The Plasmid RK2 Initiation Protein Binds to the Origin of Replication as a Monomer*

(Received for publication, October 11, 1995, and in revised form, January 4, 1996)

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The TrfA protein encoded by the broad host range bacterial plasmid RK2 specifically binds to eight direct repeats (iterons) present at the plasmid replication origin to initiate DNA replication. Purified TrfA protein is largely in the form of a dimer, and using a dimerization test system that involves the fusion of the amino-terminal domain of the λ cl repressor protein to TrfA, we show that the TrfA protein forms dimers in vivo. Because of the high stability of the dimer form of TrfA, the formation of heterodimers between the wild-type and different sized TrfA proteins requires in vivo de novo folding of the primary protein sequence or in vitro denaturation and renaturation. The results of gel mobility shift assays using in vivo or in vitro formed heterodimers indicated that the TrfA protein binds to the iteron DNA as a monomer. Furthermore, when the monomeric and dimeric forms of TrfA are separated by gel filtration chromatography, only the protein in the chromatographic position of the monomeric form demonstrated significant DNA binding activity. These results indicate that only the monomeric form of the TrfA protein is active for binding to the iterons at the RK2 replication origin.

The ability of bacterial plasmids to regulate their replication is critical to the stable maintenance of these extrachromosomal elements. RK2 and other plasmids of the incompatibility group IncP are distinguished by their ability to be stably maintained in a diverse group of Gram-negative bacteria (see Ref. 1 for references). Controlled replication of RK2 in these bacteria requires only two plasmid encoded elements: the origin of replication, oriV, and the initiation protein, TrfA (2–4). All other replication functions are provided by the host bacterium. The trfA gene encodes two forms of the RK2 initiation protein. The smaller 33-kDa protein, TrfA-33, is the result of an independent in-frame translational start in the open reading frame used for the larger 44-kDa protein, TrfA-44 (5–7). While the significance of two forms of the initiation protein may be in extending the host range of RK2, the TrfA-33 protein by itself is capable of initiating RK2 replication in a number of different Gram-negative bacteria (8–10).

The TrfA protein specifically binds to eight directly repeated sequences, called iterons, found in the origin of replication (11, 12). The iterons are arranged in oriV in groups of five and three. The eight-iteron origin is utilized in all Gram-negative bacteria examined (2), although the five-iteron origin is also functional in Escherichia coli (13). Two iterons outside of oriV have also been identified (14, 15). The iterons in RK2 were originally defined as 17-bp1 sequences separated by a 4–6-bp spacer (16). A later study demonstrated that certain bases within the less conserved spacer sequence are also required for specific binding of TrfA to the iterons (17). Binding by TrfA to the origin iterons is a key step for the regulation of replication initiation. However, binding alone is not sufficient to initiate replication, as several mutants of TrfA have been isolated that are capable of binding to oriV in vitro and in vivo, but which are defective for replication in a number of bacterial hosts (18, 19). Thus the TrfA protein by itself must carry out other functions at the origin or host proteins must interact with TrfA bound to oriV to form the prepriming nucleoprotein structure required for DNA replication to begin.

Both TrfA-33 and TrfA-44 proteins have been shown to purify as a dimer in solution by sucrose gradient analysis (18) and by chemical cross-linking (11, 18). Most prokaryotic DNA binding proteins that bind as dimers recognize sites that reflect the symmetry of the proteins by having a dyad symmetry in the DNA sequence (for review, see Refs. 20–22). The DNA binding site for TrfA has a highly conserved region (the 17-bp iteron) and a 5’ less conserved region (the 4–6-bp spacer sequence). Contacts with TrfA are made in both regions (17). The TrfA binding site does not posses any obvious dyad symmetry. However, analysis of the methylation interference pattern of TrfA-33 protein binding to a single binding site revealed putative TrfA-DNA contacts localized in two adjacent major grooves on one face of the DNA (17). These results could be an indication that TrfA binds as a dimer to DNA even if the sequence itself does not show an evident symmetry.

