Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3839–3843, April 1994
Biochemistry

Replication initiator protein RepE of mini-F plasmid: Functional differentiation between monomers (initiator) and dimers (autogenous repressor)

(DNA replication/DNA binding protein/heat shock protein)

MASAMICHI ISHIAI, CHIEKO WADA, YASUO KAWASAKI*, AND TAKASHI YURA†

Institute for Virus Research, Kyoto University, Kyoto 606-01, Japan

Communicated by E. Peter Geiduschek, January 3, 1994

ABSTRACT **Replication of mini-F plasmid requires the plasmid-encoded RepE initiator protein and several host factors including DnaJ, DnaK, and GrpE, heat shock proteins of *Escherichia coli*. The RepE protein plays a crucial role in replication and exhibits two major functions: initiation of replication from the origin, *ori2*, and autogenous repression of *repE* transcription. One of the mini-F plasmid mutants that can replicate in the *dnaJ*-defective host produces an altered RepE (RepE54) with a markedly enhanced initiator activity but little or no repressor activity. RepE54 has been purified from cell extracts primarily in monomeric form, unlike the wild-type RepE that is recovered in dimeric form. Gel-retardation assays revealed that RepE54 monomers bind to *ori2* (direct repeats) with a very high efficiency but hardly bind to the *repE* operator (inverted repeat), in accordance with the properties of RepE54 *in vivo*. Furthermore, the treatment of wild-type RepE dimers with protein denaturants enhanced their binding to *ori2* but reduced binding to the operator: RepE dimers were partially converted to monomers, and the *ori2* binding activity was uniquely associated with monomers. These results strongly suggest that RepE monomers represent an active form by binding to *ori2* to initiate replication, whereas dimers act as an autogenous repressor by binding to the operator. We propose that RepE is structurally and functionally differentiated and that monomerization of RepE dimers, presumably mediated by heat shock protein(s), activates the initiator function and participates in regulation of mini-F DNA replication.**

The mini-F plasmid, derived from the F (fertility) factor, replicates as a low-copy plasmid (one to two copies per host chromosome) in *Escherichia coli* (1, 2). The plasmid-encoded RepE initiator protein plays an essential and a specific role in initiating replication from the origin, *ori2* (3–6). RepE exhibits two major functions: initiation of DNA replication from *ori2* (initiator function) and autogenous repression of *repE* transcription (repressor function) (7, 8). These functions of RepE require its binding to the four 19-bp direct repeat sequences (iterons) found within *ori2* and to the *repE* promoter/operator, which contains an inverted repeat sequence (9–13); the half-sequence (10 bp) of the latter is similar (8-bp matches) to the 19-bp repeats (14). RepE has been purified as a dimer in several laboratories (11–13) and its binding to *ori2* iterons and the operator was demonstrated by using DNase I footprinting (9, 10) and gel-retardation (10–13) assays. Thus, RepE dimers have been thought to be involved in binding to both DNA regions, but the structural basis for each of the specific functions of RepE remained undetermined.

Besides RepE, several host factors including those involved in chromosomal DNA replication are required for mini-F plasmid replication. In particular, the heat shock σ factor (σ^{32}), which is essential for transcription of *repE* (8, 15), and a subset of heat shock proteins (DnaJ, DnaK, and GrpE) actively participate in plasmid replication (16, 17). The latter heat shock proteins are known to play specific and synergistic roles in the initiation of replication of both phage λ (see ref. 18) and plasmid mini-P1 (19–21). At least DnaK (the Hsp70 homologue) and DnaJ proteins are highly conserved and act as molecular chaperones in various other processes as well (18, 22). In mini-P1 plasmid replication, the initiator protein RepA can be activated by monomerization of dimers mediated by the heat shock proteins (23–25).

To further examine the role of heat shock proteins in mini-F plasmid replication, we have recently isolated and characterized mini-F mutants that can replicate in the *dnaJ*-defective host (26). Among them was a mutation (*repE54*) that caused production of RepE54 with a markedly enhanced initiator activity but little or no repressor activity as a result of the Arg-117 → Pro replacement (26).

