Plasmid P1 RepA Is Homologous to the F Plasmid RepE Class of Initiators*

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Suveena Sharma‡, Bangalore K. Sathyanarayana‡, Jeremy G. Bird, Joel R. Hoskins, Byungkook Lee, and Sue Wickner§,
From the Laboratory of Molecular Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892

DNA replication of plasmid P1 requires a plasmid-encoded origin DNA-binding protein, RepA. RepA is an inactive dimer and is converted by molecular chaperones into an active monomer that binds RepA binding sites. Although the sequence of RepA is not homologous to that of F plasmid RepE, we found by using fold-recognition programs that RepA shares structural homology with RepE and built a model based on the RepE crystal structure. We constructed mutants in the two predicted DNA binding domains to test the model. As expected, the mutants were defective in P1 DNA binding. The model predicted that RepA binds the first half of the binding site through interactions with the C-terminal DNA binding domain and the second half through interactions with the N-terminal domain. The experiments supported the prediction. The model was further supported by the observation that mutants defective in dimerization map to the predicted subunit interface region, based on the crystal structure of pPS10 RepA, a RepE family member. These results suggest P1 RepA is structurally homologous to plasmid initiators, including those of F, R6K, pSC101, pCU1, pPS10, pF8A, pGSH500, Rts1, RepHI1B, RepFIB, and RSF1010.

A large class of circular double-stranded DNA plasmids contain multiple repeated sequences, iterons, in their origins of replication that specifically bind plasmid encoded replication initiator proteins (recently reviewed by del Solar et al., Ref. 1). Binding of the initiator protein to the plasmid origin is an essential step in the initiation of theta-type DNA replication and triggers the assembly of a replication complex at the site through interactions between the plasmid initiator, host proteins, and the plasmid origin DNA. Plasmid initiator proteins are not only essential for DNA replication, but in most cases are also involved in the control of replication by autoregulating their gene expression. Additionally, some plasmids also possess iterons located in separate copy control loci. Binding of the initiator protein simultaneously to the control locus and the origin regulates plasmid copy number by a mechanism involving pairing of the control site and origin iterons.

In the work presented here we have studied the plasmid P1 initiator protein, RepA. RepA binds to five direct repeats of a 19-bp sequence in the P1 origin (2, 3). By binding to the origin iterons, RepA also represses transcription from the repA promoter, located within the origin repeats and it regulates copy number by binding a group of nine similar repeated sequences in a copy control locus. The purified RepA protein exists as a dimer in solution and is unable to bind DNA. The action of DnaJ and DnaK in an ATP-dependent reaction converts the inactive dimer to active monomer that is capable of binding the P1 iteron with high affinity (4–6). GrpE is required under conditions where the reaction is limited by slow nucleotide exchange (7). The RepA monomer is the active form in vivo, since DnaK, DnaJ, and GrpE are required for the stable maintenance of P1 plasmids and for repA promoter repression (8–10).

In vitro RepA can be activated for DNA binding by ClpA, a member of the Clp/Hsp100 family of ATP-dependent chaperones (11). In this case, activation is the conversion of inactive dimers to active monomers. Although both ClpA and the DnaK chaperone system convert RepA dimers to monomers, they recognize different regions of RepA (12, 13).

The initiator proteins of several other plasmids also exist as dimers yet bind to origin iterons as monomers. RepE, the initiator protein of F plasmid, requires DnaJ, DnaK, and GrpE for monomerization and activation of origin binding (14, 15). The initiator of plasmid RK2 is activated in vitro for DNA binding by monomerization by either the DnaK chaperone system in combination with ClpB or by ClpX (16, 17). The chaperone system used to activate the RK2 initiator in vivo has not been identified, suggesting the possibility that several chaperone systems are able to carry out the reaction. Recent studies have suggested that iteron DNA binding by RepA of pPS10 facilitates the conversion of inactive dimers to active monomers (18). For RepA of pSC101, the monomer is active in iteron DNA binding, but the monomer-dimer transition is independent of molecular chaperones (19, 20). Interestingly, the R6K initiator is able to bind to its iteron as a dimer or monomer (21).