The replication initiation proteins from several narrow host range iteron-containing plasmids have been studied. For plasmids P1 (23), pSC101 (24), and F (25), the wild-type Rep protein is primarily purified as a dimer in solution, but, in each case, the monomeric form of the protein binds to the iteron sequences at their replication origins (26–28). For pSC101 and F, the dimeric form of the Rep protein binds to inverted repeats that are related in sequence to the iterons and have a role in the autoregulation of rep gene expression (26, 27). The purpose of the present study was to determine the form of the TrfA protein that binds to the iterons at the RK2 origin of replication. The results presented below indicate that while purified TrfA-33 protein is largely a dimer in solution and forms, at

1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); Ni-NTA, nickel nitriolotriacetic acid; PAGE, polyacrylamide gel electrophoresis.

2 J. L. Cereghino and D. R. Helinski, unpublished results.
least to some extent, dimers in E. coli cells, it is the monomer form of this protein that is bound to the iterons of RK2 oriV.

**EXPERIMENTAL PROCEDURES**

Plasmid Constructs—Plasmid pKB277(29) carries the gene encoding the ω cl repressor protein under control of the lac promoter and was used for construction of the vector expressing the d-TrfA fusion protein. This plasmid was modified by inserting a chloramphenicol resistance gene (CmR) at a 1324-bp HaeIII fragment from plasmid pACYC184 (30), into the unique BamHI site located within the tetracycline resistance gene of pKB277 after blunting both insert and vector fragments. In the resulting construct, pKB277-CmR, expression of the cat gene is in the same orientation as the ω cl gene. Plasmid pKB277-CmR (ΔHindIII) was then constructed by deleting the 520-bp HindII fragment from pKB277-CmR in order to have a source of the ω cl repressor truncated at its carboxyl terminus. The construct for expression of the d-TrfA fusion protein was obtained by cloning into the HindII site of pKB277-CmR (ΔHindIII) the 0.9-kb Nat-HindII fragment from plasmid pEC1-33 (18). This fragment, which contains almost the entire TrfA gene sequence, was ligated to 8-bp HindII linkers (S-CAGCGCTGG-3; New England Biolabs, Beverly, MA) and after digestion with HindII, it was ligated to HindII-digested pKB277-CmR (ΔHindIII). The construct in which the ω cl and TrfA sequences were put into the same orientation as the ω cl gene, Plasmid pKB277-CmR (ΔHindIII) was discarded. The protein-resin was then washed with T/N Buffer (Tris Buffer containing 200 mM NaCl, 0.05% Triton X-100, pH 8.0) containing 50 mM NaPO4, 300 mM NaCl, pH 6.0) and then resuspended in an equal volume of Buffer H (50 mM NaPO4, 300 mM NaCl, pH 5.0). The protein-resin was then poured into an empty column at 4°C. After the column was settled, the column was washed using Buffer H and the flow of the column effluent was less then 0.01. His6-Sis was then eluted using Buffer H (50 mM NaPO4, 300 mM NaCl, pH 4.0). Peak fractions were then pooled and dialyzed against 50 mM NaPO4, 100 mM NaCl, pH 6.0, and stored at −70°C.

The His6-TrfA plus TrfA-33 or the His6-TrfA plus TrfA-N123 proteins were purified as above using E. coli BL21 (pAT35)(pKK233–2-N123) or E. coli BL23 (pAT35)(pKK233–2-N123), respectively, grown on R medium containing 200 µg/ml penicillin and 40 µg/ml kanamycin as the starting cultures.

In Vitro Heterodimer Formation—To form heterodimers in vitro, TrfA-33 or TrfA-N123 were diluted either alone or mixed in a molar ratio of 1:1 (unless otherwise noted) to final concentrations of 0.125 µM (when the two proteins were combined) in HEPES Buffer (50 mM HEPES, pH 8, 50 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40) with 4 µM guanidine HCl and incubated on ice for 30 min. The guanidine HCl was removed by dialysis at 4°C against HEPES Buffer over a 12-h period with three changes of buffer.