We now report the purification and characterization of the RepE54 mutant protein and the examination of its DNA binding properties. In contrast to the wild-type RepE, which is recovered as a dimer, RepE54 was found mostly as a monomer. RepE54 monomers bind to *ori2* with a very high efficiency but hardly bind to the *repE* operator. Furthermore, RepE monomers derived from wild-type dimers showed efficient binding to *ori2*. Thus, RepE monomers appear to represent an active form in the initiator function through binding to *ori2*, whereas dimers act as the repressor by binding to the operator.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* BL21 (λDE3) producing T7 RNA polymerase (27) was used as the host for RepE production; pLysS and the *repE* expression plasmid, pBK815, were as described (13). A similar plasmid carrying the *repE54* mutant allele, pBK815 (*repE54*), was constructed by replacing the *Xma* I–*Eco*RV fragment of pBK815 with the equivalent fragment from pKV739 carrying *repE54* (26), and the structure was confirmed by nucleotide sequencing.

Purification of RepE Proteins. RepE proteins were purified by the published procedure (12, 13) with some modifications for the mutant RepE54. Cells of BL21(λDE3) harboring plasmids pLysS and pBK815 [or pBK815 (*repE54*)] were grown in L broth containing ampicillin and chloramphenicol at 37°C. After induction of RepE by isopropyl β-D-thiogalactoside (0.5 mM) for 3 h, cells were collected, sonicated in Tris/0.45 M KCl buffer [20 mM Tris·HCl, pH 7.5/0.1 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BSA, bovine serum albumin; LALLS, low angle laser light scattering.

*Present address: Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan.
†Present address: HSP Research Institute, Kyoto Research Park, Shimogyo-ku, Kyoto 600, Japan.

EDTA/450 mM KCl/10 mM 2-mercaptoethanol/10% (vol/vol) glycerol], and centrifuged at 30,000 × *g* for 15 min. The pellet formed was solubilized with guanidine hydrochloride to obtain wild-type RepE (RepE$^+$), as described (13). The mutant RepE54 was obtained by centrifuging the supernatant at 150,000 × *g* for 1 h, passing it through a DEAE-Sephacel column (Pharmacia LKB), and collecting the flow-through fraction. The RepE$^+$ and RepE54 proteins obtained were dialyzed against Mes/0.3 M KCl buffer (20 mM Mes, pH 6.0/0.1 mM EDTA/300 mM KCl/10 mM 2-mercaptoethanol/10% glycerol) and subjected to two steps of chromatography: an FPLC anion-exchange (Mono S HR5/5, Pharmacia LKB) column and an FPLC size-exclusion (Superose 12 HR10/30, Pharmacia LKB) column. The final preparation of RepE$^+$ was used for all experiments, whereas RepE54 was dialyzed against Tris/0.45 M KCl buffer and further purified by an FPLC cation-exchange (Mono Q HR5/5, Pharmacia LKB) column. All procedures were carried out at 4°C. About 1 mg of purified RepEs (>99% purity) was obtained from 200 ml of cultures (see Fig. 1*A*).

Determination of Molecular Weight of Native RepE Proteins. Low angle laser light scattering (LALLS) (28) and velocity sedimentation (29) using an Optima XL-A analytical ultracentrifuge (Beckman) were employed. For analytical ultracentrifugation, samples were in the buffer used for gel-retardation assays (see below), omitting bovine serum albumin (BSA), poly(dI-dC), and probe DNA. Samples of 0.2–0.4 A_{280} unit were loaded for RepE$^+$ or RepE54, respectively. The rotor speed was 50,000 rpm at 25°C for 60 min, and the scanning was done every 6 min.

Gel-Retardation Assays for RepE–DNA Binding. The assay conditions were essentially as described (13). The *ori2* DNA fragment (130 bp) containing four direct repeats and the *repE* operator fragment (180 bp) containing an inverted repeat were end-labeled with [γ-^{32}P]ATP and used as probes for RepE binding. The reaction mixture contained 20 mM Tris·HCl (pH 7.5), 40 mM NaCl, 40 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, BSA (0.1 mg/ml), and poly(dI-dC) (10 μg/ml); ^{32}P-labeled DNA and RepE were added and incubated at 30°C for 30 min. After PAGE (10% gels unless otherwise indicated), gels were dried and DNA bands were quantitated with a Fujix bioimaging analyzer BAS2000 (Fuji).

