JOURNAL OF BACTERIOLOGY, Aug. 1987, p. 3737–3742
0021-9193/87/083737-06$02.00/0

Vol. 169, No. 8

P1 Plasmid Replication: Measurement of Initiator Protein Concentration In Vivo

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Received 30 December 1986/Accepted 4 May 1987

To study the functions of the mini-P1 replication initiation protein RepA quantitatively, we have developed a method to measure RepA concentration by using immunoblotting. In vivo, there are about 20 RepA dimers per unit-copy plasmid DNA. RepA was deduced to be a dimer from gel filtration of the purified protein. Since there are 14 binding sites of the protein per replicon, the physiological concentration of the protein appears to be sufficiently low to be a rate-limiting factor for replication. Autoregulation is apparently responsible for the low protein level; at the physiological concentration of the protein, the *repA* promoter retains only 0.1% of its full activity as determined by gene fusions to *lacZ*. When the concentration is further decreased by a factor of 3 or increased by a factor of 40, replication is no longer detectable.

P1 prophage is a stringently controlled plasmid replicon that is maintained at a copy number approximately equal to the number of *Escherichia coli* chromosomes (16). The basic plasmid replicon, the minimal region required to maintain the plasmid at its normal copy number, consists of an origin, the gene for the initiator protein RepA, and a control locus. The salient feature of the basic replicon is the presence of two sets of repeated, nearly identical 19-base-pair sequences. A set of five repeats is an essential part of the origin. A separate set of nine repeats constitutes the control locus (3, 4). The purified RepA protein binds to both sets of repeats (1). Extra copies of the repeats decrease the replication frequency, whereas deletion of the control locus increases the frequency (4, 14). Similar results were obtained for mini-F plasmids, whose genetic organization is very similar to that of mini-P1 (25). It has been proposed (25) that the origin and the control repeats compete for RepA, which has been shown to be limiting for replication (13, 18), implying that the physiological concentration of the protein is so low that sequestration of a few molecules would have a significant effect on the rate of replication. In other words, the number of repeats and the number of RepA molecules are expected to be comparable in the cell. In this paper we report the measurement of RepA concentration by two approaches. These measurements are consistent with the sequestration hypothesis.

The protein is also known to have two other functions. It inhibits the synthesis of the *repA* transcript and, when overproduced, inhibits replication (6). Knowledge of the protein concentration will thus be helpful in understanding how RepA functions. A preliminary report of some of this work has been presented (5).

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MATERIALS AND METHODS

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, TEMED, and glycine were electrophoresis-purity reagents from Bio-Rad Laboratories. Reagent-grade Trizma base, bovine transferrin, bovine erythrocyte carbonic anhydrase, horse heart cytochrome *c*, and immunoglobulin G-free bovine serum albumin (BSA) were from Sigma Chemical Co. Sephadex G 100 was from Pharmacia, Inc. Ultrapure urea was from Schwarz/Mann. ^{125}I-labeled protein A (specific activity, >30 mCi/mg) used in solid-phase radioimmunoassay was from Amersham Corp. ^{125}I-labeled protein A (specific activity, 70 to 100 mCi/mg) used for Western blot (immunoblot) was from New England Nuclear Corp. Nitrocellulose BA83 paper (pore size, 0.22 μm) was from Schleicher & Schuell, Inc. Extractigel D (binding capacity, 80 mg of SDS per ml of resin) was from Pierce Chemical Co.