Activation of His6-Sis—To dissociate TrfA proteins into monomers, His6-Sis was diluted to 0.27 µM (10 µM/dimer) in Tris Buffer (20 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol) with or without 4 µM guanidine HCl and incubated on ice for 30 min. The proteins were then dialyzed at 4°C against T/N Buffer (Tris Buffer containing 0.1% Nonidet P-40 and 0.5 mM NaCl) over a 12-h period with three changes of buffer.

Gel Mobility Shift Assays—Plasmids pSP25 and pSP28 (17) were used as the source of probes containing, respectively, one and two TrfA binding sites. After digestion with EcoRI and HindII, or with EcoRI and HindI, these plasmids yielded two fragments, the smaller one containing the binding site and the larger one containing the vector. The probe containing the minimal RK2 origin of replication was obtained by digestion of pSP25 (11) with EcoRI and HindII followed by isolation of the 400-bp oriV fragment. Probes were labeled using [α-32P]dATP (6000 Ci/mmol; DuPont NEN) and Klenow polymerase at 10 min at room temperature. DNA binding reactions and gel mobility shift assays were performed as described previously (11, 17), except that poly(dI-dC) was omitted in these assays involving activation of His6-Sis. When assaying binding activity of the His6-TrfA variants (see below) 10% acrylamide nondenaturing gels were used in some assays to enhance the differences in the migration of the complexes.

Cross-linking—TrfA-33 alone, TrfA-N123 alone, or an equimolar mixture of the two proteins was treated as described above for the in vitro formation of heterodimers. Controls of the three protein preparations were similarly treated but in HEPES Buffer without guanidine HCl. Proteins were cross-linked at a concentration of 3.75 µM with 0.01% glutaraldehyde for 15 min at room temperature in a 30-µl reaction volume. The proteins in the cross-linking reactions were separated by SDS-PAGE. Western blotting to an Immobilon P membrane (Millipore, Bedford, MA) was used to detect the cross-linked protein (11) at 4°C in T/N Buffer at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected, and 5-µl aliquots were analyzed for binding activity using the gel mobility shift assay with the oriV probe. His6-TrfA protein was detected in 100-µl aliquots of column fractions using an antibody capture assay (37), using the previously described anti-TrfA antiserum (36) with horseradish peroxidase-conjugated goat anti-rabbit EIA-naffinity-purified IgG and the TMB Peroxidase EIA substrate kit (Bio-Rad) for detection.

A mixture of 100 µg each of bovine serum albumin, ovalbumin, and od to least some extent, dimers in E. coli cells, it is the monomer form of this protein that is bound to the iterons of RK2 oriV.
TrfA Protein Exists as a Dimer in Vivo—It has been shown previously by sucrose gradients and glutaraldehyde cross-linking that purified TrfA proteins are dimers in solution (11, 18, 19). However, it is not known if TrfA can form dimers in vivo. The phage λ cl repressor is a DNA binding protein that needs to be dimeric to efficiently bind to the λ operators. The amino terminus of the cl protein contains the DNA binding domain; the carboxyl terminus contains the dimerization domain (38). The two domains can be separated; the carboxyl-terminal domain maintains the ability to dimerize, while the monomeric amino-terminal domain binds DNA at a significantly decreased efficiency (39). It has been shown that a fusion of the amino-terminal domain of cl with the leucine zipper region of the yeast transcriptional regulator GCN4 resulted in a stable and biologically active dimer for repression in vitro as well as in vivo (40). We used a similar approach to determine if TrfA forms dimers in vivo. The gene fragment encoding the amino-terminal domain of cl (amino acids 1–94) was fused in-frame to the TrfA-33 coding sequence, which had been deleted for the first nine amino acids of the TrfA-33 protein, thereby generating the chimeric protein designated cl-TrfA. Cells expressing the cl-TrfA fusion were tested for their sensitivity to phage λ infection. E. coli 71/18 cells expressing the full-length cl repressor from plasmid pKB277-CmR are immune to infection by the phage λref, and, therefore, no plaques are formed after infection of the strain with λref. Cells expressing only the amino terminus of cl, strain 71/18 (pKB277-CmRΔHindIII), are sensitive to λref infection, resulting in the same number of plaques as with infection of the 71/18 cells alone (>1–3 × 10⁶ plaques/ml). Finally, cells expressing the cl-TrfA fusion, from plasmid pSP32, were immune to λref infection. Thus, the cl-TrfA fusion is a biologically active dimer where the dimerization functions of TrfA restored cl affinity for the λ operators. This result indicated that TrfA is able to form dimers in vivo.