Structural studies of a monomeric mutant of RepE of F plasmid in complex with the 19-bp iteron demonstrated that RepE consists of topologically similar N- and C-terminal domains related to each other by internal pseudo 2-fold symmetry, despite the lack of amino acid similarity between the two domains (22). The two domains bind to two consecutive major grooves on one face of the helix. Amino acid homology between RepE and the initiator proteins of R6K, pSC101, pCU1, pPS10, pF8A, and pGSH500 suggests that these proteins bind their respective iterons similarly (22).

P1 RepA does not share sequence homology with RepE and could not initially be aligned with the other plasmid initiators. In this study we addressed the question of whether P1 RepA might be structurally similar to the RepE class of plasmid initiator proteins, to another class of initiator proteins, or to a known class of DNA-binding proteins by fold prediction analy-
EXPERIMENTAL PROCEDURES

Materials—ATP was obtained from Roche Applied Science. Restriction endonucleases were obtained from New England BioLabs and polymerase chain reaction reagents were obtained from PerkinElmer Life Sciences. Oligonucleotides used in DNA binding experiments were synthesized and PAGE purified by Sigma Genosys. Plasmids—Site-directed mutants of wild-type RepA and RepA-His<sub>6</sub> (15) were constructed using the QuickChange mutagenesis kit (Stratagene). RepA(H134N) and G156V were converted to Asn and Val, respectively, generating point mutants RepA(H134N) and RepA(G156V). Lys<sup>268</sup>, Arg<sup>286</sup>, Arg<sup>245</sup>, and Arg<sup>266</sup> were converted to alamines, generating RepA(K88A), RepA(K128A), RepA(R130A), RepA(R245A), and RepA(R269A). To make RepA(R3M), H134N, G156V, and N142K were introduced by megaprimer PCR. The sequences of all the mutants were verified by DNA sequencing. [3H]-labeled proteins were expressed as molar amounts of RepA dimers and ClpA hexamers. Proteins were 3H-labeled in vitro using succinimidyl propionate, N-[pro-pionate-2,3-<sup>3</sup>H] as described (15). For measuring DNA binding by gel retardation, reaction mixtures (20 μl) contained Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol (v/v)), 10 mM MgOAc, 1 mM ATP, 100 μg/ml bovine serum albumin, 0.005% Triton X-100 (v/v), 0.5 pmol of ClpA, and RepA or RepA mutant protein as indicated. Proteins were diluted in Buffer A containing 0.05% Triton X-100 (v/v). After 10 min at 24 °C, calf thymus DNA (1 μg) and 10 fmol of [H<sup>3</sup>]-labeled P1 plasmid DNA (3590 cpmp/fmol) were added. Following 5 min at 0 °C, the mixtures were filtered through nitrocellulose filters, and the retained radioactivity was measured.

For measuring DNA binding by gel retardation, reaction mixtures (20 μl) contained Buffer A, 10 mM MgOAc, 5 mM ATP, 100 μg/ml bovine serum albumin, 0.005% Triton X-100 (v/v), 0.64 pmol of ClpA, and RepA or RepA mutant protein as indicated. After 20 min at 24 °C, 2.5 pmol of double-stranded oligonucleotide, were added by annealing complementary single-stranded oligonucleotides. After 10 min at 0 °C, the samples were electrophoresed in 8% polyacrylamide Tris borate/EDTA gels and stained with SYBR green (Molecular Probes). Band intensities were determined using 300-nm transillumination and an Eagle Eye II (Stratagene) automated imaging system. Molecular Modeling of RepA—RepA was aligned to RepE using 2-fold related proteins of plasmids R6K, pSC101, pCU1, and pPS10 (22). The structural similarity of P1 RepA and F RepE with the origin proteins of plasmids R6K, pSC101, pCU1, and pPS10, previously been modeled (30) using the published alignment of the proteins and the crystal structure of RepE as the template (2, C–F). RepA of pPS10 has previously been modeled (30) using the published alignment (22). The structural similarity of P1 RepA and F RepE with the other plasmid initiator proteins is apparent. The model provides useful tools for designing site-directed mutants to elucidate the protein–protein and protein–DNA interactions involved in the process of initiation of DNA replication.