Preparation of cell extracts. Plasmid-containing *E. coli* strains were grown in L broth with 100 μg of ampicillin per ml, diluted 250-fold, and grown in the same medium to A_{590} of 0.8 as determined spectrophotometrically. Unintentionally, this value was 0.2 A_{590} units beyond the logarithmic phase of growth. The cells were washed once in 50 mM Tris hydrochloride (pH 6.8) containing 10 mM EDTA and finally suspended at 1/100 volume in 66 mM Tris hydrochloride (pH 6.8)–1 mM EDTA–10 mM NaN_3–1 mM phenylmethylsulfonyl fluoride–10% (vol/vol) glycerol. Lysozyme was added to a final concentration of 25 μg/ml. The cell suspension was subjected to three cycles of freezing and thawing (−70 to 37°C) and sonically disrupted by three 30-s bursts at 100 W from a Braunsonic 1510 sonicator with 1 min of cooling between each burst. The lysate, supplemented with $MgCl_2$ (2 mM) and $CaCl_2$ (0.1 mM), was then digested with DNase and RNase (3 μg/ml each) at 37°C for 30 min. 2-Mercaptoethanol and SDS were added to 70 mM and 1%, respectively, and the cleared lysate was boiled in a screw-cap tube for 7 min. The lysate was cooled to room temperature and centrifuged (45,000 rpm for 2 h in a Beckman 50 Ti rotor at 15°C). The

clear supernatant was removed and saved for analysis of the total protein and RepA content. Usually more than 80% of total protein and more than 90% of RepA could be recovered in soluble form. The amount of protein lost was determined by suspending the pellet in sample buffer (66 mM Tris hydrochloride [pH 6.8] containing 2% SDS, 70 mM mercaptoethanol, and 20% [vol/vol] glycerol) by sonication and boiling as described above, centrifuging the solubilized pellet at 18,000 rpm for 20 min in a Sorvall SS-34 rotor, and assaying the supernatant fluid for total protein and RepA content.

Protein determination. The protein concentration was determinated by using amido black as described by Schaffner and Weissman (20). All samples were brought to 1% SDS, and BSA was used as the protein standard. The protein concentration of purified RepA as determined by amino acid analysis was about 30% less than the concentration determined by the amido black method with BSA as standard. The discrepancy apparently reflects the different amino acid composition of RepA and BSA and not the experimental error of protein concentration measurements. The protein concentrations presented in this paper were all expressed relative to a BSA standard.

Antibody production. (i) Preparation of RepA. Highly purified RepA (about 90% pure [1]) was further purified by preparative gel electrophoresis on an SDS-polyacrylamide gel (12.5% polyacrylamide) (11). The RepA band was localized by staining a vertical section of the gel with Coomassie blue. The RepA band from the unstained portion of the gel was cut and used for the first two injections into a rabbit. For the third injection, the protein was electroeluted from the gel and renatured as described previously (10) with the following two modifications: Extractigel D was used instead of Dowex-1 X8 (Bio-Rad) to remove the SDS from the electroeluted sample, and the detergent-free sample was dialyzed for 3 h against a buffer solution of 50 mM Tris hydrochloride (pH 7.6)–200 mM NaCl–10% glycerol–1 mM dithiothreitol–1 mM EDTA. Approximately 15% of the original DNA-binding activity was recovered (data not shown).

(ii) Immunization. A New Zealand White rabbit was injected in the footpads with about 400 μg of RepA homogenized in complete Freund adjuvant. After 4 weeks a second injection of about 400 μg of RepA homogenized in incomplete Freund adjuvant was given subcutaneously. After a further 2 weeks the last injection of about 400 μg renatured RepA in incomplete Freund adjuvant was given subcutaneously. Bleedings were done 7 days after each booster injection and at weekly intervals for the next 2 months. The titer and specificity of the antisera were tested by solid-phase radioimmunoassay (assay no. 2) (23) and Western blot (24), respectively. The antiserum used in these experiments was that taken 7 days after the last injection.

Quantitative Western blot protocol. SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (11). Gels were 12.5% acrylamide and were 1.5 mm thick, 14 cm long, and 12 cm wide. Electrophoresis was performed at 10 mA per gel at 4°C for 16 h. Polypeptides in the gel were electrotransferred at 4°C to nitrocellulose paper (pore size, 0.2 μm) for 3 h to overnight at 60 V by the method of Towbin et al. (24). The nitrocellulose paper was dried in air and stored at 4°C or used immediately.

