Characterizing the Structural Features of RNA/RNA Interactions of the F-plasmid FinOP Fertility Inhibition System*

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F-like plasmid transfer is mediated by the FinOP fertility inhibition system. Expression of the F positive regulatory protein, TraJ, is controlled by the action of the antisense RNA, FinP, and the RNA-binding protein FinO. FinO binds to and protects FinP from degradation and promotes duplex formation between FinP and traJ mRNA, leading to repression of both traJ expression and conjugative F transfer. FinP antisense RNA secondary structure is composed of two stem-loops separated by a 4-base single-stranded spacer and flanked on each side by single-stranded tails. Here we show that disruption of the expected Watson-Crick base pairing between the loops of FinP stem-loop I and its cognate RNA binding partner, traJ mRNA stem-loop Ic, led to a moderate reduction in the rate of duplex formation in vitro. In vivo, alterations of the anti-ribosome binding site region in the loop of FinP stem-loop I reduced the ability of the mutant FinP to mediate fertility inhibition and to inhibit TraJ expression when expressed in trans at an elevated copy number. Alterations of intermolecular complementarity between the stems of these RNAs reduced the rate of duplex formation. Our results suggest that successful interaction between stem-loop I of FinP and stem-loop Ic of traJ mRNA requires that base pairing must proceed from an initial loop-loop interaction through the top portion of the stems for stable duplex formation to occur.

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which is a key structural motif in the loops of many antisense RNA molecules (29).

The structural features of SL-I and SL-Ic of FinP antisense RNA and traJ mRNA, respectively, that influence FinO-mediated duplex formation in vitro were characterized. Duplex analyses employing EMSAs using in vitro synthesized RNAs and purified FinO protein were used to measure apparent second-order association rate constants ($k_{\text{app}}$) of duplex formation for a variety of interacting RNA partners. Our studies demonstrate that both in vitro and in vivo, FinO can overcome a variety of sequence and structural changes to FinP SL-I and traJ mRNA SL-Ic in order to promote duplex formation.

**EXPERIMENTAL PROCEDURES**

**Preparation of in Vitro Transcription Templates**—All RNAs employed in this work are derived from F-encoded FinP and traJ mRNA. All solutions used for RNA work were treated with diethyl pyrocarbonate prior to use. Transcription of FinP SL-I, traJ SL-Ic, and their mutant derivatives employed the T7 RNA polymerase promoter to strand oligonucleotide primer MGU5 and various oligonucleotide primer templates (Table I). Oligonucleotide template primers (Molecular Biology Services Unit, University of Alberta) were electrophoresed in a 10% (29:1), 8 M urea polyacrylamide gel at a constant 300 V for ~2 h. Full-length oligonucleotides were excised from the polyacrylamide gels after ethidium bromide staining. The gel slices were crushed and eluted overnight at 37 °C in elution buffer (0.5 M NH₄OAc, 0.1 mM EDTA). The supernatants were removed and extracted with an equal volume of phenol/chloroform (1:1), followed by an extraction with chloroform-isoamyl alcohol. The RNA was heated to 95 °C for approximately 5 min and then cooled on ice. The RNA was electrophoresed on an 8% (29:1), 8 M urea polyacrylamide gel at 250 V for ~2 h. The radioactive RNA band was visualized by exposure to Kodak X-Omat R film for several minutes and then excised and purified as described above, except the purified RNA was dissolved in 10 μl of Milli-Q water after precipitation. To make unlabeled RNA, all procedures were the same, except GTP/ATP/CTP/UTP were added to transcription reactions at a final concentration of 2.5 mM. The unlabeled RNA was heated to 95 °C for approximately 5 min and then cooled on ice. The RNA was electrophoresed on an 8% (29:1), 8 M urea polyacrylamide gel at 250 V for ~2 h. The radioactive RNA band was visualized by exposure to Kodak X-Omat R film for several minutes and then excised and purified as described above, except the purified RNA was dissolved in 10 μl of Milli-Q water after precipitation. To make unlabeled RNA, all procedures were the same, except GTP/ATP/CTP/UTP were added to transcription reactions at a final concentration of 2.5 mM. The unlabeled RNA was visualized by staining in ethidium bromide and gel-purified as described above.

**In Vitro Transcriptions**—Annealed templates prepared as described above were added to a final concentration of 300 nM in 20-μl transcription reactions. For labeled reactions, GTP/ATP/CTP/UTP were added to a final concentration of each of 2.5 mM, and UTP was added to a final concentration of 0.1 mM, along with 10–50 μCi of [%32P]UTP (PerkinElmer Life Sciences). Twenty-six units of RNA Guard (Amerham Biosciences) were added to each reaction, along with 1× transcription buffer (Roche Applied Science) were added, and the reactions were incubated at 37 °C for 2 h. Ten units of DNase I (RNase Free; Roche Applied Science) were added, and the reactions were incubated for a further 15 min to digest the template DNA. One-fifth volume of RNA load dye (96% [v/v] deionized formamide, 0.05% [v/v] each xylene cyanol and bromphenol blue, 20 μl EDTA) was added, and the RNA was heated to 95 °C for approximately 5 min and then cooled on ice. The RNA was electrophoresed on an 8% (29:1), 8 M urea polyacrylamide gel at 250 V for ~2 h. The radioactive RNA band was visualized by exposure to Kodak X-Omat R film for several minutes and then excised and purified as described above, except the purified RNA was dissolved in 10 μl of Milli-Q water after precipitation. To make unlabeled RNA, all procedures were the same, except GTP/ATP/CTP/UTP were added to transcription reactions at a final concentration of 2.5 mM. The unlabeled RNA was visualized by staining in ethidium bromide and gel-purified as described above.