TrfA Dimer Are Stable in Solution—Purified wild-type and mutant TrfA proteins are largely in the form of dimers (11, 18, 19). To determine the stability of TrfA dimers, we examined the ability of TrfA-33 and a functional variant, TrfA-NΔ123, which has a deletion of 20 amino acids at the amino terminus to form heterodimers in solution. TrfA-NΔ123 is able to support replication of mini-RK2 plasmids in vivo and in vitro, to form dimers in solution, and to bind specifically to the iterons of the plasmid origin (18). The predicted molecular mass of the monomer form of TrfA-NΔ123 is 30,599 Da as compared with 33,151 Da for TrfA-33.

Samples of TrfA-33 protein alone, of TrfA-NΔ123 alone, and of an approximately 1:1 molar mixture of the two proteins were diluted to a final protein concentration of 0.125–0.25 μM and incubated on ice for 30 min and then at room temperature for 10 min. They were then tested for heterodimer formation by cross-linking with glutaraldehyde followed by SDS-PAGE and Western analysis. As shown in Fig. 1 (panel U), the mixed proteins did not form heterodimers. Similar results were obtained when the proteins were incubated on ice overnight (data not shown). However, when the proteins after mixing were diluted to the same concentration in HEPES Buffer containing 4 M guanidine HCl, incubated, and then renatured by dialysis against buffer without guanidine, glutaraldehyde cross-linking revealed the formation of heterodimers (H; Fig. 1, panel T). This indicated that once a dimer was formed, there was little dissociation of the subunits but that dimers could reform after denaturation and renaturation of the protein.

TrfA Binds as a Monomer—In order to initiate replication of plasmid RK2, it is essential that the TrfA protein binds to the iterons contained at the replication origin. The quaternary form of TrfA that binds to the iterons to promote replication initiation is not known. To determine this, the complexes formed by the binding of homodimeric and heterodimeric TrfA proteins with DNA fragments containing one or two binding sites were analyzed. We performed gel mobility shift assays using the TrfA-33 protein alone, the TrfA-NΔ123 protein alone, and the two proteins mixed. All three samples were denatured in HEPES Buffer containing 4 M guanidine HCl and then renatured by dialysis in HEPES Buffer without guanidine. The treated proteins were incubated with labeled DNA containing a single binding site (pSP25) or two binding sites (pSP28). In assays using each protein alone, a single retarded complex was observed with the single binding site probe (Fig. 2, lanes 1 and 3), while two retarded complexes were observed for the double binding site probe (Fig. 2, lanes 4–7 and 12–15). As expected, the complexes formed by TrfA-NΔ123 binding to the DNA probes were retarded less than the ones formed by wild-type TrfA-33 binding.

When the protein sample containing a TrfA-33 plus TrfA-NΔ123 mix (which should contain the TrfA-33 homodimer, the TrfA-NΔ123 homodimer, and the TrfA-33/TrfA-NΔ123 heterodimer) was used with the single binding site probe, there were only two retarded complexes that migrated to the same position in the gel as the complexes formed by each protein alone (Fig. 2, lane 2). When a TrfA-33 plus TrfA-NΔ123 mix was used with the double binding site probe a total of five complexes were formed (Fig. 2, lanes 8–11). The two fastest migrating complexes (which should represent binding to one of the two sites on the probe) ran at the same position as a complex formed by each protein alone. Of the three slowest migrating complexes (which resulted from the binding of both sites on the probe), the upper and lower band corresponded to complexes formed by each protein alone. The intermediate band was unique to the mixed protein sample. These results suggest that TrfA binds as a monomer and that the intermediate band represents the situation where both the truncated and the wild-type TrfA monomer were bound to the same DNA fragment, which contains two binding sites.
Protein was purified plus TrfA-33 proteins or the His6-TrfA plus TrfA-N heterodimers, homodimers. To test this possibility and to provide additional heterodimers were formed by renaturation of the denatured the heterodimer form is not functional for binding could not be

