarmolinsky. 1985. P1 plasmid maintenance: a paradigm of precise control, p. 355-381. In D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), *Plasmids in bacteria*. Plenum Publishing Corp., New York.
8. Chatteraj, D., K. Cordes, and A. Abeles. 1984. Plasmid P1 replication: negative control by repeated DNA sequences. *Proc. Natl. Acad. Sci. USA* **81**:6456-6460.
9. Chatteraj, D. K., R. J. Mason, and S. H. Wickner. 1988. Mini-P1 plasmid replication: the autoregulation-sequestration paradox. *Cell* **52**:551-557.
10. Chatteraj, D. K., K. M. Snyder, and A. Abeles. 1985. P1 plasmid replication: multiple functions of RepA protein at the origin. *Proc. Natl. Acad. Sci. USA* **82**:2588-2592.
11. Filutowicz, M., G. Davis, A. Greener, and D. Helinski. 1985. Autorepressor properties of the π -initiation protein encoded by plasmid R6K. *Nucleic Acids Res.* **13**:103-114.
12. Fuller, R. S., and A. Kornberg. 1983. Purified *dnaA* protein in initiation of replication at the *Escherichia coli* chromosomal origin of replication. *Proc. Natl. Acad. Sci. USA* **80**:5817-5821.
13. Galas, D. J., and A. Schmitz. 1978. DNAase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5**:3157-3170.
14. Gottesman, M. E., and M. B. Yarmolinsky. 1968. Integration-negative mutants of bacteriophage lambda. *J. Mol. Biol.* **31**:487-505.
15. Hofer, B., D. Müller, and H. Köster. 1985. The pathway of *E. coli* RNA polymerase-promoter complex formation as visualized by footprinting. *Nucleic Acids Res.* **13**:5995-6013.
16. Johnson, A. D., A. R. Poteete, G. Lauer, R. T. Sauer, G. K. Ackers, and M. Ptashne. 1981. Repressor and cro-components of an efficient molecular-switch. *Nature (London)* **294**:217-223.
17. Kamio, Y., Y. Itoh, and Y. Terawaki. 1988. Purification of Rts1 RepA protein and binding of the protein to mini-Rts1 DNA. *J. Bacteriol.* **170**:4411-4414.
18. Kamio, Y., A. Tabuchi, Y. Itoh, H. Katagiri, and Y. Terawaki. 1984. Complete nucleotide sequence of mini-Rts1 and its copy mutant. *J. Bacteriol.* **158**:307-312.
19. Kamio, Y., and Y. Terawaki. 1983. Nucleotide sequence of an incompatibility region of mini-Rts1 that contains five direct repeats. *J. Bacteriol.* **155**:1185-1191.
20. Kelley, W., and D. Bastia. 1985. Replication initiator protein of plasmid R6K autoregulates its own synthesis at the transcriptional step. *Proc. Natl. Acad. Sci. USA* **82**:2574-2578.
21. Liermo, S., C. Harrison, D. S. Cayley, R. R. Burgess, and T. Record, Jr. 1987. Replacement of potassium chloride by potassium glutamate dramatically enhances protein-DNA interaction

- in vitro. *Biochemistry* **26**:2095-2101.
22. Linder, P., G. Churchward, X. Gaijian, Y. Yi-Yi, and L. Caro. 1985. An essential replication gene, *repA* of plasmid pSC101 is autoregulated. *J. Mol. Biol.* **181**:383-393.
 23. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 24. Masson, L., and D. S. Ray. 1986. Mechanism of autonomous control of the *Escherichia coli* F plasmid: different complexes of the initiator/repressor protein are bound to its operator and to an F plasmid replication origin. *Nucleic Acids Res.* **14**:5693-5711.
 25. Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**: 499-560.
 26. Nozue, H., K. Tsuchiya, and Y. Kamio. 1988. Nucleotide sequence and copy control function of the extension of the *incl* region (*incl-b*) of Rts1. *Plasmid* **17**:46-56.
 27. Pal, S. K., and D. K. Chattoraj. 1988. P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites. *J. Bacteriol.* **170**:3554-3560.
 28. Pal, S., R. J. Mason, and D. K. Chattoraj. 1986. Role of initiator titration in copy number control. *J. Mol. Biol.* **192**:275-285.
 29. Peacock, A. C., and C. W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry* **7**:668-674.
 30. Reyes, O., M. Gottesman, and S. Adhya. 1979. Formation of lambda lysogens by IS2 recombination: *gal* operon-lambda PR promoter fusions. *Virology* **94**:400-408.
 31. Rosner, J. L. 1972. Formation, induction and curing of bacteriophage P1 lysogens. *Virology* **49**:679-689.
 32. Swack, J. A., S. K. Pal, R. J. Mason, A. L. Abeles, and D. K. Chattoraj. 1987. P1 plasmid replication: measurement of initiator protein concentration in vivo. *J. Bacteriol.* **169**:3737-3742.
 33. Tokino, T., T. Murotsu, and K. Matsubara. 1986. Purification and properties of the mini-F plasmid-encoded E protein needed for autonomous replication control of the plasmid. *Proc. Natl. Acad. Sci. USA* **83**:4109-4113.
 34. Trawick, J. D., and B. C. Kline. 1985. A two-stage molecular model for control of mini-F replication. *Plasmid* **13**:59-69.
 35. Vocke, C., and D. Bastia. 1985. The replication initiator protein of plasmid pSC101 is a transcriptional repressor of its own cistron. *Proc. Natl. Acad. Sci. USA* **82**:2252-2256.
 36. Wickner, S. H., and D. K. Chattoraj. 1987. 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*. *Proc. Natl. Acad. Sci. USA* **84**:3668-3672.