**EMSAs for Apparent Second Order Association Rate Constant Determination**—EMSAs for determination of $k_{\text{app}}$ values were performed essentially as described (20, 23). Briefly, a 50-μl aliquot of [%32P]-labeled RNA was incubated with 600 fmol of its unlabeled complementary RNA in a 50-μl reaction containing 1× TMN buffer (20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 100 mM NaCl). Plasmid R6–5 FinO, purified as described (22), was added to a final concentration of 6 μg to the reactions where appropriate. Reactions were incubated at 37 °C, and 5-μl aliquots were withdrawn at various times. Ten μl of ice-cold TMN stop solution (1× TMN containing 30% [v/v] glycerol and 0.05% [w/v] bromphenol blue), and kept on ice. The samples were then electrophoresed on 8% (29:1) nondenaturing polyacrylamide gels containing Tris/glycine buffer (25 mM Tris-HCl, pH 8.0–8.3, 190 mM glycine) at a constant 160 V for 65 min at room temperature. Gels were

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’→3’)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFR21</td>
<td>GAGGTTCCTATGTTAT</td>
<td>FinP-specific probe</td>
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<tr>
<td>MGU53</td>
<td>CCATCGGATACATAGAAGGCTGCAGCACAAGGGAGATTCTCATGGACAG</td>
<td>Mutagenic primer for creating pUC180GGA</td>
</tr>
<tr>
<td>MGU54</td>
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<td>Mutagenic primer for creating pUC180GGA</td>
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<tr>
<td>MGU5</td>
<td>TAAACTGACTCATATAG</td>
<td>Top strand of the consensus T7 RNA Polymerase promoter sequence</td>
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</tbody>
</table>

**Table I**

Primer sequence used in this study.

Base changes are underlined, and the T7 promoter sequence is in boldface type.

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In Vitro Transcriptions—Annealed templates prepared as described above were added to a final concentration of 300 nM in 20-μl transcription reactions. For labeled reactions, GTP/ATP/CTP/UTP were added to a final concentration for each of 2.5 mM, and UTP was added to a final concentration of 0.1 mM, along with 10–50 μCi of [%32P]UTP. Twenty-six units of RNA Guard (Amerham Biosciences) were added to each reaction, along with 1× transcription buffer (Roche Applied Science) supplemented with 0.01% (v/v) Triton X-100 (Sigma). Twenty-six units of T7 RNA polymerase (Roche Applied Science) were added, and the reactions were incubated at 37 °C for 2 h. Ten units of DNase I (RNase Free; Roche Applied Science) were added, and the reactions were incubated for a further 15 min to digest the template DNA. One-fifth volume of RNA load dye (96% (v/v) deionized formamide, 0.05% (v/v) each xylene cyanol and bromphenol blue, 20 μl EDTA) was added, and the RNA was heated to 95 °C for approximately 5 min and then cooled on ice. The RNA was electrophoresed on an 8% (29:1), 8 M urea polyacrylamide gel at 250 V for ~2 h. The radioactive RNA band was visualized by exposure to Kodak X-Omat R film for several minutes and then excised and purified as described above, except the purified RNA was dissolved in 10 μl of Milli-Q water after precipitation. To make unlabeled RNA, all procedures were the same, except GTP/ATP/CTP/UTP were added to transcription reactions at a final concentration of 2.5 mM. The unlabeled RNA was visualized by staining in ethidium bromide and gel-purified as described above.
dried and then exposed on Molecular Dynamics Storage Phosphor screens overnight. Free and duplexed RNA species were visualized and quantified using an Amersham Biosciences PhosphorImager 445 SI and ImageQuant software. $k_i$ values were derived from log plots of the percentage of free labeled RNA versus time of incubation to determine the time required for 50% of the free labeled RNA to form a duplex. $k_{int}$ values were then calculated from $k_i$ and the concentration of the RNA species in excess, essentially as described (23, 30).

**EMSA for Detection of FinO Binding to SL-I and SL-I(Salt1)***—The association equilibrium constant ($K_a$) for FinO binding to 32P-labeled FinP SL-I or derivatives thereof was performed as described (22), except 6 fmol of 32P-labeled RNA were used per reaction instead of 7.5 fmol. Quantification of unbound and FinO-bound RNAs and calculation of the association constants were performed exactly as described (22, 23).

**Construction of Recombinant Plasmids**—The plasmids used in this study and the relevant details and sources of each are listed in Table II. Isolation of all plasmid DNA was performed using a rapid alkaline extraction technique (31). All clones constructed during the course of this work were sequenced using the DYEnamic ET fluorescent sequencing protocol and the manufacturer's instructions (31). All clones constructed during the course of this work were sequenced using the DYEnamic ET fluorescent sequencing protocol and the manufacturer's instructions (31).

**Isolation of Mutant FinP**—Analysis—Cells pellets corresponding to 0.1 A600 equiva-

lents were boiled in SDS sample buffer (34) for 5 min, and the supernatants were electrophoresed on SDS-polyacrylamide gels using the Bio-Rad Mini-PROTEAN® system. Proteins were transferred to nitrocellulose membranes (Millipore Corp.) at 100 V for 1 h at 4 °C using Towbin buffer (35). Membranes were blocked overnight at 4 °C with 10% (w/v) skim milk (Difco) dissolved in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/w) Tween 20 (Caledon Laboratories)). Primary antibodies diluted in 10% skim milk in TBST were added to blots and incubated for 1 h at room temperature. The following dilutions of polyclonal antisera (raised in rabbits) were used: anti-FinO, 1:50,000; anti-TraJ, 1:15,000. Blots were washed at room temperature four times for 15 min each with TBST. The secondary antibody used was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) at 1:10,000 dilution. Blots were incubated for 1 h at room temperature with the secondary antibody and then washed as described above. Blots were developed with Renaissance Western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and exposed to Eastman Kodak Co. X-Omat R film for varying times to visualize the signals.