Nitrocellulose sheets were rehydrated, protein side up (if dried), prior to immunobinding. All incubations were for 2 h at 37°C unless indicated. Nitrocellulose sheets were incubated in 3% immunoglobulin G-free BSA in 0.01 M sodium phosphate (pH 7.4)–0.9% NaCl (PBS). After being rinsed

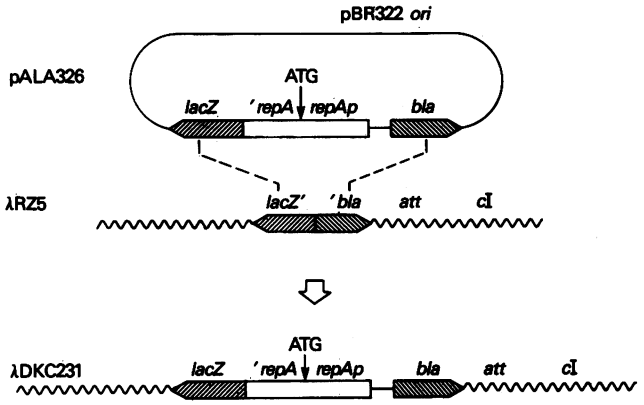

FIG. 1. Construction of *repA-lacZ* gene fusion phage λDKC231. The first 62 codons of *repA* were fused in frame to *lacZ* in a pBR322-derived vector to generate the plasmid pALA326 as described previously (6). The fused region without the pBR322 *ori* was transferred to a λ vector, λRZ5, by using *bla* and *lac* homologies (▨) to generate the phage λDKC231. The tapered ends of the hatched boxes represent the COOH terminal of the proteins. The primes represent partly deleted genes. Other symbols: ▭, P1 DNA; ∿, λ DNA; ——, pBR322 DNA; -----, connections between homology regions. Other details are described in Materials and Methods. Maps are not drawn to scale.

with PBS, the nitrocellulose sheets were incubated with a 1:2,500 dilution of anti-RepA serum in 50 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–5 mM EDTA–0.25% gelatin–0.05% Tween 20 (TGT). Titration of antiserum showed that a 1:2,500 dilution of antiserum gave the optimum signal for this method (data not shown). The blots were washed three times for 10 min each with TGT and then incubated for 2 h with 200,000 cpm of ^{125}I-labeled protein A per ml in TGT. The blots were washed three times in TGT for 10 min each and air dried. Immunoreactive proteins were localized by autoradiography with Kodak XAR 5 film. Regions of the paper that showed immunobinding to RepA were cut from the paper and counted. A corresponding region of the paper of equal size representing cells that did not have a source of RepA was used to determine the background.

Determination of native molecular weight of RepA. A 195-ml (97 by 1.6 cm) column of Sephadex G-100 was equilibrated with 50 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–1 mM 2-mercaptoethanol–10% glycerol–0.25 or 0.5 M NaCl. The flow rate was 12 ml/h, and 1-ml fractions were collected. The void volume was determined with blue dextran, and the column was calibrated with the native molecular weight standards transferrin (74,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400). Purified RepA was then chromatographed on the column. The position of the proteins was determined by using Bio-Rad protein assay kit II.

Construction of a chromosomal copy of a *repA-lacZ* gene fusion. A 563-bp *Rsa*I-*Pvu*II fragment of P1 DNA containing the promoter site and the first 66 codons of *repA* was cloned into the *Sma*I site of a pBR322-derived vector pMLB1034 (22). The cloning fuses the *repA* codons in frame with the codons of the *lacZ* gene. In the resultant plasmid, pALA326, the synthesis of the *repA-lacZ* fusion gene is now under the control of the regulatory signals of the *repA* gene. The fused region was then transferred from the plasmid to a λ vector, λRZ5, by using *bla* and *lac* homologies (Fig. 1).

The vector phage was grown lytically in a strain carrying

pALA326. The lysate was screened for blue plaques by plating on a *rec*$^+$ Δ*lac* strain, MC1000 (22), in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Usually the frequency of blue plaques was 0.1%. These plaques usually showed a mixture of blue and white plaques when replated, indicating that the original plaque came from a phage which had recombined once with the plasmid. When the blue plaques were plated again, about 50% bred true and were used to lysogenise MC4100 *recA56*, which is Δ(*argF-lac*)*U169* (22). Monolysogens were detected initially by measuring β-galactosidase as described by Miller (12). The enzyme activities fell into discrete classes apparently representing mono-, di-, and trilysogens, etc. Monolysogens were confirmed by a spot test with λ *c*I857 C17 (15, 21) and by the *ter* assay (9) with a λ Δ(*att-int*) *red imm*^{434} phage (λ DKC170). Monolysogens initially identified by the Miller assay were also confirmed to be monolysogens by these two tests in all cases.