Other Methods. Manipulation of DNA was as described (30). Purified RepE proteins were examined by SDS/PAGE, followed by staining with Coomassie brilliant blue and scanning with a densitometer, using a reference RepE that had been quantitated by a Hitachi L-8500 amino acid analyzer. Immunoblot analysis of RepE was performed essentially as described (31), but the final staining was done using ECL Western blot detection reagents (Amersham).

RESULTS

RepE54 Mutant Protein Is Found as a Monomer. The first indication that RepE54 protein was structurally distinctive came from the finding that RepE54 was recovered in a supernatant after initial centrifugation of cell extracts. Whereas wild-type and several hyperactive mutant RepE proteins that have been overproduced by the T7 expression system were found mostly in the pellet (>80%) (12, 13), >90% of RepE54 remained in the supernatant. Upon examination with an FPLC size-exclusion column (Superose 12), the elution of purified RepE54 was significantly delayed compared to that of wild-type RepE (RepE$^+$). The apparent molecular mass estimated from K_{av} values (distribution coefficient) (32) was 55 or 27 kDa for RepE$^+$ or RepE54, respectively (data not shown).

We then determined the molecular mass of native RepE proteins by using the LALLS technique, which was expected to yield results virtually unaffected by protein shape. We found that RepE$^+$ was 58.6 (±3.0) kDa and RepE54 was 28.3 (±1.5) kDa (Fig. 1*B*). Sedimentation coefficients of RepE were also determined by ultracentrifugation and shown to be 4.1 S for RepE$^+$ and 2.8 S for RepE54. Since the molecular mass of RepE monomer predicted from the DNA sequence is ≈29 kDa (14), the wild-type RepE must be a dimer as reported (11–13) and RepE54 must be a monomer. Evidently, RepE54 cannot form stable dimers under the conditions employed.

Binding of RepE54 to *ori2* and Operator DNA. The activity of RepE$^+$ and RepE54 in binding to ^{32}P-labeled *ori2* DNA was first analyzed by gel-retardation techniques. With increasing amounts of RepE used, four bands with decreasing mobilities appeared that corresponded to DNA fragments in which one, two, three, or four iterons were bound to RepE, judging from their mobilities and relative intensities observed (Fig. 2 and Table 1). Apparently, the wild-type RepE bound to *ori2* DNA with a very low efficiency as reported (11–13), whereas RepE54 bound with a much higher efficiency (Fig. 2), in agreement with the high initiator activity of RepE54 found *in vivo* (26). The amount of RepE required for shifting 50% of *ori2* DNA was 450 fmol for RepE$^+$ and 0.9 fmol for RepE54 under the conditions used, indicating an ≈500-fold increase in binding efficiency. It should be noted that the molar concentrations (or amounts) of RepE referred to throughout this paper are expressed in terms of monomers.

In contrast to the markedly enhanced binding to *ori2*, no significant binding to the operator DNA was detected with RepE54 under the conditions that permit binding of most (96%) DNA by RepE$^+$ (10 pmol) (Fig. 3). The amount of RepE required for shifting 50% of DNA was 210 fmol for RepE$^+$, indicating that the binding efficiency of RepE54 was reduced by at least 100-fold. These results agreed well with the finding that RepE54 exhibits little or no repressor activity *in vivo* (26). They also raised the interesting possibility that RepE monomers normally represent an active form in binding to *ori2* and initiating replication, whereas only dimers bind to the operator and act as the autogenous repressor.