However, the possibility that TrfA binds as a dimer and that the heterodimer form is not functional for binding could not be ruled out from this experiment alone, particularly since the heterodimers were formed by renaturation of the denatured homodimers. To test this possibility and to provide additional evidence for binding by the monomer form, we decided to form heterodimers, in vivo, using a derivative of TrfA-33 that has 36 additional amino acids at the amino terminus, including a stretch of six histidine residues. This protein, designated His6-TrfA, is functional in vivo and in vitro for mini-RK2 replication.4

Using compatible co-resident vectors, either the His6-TrfA plus TrfA-33 proteins or the His6-TrfA plus TrfA-N123 proteins were expressed in the same cell. Protein was purified from cleared lysates of these E. coli cultures using a Ni-NTA affinity column to specifically bind His6-TrfA. This scheme results in the purification of protein samples that have His6-TrfA homodimers (or monomers) and His6-TrfA/TrfA-33 or His6-TrfA/TrfA-N123 heterodimers but no TrfA-33 or TrfA-N123 homodimers (or monomers). While these latter homodimers are formed in the cell, they cannot be purified on

The affinity-purified protein preparations were then used in gel mobility shift assays with either a single binding site or a double binding site probe. For the single binding site probe, each protein preparation has only two dimeric forms of TrfA protein, as compared with the three forms obtained when the heterodimer was prepared in vitro (Fig. 1).

The two binding site probe, there was a maximum of five retarded complexes for either protein preparation (Fig. 4, lanes 6 and 7). These five bands run at the exact same position of the purified proteins when they are mixed together in vitro; four of the bands line up with bands obtained when each of the two proteins were purified separately and then bound to the DNA (data not shown). The only consistent interpretation of these results is that TrfA binds as a monomer.

TrfA Can Be Activated for Binding—Since TrfA binds as a monomer to the iterons and since the protein is a dimer in solution, then any treatment that results in an increase in the concentration of monomers in a sample of TrfA protein should result in an apparent increase in activity of the TrfA protein sample for binding to the iterons. His6-TrfA at 0.27 μM was denatured with Tris Buffer containing 4 M guanidine HCl and then allowed to renature in Tris Buffer without guanidine but with 0.5 M NaCl and 0.1% Nonidet P-40 present in order to reduce the formation of dimers from the refolded monomers. As shown in Fig. 5, such treatment resulted in an approximately 40-fold decrease in the amount of His6-TrfA required to bind approximately 50% of the oriV probe fragment as compared

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His6-TrfA homodimers and His6-TrfA/TrfA-33 heterodimers from cells expressing both His6-TrfA and TrfA-33 proteins. A. Western blot using anti-TrfA antiserum of 160 μg of cleared lysates, separated by SDS-PAGE, prepared from cultures of E. coli BL21(pBK3), which expresses TrfA-33 (lane 1); BL21(pAT30), which expresses His6-TrfA (lane 2); BL21(pAT35)(pBK3), which expresses His6-TrfA and TrfA-33 (lane 3); and BL21(pBK3) plus BL21(pAT30) cells grown separately but mixed prior to purification (lane 4). B, proteins purified from the cleared lysates by affinity to a Ni-NTA resin separated by SDS-PAGE and then stained with Coomassie Blue: lane 1, BL21(pBK3); lane 2, BL21(pAT30); lane 3, BL21(pAT35)(pBK3); lane 4, BL21(pBK3) plus BL21(pAT30) cells grown separately but mixed prior to purification; and lane 5, purified His6-TrfA (striped arrowhead) and TrfA-33 (white arrowhead) proteins. 20 μg of protein was loaded per lanes 2, 3, and 4. Lane 1 had 2 × the amount of column eluate as lane 4.

