Specific binding of MobA, a plasmid-encoded protein involved in the initiation and termination of conjugal DNA transfer, to single-stranded oriT DNA

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ABSTRACT
MobA protein, encoded by the broad host-range plasmid R1162, is required for conjugal mobilization of this plasmid. The protein is an essential part of the relaxosome, and is also necessary for the termination of strand transfer. In vitro, MobA is a nuclease specific for one of the two DNA strands of the origin of transfer (oriT). The protein can cleave this strand at the same site that is nicked in the relaxosome, and can also ligate the DNA. We show here that purified MobA protein forms a complex that is specific for this single oriT strand. The complex is unusually stable, with a half-life of approximately 95 min, is not disrupted by hybridization with the complementary strand, and reforms rapidly after boiling. Both the inverted repeat within oriT, and the eight bases between this repeat and the site cleaved by MobA, are required for binding by the protein. Mutations reducing base complementarity between the arms of the inverted repeat also decrease binding. This effect is partially suppressed by second-site mutations restoring complementarity. These results parallel the effects of these mutations on termination. Footprinting experiments with P1 nuclease indicate that the DNA between the inverted repeat and the nick site is protected by MobA, but that pairing between the arms of the repeat, which occurs in the absence of protein, is partially disrupted. Our results suggest that termination of strand transfer during conjugation involves tight binding of the MobA protein to the inverted repeat and adjacent oriT DNA. This complex positions the protein for ligation of the ends of the transferred strand, to reform a circular plasmid molecule.

INTRODUCTION
The broad host-range, IncQ plasmid R1162, essentially identical to the plasmid RSF1010, is efficiently co-transferred during conjugation by the self-transmissible, IncP-1 plasmids R751 and RK2 (1). R1162 encodes three proteins that are required for this mobilization (2). The largest of these proteins is MobA, which is composed of a polypeptide chain of 709 amino acids (3); however, only an amino-terminal segment of approximately 284 amino acids is required for conjugal transfer (2). MobA is an essential component of the relaxosome, a plasmid DNA—protein complex assembled at a specific region of the plasmid called the origin of transfer (oriT) (4). An active relaxosome is required for an initial step in transfer, the strand-specific nicking of the plasmid DNA at a unique site within oriT (4). After nicking, MobA probably becomes covalently attached to the 5' end of the cleaved strand (4,5).

MobA is also involved in the termination of transfer (6,7). It is likely that the nicked plasmid DNA is unwound, and the linear strand transferred into the recipient cell. The strand is then ligated by the covalently attached MobA protein to reform a circular molecule (5,7). In support of this, a purified, amino-terminal fragment of MobA (termed MobA*), fused to β-galactosidase, can cleave single-stranded oriT DNA at the nick site, and can also ligate this cleaved DNA (5). Only one of the two strands is cleaved, the strand that is nicked during conjugation.

The oriT of R1162 is small, consisting of no more than 38 base-pairs of DNA (Fig. 1) (8). Within this DNA there is an inverted repeat, with 10 base-pair arms containing a single mismatch, located 8 base-pairs away from the nick site. The arm distal to the nick site is not required for relaxosome-induced nicking, but is required for the termination of transfer (7,9). In vitro, MobA* protein will specifically cleave single-stranded oriT DNA at the nick site, whether or not an inverted repeat is present (9). However, intact oriT strands are preferentially cleaved in competition assays, suggesting that the inverted repeat is required for tight binding by MobA*, but not for its recognition of the correct cleavage site. In this paper we report the results of experiments designed to examine directly the binding of full-length MobA protein to oriT DNA. We have found that MobA forms a very stable complex with this DNA. This stability depends on the structure of the inverted repeat, as well as on the base sequence of DNA lying between the inverted repeat and the nick site.

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MATERIALS AND METHODS

Plasmids and bacteriophage

Bacteriophage are derivatives of M13mp19 (10) containing cloned oriT DNA. The construction of most of these, and the locations of the mutations within the oriT DNA (shown in Fig. 1), have been described in previous publications (6, 7, 9). Phage φrm321 was obtained by cloning the 32 base-pair BamHI–Hpal oriT fragment from φrm136 (9) into M13mp9 (11).

Plasmid pUT1151 was constructed for the overproduction of MobA protein. This plasmid consists of a 3000 base-pair fragment of R1162 DNA, containing the mobA gene, that was first fused to the tac promoter (12) and then inserted upstream of the transcription termination sites in the vector pKK232-8 (13).