Site-directed RepA Mutants Defective in DNA Binding—We next wanted to test the validity of the model experimentally. One prediction is that RepA binds to one face of the DNA. This was confirmed by previous DNA footprinting experiments showing that RepA binds exclusively to one face of the DNA, through a major groove in the DNA binding site through a C-terminal and a N-terminal domain. To test the model, we constructed site-directed mutations in RepA, two in the predicted N-terminal recognition helix, RepA(K128A) and RepA(R130A), and one in the predicted C-terminal recognition helix, RepA(R245A) (indicated in Figs. 1 and 2A). We purified the proteins and measured DNA binding both by a gel shift assay using a 33-bp double-stranded oligonucleotide containing the RepA consensus binding site and by nitrocellulose filter binding assay using plasmid DNA containing the P1 origin of replication. As a control, and as previously seen, RepA bound specifically to the consensus iteron in a reaction requiring chaperone activation, either ClpA (Ref. 11 and Fig. 3) or DnaJ and DnaK (5). When the mutant proteins were tested for DNA binding, RepA(R130A) and RepA(R245A) bound P1 iteron DNA with about 10% the affinity of wild type following activation by ClpA.
DNA binding recognition helices, the validity of the model and the importance of residues in both formations similar to wild type. Thus these results support the ClpA for activation suggested that the proteins folded in containing DNA, albeit poorly in some cases, and required proximity to the DNA.

These results are consistent with these regions being in close proximity to the DNA.

The model predicts that RepA should make contacts with the phosphate backbone of the DNA outside of the recognition helices in the N-terminal region of a2 (residues 87–89), the N-terminal region of a2′ (residues 204–206), between β3 and β4 (residues 170–173), and between β3′ and β4′ (residues 266–269). We constructed and tested mutants in two of these regions. RepA(K88A) was tested because it aligns with an arginine of RepE that contacts the phosphate backbone, R33 (shown in Figs. 1 and 2). We found that it bound oriB with about 60% lower affinity than the wild type (Fig. 3B). These results strongly suggest that Arg130 and Arg245 participate in RepA-DNA interactions. RepA(K128A) bound oriB DNA with about 60% the affinity of wild type after activation (Fig. 3B). Although Lys128 is predicted to be in the DNA recognition helix of RepA, the biochemical results indicate that it is not as involved in DNA binding as Arg130.

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The observations that the mutant proteins bound P1 iteron containing DNA, albeit poorly in some cases, and required ClpA for activation suggested that the proteins folded in conformations similar to wild type. Thus these results support the validity of the model and the importance of residues in both DNA binding recognition helices, a4 and a4′. They also suggest that the residues in the N-terminal region of a2 and between β3′ and β4′ are important for DNA binding.

DNA Binding by RepA to the 19-bp Binding Site and to the Half-Sites—The model predicts that RepA should contact both halves of the iteron. The structure data shows that the C-terminal DNA binding domain of RepE makes stronger protein-DNA contacts with the first half of the iteron than the N-terminal domain makes with the second further suggests similarity between RepE and RepA.

DNA Binding by RepA to the 19-bp Binding Site and to the Half-Sites—The model predicts that RepA should contact both halves of the iteron. The structure data shows that the C-terminal DNA binding domain of RepE makes stronger protein-DNA contacts with the first half of the iteron than the N-terminal domain makes with the second half of the iteron (22). The two halves of the iterons were referred to as “first” and “second” before the structure of RepE demonstrated that the C-terminal domain of RepE binds to the first half-site and the N-terminal domain binds to the second half-site (22). In the N-terminal recognition helix of RepE, a4, only two residues contact the second half of the iteron directly while in the C-terminal recognition helix, a4′, four residues contact the first half of the iteron (22). We wanted to test whether or not P1 RepA binds to the separate halves of the P1 iteron and whether RepA has a higher affinity for the first half-site of the site than the second. We compared DNA binding to 33-bp double-stranded oligonucleotides containing either the 19-bp consensus RepA binding site, the first half of the site, or the second half of the site (shown in Fig. 4). When DNA binding to the consensus site was measured using RepA activated by ClpA, 50% retardation was seen with 80 nM RepA and 125 nM DNA fragment (Fig. 5A).