TrfA Binds as a Monomer

![Fig. 2. Binding of in vitro guanidine-treated TrfA proteins to DNA probes containing one or two binding sites.](http://www.jbc.org/)

In all lanes is the vector fragment oriV probe fragment as compared oriV probe fragment as compared

![Fig. 3. Purification of His6-TrfA homodimers and His6-TrfA/TrfA-33 heterodimers from cells expressing both His6-TrfA and TrfA-33 proteins.](http://www.jbc.org/)

Using compatible co-resident vectors, either the His6-TrfA plus TrfA-33 proteins or the His6-TrfA plus TrfA-N123 proteins were expressed in the same cell. Protein was purified from cleared lysates of these E. coli cultures using a Ni-NTA affinity column to specifically bind His6-TrfA. This scheme results in the purification of protein samples that have His6-TrfA homodimers (or monomers) and His6-TrfA/TrfA-33 or His6-TrfA/TrfA-N123 heterodimers but no TrfA-33 or TrfA-N123 homodimers (or monomers). While these latter homodimers are formed in the cell, they cannot be purified on
Fragment, which was also labeled during probe construction.

protein (●) to the two binding site probe. The lanes is the free probe; the upper band

position of a complex resulting from the binding of TrfA-N123 protein; and

indicates the position of a complex resulting from the binding of one His6-TrfA and

● indicates the position of a complex resulting from the binding of one His6-TrfA plus

TrfA-33; and of the treated but nondenatured control (lanes 2–6) were incubated

with 0.4 fmol of oriV probe in the absence of nonspecific competitor DNA. The
denatured His6-TrfA protein was added at 1.65 ng (lane 2), 5 ng (lane 3), 10 ng (lane 4), 20 ng (lane 5), and 50 ng (lane 6). Denatured/renatured His6-TrfA was added at 0.25 ng (lane 8), 0.5 ng (lane 9), 1 ng (lane 10), 5 ng (lane 11), and 10 ng (lane 12). Lanes 1 and 7 have no protein added.

with His6-TrfA protein that had been similarly diluted and treated but in Tris Buffer without guanidine. Similar activation was seen for the TrfA-33 protein (data not shown).

Activated TrfA Is a Monomer—10 μg of His6-TrfA protein at 1.35 μM that had been activated for binding by treatment with guanidine HCl was run on a Superose 12 gel filtration column. As shown in Fig. 6, the peak fraction of protein was at the approximate position of a dimer (54 kDa) (Fig. 6A) while DNA binding activity was principally associated with protein running at the approximate position of a monomer (29 kDa) (Fig. 6B). When 10 μg of His6-TrfA protein, which was not activated, was run on the column, the majority of protein ran at the position of a dimer, and again activity, which was significantly reduced, ran at the position of a monomer on the Superose 12 sizing column (Fig. 6C).

**FIG. 4.** Binding of Ni-NTA purified co-expressed proteins to DNA probes containing one or two binding sites. Gel mobility shift assays with the one binding site probe (lanes 1–5; duplicate assays) or the two binding site probe (lanes 6 and 7) were performed using either co-expressed or previously purified protein samples. Purification of co-expressed His6-TrfA plus TrfA-N123 proteins results in His6-TrfA homodimers and His6-TrfA/TrfA-N123 heterodimers. Purification of co-expressed His6-TrfA plus TrfA-33 proteins results in His6-TrfA homodimers and His6-TrfA/TrfA-33 heterodimers (see Fig. 3). Protein was added to each assay as follows: lane 1, 1 μg co-expressed His6-TrfA plus TrfA-N123 proteins; lane 2, 100 ng of purified TrfA-N123; lane 3, 100 ng of purified His6-TrfA; lane 4, 100 ng of purified TrfA-33; lane 5, 1 μg of co-expressed His6-TrfA plus TrfA-33 proteins; lane 6, 268 ng (a) or 535 ng (b) of co-expressed His6-TrfA plus TrfA-N123 proteins; and lane 7, 308 ng (a) or 615 ng (b) of co-expressed His6-TrfA plus TrfA-33 proteins. Black arrowhead indicates the position of a complex resulting from the binding of TrfA-N123 protein; white arrowhead indicates the position of a complex resulting from the binding of TrfA-33 protein; striped arrowhead indicates the position of a complex resulting from the binding of one TrfA-N123 protein; and ⬤ indicates the position of the vector fragment, which was also labeled during probe construction.