Purification of MobA protein

A three liter culture of E.coli K-12 strain JM103 (14) containing plasmid pUT1151 was grown at 37°C to mid-log phase (A600 = 1) in 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl. IPTG was then added to 1 mM, and incubation continued for three hours. The cells (7 g wet weight) were harvested by centrifugation and resuspended in 20 ml buffer A (25 mM Tris HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol, 0.5% Brij 58). The cells were lysed in a French pressure cell, phenylmethylsulfonyl fluoride was added to 1 mM, and the debris pelleted by centrifugation at 100,000×g for 45 min. The supernatant was applied to a DEAE Sephacel column (3.5×30 cm). The column was washed with 300 ml buffer A, and the MobA protein eluted with 500 ml buffer A containing 0.1 M NaCl. This eluent was then applied to a heparin-Sepharose column (1.5×20 cm), the column washed with buffer A containing 0.3 M NaCl, and the MobA protein eluted with buffer A containing 0.35 M NaCl. The preparation was then applied to an hydroxyapatite column (1.5×10 cm), which was then washed with 100 ml buffer A containing 0.1 M potassium phosphate (pH 7.5). The MobA protein was eluted with 200 ml buffer A containing 0.2 M potassium phosphate, and fractions containing MobA activity pooled and concentrated by ultrafiltration (Diaflo membrane YM10, Amicon). Finally, the pooled preparation was fractionated by gel filtration (BioGel A 1.5m column, 1.5×100 cm). Fractions with MobA activity were pooled. Thirty mg of protein, determined by the Bradford procedure, were obtained by this method. The preparation appeared homogeneous after SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue.

During purification, MobA was assayed by specific cleavage of end-labeled, single-stranded oriT DNA, as previously described (5).

Footprinting with PI nuclease

Reactions were carried out with 50 pg of a single-stranded oriT DNA fragment end-labeled with α-[32-P]-dATP and Klenow fragment. The single-stranded DNA was derived from a 70 base-pair duplex oriT fragment generated by digestion of φrm18 (6)
RFI DNA with EcoRI. The DNA was incubated in 6 μl 0.1 M Tris-HCl (pH 7.0), with or without 0.5 μg MobA protein, at 0°C for 5 min. P1 nuclease in 1 μl 0.1 M Tris-HCl (pH 7.0), 12 mM ZnCl₂, was then added and incubation continued at the same temperature for an additional 5 min. The reaction was stopped by the addition of 1 μl 100 mM EDTA. Samples were loaded after heating onto a 9.5% acrylamide, 0.5% bis-acrylamide, 8 M urea gel (33 × 41 × 0.035 cm). Electrophoresis was carried out at 130 W for 90 min.

Other procedures

The two oriT DNA strands were separated by differential precipitation with polyethylene glycol essentially according to the procedure of Fernandez-Busquets and Daban (15). The strands were derived from duplex DNA fragments, 61 to 81 base-pairs in size (6, 7, 9), derived by restriction endonuclease digestion of the RFI DNA of the appropriate M13mp19 derivative. Prior to separation of the strands, the duplex DNA was end-labeled with Klenow fragment and α-[32P]-dATP. The single strands obtained were generally contaminated with variable amounts of duplex DNA, which however had a distinct migration rate and could be clearly distinguished during gel electrophoresis (see figures).

Gel retardation assays (16) were carried out by mixing protein and DNA in 0.1 M Tris HCl buffer (pH 8.0) and applying to gels polymerized from a solution of 9.5% acrylamide and 0.5% bis-acrylamide. Electrophoresis was in Tris-borate buffer (89 mM Tris HCl, 89 mM boric acid, 2 mM EDTA, pH 8) at 25 volts/cm and at room temperature. Under our conditions, duplex oriT DNA present in our preparations of single-stranded DNA did not bind significant amounts of MobA protein.

RESULTS

MobA protein binds specifically to one of the two oriT DNA strands to form a stable complex

A 70 base-pair DNA fragment, containing the R1162 oriT, was excised from phage φrm18 (6) by digestion of RFI DNA with EcoRI, and end-labeled by filling in the recessed ends with Klenow fragment. The two oriT strands were then separated by differential precipitation with polyethylene glycol (15). Each labeled strand was mixed with increasing amounts of purified MobA protein and subjected to polyacrylamide gel electrophoresis (Fig. 2). The migration of the oriT 'positive' strand, the strand normally cleaved by MobA protein (5), is shown in lanes a–e. When MobA protein was mixed with this strand, migration of the DNA in the gel was retarded. In contrast, when similar amounts of protein were mixed with the other ('negative') strand, no slowly-migrating band appeared (Fig. 2, lanes f–h). We conclude that MobA binds specifically to the strand that it cleaves.