When we measured RepA binding to the first half of the iteron, we found that RepA bound, but the apparent affinity was about 60-fold lower than that for the full site (Fig. 5B). DNA binding was specific and required activation by ClpA. In contrast we were unable to detect binding of activated RepA to the second half of the iteron, suggesting that the affinity was at least 300-fold lower than that for the full site (Fig. 5C). DNA binding was also not detected when we used an oligonucleotide containing the second half-site plus the T that is located in the middle of the 19 bp iteron (data not shown). The observation that RepA binds with higher affinity to the first half of the site than the second further suggests similarity between RepE and RepA.

The model predicts that the C-terminal DNA binding domain of RepA interacts with the first half of the iteron. To test this we reasoned in the following way. Since RepA binds, although with lower affinity, to the first half of the iteron, mutants in the N-terminal DNA binding domain might bind the first half-site through interactions involving their wild-type C-terminal DNA binding domain with similar affinity as wild-type RepA. DNA binding studies confirmed this (Fig. 6). Both RepA(R130A) and wild-type RepA bound the first half-site with similar relative affinity, suggesting that they were both binding to the first half-site through interactions with their C-terminal DNA binding domain. Moreover the RepA(R130A) bound the first half-
site DNA with similar affinity as it bound the full iteron (Fig. 3), suggesting that the defect in DNA binding to the full site reflects a defect in interaction of the second half-site with the N-terminal domain of RepA. In addition, the model predicted that RepA(R245A) would have weaker binding to the first half-site than wild-type RepA. When DNA binding was tested, there was no detectable binding by RepA(R245A) to the first half-site (Fig. 6). These results also support the validity of the molecular model.

The dimeric forms of the initiators of plasmids F, pSC101, R6K, and pPS10 are known to bind as dimers to the promoters of the initiator genes and repress transcription (1). For these plasmids the operator site is in a separate location from the origin of replication and consists of the first half of the iteron followed by an inverted first half-site. The two inverted repeat sequences are separated by roughly one turn of the helix, 9 bp. Although the promoter for the repA gene of P1 is embedded within the origin iterons and repression of repA expression results from the binding of monomers to the origin iterons, it remained possible that RepA might bind to inverted half-sites as a dimer, by analogy with RepE, and perhaps function elsewhere in the P1 genome. We used an oligonucleotide in which the first half-site was followed by an inverted first half-site and the two half-sites were separated by 9 bp (Fig. 4). When we measured DNA binding by dimeric RepA, there was no detectable binding (data not shown). After chaperone activation, RepA bound the DNA with about the same affinity as the first half-site (data not shown). Although it remains possible that RepA might bind to an inverted repeat of the first half-site if a different spacer were used, the results suggest that dimeric RepA does not possess the same ability as some other plasmid initiators to act as a dimeric repressor. This difference may be
because it contains a N-terminal domain of 70 amino acids not present in the other initiators.

RepA Mutants with Increased Monomer/Dimer Ratios—The crystal structure of a dimer of the N-terminal domain of RepA of plasmid pPS10 was solved recently (28) (shown in Fig. 7A). The structure demonstrates that the conformation of the N-terminal domain in the dimer protomers is similar to that in the monomer. The dimerization interface is between $\beta_2$-$\beta_3$ of one protomer and $\beta_3$-$\beta_4$ of the other, forming a five-stranded antiparallel pleated sheet of $\beta_2$-$\beta_4$-$\beta_3$ from one protomer and $\beta_2$-$\beta_2$-$\beta_3$ from the other, using the secondary structure nomenclature used in Fig. 1 and by Komori et al. (Ref. 22 and shown in Fig. 7A). Because pPS10 RepA is a member of the RepE family of initiator proteins, the other members of the family are expected to have similar dimeric structures.