**FIG. 5.** Activation of His6-TrfA for binding to oriV. Increasing amounts of guanidine-treated/renatured His6-TrfA protein (lanes 8–12) and of the treated but nondenatured control (lanes 2–6) were incubated with 0.4 fmol of oriV probe in the absence of nonspecific competitor DNA. The nondenatured His6-TrfA protein was added at 1.65 ng (lane 2), 5 ng (lane 3), 10 ng (lane 4), 20 ng (lane 5), and 50 ng (lane 6). Denatured/renatured His6-TrfA was added at 0.25 ng (lane 8), 0.5 ng (lane 9), 1 ng (lane 10), 5 ng (lane 11), and 10 ng (lane 12). Lanes 1 and 7 have no protein added.

**FIG. 6.** Separation of His6-TrfA protein on Superose-12 sizing column. **A**, protein profiles of guanidine-treated/renatured His6-TrfA (●) and of nondenatured but treated His6-TrfA (□) run on a Superose-12 column. The relative amount of His6-TrfA was determined in 100-μl aliquots from each fraction by an antibody capture assay using anti-TrfA antiserum. The position of the bovine serum albumin (66 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa) protein standards run on the column are indicated with arrows at the top of the profile. **B**, gel mobility shift assay using the oriV DNA probe with 5-μl aliquots of fractions from the guanidine-treated/renatured His6-TrfA column separation. **C**, gel mobility shift assay using the oriV DNA probe with 5-μl aliquots of fractions from the nondenatured His6-TrfA column separation.

**DISCUSSION**

We have previously shown that the replication initiation protein, TrfA, of the broad host range plasmid RK2, binds specifically to the iterons present at oriV (11). Even though the protein is isolated largely as a dimer (11, 18, 19), in this paper we show that TrfA binds to iteron DNA as a monomer (Figs. 2, 4, and 6). Moreover, TrfA dimers are very stable (Figs. 1 and 3), at least in the various buffers used, with no detectable exchange of monomers when different sized TrfA molecules, either purified or in crude cell lysates, are mixed. Formation of

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heterodimers of different sized TrfA proteins requires either in vitro renaturation of chemically denatured proteins (Fig. 1) or co-expression of the different sized proteins in vivo (Fig. 3).

The d-TrfA fusion protein constructed in this study was used in a biological assay for λ repressor activity to demonstrate that TrfA can form dimers in vivo. However, since the construct expressing the d-TrfA fusion protein expressed significantly more protein than native RK2 (data not shown), it is not clear whether or not the TrfA protein is dimeric in E. coli cells carrying intact RK2. An analysis of exponentially growing E. coli cells carrying RK2 determined that there are 220 monomers of TrfA-33 and 80 molecules of TrfA-44 per cell (36). Assuming a cell volume for exponentially growing E. coli cells of 4.6 \( \times 10^{-10} \) \( \mu \)l (41), the in vivo concentration of TrfA is 1.08 \( \mu \)M. The results presented in Fig. 5 show that protein at 0.27 \( \mu \)M could be activated for binding approximately 40-fold; this activation is presumably the result of the conversion of dimers to monomers. Also, as shown in Fig. 6, His6-TrfA at 1.35 \( \mu \)M was primarily dimeric even after treatment to enhance monomer formation. Thus, assuming there is no active process in the cells that converts dimers to the monomer form, it seems likely on the basis of the in vivo estimates of TrfA concentration and the in vitro observations presented here that unbound TrfA is largely present as a dimer in E. coli.