Quantitation of RepA by measurement of β-galactosidase activity from a single chromosomal copy of a *repA-lacZ* gene fusion. The number of RepA monomers per cell was also estimated by measuring the β-galactosidase activity of a RepA-LacZ fusion protein essentially as described by Raleigh and Kleckner (17). Units of β-galactosidase activity were converted to number of monomers of the fusion protein as follows: number of monomers per cell = (1.04×10^{10} monomers per enzyme unit) × (enzyme units/amount of cell protein [milligrams]) × (amount of cell protein [milligrams]/number of cells). The value for the monomers/enzyme unit was determined previously (19). Enzyme units/milligram of cell protein (i.e., specific activity) was obtained from the relationship

$$\frac{\text{enzyme units}}{\text{amount of cell protein (milligrams)}} = \frac{A_{420} \times 213(\text{nanomoles of ONP/ml per } A_{420}) \times \text{final volume (milliliters)}}{\text{time of assay (minutes)} \times \text{amount of protein in initial volume (milligrams)}}$$

The protein concentration was determined by using amido black (20). The number of cells was measured microscopically with a Petroff-Hausser counter at a cell density below 10^8/ml. To stop the cells from swimming, the culture was made 10 mM in NaN_3 before being counted. For viable counts the L-broth cultures were diluted in chilled L broth and plated with top agar.

Determination of chromosomal DNA content of a culture. To relate the amount of RepA protein to the concentration of *repA* gene, the total amount of *repA* DNA in the cultures was determined. The *repA* gene was present in a mini-P1 prophage, λ-P1:5R, integrated at the *attP1* site of the chromosome (4).

The recovery of DNA was estimated by using an internal standard (φX174 replicative-form DNA), and the concentration of the *repA* gene was determined by blot hybridization with a pure *repA* DNA preparation (pSP120, see below) of known concentration. DNA isolation and blot hybridization experiments have been described recently (14).

Briefly the cells were suspended in 50 mM Tris hydrochloride (pH 8.0)–50 mM EDTA and lysed with lysozyme and SDS. After treatment with proteinase K, the lysate was divided into two fractions, A and B. Known amounts of purified φX174 replicative-form DNA were added to fraction A. Both fractions were treated with phenol and chloroform, and the aqueous phase was denatured with alkali. Just before the fractions were loaded in the slots of the blotting apparatus (Manifold II; Schleicher & Schuell), various amounts of alkali-denatured φX174 replicative-form DNA were added to extract B. With nick-translated φX174 DNA as probe, the amounts of φX174 DNA present in the two extracts were determined. The amount of φX174 DNA recovered in extract A was 85 to 90% of that added initially, indicating that about 10 to 15% of the DNA could be lost during the phenol extraction step. The estimate of total DNA was corrected accordingly.

To determine the concentration of the *repA* gene, extract A was immobilized on another filter. Known concentrations of pure pSP120 DNA, a pBR322 derivative carrying the 924-base-pair *Hin*dIII-*Bam*HI fragment of pSP102 containing the *repA* gene (14), were mixed with an identically treated extract from cells without λ-P1:5R but otherwise isogenic, and the mixture was immobilized alongside extract A on the same filter. The probe used was the nick-translated 924-base-pair *Hin*dIII-*Bam*HI fragment of pSP120 carrying only the *repA* gene.

RESULTS

Quantitation of RepA by Western blot analysis. In this study we determined that 5 ng or more of RepA protein can be measured by Western blotting. The feasibility of quantification of such small amounts of RepA was determined by mixing known quantities of purified RepA with extracts of cells lacking RepA. The RepA protein was purified to 90% homogeneity (1). The RepA signal, however, was dependent on the quantity of cell extracts used. The RepA signal diminished in backgrounds of increasing *E. coli* proteins, probably owing to competition of these proteins with RepA for binding to the nitrocellulose paper. As a result, all quantitative assays included a standard curve of authentic RepA in the appropriate amount of cell extract lacking RepA (Fig. 2). The use of the standard curve also eliminates the necessity of establishing whether the Western transfer is complete.

