MobA, the DNA Strand Transferase of Plasmid R1162
THE MINIMAL DOMAIN REQUIRED FOR DNA PROCESSING AT THE ORIGIN OF TRANSFER*

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MobA is a DNA strand transferase encoded by the plasmid R1162 and required for plasmid DNA processing during conjugal transfer. The smallest active fragment was identified using phage display and partial enzymatic digestion of the purified protein. This fragment, consisting of approximately the first 184 amino acids, is able to bind and cleave its normal DNA substrate, the origin of transfer (oriT). Smaller fragments having one of these activities were not obtained. An active intermediate consisting of MobA linked to DNA was isolated and used to show that a single molecule of MobA is sufficient to carry out all of the DNA processing steps during transfer. These results, along with those obtained earlier, point to a single large, active site in MobA that makes several different contacts along the oriT DNA strand.

Prior to conjugal transfer of the broad-host-range plasmid R1162, three plasmid-encoded proteins assemble at a unique site, the origin of transfer (oriT), to form the relaxosome (1). One component of the relaxosome is MobA, a large (708-amino acid) protein that consists of two domains. The carboxyl-terminal region is a primase and is not required for the interaction of the protein with oriT (2, 3). The primase is also translated separately (2), and the gene for this protein probably became fused to mobA as a secondary adaptation that increased the frequency of transfer (4). In agreement with this, pSC101 as well as other plasmids contain MobA homologs lacking the primase domain (5).

The principal DNA processing reactions carried out by MobA at initiation and termination of transfer are shown in Fig. 1. The amino-terminal region of MobA, a strand transferase consisting of about 250 amino acids, cleaves one of the DNA strands at oriT and forms a tyrosyl phosphodiester bond with the 5′ end (1, 6). The cleavage reaction is reversible and does not result in the loss of plasmid superhelicity (1). The two ends of the cleaved strand are probably held together by MobA, which prevents relaxation of the plasmid DNA. In the cell, cleavage and rejoining of the oriT DNA strand might occur as an idling reaction, awaiting a hypothetical signal to direct the complex into a productive round of transfer.

Actual DNA transfer involves the unwinding of the cleaved strand and its passage, in the 5′ to 3′ direction (7), through an intercellular pore by a transporting machine assembled at this site. At the end of this process, the two ends of the strand are rejoined, presumably by a second trans-esterification carried out by the covalently linked MobA. This reaction has not been demonstrated to occur in the cell, but in vitro MobA can both cleave and rejoin single-stranded oriT DNA (8). Moreover, the putative intermediate, MobA covalently linked to single-stranded DNA, is stable and able to rejoin the strands (this work).

During both initial strand cleavage within the relaxosome and subsequent strand rejoining after a round of transfer, MobA probably interacts with an oriT DNA structure made up of both double- and single-stranded domains (9, 10) (Fig. 1). In the relaxosome, the DNA duplex in the AT-rich region of oriT is disrupted by MobA and a second protein, MobC (10). MobB, another component of the relaxosome but not shown in the figure, stabilizes the interaction of these proteins with the DNA (11). At the termination of a round of transfer, when the mobilized DNA is single-stranded, the double-stranded domain is formed by hybridization of the two arms of the inverted repeat in oriT. MobA must bind to both domains in order to function properly during transfer and to cause a gel mobility shift of single-stranded oriT DNA in vitro (12, 13). The site of cleavage is located several bases from the AT-rich region (Fig. 2A). Thus, during conjugal transfer, MobA must contact a large fraction of the 38-bp oriT.

We have identified the minimal region of MobA required for DNA processing at oriT. We also show that a single molecule is able to make all the necessary contacts required for this processing. Our results suggest that a single large functional domain of the protein contacts the origin of transfer.

EXPERIMENTAL PROCEDURES

Construction of Phage Display Library—The display vectors used in this experiment are derivatives of the plasmid pCDRR (14) modified to contain restriction sites for EcoRV, SmaI, and BstBI positioned so that pelB and gplII are in different relative reading frames. A fragment of mobA encoding the first 391 amino acids of the protein was amplified by PCR, and then portions of the product were digested separately with MnlI, BglI, RsaI, Eco47III, or Bst1107I. We then combined the products of these digestions and ligated this DNA to display vectors linearized at each of the three newly introduced restriction sites in order to increase the probability that in-frame trihybrid proteins will be encoded by the recombinant phage. Competent cells of Escherichia coli JMI093(pREP4) (15, 16) were transformed with the ligated DNA by the method of Cohen et al. (17) and then allowed to grow in 2× YT medium (18) supplemented with 1% glucose at 37 °C for 90 min. We found that the additional copies of lacI* contained in pREP4 as well as glucose in the medium were necessary to suppress background expression of gplII fusion proteins to the level necessary for good growth. Transformation resulted in ∼30,000 transformed cells, which were enumerated by plating on medium containing ampicillin.