In all experiments, only a single band with retarded mobility was found when the positive strand of oriT DNA was mixed with MobA. This was true even when an excess amount of protein was used. When 25 pg of labeled positive strand was mixed with 2.5 μg MobA protein, all of the DNA showed a mobility shift (Fig. 2, lane e). No additional, discrete bands were observed when the same amount of DNA was mixed with 30 μg of protein, although some nonspecific smearing of the negative strand was detected under these conditions (data not shown).

The MobA–oriT (positive strand) complex is very stable. We allowed the complex between MobA and labeled, positive strand oriT DNA to form, and then added a molar excess of unlabeled φrm18 single-stranded phage DNA, which contains the oriT positive strand. At different times the mixture was sampled, and the complexed and free labeled DNA separated by gel electrophoresis (Fig. 3). The relative amounts of label in each band on the gel were determined by densitometry of the autoradiogram. We calculated from these measurements that the complex has a half-life of approximately 95 minutes. The addition of the complementary strand also did not displace bound MobA protein from the oriT positive strand. MobA–oriT DNA complex was incubated with a six-fold excess of complementary strand DNA. Subsequent gel electrophoresis showed no decrease in the amount of label in the band with retarded mobility (data not shown).

MobA protein that has been heated to 100°C is still able to bind oriT positive strand DNA (Fig. 4). A protein–DNA complex was observed during gel electrophoresis even after the mixture had been heated to 100°C (Fig. 4, compare lanes b and c). Complex was also formed when protein was heated to 100°C prior to mixing with DNA (lane d). In contrast, when labeled complex was heated in the presence of excess, unlabeled competitor DNA, the complex was no longer observed in the gel (lane e). The protein had therefore dissociated from the labeled DNA at the high temperature, and had then been trapped by the competitor DNA. No competition was observed for a sample that
had not been heated (lane f). Our results indicate that although the complex is not stable at 100°C, the protein is resistant to thermal denaturation at this temperature. Thus, complexes disrupted at high temperature are able to rapidly reform upon cooling.

In cells providing MobA protein, M13 derivatives containing two, directly-repeated copies of oriT undergo site-specific recombination, resulting in phage molecules having a single, hybrid oriT (6). In vitro, different oriT DNAs cleaved by MobA at the nick site can also be ligated by this protein, to form a recombinant oriT molecule (5). Since two different oriT DNAs are involved in this recombination, we asked whether the MobA—oriT complex detected on our gels contained more than one molecule of DNA. We isolated 61-base and 81-base oriT fragments in lanes b and c, and the two fragments mixed together in lanes e and f.

MobA shows altered binding to oriT DNAs containing deletions and other mutations

We initially used a competition assay to examine the effect of deletions and other mutations in oriT on the binding of MobA protein. Labeled, oriT positive strand DNA was mixed with MobA protein in the presence of an approximately two-fold molar excess of unlabeled, M13mp19 phage DNA containing the cloned, test oriT. Inhibition of complex formation by the test oriT was then assessed by observing the amount of label in the complexed and protein-free bands after gel electrophoresis. The mutations tested and the names of the corresponding bacteriophages are listed in Fig. 1, and the results of this analysis are shown in Fig. 6. Viral DNA by itself, lacking a cloned oriT, had very little effect on binding of MobA to the labeled oriT fragment (lane h), compared to the control (no competitor, lane i). In contrast, φrm20 DNA, containing an unmutated oriT, competed with the labeled fragment, so that radioactivity was now present in both the bound and unbound positions on the gel (lane g). Deletion of either the entire inverted repeat (φrm13, lane e), or just the outer arm of this repeat (φrm147, lane f), resulted in failure of the test DNA to bind MobA effectively. However, the inverted repeat was not sufficient for binding. Deletion of oriT DNA to the right of the inverted repeat, with the inverted repeat itself remaining intact, also greatly reduced binding by the test DNA (φrm321, lane d). Two point mutations at positions 25 and 28, between the inverted repeat and the nick site, reduced but did not eliminate binding by the competitor (φrm136, lane c). Competition was abolished by a 6 base-pair insertion between these two mutations (φrm293, lane b). Point mutations at 30 and 32 in the test oriT DNA (φrm74, lane a) had little effect on complex formation. These point mutations have been previously shown not to affect the termination step in DNA transfer, or the cleavage of oriT single-stranded DNA by MobA (7,9).