Our primary interest was to quantitate the RepA protein produced from the unit-copy plasmid λ-P1:5R and to compare this amount with that supplied by various other sources of RepA that have been made by cloning the gene behind promoters in pBR322 vectors (Table 1; Fig. 2). The *repA* gene was cloned behind the *bla-p2* promoter of pBR322 to generate the plasmid pALA162 (Table 1, line 3). From maxicell experiments as described previously for other plasmids (2), it was apparent that pALA162 was producing substantially more RepA than λ-P1:5R was (unpublished results); in fact this level was inhibitory to the replication of λ-P1:5R (6). The amount of protein was reduced by interposing a transcription terminator (T1 of the *E. coli rrnB* operon [6]) between the promoter and the gene; in the inverted orientation, the terminator was less active than in the proper orientation (compare pALA176 with pALA177: Table 1, lines 4 and 5). The protein level was further reduced with two terminators as in pALA178 (Table 1, line 6). For comparison, the protein level is also shown for pALA69, for which the gene is transcribed from unknown pBR322 sequences (Table 1, line 7). pALA69 has been used in the past as a standard *trans*-acting source of RepA (3, 6). From the knowledge of protein levels of the above plasmids and from

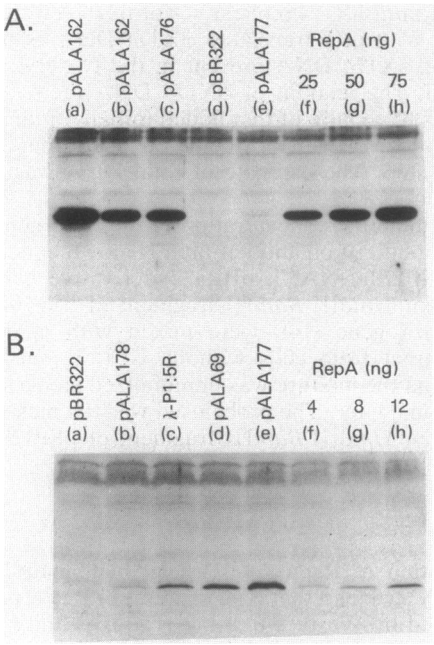

FIG. 2. Quantitative Western blot analysis of RepA produced from plasmids identified in the top line. (A) Cell extract protein (200 μg) was added in each lane. In lanes f to h, pure RepA was added as a standard to the extract made from cells carrying pBR322. Intensities of the RepA band in lanes a and e were outside the range of the standards and were ignored. In lane b, extracts from pALA162 carrying cells were diluted fivefold with extract from pBR322-carrying cells. (B) As panel A, except that 400 μg of cell extract was added per lane. Lanes d and e were ignored for the reasons described for lanes a and e in panel A. Other details are described in Materials and Methods.

our earlier results (6) on the effect of the above plasmids on mini-P1 replication, we can conclude that sevenfold overproduction of RepA is still tolerated by mini-P1 plasmids. In the absence of selection, however, mini-P1 plasmids were more unstable in the presence of pALA176 (about a 75% loss in 10 generations) than in the presence of pALA69 or pALA177 (<1% loss in 20 generations) (6), indicating that the amount of protein produced from pALA176 exceeds the optimum for replication. Cells carrying pALA162, which made 42-fold more protein than does λ-P1:5R, failed to support replication of incoming mini-P1 plasmids (6). The results of preliminary studies of the relationship of RepA concentration to copy number are consistent with the conclusion that instability at high RepA concentration is indeed due to decreased replication (13). At the other extreme, cells carrying pALA178, which made one-third as much RepA as does λ-P1:5R, could not be transformed with a mini-P1 plasmid whose replication was dependent upon an external source of RepA (6).