Attempts to utilize the d-TrfA fusion protein system to isolate TrfA dimerization-defective mutants by replacing the wild type trfA sequence in pSP32 with previously mutated trfA sequences (31) have not been successful. The use of this dimerization reporter system for this type of screen was described in the original report of the system (40). Subsequent studies have used d-reporter systems to isolate or characterize cellular proteins that by their binding to the d-fusion protein interfere with the dimerization of yeast GCN4 (40), HIV Tat (42), or Myc (43, 44). With the TrfA dimerization mutant screen, the few E. coli(pSP28-mutant) clones isolated initially that expressed full-length d-TrfA fusion protein and were consistently defective in dimerization as determined by their sensitivity to λ infection proved upon DNA sequencing to have large regions of DNA sequence rearrangements in trfA. The same bank containing the mutated trfA genes has been utilized successfully in other mutant screens (18, 31, 45–47), where all mutants isolated were found to result from single point mutations. While more can be done with this approach, the results to date suggest that a single point mutation is not likely to abolish the ability of the TrfA protein to dimerize.

Functional roles have been shown for both the dimer and monomer forms of the initiation proteins of plasmids F and pSC101, with dimers binding to direct repeats at the rep promoter and monomers binding to the origin iterons (26, 27). Evidence has also been obtained for pSC101 indicating that dimeric RepA bound to the inverted repeat sequence, IR1, interacts with monomeric RepA bound to the iterons, and this interaction is an element in the control of plasmid copy number (48). For plasmid P1, a role has not yet been identified for the dimer form of the RepA protein, and results have been obtained that suggest that in vivo RepA is primarily monomeric (49).

The functional significance of the dimer form of TrfA is unclear. Expression of trfA is not subject to auto-regulation, and no indirect repeats related to the iteron sequences are present upstream of trfA. Two possible half-sites related to the RK2 iterons have been identified elsewhere in RK2 (15) however, there is no evidence for the binding of TrfA protein to these half-sites.

TrfA protein has been shown to have both a positive and a negative activity in regulating replication from the RK2 origin (31, 50). By binding to the origin iterons, TrfA activates replication. The kinetics of TrfA dimer dissociation into monomers might influence the rate of assembling of the replication initiation complex on oriV, thus adding an additional level of control for replication initiation. However, the copy number of plasmid RK2 is not increased in response to increases in TrfA protein levels (36). Alternatively or additionally, TrfA dimerization may be involved in the regulatory function of TrfA in plasmid copy number control. It has been proposed that, when the concentration of RK2 iteron-containing origins in the cell exceeds the typical plasmid copy number, all of the TrfA-oriV complexes are reversibly coupled at their origins thereby preventing DNA replication (handcuffing model) (31, 36, 50). It is possible that the dimeric form of TrfA plays a role in the reversible coupling of the monomeric TrfA-oriV complexes.

The E. coli chaperon proteins DnaJ and DnaK are apparently involved in the formation of monomers from dimers of the RepA initiation protein of P1 (28) and the RepE initiation protein of plasmid F (27), although an argument has been made for P1 RepA that the role of the chaperons is to activate inactive monomers (49). The DnaK protein may also be important for pSC101 replication (26). Unlike the case with these other initiator proteins, incubation of His6-TrfA or TrfA-33 with the E. coli DnaJ and DnaK proteins in vitro has no affect on the activity of the TrfA protein in binding to the iterons at the origin of replication. In addition, studies with E. coli DnaJ and DnaK deletion mutants indicate that these two chaperones are not required for RK2 replication in vivo. It is possible, however, that other E. coli chaperone proteins, such as GroEL, are involved in TrfA activation. Alternatively, the conversion of TrfA dimers to monomers in vivo may simply be passive. Given the stability of TrfA dimers in solution, it is possible that it is not the dissociation of dimeric TrfA that provides the source of monomeric TrfA available for DNA binding, rather it is denovo synthesized TrfA that binds DNA prior to dimerization. The isolation of a dimerization defective mutant of TrfA would, therefore, be important for determining the exact mechanism of dimer to monomer conversion and certainly would be very useful in determining the role of TrfA dimers in RK2 replication.

Acknowledgment.—We thank Dr. Piero Battaglia for plasmid pBK277 and for the λ ref. phage.

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TrfA Binds as a Monomer

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J. Biol. Chem. 1996, 271:7072-7078.
doi: 10.1074/jbc.271.12.7072

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