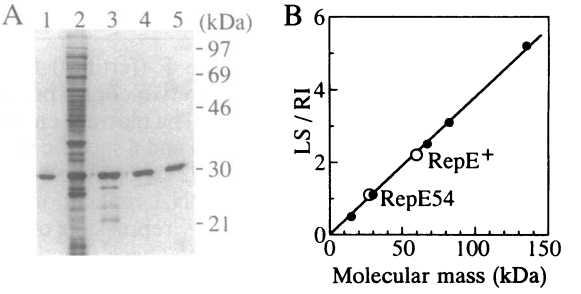

FIG. 1. Determination of molecular mass for RepE proteins. (*A*) SDS/PAGE (12% gel) analysis of purified RepE. Lanes: 1, wild-type RepE purified through Superose 12 column; 2–5, RepE54 purified through DEAE-Sephacel, Mono S, Superose 12, and Mono Q columns, respectively. Numbers to the right indicate positions of size markers. (*B*) Purified RepE was applied to a Superdex 75 HR10/30 (Pharmacia LKB) column with Mes/0.5 M KCl buffer at a flow rate of 0.5 ml/min, and the effluent was successively monitored by an SPD-2A UV detector (280 nm) (Shimadzu), a KMX-6 LALLS photometer (Chromatix, Sunnyvale, CA) with a He-Ne laser (633 nm), and an R-401 differential refractometer (Waters). All measurements were carried out at 23°C, and authentic proteins were used as standards, omitting direct measurement of the dn/dc value for RepEs. The vertical axis represents the ratio between LS (output of LALLS photometer) and RI (output of differential refractometer). The horizontal axis represents the molecular mass. ○, RepE proteins; ●, BSA dimer (132 kDa), creatine kinase (82 kDa), BSA monomer (66 kDa), carbonic anhydrase (29 kDa), and RNase A (13.7 kDa).

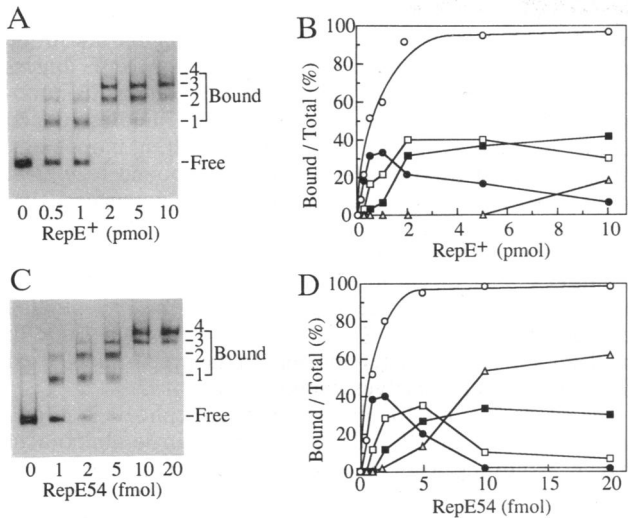

FIG. 2. Gel-retardation analysis of RepE binding to *ori2* DNA. (*A* and *C*) Autoradiographs of DNA bands. The reaction mixture (15 μl) containing 2 fmol of *ori2* DNA and indicated amounts of RepE was analyzed. (*B* and *D*) Quantitation of the assay: Gels were analyzed with a bioimaging analyzer, and the ratios of bound DNA to total DNA are plotted as a function of the amount of RepE+ (*B*) or RepE54 (*D*) used. The values are averages of four experiments. ○, Sum of 4 bands; ●, band 1; □, band 2; ■, band 3; △, band 4.

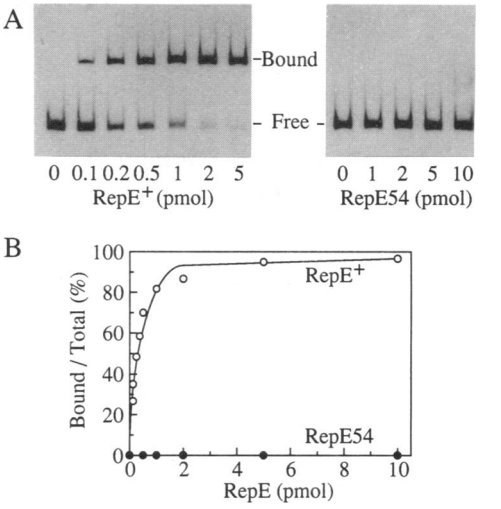

FIG. 3. Gel-retardation analysis of RepE binding to the operator DNA. (*A*) Autoradiographs of DNA bands observed with RepE+ (*Left*) or RepE54 (*Right*). (*B*) Quantitation of the assay. The procedures are the same as in Fig. 2. The values are averages of four or two experiments for RepE+ (○) or RepE54 (●), respectively.