RepA protein exists as a dimer in solution. The native form of the RepA protein was determined by gel filtration over a column of Sephadex G-100. RepA migrated as a single species with an apparent M_r of 66,000 in buffer solutions containing 0.25 M or 0.5 M NaCl. Although RepA appeared to be soluble in the presence of 0.25 M NaCl, an even higher salt concentration was used to ensure better solubility, since the protein in the presence of 0.1 M NaCl forms a precipitate which can be dissolved readily with 0.5 M NaCl (1). Since the M_r of monomeric RepA is 32,000 from DNA and protein

TABLE 1. Concentration of RepA in cells carrying different RepA-producing plasmids

Source plasmid[a]	Promoter	Terminator	Amt of RepA (ng/mg of cell protein)[b] (mean ± SD)	Relative amt of RepA protein
N100(λ-P1:5R)$_{attP1}$	*repAp*		24 ± 3	1
N100/λ-P1:5R	*repAp*		23 ± 4	1.0
N100/pALA162	*bla-p2*		1,006 ± 76	42
N100/pALA176	*bla-p2*	T1 (inverted)	175 ± 16	7
N100/pALA177	*bla-p2*	T1	73 ± 10	3.0
N100/pALA178	*bla-p2*	T1 (two copies)	9 ± 1	0.4
N100/pALA69			42 ± 3	1.8

[a] In line 1 the wild-type mini-P1 plasmid λ-P1:5R was integrated into the chromosome at the *attP1* site at 66 min. The prophage was present as a plasmid in line 2 (slash). In the other lines the *repA* gene was present in pBR322 derived vectors and was expressed variously from foreign promoters (see Results and reference 6 for plasmid maps).

[b] Cell protein is the amount present in the supernatant as determined by the amido black assay (20); it varied within 10% from each other in extracts from different strains. The RepA concentration in the supernatant was determined by Western blot analysis (see Fig. 2 for details).

sequencing, it appears that RepA exists as a homodimeric species. It should be noted that although 1 mM 2-mercaptoethanol was present during RepA purification and gel filtration, we have not shown that all reactive sulfhydryl groups were maintained in the reduced form and that the homodimeric state was not an artifact of altered S—S bonding.

Determination of chromosomal copy number and relative concentration of RepA protein with respect to the *repA* gene. We endeavored to relate the amount of RepA produced from its natural source, λ-P1:5R, to the number of copies of the *repA* gene to determine the relative concentration of the protein with respect to its binding sites. The number of *repA* genes in the culture was determined by probing with *repA* DNA in blot hybridization experiments. Total DNA from N100 and N100(λ-P1:5R)$_{attP1}$ was isolated from known volumes of the culture. Recovery of DNA was 85 to 90% as discussed in Materials and Methods. N100(λ-P1:5R)$_{attP1}$ DNA was then compared with known amounts of pure *repA*

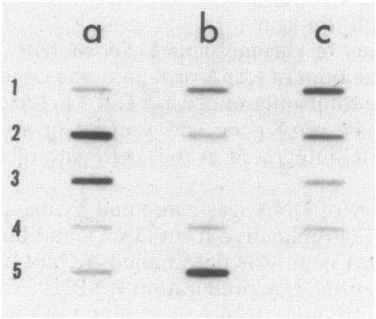

FIG. 3. Autoradiogram of slot blots to determine chromosomal copy number. Crude lysates from N100 and N100(λ-P1:5R)$_{attP1}$ cells containing equal amounts of total DNA as determined spectrophotometrically were loaded in different slots. Lysates from N100(λ-P1:5R)$_{attP1}$ were loaded in 1a, 2b, 3c, 4b, and 5a. In the remaining slots, lysates from N100 were loaded along with following amounts (in nanograms) of denatured pSP120 DNA: none in 3b and 5c, 0.057 in 4a and 4c, 0.114 in 2c and 1b, 0.190 in 1c and 3a, and 0.285 in 5b and 2a. The intensity of pSP120 DNA bands was plotted against the amount of pSP120 DNA added, and from this standard curve the amount of λ-P1:5R DNA was determined.