**Northern Blot Assays**—Total RNA was isolated using a modified hot phenol method as described (23, 24) from strains grown in liquid cultures (LB broth) at 37 °C for an A600 of 0.8–1.0. RNA (30 μg) was denatured for 5 min at 95 °C in formamide RNA load dye and then electrophoresed on an 8% (29:1), 8 M urea polyacrylamide gel and transferred to Zeta-PROBE nylon membranes (Bio-Rad) as described (24). The blots were prehybridized for 4 h using the same conditions as described (24), except 200 μg/ml of each of boiled E. coli strain W tRNA type XX and sonicated calf thymus DNA (Sigma) were added to the hybridization solution. The FinP-specific probe primer A (Table I) was end-labeled with γ-32PATP (PerkinElmer Life Sciences), and 10 pmol of the probe was added to the blots in fresh hybridization solution. Incubation proceeded overnight at 37 °C, and the blots were then washed as described (23) and exposed on an Amersham Biosciences storage phosphor screen. Bands corresponding to FinP were visualized using an Amersham Biosciences PhosphorImager 445 SI and ImageQuant software.

**RNA Secondary Structure Predictions**—Secondary structure predic-

TABLE II

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
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<th>Source or reference</th>
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<tr>
<td>pUC88-Km</td>
<td>Km' F tra region, Rep FIA replicon, 55-kb HindIII F fragment</td>
<td>Ref. 56</td>
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<tr>
<td>pUC18</td>
<td>Ap' general cloning vector, modified ColE1 replicon</td>
<td>Ref. 57</td>
</tr>
<tr>
<td>pT7-3</td>
<td>Ap' general cloning vector, ColE1 replicon</td>
<td>Ref. 33</td>
</tr>
<tr>
<td>pLT180</td>
<td>Ap' pT7-3 derivative expressing wild-type FinP</td>
<td>Ref. 24</td>
</tr>
<tr>
<td>pLT180GGA</td>
<td>Ap' pT7-3 derivative expressing wild-type FinP</td>
<td>Ref. 24</td>
</tr>
<tr>
<td>pCR4Blunt-TOP4</td>
<td>General cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSLF20</td>
<td>finP' F derivative</td>
<td>Ref. 25</td>
</tr>
<tr>
<td>pUC180</td>
<td>CM' finO' pACYC184 derivative</td>
<td>Ref. 25</td>
</tr>
<tr>
<td>Strains</td>
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<tr>
<td>MC4100</td>
<td>F' araD139 ∆argF-lac U180 rpsL150 (Str') relA1 fts5301 deoC1 ptsF25 rbsR</td>
<td>Ref. 58</td>
</tr>
<tr>
<td>ED24</td>
<td>F' lac' Spc'</td>
<td>Ref. 59</td>
</tr>
</tbody>
</table>

**Plasmids and bacterial strains used in this study**

Plasmid or strain: pUC18, pUC180, pLT180, pLT180GGA, and pCR4Blunt-TOP4 were transformed with the control plasmid pSLF20 (Table II) was transformed with the control plasmid pSLF20 alone, which was set at 100% mating efficiency.

**Immunoblot Analysis**—Cell pellets corresponding to 0.1 A600 equivalents were boiled in SDS sample buffer (34) for 5 min, and the supernatants were electrophoresed on 15% (29:1) SDS-polyacrylamide gels using the Bio-Rad Mini-PROTEAN® system. Proteins were transferred to nitrocellulose membranes (Millipore Corp.) at 100 V for 1 h at 4 °C using Towbin buffer (35). Membranes were blocked overnight at 4 °C with 10% (w/v) skim milk (Difco) dissolved in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/w) Tween 20 (Caledon Laboratories)). Primary antibodies diluted in 10% skim milk in TBST were added to blots and incubated for 1 h at room temperature. The following dilutions of polyclonal antisera (raised in rabbits) were used: anti-FinO, 1:50,000; anti-TraJ, 1:15,000. Blots were washed at room temperature four times for 15 min each with TBST. The secondary antibody used was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) at 1:10,000 dilution. Blots were incubated for 1 h at room temperature with the secondary antibody and then washed as described above. Blots were developed with Renaissance Western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and exposed to Eastman Kodak Co. X-Omat R film for varying times to visualize the signals.
RESULTS

FinO from the related F-like plasmid R6-5 has been determined to function in vivo to repress F transfer and in vitro to both bind FinP antisense RNA and promote duplex formation between FinP and traj mRNA (10, 20, 22). In the absence of FinO, FinP/traj184 mRNA duplex formation occurs in vitro with a $k_{app}$ of $5 \times 10^9$ M$^{-1}$ s$^{-1}$, and in the presence of wild-type R6-5 FinO, the $k_{app}$ increases to $2.5 \times 10^9$ M$^{-1}$ s$^{-1}$ (20). In the present study, a variety of RNA stem-loop constructs derived from FinP and traj184 mRNA were synthesized in vitro and subjected to EMSA analysis to determine their apparent second order association rate constants in the presence and absence of FinO. In order to determine specific regions of FinP SL-I were chosen to test for their contribution to SL-I/SL-Ic duplex formation. All mutations were transversions that disrupted the consensus 5'-UUGG-3' motif, which has been shown to make important structural contributions to RNA/RNA interactions (29). SL-I exhibits higher conservation in the loop nucleotides than SL-II, among the known alleles of FinP (8, 12, 21). These observations suggest that the more highly conserved loop of SL-I is important in the initial interaction between FinP and traj mRNA molecules and in the inhibition of traj mRNA translation.