Stoichiometry of the RepE Binding to *ori2* DNA. The stoichiometry of RepE binding to *ori2* iterons was determined directly by the double-labeling method (33, 34) using ^{3}H-labeled RepE54 and ^{32}P-labeled *ori2* DNA. Each of the four bands of RepE–DNA complexes obtained by gel-retardation assay was examined. As summarized in Table 1, the molar ratios of RepE54 to DNA were approximately 1, 2, 3, and 4 for bands 1, 2, 3, and 4, respectively, as expected from their dependencies on RepE concentration as well as gel mobilities (Fig. 2). These results clearly indicated that RepE54 specifically binds to iterons within *ori2* DNA, 1 mol of RepE54 monomer binding to each iteron. Because four bands with the same mobilities are obtained for RepE+, albeit with much lower efficiencies (Fig. 2), the stoichiometry is probably the same, 1 mol of monomer per iteron.

Effects of Protein Denaturants on DNA Binding Activities of RepE Dimers. To further explore the function of RepE dimers and monomers, RepE+ dimers were treated with protein denaturants and examined for DNA binding activities (Table 2). Treatment with guanidine hydrochloride (2–6 M) enhanced binding to *ori2* DNA significantly but reduced binding to the *repE* operator, provided that the RepE concentration was kept relatively low: high protein concentrations markedly reduced the observed effects on the binding to either DNA. Treatments of RepE dimers with 2–6 M urea or 1–4 M NaCl showed similar effects (data not shown), whereas Triton X-100 or sarcosyl failed to affect DNA binding appreciably. The observed effect of guanidine hydrochloride depended on incubation at 30°C (Table 2), indicating that it directly affects RepE and not the subsequent DNA binding or other reactions during gel retardation assays.

The *ori2* Binding Activity Associated with RepE+ Monomers. The guanidine-treated RepE+ dimers (15 pmol) were then applied to a size-exclusion column to find the extent of conversion to monomers. The effluents were examined for RepE by immunoblot analysis and for DNA binding activities by gel retardation. Two peaks that corresponded to RepE dimers and monomers were obtained at fractions 41–43 and 49–51, respectively (Fig. 4*A*). In contrast, essentially a single peak of dimers with a small fraction of monomers was obtained from the untreated control (Fig. 4*B*). The RepE54 protein either untreated (Fig. 4*C*) or treated with 4 M

Table 1. Stoichiometry of binding for RepE54–*ori2* DNA complexes

Complex	Molar ratio of RepE54 bound per *ori2* DNA	Deduced no. of RepE54 monomers bound per iteron
Band 1	1.15 (1.00)	1.15
Band 2	2.42 (2.11)	1.21
Band 3	3.10 (2.70)	1.03
Band 4	4.67 (4.07)	1.17
Free DNA	0.07	—

Reaction mixture (50 μl) containing 3 pmol of [^{32}P]*ori2* DNA (630 cpm/pmol) and 5.5–28 pmol of [^{3}H]RepE54 (labeled with [^{3}H]Leu and [^{3}H]Lys, each at 70 cpm/pmol) was analyzed by gel-retardation techniques using a 6% gel. The RepE-bound DNAs that correspond to bands 1–4 in Fig. 2 were detected by autoradiography, excised from the gel, and treated with Solvable (DuPont). The ^{3}H and ^{32}P radioactivities were determined in Atomlight (DuPont) and converted to molar ratios. Averages of two experiments are presented. Values in parentheses are relative to band 1.

Table 2. Effect of protein denaturants on DNA binding activities of wild-type RepE

Treatment	DNA binding efficiency, %	
	ori2	*repE* operator
None	9.4 (1.0)	45.9 (1.0)
Guanidine hydrochloride (2 M)	27.9 (3.0)	0.9 (0.02)
Guanidine hydrochloride (4 M)	53.8 (5.7)	1.3 (0.03)
Guanidine hydrochloride (6 M)	68.3 (7.3)	<0.03 (<0.01)
Guanidine hydrochloride (4 M)*	11.0 (1.2)	53.5 (1.2)
Triton X-100 (0.5%)	8.8 (1.0)	60.4 (1.3)
Sarcosyl (0.1%)	14.3 (1.5)	24.6 (0.5)

Reaction mixture (12 μl) contained Mes/0.5 M KCl buffer, 0.1 μg of BSA, and 8 pmol of RepE+. The mixture was incubated at 30°C for 30 min, then diluted 1:40 with buffer, and subjected to gel-retardation assay; samples containing 50 or 200 fmol of RepE were analyzed for binding to *ori2* or *repE* operator DNA (2 fmol), respectively. Quantitation of DNA binding was performed as in Fig. 2. The values are averages of at least two experiments; those in parentheses represent binding activities relative to that of untreated RepE. Sarcosyl, *N*-lauroylsarcosine sodium salt.
*Not incubated at 30°C (zero time control).