TABLE 2. Activities of *repA* promoter in the absence and presence of RepA protein as determined by gene fusion to *lacZ*

Strain	Sp act of β-galactosidase		No. of β-galactosidase monomers/cell
	Miller units	nmol of ONPG[a] hydrolyzed/min at 28°C per mg of protein	
BR2816, MC4100 *recA56*(λDKC231)[b]	568 ± 32	1,160 ± 34	3,550 ± 240
BR2818, BR2816 (λ-P1:5R)$_{attP1}$	0.63 ± 0.03	1.31 ± 0.03	5.0 ± 0.6[c]
BR2816/BR2818 ratio	905	886	720

[a] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

[b] The *repA-lacZ* fusion phage λDKC231 is described in Fig. 1. β-Galactosidase activities were measured exactly as described by Miller (12). A_{420} values were used to determine specific activities and monomers per cell, as discussed in Materials and Methods. Typically cells were grown in L broth to an A_{600} of 0.8 (corresponding to about 3×10^8 cells per ml, of which about 20% were viable) and chilled in ice for 30 min, and the A_{600} was measured again. The latter A_{600} value was used in quantitation. The chilled culture was used for viable and microscopic counts. For protein determination, 1 ml of the culture was spun in an Eppendorf centrifuge for 45 s, suspended in 600 μl of sample buffer, sonicated at 50 W for 15 s, boiled for 5 min, and spun for 5 min. The supernatant was used for the protein measurement as described previously (20).

[c] Because of the low values of β-galactosidase monomers for BR2818 cells, the assays were done with sonicated cell extracts in addition to the usual permeabilized whole cells. The results were within 10%, indicating that there were at least four monomers per cell. β-Galactosidase activities in whole-cell assays can be lower than in cell extracts if there are fewer than four polypeptide chains per cell, as these chains can assemble into active tetramers only after cell disruption (17).

DNA mixed with N100 DNA by using an α-^{32}P-labeled *repA* probe (Fig. 3). Typically 1 ml of N100(λ-P1:5R)$_{attP1}$ culture at an optical density at 590 nm of 0.8 was found to have 1.3×10^9 *repA* genes integrated at the *attP1* site at 66 min of the *E. coli* chromosome. In the same culture, the number of RepA monomers was 5.2×10^{10}/ml. Thus there were about 40 RepA monomers or 20 RepA dimers present per *repA* gene in an L-broth culture, which corresponds to about 160 monomers per cell. The number of cells in the culture was about 3.2×10^8/ml by microscopy; 20% of these were colony formers. The inviability was probably due to the presence of the *recA3* mutation in N100 cells. Thus there could be four copies of the *repA* gene per cell in an L-broth culture when integrated near 66 min. It should be noted that intracellular RepA concentration was estimated by comparison with authentic RepA, whose concentration was determined by the amido black assay with BSA as a standard (see Materials and Methods). It should also be noted that in estimating the RepA/*repA* value we have assumed the inviable cells of the *recA* culture to have the same DNA and protein contents as the viable cells.

Repressor activity of RepA in vivo as determined from β-galactosidase activity of a *repA-lacZ* fusion gene. *repA* expression is autoregulated at the level of transcription; the activity of the fusion gene depended upon the presence of authentic RepA (Table 2). The authentic RepA was supplied from λ-P1:5R, so that the repressed level of activity of the fused gene could be taken to correspond to the steady-state level of RepA from λ-P1:5R. From the data in Table 2 it appears that in the presence of physiological concentrations of RepA, the *repA* promoter (*repAp*) retains only 0.1% of its full activity. We note that the fusion activity shown in Table 2 corresponds only to five LacZ monomers per cell (Materials and Methods; 17). We have determined earlier that there are four *repA* genes per cell at *attP1* under our culture conditions. The number of *lacZ* genes per cell at *att*λ has not been determined directly, but is expected to be somewhat less than four, since *att*λ (at 17 min) is further removed from *oriC* (at 84 min) than *attP1* is (at 66 min). Therefore the present estimate that there are five *lacZ* monomers per cell (Table 2) would correspond at most to one RepA dimer per *repA* gene. The number is at least 20-fold lower than what was obtained before by Western blots and is not due to repressor activity of the fusion protein itself in the absence of authentic RepA (unpublished results). The Western blot data should be subject to fewer possible artifacts, and these will be discussed below.