We have illustrated the P1 RepA dimer by superimposing the modeled monomer structure of RepA on each of the protomers of the pPS10 RepA dimer structure (28) as described under “Experimental Procedures” (Fig. 7B). In the figure the dimerization interface of P1 RepA is very similar to that of pPS10 RepA, involving the $\beta_2$-$\beta_3$-sheets in the N-terminal domain. The monomer form of P1 RepA and the other plasmid initiator proteins differ from the x-ray crystallographic dimer structure of the N-terminal domain of pPS10 RepA in that the monomer has a $\beta$-sheet at the N terminus where the dimer has a long $\alpha$-helix, and the monomer has a much shorter $\alpha$-helix than the dimer (Fig. 7, A–D). In addition, the antiparallel $\beta_2a$-$\beta_2b$-sheet is bent inwards in the monomer compared with the dimer. The C-terminal domain of the RepA monomer model is included in the illustration, although its location relative to the N-terminal domain is not known.

Previously, mutations in P1 RepA were isolated that resulted in increased monomer/dimer ratios. We have investigated the DNA-binding properties of these mutants (Fig. 3). The mutations that resulted in increased monomer/dimer ratios (R130A, R245A, K128A, K88A, R269A) were all located in the N-terminal domain. The DNA-binding properties of these mutants were similar to those of wild-type RepA, with the exception of RepA(R245A), which was significantly less active than wild-type RepA.

FIG. 3. DNA binding by RepA mutants predicted to be DNA binding defective. A, RepA (diamonds), RepA(R130A) (squares), or RepA(R245A) (triangles), at the concentrations indicated, was incubated with ClpA in the presence (closed symbols) or absence (open symbols) of ATP and DNA binding was measured using gel shift assay as described in “Experimental Procedures.” B, RepA (diamonds), RepA(K128A) (squares), or RepA(K88A) (circles) was incubated with ClpA (filled symbols) or without ClpA (open symbols), and DNA binding was measured using nitrocellulose filter binding assay as described under “Experimental Procedures.” C, RepA (diamonds) or RepA(R269A) (triangles) was incubated with ClpA (filled symbols) or without ClpA (open symbols), and DNA binding was measured using nitrocellulose filter binding assay as in Fig. 3B.
in a high copy phenotype and some of these were shown to be chaperone independent in vivo, suggesting that they may be dimerization defective (33–35). The region on the P1 RepA model that contains the known high copy mutations is colored in yellow. Importantly, it is the region predicted to be involved in dimerization (Fig. 7B).

As a further test that P1 RepA is structurally related to the other plasmid initiators, we tested two previously isolated high copy RepA mutants, RepA(H134N) and RepA(G156V) (Refs. 33 and 34 shown in Figs. 1 and 7B), for their ability to bind DNA in the absence of chaperones. The mutant proteins bound oriP1 DNA well in the absence of chaperones (Fig. 8A), although both were further activated about 2-fold by ClpA (Fig. 8A) and by the DnaK chaperone system (data not shown). By gel filtration analysis, the mutant proteins existed predominantly as monomers in the low nanomolar range and existed as mixtures of monomers and dimers in the low micromolar range (data not shown). To obtain a RepA mutant with a higher monomer/dimer ratio we constructed a multiple mutant, RepA(3M), containing both H134N and G156V substitutions and N142K, another substitution that results in a high copy phenotype (Ref. 36 shown in Figs. 1 and 7B). RepA(3M) bound oriP1 DNA well without activation by chaperones and was not significantly further activated by chaperones (Fig. 8B). By gel filtration analysis RepA3M existed mainly as a monomer at concentrations as high as 1.7 μM (Fig. 9A). At concentrations below 0.8 μM, the dimer shoulder was not present. In contrast, RepA wild type existed as a dimer at 20 nM, the lowest concentration that could be measured (Fig. 9B). The observation that mutants predicted to be in the subunit interface region are defective in dimerization further supports the validity of the alignment of RepA with the RepE family of initiators.

RepE mutants that are defective in dimerization have also been reported, one of which was used in the crystallization studies (15, 22, 37). These mutations map to a region of RepE where high copy mutations map (38, 39). We have represented F plasmid RepE as a dimer using the method used for the P1 RepA dimer and indicated the region of the known high copy mutants. It can be seen that the mutants are in the predicted subunit interface region (Fig. 7C). We have similarly represented R6K pi protein as a dimer and colored the region that contains high copy mutations (Fig. 7D). These also are located in the dimerization region, although none reported to date are in the β-sheets expected to be involved directly in subunit interactions (21, 40).