An intact inverted repeat is required for binding by MobA protein. We asked how base-pairing between the arms of this repeat affected complex formation. In the normal oriT, these arms are complementary except at positions 3 and 21 (Fig. 1). We first determined how repairing this mismatch affected binding of MobA to the oriT positive strand. The oriT DNA in φrm236 (Fig. 1) contains G instead of A at position 3, and is thus able to pair with the C at position 21. A labeled, positive strand oriT fragment containing this mutation was incubated with a limiting
Figure 7. Effect of point mutations on formation of the MobA–oriT complex. Panel A: Gel lanes contained 25 pg, end-labeled oriT positive strand DNA derived from £rm20 (lanes a and b) and £rm236 (lanes c and d). In lanes b and d, DNA was mixed with 0.2 µg MobA protein. Panel B: Gel lanes contained 25 pg, end-labeled oriT positive strand DNA derived from £rm20 (lanes a and b), £rm236 (lanes c and d), £rm211 (lanes e and f), £rm228 (lanes g and h), £rm233 (lanes i and j), and £rm229 (lanes k and l). MobA protein (0.3 µg) was present in lanes b, d, f, h, j and l.

Figure 8. MobA protects oriT single-stranded DNA from digestion by P1 nuclease. Polyacrylamide gel electrophoresis of 50 pg end-labeled, positive strand oriTDNA digested with 0 U (lane a) 0.002 U (lane b), 0.004 U (lane c), 0.008 U (lane d) and 0.012 U (lane e) P1 nuclease. In lanes f—i, the DNA was incubated with 0.5 µg MobA protein prior to digestion with 0 U (lane f), 0.004 U (lane g), 0.012 U (lane h) and 0.020 U (lane i) of the enzyme. Size markers at the left of the figure were derived by digestion of the end-labelled duplex oriT fragment with different restriction endonucleases prior to strand separation. The indicated fragments of 63, 47 and 27 bases consist of positive strand DNA and were generated by digestion with BamHI, TaqI and HinPI, respectively.

amount of MobA protein, and the resulting mixture examined by gel electrophoresis to determine the relative amounts of DNA at the complexed and free positions (Fig. 7, Panel A). Under conditions where only part of the unmutated DNA is shifted to the complexed position (lane b), all of the mutated DNA appears in the complexed form (lane d). This result suggests that the MobA protein has a greater apparent affinity for the mutated oriT.

This must reflect an increase in the association rate of protein and DNA, because the extremely slow dissociation rate of the complex indicates that equilibrium would be reached only very slowly.

We also tested whether mutations reducing base complementarity between the arms reduced binding by MobA, and whether second-site mutations restoring complementarity increased binding (Fig. 7, Panel B). The oriT DNAs derived from £rm211 and £rm233 contain single point mutations in the inner arm of the inverted repeat (Fig. 1). In each case, binding of MobA protein to these DNAs was reduced (Fig. 7B, lanes f and j), compared to binding to the unmutated oriT DNA control (lane b), or to the oriT DNA with the mutation restoring the natural mismatch (lane d). When mutations located in the outer arm of the inverted repeat and restoring base complementarity were also present (£rm228 and £rm229, Fig. 1), the proportion of the labeled DNA bound by MobA increased in each case (Fig. 7B, lanes h and l). We conclude that base complementarity between the arms of the oriT inverted repeat is important in the formation of the MobA–oriT (positive strand) complex.

MobA protein protects single-stranded oriT DNA between the inverted repeat and the nick site from cleavage by P1 nuclease

We probed the MobA–oriT positive strand complex for resistance to digestion by the single-strand-specific nuclease P1. Under conditions of limited digestion with this enzyme, a ladder of DNA fragments differing in length by a single nucleotide were observed, although not all positions were cleaved at equal frequency by the enzyme (Fig. 8). However, even in the absence of MobA protein, DNA making up the inverted repeat was relatively resistant to digestion, compared to the rest of the oriT DNA (Fig. 8, lanes b–e). In particular, there was a noticeable transition from sensitivity to resistance at the end of the inverted repeat closest to the nick site. These results suggest that a hairpin loop had formed under the conditions of our assay. An alternative explanation, that the resistance was due to intermolecular base-pairing between the arms, seemed unlikely since resistance to digestion was independent of DNA concentration (results not shown).

In the presence of MobA protein, protection to P1 was extended from the end of the inverted repeat to base 30, just before the cleavage site (between bases 31 and 32) in oriT (Fig. 8, lanes g–i). These results reinforce our conclusion, based on the behavior of the oriT DNA fragments in the gel retardation assay, that the DNA between the inverted repeat and the nick site is part of the binding site of MobA, and is thus included in the footprint produced by this protein. In addition, 4–6 bases in the outer arm of the inverted repeat, distal to the nick site, show increased sensitivity to enzyme.