DISCUSSION

We have used a solid-phase immunobinding assay involving the immobilization of epitope-containing peptides on nitrocellulose. In principle this technique is analogous to the well known enzyme-linked immunosorbent assay technique. In the present work there is the added advantage that the proteins being quantified are first separated in a gel from other cross-reacting materials of different molecular weight (24). The technique has been used to quantify an *E. coli* protein which makes up only 0.002% of the total cell protein. The estimates are reproducible to within 20% (Table 1). We find that for a wild-type mini-P1 plasmid with 14 binding sites there are about 20 RepA dimers. Thus it is quite plausible that enough RepA could be sequestered by the nine control repeats to influence replication significantly.

From the present study it appears that the RepA promoter is repressed about 900-fold under physiological conditions. Previous studies indicated that the repression is at the level of transcription (6). In the presence of authentic RepA, the *repAp* activity was repressed equally both in an operon and a protein fusion (6). The situation is comparable to that of the *lac* operon, in which a 1,000-fold repression of the *lac* promoter is achieved by about 10 to 20 *lac* repressor molecules (8). Also it should be noted that despite the high degree of repression, the steady-state level of RepA is not unusually low, as there are several important *E. coli* proteins that are present in 20 to 100 copies per cell (17).

In Western blot analysis, the loss of protein as a result of incomplete extraction and protease activity during the isolation procedure remains an unknown factor. An independent estimate of RepA protein concentration that did not depend upon cell lysis could be made from *lacZ* fusion data. The latter assay gave, in fact, a 20-fold-lower value of RepA concentration and so far is our only evidence against appreciable protease activity and incomplete extraction. The fusion method is, however, vulnerable to a number of possible artifacts: the activity of *repA-lacZ* transcript could differ from that of *repA* due to degradation; secondary structure of the fusion transcript could interfere with translation; the fusion protein could be degraded more rapidly than authentic RepA (or β-galactosidase); the specific activity of the fusion protein could be lower than that of authentic β-galactosidase; the presence of the RepA polypeptide portion could interfere with the state of oligomerization of the β-galactosidase monomers, etc. Some of these factors must be operative, since the specific activity of a second *repA-lacZ* gene fusion containing the first 113 amino acids of RepA was about sixfold lower than that of the smaller fusion protein containing 62 amino acids (unpublished results). Because of these uncertainties, we consider the estimates from Western blots to be more reliable. When the protein

levels from plasmids pALA162, pALA176, pALA177, and pALA178 were compared with the degree of *repA* transcription from the above plasmids, a close correspondence was obtained between the protein and RNA values, giving further confidence in the Western blot method (5). It should be noted that the limitations of the gene fusion method in the quantitation of absolute protein concentration are not applicable to measurement of relative promoter strengths. Hence our conclusion regarding the degree of repression of *repAp* should be considered reliable (Table 2).

Initiator protein concentration of another plasmid, R6K, has been recently measured and is about 2 orders of magnitude higher than that reported for mini-P1 in the present work (7). This difference is interesting, since both plasmids have repeat sequences at the origin and an autoregulated initiator protein that binds to the repeats, and when overproduced, the initiators actually inhibit replication. The relationship of initiator protein concentration to the copy number is complex for R6K (7).

The ability to quantitate the in vivo concentration of RepA protein will allow us to determine how the plasmid copy number depends upon RepA concentration and to compare the data with the results of studies on R6K. Such studies are in progress.

ACKNOWLEDGMENTS

We are indebted to Lise Raleigh for counsel and encouragement in the fusion analysis, to Terry Copeland for amino acid analysis of purified RepA and, to Bob Zagursky and Mike Berman for use of the vector λRZ5. We are also indebted to Sam Wilson and George Weinstock for many helpful discussion and to Barbara Funnell, Sue Wickner, and Michael Yarmolinsky for critical reading of the manuscript.

Research at Frederick was sponsored in part by the National Cancer Institute under contract no. 1-CO-23909 with Litton Bionetics, Inc.

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