The transformed cells were diluted 2-fold into 2× YT medium containing 1% glucose and 100 μg/ml ampicillin and incubated overnight. Cells were then diluted 20-fold into the same medium lacking glucose

Received for publication, November 8, 2001
Published, JBC Papers in Press, February 11, 2002, DOI 10.1074/jbc.M110759200
and incubated for 30 min at 37 °C. These cells were infected with ~10^10 plaque-forming units of the helper phage R408 (19). The infected culture was incubated at 37 °C for an additional 16 h. Phagemid particles in the medium were concentrated with polyethylene glycol after removing the cells by centrifugation. The particles were stored in 400 μl of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl (TBS buffer). The suspension contained 4.7 × 10^11 transducing units/ml.

Selection by Biopanning for Phagemid Particles That Bind oriT—Biopanning was done by the method of Scott and Smith (20) with some modifications. Streptavidin (2 μl of a 200 μg/ml solution) was diluted in 40 μl of 0.1 M NaHCO_3 and then added to each well of a 96-well polystyrene microtiter dish. The dish was sealed with tape and incubated with rocking at 4 °C for 16 h. The wells were then emptied by vacuum aspiration, filled with about 20 μl of blocking solution (0.1 M NaHCO_3, 5 mg/ml dialyzed bovine serum albumin, 0.1 μg/ml streptavidin, and 0.02% NaN_3) and further incubated at room temperature for 1 h, and then washed four times with 400 μl of TBS containing 0.5% Tween 20. We then added to each well the 3'-biotinylated oriT oligonucleotide 5'-GGCCAGTTTCTCGAAGAGAAACCGGTAAATGCG-CCCTCCCCTACAAAGTAG (14 pmol of DNA in 50 μl of TBS/Tween containing 0.2% NaN_3 and 1 mg/ml bovine serum albumin). After 2 h of incubation at 4 °C, the wells were vacuum-aspirated and washed six times with TBS/Tween.

Sixty μl of the phagemid suspension (10^11 infecting particles/ml) was added to the DNA-containing well, and the plate was gently agitated for 2 h at room temperature. Nonbinding particles were then removed by aspiration, and the well was washed 10 times with 400 μl of TBS/Tween. We eluted the bound particles by adding to each well 40 μl of a solution containing 0.1 M HCl (pH adjusted to 2.2 with glycine), 1 mg/ml bovine serum albumin and 0.1 mg/ml phenol red. After 10 min at room temperature, the eluate was removed and neutralized with 7.5 μl of 1 M Tris base (pH 9.1). Approximately 2.5 ml of early log-phase cells of JM103 (pREP4), grown in 2 × YT broth containing glucose were then infected with 20 μl of the neutralized phage solution, and the culture was further incubated for 1 h at 37 °C with shaking. The cells were then diluted to 30 ml in 2 × YT containing ampicillin, and the phagemids were packaged by infecting the culture with 10^9 plaque-forming units of R408. After incubation overnight at 37 °C, cells were removed by centrifugation. Ten μl of the supernatant was heated to 70 °C for 20 min and then mixed with 50 μl of TBS and subjected to two additional rounds of biopanning.

We estimated by a gel shift assay the proportion of phagemids binding to DNA after each cycle of enrichment. Five μl of the phagemid suspension for each cycle were mixed with 2.5 ml of JM103 cells, which were then incubated at 37 °C for 1.5 h. The cells were then diluted into 10 ml 2 × YT medium containing 100 μg/ml ampicillin and R408 (10^10 particle/ml) and incubated overnight. Binding reactions consisted of 5 μl of the supernatant of the overnight culture, 50 fmol of 5'-P-labeled oriT oligonucleotide (above) and 15 μl of TBS. Bound and free oligonucleotide were separated by electrophoresis through a 10% polyacrylamide gel and visualized by autoradiography.

**Purification of Moba—**A protein fragment containing the first 321 amino acids of Moba was purified by first fusing it to an intein and a chitin binding domain (21). DNA encoding the fragment was amplified by PCR and cloned into the vector pTYB2 (New England Biolabs) by
standard methods. Cells were grown at 37 °C in 1 liter of broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) to ~4 × 10^8 cells/ml isopropyl-1-thio-β-D-galactopyranoside was then added to 0.3 ml, and the culture was incubated overnight at 20–25 °C. Protein was prepared from the induced culture by affinity chromatography with a column containing chitin, essentially according to the procedures described by New England Biolabs. This protein is designated MobA*.