A single base pair mismatch in the stem of SL-I and two single base pair mismatches in the stem of SL-Ic results in lower stability of these stems compared with the more extensively base-paired stems in SL-II and SL-Ic (Fig. 1). The lower predicted free energy of unfolding of SL-I ($\Delta G = -10.1$ kcal/mol) and SL-Ic ($\Delta G = -8.6$ kcal/mol) compared with SL-II ($\Delta G = -28.2$ kcal/mol) and SL-IIc ($\Delta G = -23.3$ kcal/mol) suggests that intermolecular base pairing between the stems of SL-I and SL-Ic during the formation of a stable FinP/traj184 mRNA duplex are more likely to occur than between the stems of SL-II and SL-Ic (10).

Contribution of the Loop Residues of SL-I to RNA/RNA Duplex Formation—Three regions of the loop of SL-I were chosen to test for their contribution to SL-I/SL-Ic duplex formation. All mutations were transversions that disrupted the expected Watson-Crick base pair interactions between the loops. The predicted secondary structures of all of these constructs are shown in Fig. 2. One- and two-base transversion mutations in these regions resulted in no noticeably obvious alterations to duplex formation ability (data not shown); therefore, 3- and 4-base transversion mutations were examined. The first mutation examined lies within the 5’ side of the loop of SL-I, 5'-C16G/C17G/U18A-3’, which is referred to as SL-I (16–18) throughout this work. This mutation alters 3 of the 6 bases that comprise the predicted anti-RBS of FinP (Fig. 1). The second mutation is located on the 3’ side of the loop of SL-I, 5’-C21G/A22U/A23U-3’, which is referred to as SL-I(21–23). The last extends across the top of the loop of SL-I, 5’-U18A/C19G/A20U/C21G-3’, which is referred to as SL-I(18–21). When compared with the $k_{app}$ for SL-I/SL-Ic duplex formation under identical conditions, the $k_{app}$ for SL-I(16–18)/SL-Ic duplex formation was reduced by ~52% in the absence of FinO and by 55% in the presence of FinO (Fig. 3, A and B, Table III). SL-I(18–21)/SL-Ic duplex formation exhibited a $k_{app}$ reduced by 35% in the absence of FinO and by 60% in the presence of FinO, whereas SL-II(21–23)/SL-Ic revealed a $k_{app}$ reduced by 55% in the absence of FinO and by 51% in the presence of FinO, when compared with the $k_{app}$ for SL-I/SL-Ic duplex formation under the same conditions (Fig. 3, A and B; Table III). These results suggest that the level of complementarity between loop residues of SL-I and SL-Ic affects FinO-mediated duplex formation in vitro. The observation that the $k_{app}$ values for duplex formation of all of the interactions tested between the SL-I loop mutants and SL-Ic were 10–19-fold higher in the presence of FinO than in the absence of FinO (Table III) reveals that FinO can overcome as many as four mismatches in the loop-loop base pairing interaction to promote duplex formation in vitro.

The Effect of Stem Mutations on SL-I and SL-Ic Duplex Formation—The bulged A12:A27 base pair mismatch in SL-I and the corresponding U85:U100 mismatch in SL-Ic (Fig. 1) were examined for their contribution to duplex formation. SL-I(A27U) ($\Delta G = -14.3$ kcal/mol) and SL-Ic(U85A) ($\Delta G = -12.1$ kcal/mol) were made to increase the stability of the stems while maintaining full intermolecular complementarity between the stems of the two RNAs. The $k_{app}$ for SL-I(A27U)/SL-Ic(U85A) duplex formation in the absence of FinO was reduced by 32%, whereas in the presence of FinO, the $k_{app}$ showed a 74% reduction, compared with the $k_{app}$ for SL-I/SL-Ic duplex formation (Fig. 4A, Table III). These results suggest that the overall stability of the stem regions of SL-I and SL-Ic influences their transition to a stable duplex. To create more drastic mutations affecting stem complementarity and to provide insight into the direction of progression of duplex formation, SL-Ic(TSR) and SL-Ic(BSR) were constructed. SL-Ic(TSR) has 5 base pairs in the stem immediately below the loop reversed in orientation, resulting in noncomplementarity with the corresponding region in SL-I (Fig. 2). SL-Ic(BSR) has 6 base pairs at the bottom of the stem reversed in sequence in the same fashion (Fig. 2). The single-stranded tail regions were not included in these constructs, ensuring that only the effects on intermolecular stem/stem interactions were examined. SL-I/SL-Ic(TSR) du-
plex formation in both the presence and absence of FinO was minimal, and a k_{app} could not be calculated in either case because less than 20% of the ^32P-labeled free RNA in the reactions was converted to a duplex (Fig. 4A). SL-I SL-Ic(BSR) duplex formation in the absence of FinO revealed a k_{app} that was reduced by 84% relative to the k_{app} for SL-I SL-Ic duplex formation (Fig. 4A; Table III). In the presence of FinO, the k_{app} for SL-I SL-Ic(BSR) duplex formation was reduced by 66% compared with the k_{app} for SL-ULSL-Ic duplex formation (Fig. 4A; Table III). These results suggest that stable duplex formation between SL-I and SL-Ic can proceed only if intermolecular complementarity extends from the loop through the top of the stem. The virtually identical k_{app} values for SL-I SL-Ic(BSR) and SL-ULSL-Ic(BSR) duplex formation also suggests that a region of noncomplementarity at the bottom of the stem has no significant effect on the ability of FinO to promote duplex formation between these constructs in vitro.