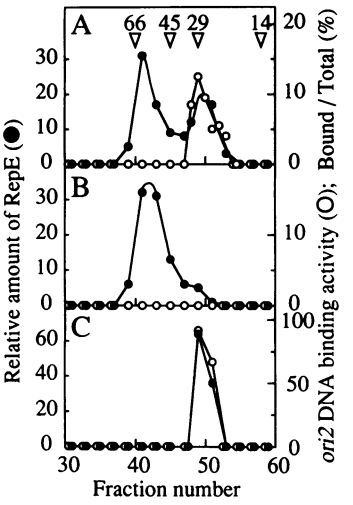

FIG. 4. Separation of monomers from guanidine-treated RepE dimers. (*A*) A 12-μl sample of RepE$^+$ (15 pmol) in Mes/0.5 M KCl buffer containing 0.1 μg of ovalbumin was treated with 4 M guanidine hydrochloride at 30°C for 30 min, diluted 1:50, applied to an FPLC size-exclusion column (Superdex 75), and eluted with Mes/0.5 M KCl buffer at 0.5 ml/min. Fractions (0.25 ml) were collected, and RepE was determined by immunoblot analysis with a specific antiserum. Approximately 80% of RepE was recovered. The *ori2* DNA binding activity was determined by gel-retardation assay. The reaction mixture (30 μl) contained 4 or 0.4 μl of each fraction for RepE$^+$ or RepE54, respectively, and 10 fmol of labeled *ori2* DNA. Quantitation was performed as in Fig. 2, and averages of two experiments are shown. Arrowheads indicate the positions of size markers: BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and RNase A (13.7 kDa). (*B* and *C*) Untreated wild-type RepE and RepE54, respectively, were similarly analyzed. ●, Relative amounts of RepE (% of total); ○, *ori2* DNA binding activity.

guanidine hydrochloride (data not shown) was eluted in fractions 49–51 under the same conditions. Most interestingly, the *ori2* DNA binding was uniquely associated with monomers (Fig. 4*A*). The *ori2* binding activity of monomers derived from the untreated control was too low to be detected under the assay conditions used (Fig. 4*B*). Also, the operator DNA binding was not detected with any of the fractions tested: not enough RepE dimers were recovered under these conditions.

Similar conversion to monomers was observed when RepE dimers were treated with 4 M NaCl or 4 M urea: a distinct peak at fractions 49–51 appeared that coincided with the *ori2* binding activity, although the yield of monomers was less than that obtained with guanidine hydrochloride (data not shown). Moreover, the *ori2* binding activities of monomers obtained by these diverse treatments appeared to be quantitatively similar: they were active in *ori2* binding though the activity was an order of magnitude lower than that of the RepE54 monomers (Fig. 4 *A* and *C*).

Finally, RepE$^+$ dimers at various concentrations (20–2600 nM) were examined by size-exclusion chromatography to obtain a rough estimation of the dissociation constant (K_d), which was found to be 3×10^{-10} M, indicating that the equilibrium strongly favored dimerization under the conditions used.

DISCUSSION

We have shown that the mini-F RepE protein can assume at least two functionally distinct forms, monomers and dimers. The low dissociation constant observed is consistent with the fact that wild-type RepE is found mostly as dimers. Such dimers bind preferentially to the *repE* operator but only inefficiently to *ori2* iterons (9–12), whereas the RepE54 mutant protein was obtained mostly as monomers and bound to *ori2* DNA at a very high efficiency (Fig. 2) but not to the operator (Fig. 3). Furthermore, treatments of RepE dimers with protein denaturants partially converted dimers to monomers and led to an increased binding to *ori2* and a reduced binding to the operator (Table 2). The resulting monomers, when separated from dimers, specifically bound to *ori2* at high efficiency (Fig. 4). These results strongly suggest that RepE monomers normally represent an active form in *ori2* DNA binding. One mole of RepE54 binds to each of the iterons (Table 1); this probably applies to RepE$^+$ as well (Fig. 2). These results indicate that the apparent *ori2*-binding activities observed with RepE$^+$ dimers are actually due to small amounts of monomers present in the preparations used and that dimers themselves cannot bind to *ori2*.