**DISCUSSION**

By using fold recognition programs we are able to align P1 RepA with the F plasmid RepE family of initiator proteins and construct a molecular model of a monomer bound to DNA. All members of the class contain N- and C-terminal domains related to each other by 2-fold structural symmetry and each domain contains an HTH DNA binding motif. To verify the
model, we have shown that RepA mutants in each of the two recognition α-helices are defective in DNA binding as are two other mutants in regions that are predicted to contact the phosphate backbone of the DNA. In addition we found that RepA is able to bind to the first half of the binding site alone through interactions with the C-terminal HTH domain. Further support that P1 RepA is a member of the RepE family of initiator proteins was obtained by comparing the model of P1 RepA with the crystal structure of a dimer of plasmid pPS10 RepA.

The subunit interface was predicted from the comparison of mutants that were made in that region. We found that the mutants were more monomeric in solution and less chaperone dependent for activation of oriP1 DNA binding.

Our results suggest P1 RepA is homologous to F plasmid RepE and the other plasmid initiator proteins in the RepE family, including the initiators of F, R6K, pSC101, pCU1, pPS10, pFA3, and pGSH500. The fold recognition programs used to place P1 RepA in the family of plasmid initiators with RepE also placed the initiators of Rts1, RepH1B, RepFIB, and RSF1010 in the same family. The initiator proteins of Rts1, RepH1B and RepFIB were expected to be in a class with P1 RepA since they are known to be 60, 40, and 40% identical to P1 RepA at the amino acid level, respectively. In contrast, RSF1010 RepC shares no sequence homology with either F RepE or P1 RepA. No fold similarity was detected between this family and the initiators of RK2, R1 (R100, NR1), and d d

It is conspicuous that P1 RepA and pGSH500 RepC have 70 amino acids at their N termini that are not present in the other plasmid initiators and the initiators of R6K, pSC101, and pFA3 have 30–100 additional amino acids on their C termini that are not present in the other initiators. Presumably these extra domains carry out activities specific for these plasmids.

Mutants and truncations of plasmid initiators have identified potential regions of interaction between the initiator and other components of the replication machinery. For example, DnaJ, which interacts with P1 RepA and targets RepA to DnaK, interacts with RepA through residues between 185 and 200 (13). In the monomer model the DnaJ site, composed of α5 and β1, is in a region of hydrophobic residues typically found in DnaJ binding sites (41). Importantly, in the molecular model, this region is on the surface. Based on the dimer structure of pPS10 RepA, the DnaJ binding site will be in α5 in the P1 RepA dimer. DnaJ forms complexes with monomers as well as dimers, suggesting that the DnaJ site is exposed in both forms of RepA. No insight into the DnaK and ClpA recognition sites was gained from the modeling work since both sites are located in the N-terminal 70 amino acid portion of RepA for which there is no predicted structural information (13, 12). Studies of deletion mutants have demonstrated that the initiator of pSC101 interacts with DnaB through residues in the N-terminal domain, in the region of residues 64–142 (42) and with DnaA through residues 1–142 (43). The R6K initiator has also been found to interact with DnaA and DnaB through contacts in the N-terminal domain (44, 45). By analogy, the other plasmid initiators in the RepE class are expected to make similar contacts with DnaA and DnaB through the N-terminal domain and those that interact with DnaJ are expected to do so in the region between the N- and C-terminal domains.

From the illustration of RepA dimers, it can be seen that a dimer could form from a pair of monomers having the structure determined for the RepE monomer without distortion of the structure, assuming the N-terminal 70 amino acids not represented in the model are not in the way (Fig. 7). Dimers with this structure would be able to bind two iterons oriented in an antiparallel fashion, thereby pairing DNA. However, dimers of this type would be physically unable to bind to inverted half
terons and it is known that many plasmid initiator proteins bind to such sites in the promoter regions of the initiator genes and repress gene expression. Thus the structure of the dimer when bound to inverted half iterons must be significantly different than the known structure of the monomer bound to the full iteron. It remains possible that there are two forms of iterons and replication factors recruited from the plexes, consisting of multiple initiator protein monomers bound also needed to understand the architecture of initiation complexes, consisting of multiple initiator protein monomers bound to multiple iterons and replication factors recruited from the host.

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