Detection of SL-I SL-Ic Kissing Complexes—Since kissing between loop regions is normally the first interaction in most antisense/sense RNA-pairing reactions, it was decided to determine whether a SL-I SL-Ic kissing dimer could form and be detected by EMSA analysis. SL-IcR was created such that the loop region was completely complementary to SL-I, but the stems and tails were not complementary (Fig. 2). In the presence and absence of FinO, no stable kissing intermediate was detectable (Fig. 4B), suggesting that that any initial kissing complex that forms between SL-I and SL-Ic is transient and unstable and is not detectable by EMSA analysis. These results also confirm the observations resulting from the SL-I SL-Ic(TSR) duplexing experiments described above. The formation of a stable SL-I SL-Ic duplex requires complementarity in both the loops and as much as half of the stem in the region immediately below the loops of both RNA molecules.

Contribution of the Single-stranded Tail Regions of SL-I to RNA/RNA Duplex Formation—Since the single-stranded tails of FinP SL-I and SL-II have been shown to influence the ability of FinO to bind FinP with high affinity (21), the contribution of these regions to duplex formation in vitro was tested. SL-I SL-Ic(Δtails) duplex formation showed a k_{app} reduced by 68% in the absence of FinO, and a k_{app} reduced by 72% in the presence of FinO, relative to the k_{app} for SL-I SL-Ic duplex formation under identical conditions (Fig. 5A; Table III). Analysis of SL-I(Δtails) duplex formation with SL-Ic(Δtails) revealed a 55% decrease in k_{app} in the absence of FinO and an 81% reduction in k_{app} in the presence of FinO, compared with the k_{app} for SL-U SL-Ic duplex formation under identical conditions (Fig. 5A; Table III). These values are comparable with the values obtained for SL-I SL-Ic(Δtails) duplex formation, suggesting that complementarity of the single-stranded tail regions, rather than possible structural alterations to the molecules upon removal of these regions, affects duplex formation in vitro. These results suggest that the presence of the single-stranded regions flanking SL-I and SL-Ic makes important contributions to the FinO-mediated formation of the RNA/RNA duplex in vitro. In order to ensure that any decrease in k_{app} was the result of alterations in complementarity of the interacting RNAs and not due to an inability of FinO to bind them, EMSA analysis was performed to determine whether FinO could bind to SL-I and SL-I(Δtails). As shown in Fig. 5B, FinO was able to bind to both RNA molecules, with a K_{d} of 8.6 × 10^{6} M^{-1} and 3.5 × 10^{6} M^{-1}, for binding SL-I and SL-I(Δtails), respectively. These K_{d} values are higher than those reported in a previous study, which may be attributable to the fact that our study employed native FinO, whereas the previous study employed a glutathione S-transferase-FinO fusion protein (21). Regardless, our results confirm that FinO could bind the SL-I constructs employed in the duplex assays.

Contribution of the Anti-RBS of FinP to Its in Vivo Function—As described above, alteration of a portion of the anti-RBS of FinP within the loop of SL-I moderately reduced the
that the ability of FinP to inhibit conjugative transfer of F-like plasmids is dependent upon gene dosage (15). They also confirm the results from the in vitro duplex formation assays described above, which showed that FinO can overcome multiple base mutations in FinP SL-I and promote SL-I/SL-Ic duplex formation in vitro when complete loop-loop complementarity is absent. In vivo, it also appears that FinO can compensate for suboptimal loop-loop base complementarity and promote fertility inhibition when the tested FinP loop mutant is supplied at an elevated copy number. Experiments with the very high copy number construct pUC180 gave similar results as with pLT180 (data not shown).

**DISCUSSION**

This report describes the structural features of FinP antisense RNA and traJ mRNA that influence FinO-mediated duplex formation. A common theme among antisense-sense pairing is the important initial interaction between single-stranded complementary loops of the RNA molecules (4, 30, 38, 39) (reviewed in Ref. 28). For example, in the case of plasmid ColB-P9, multiple single-base mutations that altered canonical loop-loop base pairing between Inc RNA and RepZ mRNA significantly decreased their in vivo function and impaired RNA/RNA duplex formation in vitro (40). Similarly, a single base mutation that interrupted expected loop-loop pairing between plasmid R1 CopA antisense RNA and its target, CopT, inhibited the formation of a duplex in vitro (30). Three regions of the loop of FinP SL-I were examined for their contribution to FinP traJ mRNA duplex formation in vitro. Three-base transversion mutations that disrupted expected Watson-Crick base pairing were made in FinP SL-I on the 5’ and 3’ sides of the loop, whereas a 4-base transversion was made across the top of the loop. In each case, the $k_{app}$ for duplex formation decreased by a moderate amount, in both the presence and absence of FinO (Table III). This observation suggests that other factors, separate from loop-loop interactions between FinP and traJ mRNA, affect duplex formation.