The majority of mini-F mutants that can replicate in *E. coli* strains defective in σ^{32} or DnaJ carried a single amino acid change within the RepE segment spanning residues 92–134 and exhibited increased initiator activity and reduced repressor activity (26, 31). All these RepE mutants except RepE54 have been purified as dimers that exhibited enhanced *ori2* binding (3- to 12-fold of wild-type binding) and concomitantly reduced operator binding (0.3- to 0.5-fold of wild-type binding), in agreement with their properties *in vivo* (ref. 13; M.I., unpublished results). Perhaps, these dimers are converted to monomers more readily than are wild-type dimers. Since *repE54* is a missense mutation that replaces Arg-117 with Pro, the Arg-117 residue may be involved in dimerization, but the mutation could affect dimerization indirectly by altering protein conformation. Determination of the specific domain involved in dimerization should be the subject of a further study.

For the mini-P1 plasmid, a RepA dimer and a DnaJ dimer form a complex that is inactive in binding to the origin (direct repeat) (21) and was thought to be converted to active RepA monomers by DnaK, GrpE, and ATP (23–25). However, a recent report suggested that RepA might exist mostly as a monomer and be further activated by heat shock proteins (35). The initiator protein (RepA) of another plasmid pSC101 was also reported to bind to the operator (inverted repeat) as a dimer and to the origin (direct repeat) as a monomer, on the basis of gel-retardation analysis of crude lysates containing putative heterodimers formed between normal and truncated RepA molecules *in vivo* (36).

The fact that the *repE54* and other hyperactive RepE mutants can replicate in the *dnaJ*, *dnaK*, or *grpE*-defective host as well (26, 31) is consistent with the notion that these heat shock proteins work cooperatively in replication of mini-F plasmid, as has been found with phage λ and plasmid mini-P1 (18–25). Thus, these heat shock proteins may be involved in the conversion of RepE dimers to monomers. RepE exists mostly as dimers, functions as a repressor, and thus limits the cellular levels of RepE within a certain range. Depending on the set of presently undefined conditions, a small fraction of RepE dimers may be converted to monomers that are active as an initiator, and this conversion may be mediated by DnaJ, DnaK, and GrpE proteins. In this connection, the *ori2* binding activity of RepE$^+$ monomers derived from dimers is appreciably lower than that of RepE54 (Fig. 4 *A* and *C*), suggesting that activation of RepE might involve a mechanism in addition to monomerization, that may be dependent or independent of the heat shock proteins. RepE54 might then represent at least partially activated monomers. This is in line with the recent suggestion that the inactive RepA monomers of mini-P1 plasmid can be converted to active form (35).

The functional differentiation between the two forms of RepE is reminiscent of the "two-stage molecular model" proposed by Trawick and Kline (37) to explain the bifunctional nature of RepE protein. The normal or unmodified

RepE that functions as an autogenous repressor is now considered to be dimeric, whereas a small fraction of modified RepE postulated to function as an initiator is considered to be monomeric. Our results are thus consistent with, and provide at least partial structural basis for, their model. In any event, the present findings shed some light on our understanding of regulation of mini-F plasmid replication. Specifically, interconversion between dimers and monomers as well as the amount of RepE must play critical roles in plasmid replication. At present, the question of how the control of RepE conversion (monomerization) and activation can be related to mini-F plasmid replication is not known, and further work is required to elucidate mechanisms that control the plasmid copy number, possibly coupled with that of the host cell cycle (38, 39).

We are grateful to B. Kline for bacterial strains and plasmids, K. Matsubara for antiserum, K. Shigesada and T. Nagata for advice and encouragements, and M. Mihara for laboratory supplies. We also thank A. Kinugawa, T. Takagi, M. Koizumi, and Y. Yagi for kind advice and assistance in determination of molecular masses of proteins. This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan.