**Purification and Analysis of Noncovalent MobA Peptide-oriT DNA Complexes**—We first determined the conditions required to visualize and separate complexes of oriT DNA and MobA polypeptides generated by partial digestion with chymotrypsin. Twenty-μg amounts of MobA dissolved in 20 μl of buffer containing 50 mM Tris-HCl, pH 8, 50 mM NaCl, 20 mM CaCl₂, and 1 mM EDTA were digested with chymotrypsin (final concentrations 0.25, 2.5, and 50 μg/ml) for 16 h at 25 °C. The locations of the sites in MobA that are cleaved by this protease are shown in Fig. 2C. The sample was then mixed with the oligonucleotide 5'-GGGCGGATCCAAA-3' with 120 μg of purified MobA for 1 h at 37 °C in 125 μl of reaction buffer consisting of 50 mM Tris-HCl, pH 8, 10 mM MgCl₂, 0.5 mM EDTA, and 15% (v/v) glycerol (1). EDTA was then added to 100 mM and a final volume of 150 μl. Fifty μl of this reaction was mixed with 50 μl of 2× NaCl and added to 100 μl of the bead preparation (above). The complex binds to the beads because the DNA hybridizes to the short oligonucleotide attached by the biotin label. The mixture was then adjusted to 1× NaCl and a final volume of 400 μl, with the high salt concentration preventing further binding of free MobA to the oligonucleotide. The beads were chilled in an ice-water bath and washed 15 times with 400 μl of buffer containing 50 mM Tris-HCl, pH 8, 1× NaCl, and 2% Triton X-100. Finally, the beads were washed two times with 60 μl of 10× Tris-HCl (pH 8, 1× NaCl), and resuspended in 30 μl of the same buffer. The complex was eluted from the beads by heating at 65 °C for 1 min followed by collection of the beads with a magnet and washing twice with 60 μl of lysis buffer consisting of 50 mM Tris-HCl, pH 8, 0.1% SDS, 1 mM EDTA, PH 7.5) and visualized by autoradiography. Blotted peptides were detected by soaking the membrane for 5 min in 50 ml of 40% MeOH solution containing 0.025% Coomassie Blue R-250 and then destaining for 15 min in 200 ml of 50% MeOH. The membrane was dried in a stream of argon. The positions of the oriT-polypeptide complexes were then determined by comparison of the Coomassie-stained bands with those appearing after autoradiography of the membrane. The polypeptide-oriT DNA complexes were cut from the membrane and submitted for peptide sequencing. In each case, the cloned DNA was identical and confirmed by amino-terminal amino acid sequencing and electrospray analysis.

**RESULTS**

**A Minimal Binding Fragment Identified by Phage Display**—We used phage display to identify the minimal MobA fragment able to bind an oriT oligonucleotide. DNA encoding the protein was amplified by PCR, digested with different restriction enzymes, and then cloned into derivatives of the display phagemid pCDLRR (14). In this vector, fragments are cloned between genes encoding the amino terminus of gpIII, a minor coat protein of M13, and pelB, a signal sequence. Plasmid molecules containing cloned fragments of mobA DNA were introduced by transformation into JM103 (15) followed by selection for ampicillin resistance. We then generated a phagemid library by infecting these transformed cells with the partially defective helper phage, R408 (19). The resulting phagemid particles were subjected to biopanning in microtiter wells coated with single-stranded oriT DNA. Particles remaining bound to the DNA after repeated washing were eluted with acidic buffer and amplified by infection of JM103. After two additional cycles of biopanning, the infected cells were plated for resistance to ampicillin.

For each cycle of biopanning, phagemids were assayed for binding to oriT DNA. The medium from the infected culture was mixed with radiolabeled DNA and applied to a polyacrylamide gel. This medium contains phagemid proteins in sufficient amounts to allow the detection of an active MobA fusion protein by a gel mobility shift assay. Binding to oriT was easily detected in culture supernatants from phagemids that had undergone three rounds of biopanning.

We isolated the phagemid DNA from 20 colonies and characterized the cloned DNA by restriction analysis and DNA sequencing. In each case, the cloned DNA was identical and

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1 The abbreviations used are: HPLC, high-pressure liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminoethanesulfonic acid.
encoded the amino-terminal 184 amino acid residues of MobA (Fig. 2B).