The plasmid R1 encodes a FinOP system very similar to the F plasmid. Single-base mutations in the top portions of the loops of R1 FinP SL-I or SL-II that altered potential loop-loop base interactions with traJ mRNA significantly inhibited FinO-mediated repression of conjugative transfer of R1, when these mutant FinP molecules were supplied in trans at elevated copy number. However, FinO was able to mediate repression of traJ expression under the same conditions, as measured by $\beta$-galactosidase assays of a traJ-lacZ translational fusion reporter construct (15). Whereas mutations in FinP can severely inhibit R1 fertility inhibition, FinO appears to be able to promote direct inhibition of translation of traJ by mutant FinP RNA. When single base transversions were made in the loops of SL-I and SL-II simultaneously, FinO-mediated repression of both traJ expression and conjugative plasmid transfer were significantly reduced, suggesting that both loops play a role in FinP traJ mRNA duplex formation (15). Interestingly, a single-base transversion mutation made in the 3’ portion of the loop of FinP SL-I had no negative effect on FinO-mediated inhibition of traJ expression or plasmid transfer (15). These results suggest that the interaction of FinP and traJ mRNA in vivo relies more on the bases located at the top of the loops than those situated on the 3’ side (15). In the present study, the inhibitory function of FinP in vivo was shown to rely on interactions between the anti-RBS of FinP and the RBS of traJ mRNA. When supplied in trans at medium copy number, FinP (16–18) exhibited full negative regulatory function only in the presence of FinO. These observations support the finding that FinO can compensate for loop mutations in its RNA targets and promote duplex formation in vitro and confirm that loop-loop base pair-
FinOP RNA/RNA Interactions

**TABLE III**

<table>
<thead>
<tr>
<th>RNAS present</th>
<th>$k_{app}$ values</th>
<th>Relative $k_{app}$</th>
<th>$k_{app}$ values</th>
<th>Relative $k_{app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-I-SL-Ic</td>
<td>$3.1 \pm 0.7$</td>
<td>100</td>
<td>$5.3 \pm 1.0$</td>
<td>100</td>
</tr>
<tr>
<td>SL-I(16–18)/SL-Ic</td>
<td>0.15</td>
<td>48</td>
<td>2.4</td>
<td>45</td>
</tr>
<tr>
<td>SL-I(18–21)/SL-Ic</td>
<td>0.2</td>
<td>65</td>
<td>2.1</td>
<td>40</td>
</tr>
<tr>
<td>SL-I(21–23)/SL-Ic</td>
<td>0.14 $\pm 0.2$</td>
<td>45</td>
<td>2.6 $\pm 0.3$</td>
<td>49</td>
</tr>
<tr>
<td>SL-I(A27T)/SL-Ic(U85A)</td>
<td>0.21 $\pm 0.3$</td>
<td>68</td>
<td>1.4</td>
<td>26</td>
</tr>
<tr>
<td>SL-I/SL-IcR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SL-I/SL-Ic(TSR)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SL-I/SL-Ic(BSR)</td>
<td>0.051</td>
<td>16</td>
<td>1.8 $\pm 0.2$</td>
<td>34</td>
</tr>
<tr>
<td>SL-I/SL-Ic(\Delta tails)</td>
<td>0.1 $\pm 0.03$</td>
<td>32</td>
<td>1.5</td>
<td>28</td>
</tr>
<tr>
<td>SL-I(\Delta tails)/SL-Ic(\Delta tails)</td>
<td>0.09 $\pm 0.05$</td>
<td>30</td>
<td>1.0</td>
<td>19</td>
</tr>
</tbody>
</table>

* $k_{app}$ values are an average of at least two or more independent EMSA analyses ± S.D. where appropriate.

Relative $k_{app} = \frac{[k_{app}(variant pair)]}{[k_{app}(SL-I-SL-Ic)]} \times 100$.

N.D., not determined.

**Fig. 4.** Mutations in the stem regions of SL-I and SL-Ic decrease duplex formation rates. A. EMSA analysis of duplex formation between 32P-labeled SL-I or SL-I(A27U) and their unlabeled SL-Ic-derived cognate binding partners (indicated above each panel). In all reactions without FinO (−FinO), samples were taken at 0, 15, 30, 60, and 120 min. In reactions containing FinO (+FinO), samples were taken at 0, 1, 2, 3, 4, 6, 10, and 15 min (SL-I(A27U)/SL-Ic(U85A)) and at 0, 1, 2, 5, 10, 15, 30, and 60 min (SL-I/SL-Ic(BSR) and SL-I/SL-Ic(TSR)). Procedures for EMSA analysis and determination of $k_{app}$ are described in detail under “Experimental Procedures.” The open arrows indicate free RNA, and closed arrows indicate RNA duplexes. B. EMSA analysis of duplex formation between 32P-labeled SL-I or SL-I(A27U) and their unlabeled SL-Ic-derived cognate binding partners (indicated above each panel). In all reactions without FinO (−FinO), samples were taken at 0, 15, 30, 60, and 120 min. In reactions containing FinO (+FinO), samples were taken at 0, 1, 2, 3, 4, 6, 10, and 15 min (SL-I(A27U)/SL-Ic(U85A)) and at 0, 1, 2, 5, 10, 15, 30, and 60 min (SL-I/SL-Ic(BSR) and SL-I/SL-Ic(TSR)). Procedures for EMSA analysis and determination of $k_{app}$ are described in detail under “Experimental Procedures.” The open arrows indicate free RNA, and closed arrows indicate RNA duplexes. C. EMSA analysis of duplex formation between 32P-labeled SL-I or SL-I(A27U) and their unlabeled SL-Ic-derived cognate binding partners (indicated above each panel). In all reactions without FinO (−FinO), samples were taken at 0, 15, 30, 60, and 120 min. In reactions containing FinO (+FinO), samples were taken at 0, 1, 2, 3, 4, 6, 10, and 15 min (SL-I(A27U)/SL-Ic(U85A)) and at 0, 1, 2, 5, 10, 15, 30, and 60 min (SL-I/SL-Ic(BSR) and SL-I/SL-Ic(TSR)). Procedures for EMSA analysis and determination of $k_{app}$ are described in detail under “Experimental Procedures.” The open arrows indicate free RNA, and closed arrows indicate RNA duplexes. D. EMSA analysis of duplex formation between 32P-labeled SL-I or SL-I(A27U) and their unlabeled SL-Ic-derived cognate binding partners (indicated above each panel). In all reactions without FinO (−FinO), samples were taken at 0, 15, 30, 60, and 120 min. In reactions containing FinO (+FinO), samples were taken at 0, 1, 2, 3, 4, 6, 10, and 15 min (SL-I(A27U)/SL-Ic(U85A)) and at 0, 1, 2, 5, 10, 15, 30, and 60 min (SL-I/SL-Ic(BSR) and SL-I/SL-Ic(TSR)). Procedures for EMSA analysis and determination of $k_{app}$ are described in detail under “Experimental Procedures.” The open arrows indicate free RNA, and closed arrows indicate RNA duplexes.