1. Timmis, K., Cabello, F. & Cohen, S. N. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2242–2246.
2. Lovett, M. A. & Helinski, D. R. (1976) *J. Bacteriol.* **127**, 982–987.
3. Watson, L. A., Phua, S. H., Bergquist, P. L. & Lane, H. E. D. (1982) *Gene* **19**, 173–178.
4. Tolun, A. & Helinski, D. R. (1982) *Mol. Gen. Genet.* **186**, 372–377.
5. Maki, S., Kuribayashi, M., Miki, T. & Horiuchi, T. (1983) *Mol. Gen. Genet.* **191**, 231–237.
6. Muraiso, K., Tokino, T., Murotsu, T. & Matsubara, K. (1987) *Mol. Gen. Genet.* **206**, 519–521.
7. Søgaard-Andersen, L., Rokeach, L. A. & Molin, S. (1984) *EMBO J.* **3**, 257–262.
8. Wada, C., Imai, M. & Yura, T. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8849–8853.
9. Tokino, T., Murotsu, T. & Matsubara, K. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4109–4113.
10. Masson, L. & Ray, D. S. (1986) *Nucleic Acids Res.* **14**, 5693–5711.
11. Masson, L. & Ray, D. S. (1988) *Nucleic Acids Res.* **16**, 413–424.
12. Kline, B. C., Sandhu, G. S., Eckloff, B. W. & Aleff, R. A. (1992) *J. Bacteriol.* **174**, 3004–3010.
13. Kawasaki, Y., Wada, C. & Yura, T. (1992) *J. Biol. Chem.* **267**, 11520–11524.
14. Murotsu, T., Matsubara, K., Sugisaki, H. & Takanami, M. (1981) *Gene* **15**, 257–271.
15. Wada, C., Akiyama, Y., Ito, K. & Yura, T. (1986) *Mol. Gen. Genet.* **203**, 208–213.
16. Ezaki, B., Ogura, T., Mori, H., Niki, H. & Hiraga, S. (1989) *Mol. Gen. Genet.* **218**, 183–189.
17. Kawasaki, Y., Wada, C. & Yura, T. (1990) *Mol. Gen. Genet.* **220**, 277–282.
18. Georgopoulos, C., Ang, D., Liberek, K. & Zylicz, M. (1990) in *Stress Proteins in Biology and Medicine*, eds. Morimoto, R. I., Tissieres, A. & Georgopoulos, C. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 191–221.
19. Tilly, K. & Yarmolinsky, M. (1989) *J. Bacteriol.* **171**, 6025–6029.
20. Bukau, B. & Walker, G. C. (1989) *J. Bacteriol.* **171**, 6030–6038.
21. Wickner, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2690–2694.
22. Gross, C. A., Straus, D. B., Erickson, J. W. & Yura, T. (1990) in *Stress Proteins in Biology and Medicine*, eds. Morimoto, R. I., Tissieres, A. & Georgopoulos, C. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 167–189.
23. Wickner, S., Hoskins, J. & McKenney, K. (1991) *Nature (London)* **350**, 165–167.
24. Wickner, S., Hoskins, J. & McKenney, K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7903–7907.
25. Wickner, S., Skowyra, D., Hoskins, J. & McKenney, K. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 10345–10349.
26. Ishiai, M., Wada, C., Kawasaki, Y. & Yura, T. (1992) *J. Bacteriol.* **174**, 5597–5603.
27. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
28. Takagi, T. (1981) *J. Biochem.* **89**, 363–368.
29. Olsen, P. H., Esmon, N. L., Esmon, C. T. & Laue, T. M. (1992) *Biochemistry* **31**, 746–754.
30. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
31. Kawasaki, Y., Wada, C. & Yura, T. (1991) *J. Bacteriol.* **173**, 1064–1072.
32. Laue, T. M. & Rhodes, D. G. (1990) *Methods Enzymol.* **182**, 566–587.
33. Yang, C.-C. & Nash, H. A. (1989) *Cell* **57**, 869–880.
34. Schneider, G. J. & Geiduschek, E. P. (1990) *J. Biol. Chem.* **265**, 10198–10200.
35. DasGupta, S., Mukhopadhyay, G., Papp, P. P., Lewis, M. S. & Chattoraj, D. K. (1993) *J. Mol. Biol.* **232**, 23–34.
36. Manen, D., Upegui-Gonzalez, L.-C. & Caro, L. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8923–8927.
37. Trawick, J. D. & Kline, B. C. (1985) *Plasmid* **13**, 59–69.
38. Keasling, J. D., Palsson, B. O. & Cooper, S. (1991) *J. Bacteriol.* **173**, 2673–2680.
39. Koppes, L. J. H. (1992) *J. Bacteriol.* **174**, 2121–2123.