**DISCUSSION**

The MobA protein is a strand-specific DNA nuclease and ligase that is centrally involved in the termination of the conjugal transfer of R1162 DNA. During transfer, it is likely that MobA,
covalently linked to the 5' end of the transferred single strand, subsequently binds to the trailing portion of oriT and recircularizes the DNA. If an intact oriT is present in the transferred strand, which would be the case if there was rolling-circle replication from the 3' end of the cleaved oriT(17), then MobA can cleave the intact oriT to regenerate a monomeric molecule prior to ligation (5).

We have shown previously that specific cleavage of oriT by MobA did not require an inverted repeat, but that oriT DNA with an intact repeat was preferentially cleaved in a competition assay (9). The results of the studies reported here indicate that both the inverted repeat, and the DNA between the inverted repeat and the nick site, are required for formation of a specific complex between MobA and the positive strand of oriT. We conclude that the 8 base-pairs between the inverted repeat and the nick site bind MobA sufficiently well to allow specific cleavage, but that this association between protein and DNA is not sufficiently stable to be detected in the gel retardation assay. It is the inverted repeat that provides the necessary stability to the complex to allow it to be detected in a gel. However, although the inverted repeat is required for formation of a stable MobA—oriT DNA complex, it is not sufficient. The DNA between the inverted repeat and the nick site is also necessary (Fig. 6). The association between MobA and this DNA was visualized by footprinting with PI nuclease: oriT nucleotides to within 2 bases or less of the nick site were protected by the protein. Thus, in the complex the nick site is brought close to bound MobA, but is probably not itself covered by the protein. In agreement with thenick site being at one end of the oriT footprint, we have found that mutations in bases 33—38 in oriT, at the uncovered 5' side of the nick site, do not strongly affect cleavage (unpublished results). Interestingly, this has also been reported for the unrelated oriT of the broad host-range, IncP-1 plasmid RK2 (18).

The P1 protection assay revealed also that binding of MobA protein increased the nuclease sensitivity of the DNA in the outer arm of the inverted repeat. One explanation for this result is that duplex DNA, formed in the case of single-stranded DNA by intramolecular base-pairing between the arms of the inverted repeat, is required for formation of the primary contacts with MobA protein. This is supported by our observation that point mutations in the inverted repeat reduce the affinity of MobA for oriT DNA, and binding is partially restored when suppressor mutations are also present (Fig. 7). As the contacts between MobA and duplex DNA are formed, base-pairing between the arms might then be disrupted. One speculation is that this displacement reflects a biological role of MobA. Nicking within the relaxosome might require localized melting of the DNA, and this could be facilitated by binding of MobA to the duplex oriT DNA at the inner arm of the inverted repeat, and displacement of one of the two strands.

The inverted repeat contains one mismatch between its arms, at base-pairs 3 and 21. We have shown elsewhere that repairing this mismatch, by means of an A to G transition at position 3, results in a higher rate of termination, measured by phage recombination (9). In addition, MobA appears to have a higher affinity in vitro for oriT single-stranded DNA with this mutation (Fig. 7A). It is therefore likely that the oriT base sequence is not optimal for complex formation. The affinity of MobA for oriT with the normal sequence might be sufficiently great so that complex formation does not determine the overall efficiency of transfer, and thus there would be no selection for higher-affinity complexes. It is possible too that the higher-affinity complex is

more stable, and would present a problem for subsequent dissociation. It is noteworthy in this regard that the normal complex is very stable in vitro, having a half-life of approximately 95 minutes. In comparison, the half-life of the λ repressor—operator complex is 0.5 min (19), the lac repressor—operator complex 5 minutes (20), and the RNA polymerase—UV5 promoter complex 40 minutes (16). The stability of the normal complex, with its long half-life, suggests that after transfer some active mechanism is employed to disengage MobA from the DNA. Possibly, synthesis of the complementary plasmid strand accomplishes this purpose.

MobA, as part of the relaxosome, generates an initial nick in the strand to be transferred, and becomes covalently linked to the 5' end of this DNA. It is attractive to think that there is an underlying similarity, in terms of the activities of MobA, between the initial nicking and the subsequent termination steps. Initial nicking requires the inner but not the outer arm of the inverted repeat (9); possibly this duplex DNA is equivalent to the hairpin loop present during the termination reaction and is required for tight binding by the protein. A possible function of the additional proteins in the relaxosome would be to promote helical distortion, favoring formation of a single-stranded region that is also part of the MobA recognition and cleavage site. In addition, as suggested above, binding of MobA itself could also participate in this local strand denaturation, by disrupting base-pairing within the inner arm of the inverted repeat.

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