**Fig. 5.** Removal of the single-stranded tails of SL-I and SL-Ic reduces the rate of duplex formation. A. EMSA analysis of duplex formation between 32P-labeled SL-I or SL-I(A27U) and unlabeled SL-Ic(\Delta tails) as indicated above each panel. Samples were taken at 0, 15, 30, 60, and 120 min (−FinO) and at 0, 1, 2, 3, 4, 6, 10, and 15 min (+FinO) after the initiation of duplex formation. EMSA analysis and determination of $k_{app}$ are described in detail under “Experimental Procedures.” The open arrows indicate free RNA, and closed arrows indicate RNA duplexes. B. EMSA analysis of duplex formation between 32P-labeled SL-I or SL-I(A27U) and unlabeled SL-Ic(\Delta tails) as indicated above each panel. Samples were taken at 0, 15, 30, 60, and 120 min (−FinO) and at 0, 1, 2, 3, 4, 6, 10, and 15 min (+FinO) after the initiation of duplex formation. EMSA analysis and determination of $k_{app}$ are described in detail under “Experimental Procedures.” The open arrows indicate free RNA, and closed arrows indicate RNA duplexes.
TABLE IV

<table>
<thead>
<tr>
<th>Plasmid in pSLF20/MC4100</th>
<th>FinP expressed</th>
<th>Transconjugants/100 donors</th>
<th>Mating efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No FinO + FinO&lt;sup&gt;b&lt;/sup&gt;</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>27 35</td>
<td>100 100</td>
</tr>
<tr>
<td>pT7–3</td>
<td>Wild type</td>
<td>24 34</td>
<td>99 97</td>
</tr>
<tr>
<td>plLT180</td>
<td></td>
<td>0.19 0.015</td>
<td>0.7 0.04</td>
</tr>
<tr>
<td>plLT180GGGA</td>
<td>FinP(16–18)</td>
<td>19 2.5</td>
<td>70 7.1</td>
</tr>
</tbody>
</table>

* The ratio of transconjugants/100 donors for each mating assay was determined and compared with the same ratio obtained for pSLF20, which was set as 100% mating efficiency. Values were determined from two separate experiments (performed in duplicate) and averaged.

** FinO was provided in trans by the plasmid pSno104, expressing plasmid R6–5 FinO.

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**Fig. 6. Mutation of the anti-RBS of FinP alters its regulatory function in vivo.** Shown is immunoblot analysis of Traf expressed from pSLF20 in the presence and absence of wild-type FinP and FinP (16–18) expressed in trans at moderate copy number. The FinP F-derivative plasmid pSLF20 is present in all strains (+) except the negative control lane containing MC4100, which contains no plasmids (−). The presence (+) or absence (−) of pSno104, which expresses plasmid R6–5 FinO in trans, is indicated above each lane. The presence of the control plasmid pT7–3 or the FinP-expressing plasmids, pLT180 and plLT180GGGA, is indicated above the relevant lanes. The location of Traf and FinO are indicated to the right, and relevant molecular mass protein markers (kDa) are listed on the left. The bottom panel is a Northern blot of total cellular RNA isolated from the same strains, probed with a FinP-specific probe. The location of FinP is indicated on the right. Procedures for immunoblotting and Northern blot analysis are described in detail under “Experimental Procedures.”

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SL-I performed in this work altered the YUNR motif and significantly disrupted complementary Watson-Crick base-pairing interactions but led to only moderate decreases in duplex formation rates (Table III). Whether or not the loop mutations disrupted the YUNR motif, the decrease in in vitro duplex formation rates was approximately equivalent. In all cases, the presence of FinO resulted in higher k<sub>app</sub> values for duplex formation, demonstrating its ability to promote duplex formation in vitro between RNAs with suboptimal complementarity in loop regions. These results suggest that whereas loop-loop pairing between FinP and traJ mRNA is important, the sequence, and possibly the structure, of the YUNR motif in the loops may play a smaller role than in other systems.

The presence of short single-stranded tails flanking both the 5′ and 3′ sides of SL-I influenced the ability of SL-I to duplex with SL-Ic in vitro. The removal of both single-stranded tails from SL-I and SL-Ic led to a decrease in FinO-catalyzed duplex formation, which was more significant than any of the loop mutations that were tested, suggesting that single-stranded regions in FinP and traJ mRNA are critical for efficient duplex formation. However, a reduced affinity between FinO and these RNA constructs cannot be ruled out as having an effect on duplex formation at this time. RNA I RNA II interaction in ColE1 replication control (4) as well as the Cpa/CopT interaction of plasmid R1 (38, 42, 43) rely on interactions between single-stranded regions for full activity, demonstrating the importance of such regions to antisense-sense RNA pairing. Considering the short length of complementary single-stranded regions in FinP and traJ mRNA and the importance of such regions to stable duplex formation in other systems (38, 42, 43), the requirement for complementarity in both the loop and single-stranded tail regions of these RNAs is not unexpected.