Could we isolate additional phagemids expressing smaller MobA fragments? To answer this question, the mobA DNA in the phagemid was amplified by PCR, and the product was digested for various times with exonuclease Bal31. The DNA from each time point in the digestion was then cloned separately into the display system, and biopanning was carried out for three cycles as before. Protein binding to oriT DNA was detected for each time point (data not shown), but in each case only the input phagemid was recovered. We conclude therefore that the trihybrid protein expressed by the phagemid probably contains the smallest fragment of MobA capable of optimal binding of oriT DNA.

The Minimal Binding Domain of MobA Determined by Partial Enzymatic Digestion—The result from the phage display experiment suggested that a large fragment of MobA, beginning at the amino-terminal end, is required for binding to oriT. However, this does not exclude the possibility that other phagemid particles expressing smaller fragments of MobA might also bind oriT DNA but are outcompeted during the cycles of biopanning. Moreover, in MobA the nucleophile active for cleavage of oriT DNA is a tyrosine near the amino-terminal end of the protein, at position 25 in Fig. 2B (counting from the amino-terminal methionine) (1). Our earlier studies (13) indicated that the oriT base sequences required for strong binding by MobA are not located around the cleavage site. It was possible that the active binding region was smaller than indicated by phage display, with the larger MobA fragment required only for proper folding within the trihybrid protein. We therefore used an independent method involving partial enzymatic cleavage of MobA to map the minimal binding domain.

We purified a MobA fragment that is competent for transfer, but lacks the primase domain. This fragment, termed MobA*, consists of the first 321 amino acids of MobA, and the sequence is shown in Fig. 2B. The amino-terminal methionine is cleaved from the protein in the cell, and the three carboxyl-terminal amino acids, Thr-Pro-Gly, were added as the result of cloning in the expression vector. We partially digested this protein with different amounts of chymotrypsin. The enzyme was then inactivated, and the samples were mixed with a radiolabeled, single-stranded oriT oligonucleotide and applied to a 10% polyacrylamide gel (Fig. 4A). During electrophoresis MobA* binds to the DNA to form a slowly migrating complex (Fig. 4A, leftmost lane), whereas partial digestion of the protein resulted in additional complexes with greater mobility. We transferred the electrophoretically separated complexes to a polyvinylidene membrane by electroblotting and located the DNA-protein complexes by autoradiography and staining (Fig. 4B). The amino-terminal sequence of the polypeptide in each complex was then determined by the Edman procedure. The same experiment was carried out with protein digested instead of trypsin, and in this case the two complexes with the greatest mobility were collected on the membrane (data not shown). For the five complexing polypeptides (two from the trypsin digestion and three from the chymotrypsin digestion), the amino-terminal sequences were the same and were identical to the amino-terminal sequence of MobA* (Fig. 2B). We thus conclude that the minimal binding region of MobA* includes the active tyrosine.

We determined the minimal binding domain of MobA* by again partially digesting this protein with trypsin but then separating the fragments by HPLC (Fig. 3), and testing fractions for binding to the oriT oligonucleotide. The polypeptides in the peak fractions, which are indicating by the black bars at the bottom of the chromatographic profile in Fig. 3, were tested for binding by a mobility shift assay. No binding was observed except for the fractions shown in Fig. 5A. In each case, the polypeptide-oriT DNA complex migrated more rapidly than the MobA*-DNA complex (lane marked +/+).

The peak fractions were also tested for cleavage of oriT single-stranded DNA (13). Those fractions showing a mobility shift, but none of the other fractions tested cleaved the DNA (Fig. 5B). Fraction 48, which formed the least amount of complex, also generated little or no cleavage product. Thus, although strong binding is not required for proper cleavage of the oriT DNA strand (8), there were no polypeptides active in cleavage but unable to cause a mobility shift.

We examined the proteins in fractions 48–53 by SDS-polyacrylamide gel electrophoresis. Although most fractions contained more than one protein fragment, only one of these fragments was common to all the fractions active in binding (Fig. 5C). The migration rate of this fragment indicated that it consisted of most of MobA*. To identify this polypeptide, we submitted a sample of fraction 49 for automated amino-terminal sequencing to verify that it included the amino-terminal end of MobA, and we also determined the mass by electrospray ionization as expected, the amino-terminal sequences of MobA and the binding polypeptide were the same. Two determinations by electrospray analysis gave molecular mass determinations of 21121.5 and 21124.0 daltons. The amino-terminal MobA fragment closest in mass, with a calculated molecular size of 21121.5 daltons, consists of 188 amino acids and is underlined in Fig. 2B. This fragment is almost the same mass as that identified by phage display.