The presence of bulged nucleotides and mismatched bases in the stems of interacting RNAs is critical for antisense/sense RNA interactions both in vitro and in vivo for several plasmids. These regions are thought to allow breathing of the stems immediately below the loops in order to allow for efficient progression of stable duplex formation (44–46). When the purine-purine mismatches in SL-I and SL-Ic were altered to A:U base pairs, maintaining their complementarity but increasing the predicted free energy of unfolding of the stems, the k<sub>app</sub> for FinO-mediated duplex formation decreased relative to duplex formation between wild-type SL-I and SL-Ic. These results indicate that the bulged mismatched base pairs in the stems of SL-I and SL-Ic influence the progression of duplex formation. Alteration of complementarity between the stems of SL-I and SL-Ic revealed that, provided at least 5 base pairs immediately below the loops are complementary, stable duplex formation could occur, albeit at a reduced rate. Interaction of the anti-sense regulatory RNA DsAR with one of its targets, rpoS mRNA, exhibits a similar requirement. Complementarity between bases in the top of the stem of SL-I in DarA and a specific region of the upstream leader of rpoS mRNA is required for efficient intermolecular pairing in order to promote translation of rpoS (47). Kissing intermediates formed by interacting RNA stem-loop constructs can often be detected readily by EMSA analysis (48, 49). Our inability to detect a SL-I/SL-Ic kissing intermediate by EMSA analysis under the conditions tested suggests that such a complex may be unstable and short-lived, unless initial loop-loop pairing can progress through the stems to form a more stable duplexing intermediate. Alternatively, a stable kissing intermediate may form but might only be detectable using more sensitive means, such as NMR analysis (50). One cannot exclude the possibility that a stable kissing intermediate, mediated by SL-I/SL-Ic and SL-II/SL-IIc interactions between whole FinP and traJ RNAs, may occur, although this possibility remains to be tested.

Several biological systems employ an accessory protein to promote RNA duplex formation, each using a different mechanism. The Rom protein of ColE1 binds to and stabilizes an initial RNA complex between RNAI and RNAII, driving the reaction toward stable duplex formation (5, 51). The TreA Hfq protein is thought to form a nucleoprotein complex with Spot42 antisense RNA and its target, gatF mRNA, cooperatively facilitating RNA/RNA pairing (52). The NCp7 nucleaseapd protein of HIV-1 has been shown to facilitate dimerization between the stem-loops of the dimerization initiation site of the HIV-1 genomic RNA by converting an initial unstable RNA loop-loop complex to a stable dimer (48, 49, 53). More recently, NCp7 was also shown to transiently melt the secondary structure of portions of the stems of human immunodeficiency virus TAR RNA and its DNA complement, cTAR (54). Clearly, accessory proteins that mediate RNA/RNA interactions use a variety of mechanisms to promote RNA pairing. Based upon its similarities to such systems, previous work done on the FinOP system, and the results presented in this work, we present a preliminary model of the mechanism of FinO-mediated duplex formation. FinO is able to destabilize double-stranded RNA, which, along with its RNA-RNA duplex catalysis activity, has been localized to a lysine-rich region within the N-terminal 44 amino acid region.
acids of the protein. The highest affinity binding sites of FinO are SL-II of FinP and SL-Ic of traJ mRNA, although SL-I is also a target for binding (Fig. 5B) (21). Initial binding of FinP and traJ mRNA by FinO allows their loops to come into close proximity and begin base pairing, whereas its RNA destabilization activity begins to open the stems of both SL-I and SL-II. This destabilization of the stems should alleviate the topological restraints inherent in such RNA/RNA interactions, which impose a kinetic barrier to extended duplex formation (reviewed in Ref. 28). Thus, more extensive intermolecular interactions between FinP and traJ mRNA should occur in the presence of FinO. It is likely that destabilization of SL-II and SL-Ic by FinO is more critical than for SL-I and SL-Ic, considering the lower thermal stability of SL-I and SL-Ic imposed by the presence of mismatches in both of their stems. Once duplex formation initiates at the loops and tops of the stems, the single-stranded tail regions of both RNAs may begin to base pair, leading to extended duplex formation. Alternatively, once it has bound to each of its RNA targets, FinO might induce extended regions of single-stranded RNA via its destabilization activity. This function might be similar to that of the extended regions of single-stranded RNA via its destabilization activity. This function might be similar to that of the extended regions of single-stranded RNA via its destabilization activity. This function might be similar to that of the E. coli Hfq RNA chaperone protein, which binds to and partially destabilizes the secondary structure of stem-loops in the small, untranslated regulatory RNA OxyS. This destabilization has been hypothesized to facilitate interaction of OxyS with one of its targets, hflA mRNA (55). Once a critical level of single-stranded RNA is achieved, rapid formation of a stable duplex between the FinP and traJ mRNA can occur.

An interesting observation that emerges from this work is that in vivo, minor changes to the RNA components of the FinOP system can cause significant changes to its function, although in vitro, structural changes to the portions of the RNAs are tolerated during duplex formation. The interaction between FinP, FinO, and traJ mRNA occurs concurrently with transcription of the tra operon, which is in turn influenced by a variety of other factors, including the concentration of these molecules (8). Likewise, the ability of FinOP to inhibit plasmid transfer does not perfectly correlate with the ability of the system to prevent traJ expression (15). It is likely that a delicate balance of factors influences the ability of FinO to promote the formation of a FinP/traJ mRNA duplex in vivo that ultimately results in inhibition of transcription of the tra operon. The exact mechanism underlying FinO-mediated FinP/traJ mRNA duplex formation remains to be elucidated.

REFERENCES