Stoichiometry of DNA Processing by MobA—Our results indicate that a MobA fragment of about 180 amino acids is required for strong binding to oriT and for cleavage of this DNA. A fragment of this size, folded according to several dif-
different predictions of general structure based upon amino acid sequence, would be sufficiently large to contact both the inner arm of the inverted repeat, with the outer arm folded to form a hairpin, the AT-rich region, and the correct site of cleavage (Figs. 1 and 2A). Moreover, the relaxosome has been estimated to contain one to two molecules of MobA, based on the protein stoichiometry required for optimum activity of relaxosomes reconstituted in vitro (1). We asked whether only one molecule of MobA* is required for the DNA processing reactions at oriT.

For a single molecule of MobA* to be sufficient, it must be able to cleave the oriT DNA, remain covalently attached to the 5’ end of the DNA during passage into the recipient cell, and then bind the trailing part of oriT in a manner allowing the strand-joining second trans-esterification (Fig. 1). We first generated the presumed intermediate in this series of reactions by incubating purified MobA* with an oriT oligonucleotide lacking the outer arm of the inverted repeat. In the presence of large amounts of protein, the oligonucleotide is correctly cleaved (8), but there is no inverted repeat and the protein is unable to bind strongly to the DNA, the reverse reaction is inhibited. This allows the accumulation of large amounts of the intermediate. The protein-DNA complex is then collected using streptavidin-coated magnetic beads. The complex is purified from free MobA* by extensive washing (Fig. 6A).

We incubated the complex with a 5’ end-labeled oligonucleotide having the correct sequence at the 3’ end for strand rejoining. This DNA was rejoined and the DNA of the complex in the presence of MgCl₂, as shown by the slower mobility of the labeled DNA during gel electrophoresis. The complex was purified free of free MobA by extensive washing in low stringency buffer (0.2% Triton X-100; lane e), five washes in high stringency buffer (2% Triton X-100; lane d), 15 washes in high stringency buffer. Samples were analyzed by 8% polyacrylamide-SDS gel electrophoresis (Tricine-buffered). B, ephoreptetic migration (10% polyacrylamide-6 M urea) of 5’ end-labeled trailing oriT fragment after incubation with covalent MobA*-DNA complex in the presence (lane a) or absence (lane b) of 10 mM MgCl₂. C, electrophoretic migration (12% polyacrylamide) of 200 fmol of 5’ end-labeled, single-stranded oriT oligonucleotide after incubation with 6 pmol of covalent MobA*-DNA complex (lane a), complex with 1.4 and 5.6 pmol of free MobA* (lanes b and c), and 1.4 and 5.6 pmol of free MobA* (lanes d and e).

DISCUSSION

Conjugal mobilization of R1162 requires that the MobA protein contact oriT at the inner arm of the inverted repeat, the AT-rich region, and the cleavage site. It seemed possible, therefore, that MobA could contain more than one distinct functional domain able to interact with oriT. Several observations are consistent with this possibility. First, in vitro the inverted repeat is not required for cleavage of oriT DNA at the correct site (8). Second, the distance between the inverted repeat and the other sites can be increased by one base without loss of activity (13), and this flexibility could reflect the presence of two independent binding domains on the protein. Third, in plasmids such as RK2, the small TraD protein binds to the inner arm of the inverted repeat (22). This complex is necessary for subsequent docking of the relaxase, TraI. Although in R1162 one protein is responsible for both docking and cleavage, the two required domains might still be functionally independent. Finally, within the oriTs of plasmids having Mob systems in the R1162/RSF1010 family, the inverted repeat is highly variable in sequence, whereas the sequence of the DNA making up the AT-rich region and the cleavage site is highly conserved. This suggests that there have been different selective pressures during the evolution of these two segments of oriT, a
situation most easily accommodated by two separate domains.

We found, however, that the smallest fragment capable of strong binding to oriT DNA is also the smallest fragment that cleaves this DNA. This minimal fragment consists of $\sim 184–188$ residues and includes the amino-terminal end of the protein. Fragments smaller than this but able to bind oriT were not detected either by phage display or after a partial tryptic digest of the protein. Moreover, among the fragments generated by digestion with trypsin, only those fragments able to bind could cleave oriT DNA. The data therefore suggest a single, large MobA domain that makes all of the necessary contacts with oriT DNA. In this context, it is interesting to compare the minimal MobA with the homologous protein of another plasmid, pSC101. The oriTs of R1162 and pSC101 are almost identical in the region of the cleavage site and the activity of MobA (23). We found that a MobA amino-terminal fragment extending to residue 179 in MobA was inactive in strand rejoining.2 Thus, either a second molecule of MobA can be recruited, or MobA has a second, cryptic nucleophile that is activated during transfer.

**Acknowledgments**—We thank Klaus Linse (Institute for Cellular and Molecular Biology, University of Texas at Austin) for advice and assistance.

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