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Stabilization of the Relaxosome and Stimulation of Conjugal Transfer Are Genetically Distinct Functions of the R1162 Protein MobB

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MobB is a small protein encoded by the broad-host-range plasmid R1162 and required for efficient mobilization of its DNA during conjugation. The protein was shown previously to stabilize the relaxosome, the complex of plasmid DNA and mobilization proteins at the origin of transfer (*oriT*). We have generated in-frame *mobB* deletions that specifically inactivate the stabilizing effect of MobB while still allowing a high rate of transfer. Thus, MobB has two genetically distinct functions in transfer. The effect of another deletion, extending into *mobA*, indicates that both functions require a specific region of MobA protein that is distinct from the nicking-ligating domain. The *mobB* mutations that specifically affected stability also resulted in poor growth of cells, due to increased transcription from the promoters adjacent to *oriT*. The effects of the mutations could be suppressed not only by full-length MobB provided in *trans*, as expected, but also by additional copies of *oriT*, cloned in pBR322. In addition, in the presence of MobA both the full-length and truncated forms of MobB stimulated recombination between *oriT*-containing plasmids. We propose a model in which MobB regulates expression of plasmid genes by altering the stability of the relaxosome, in a manner that involves the coupling of plasmid molecules.

Proteins required to process plasmid DNA for conjugal transfer assemble at a unique locus, the origin of transfer (*oriT*), to form a complex called the relaxosome. For the broad-host-range plasmid R1162, and the nearly identical RSF1010, there are three mobilization (*mob*) genes (Fig. 1) (4, 7), each encoding a protein important for the activity of the relaxosome. The largest and best characterized of these proteins, MobA, locally disrupts the helical structure of the *oriT* DNA in the relaxosome and then cleaves one of the strands (31). MobC, a second component of the relaxosome, enhances strand separation at the site of cleavage (32). The linear strand, with MobA covalently attached to the 5′ end (1, 28), is probably unwound from its complement and inserted into a recipient cell in the 5′-to-3′ direction (14). During a late stage in transfer, MobA rejoins the ends of this strand to regenerate a circular molecule.

Reconstitution experiments in vitro have indicated that the third mobilization protein, MobB, stimulates nicking of *oriT* DNA in the relaxosome (28). In agreement with this, we have found that in vivo MobB stabilizes the assembly of MobA and MobC at *oriT* (23). This stabilization is shown by the greater proportion of *oriT* DNA sensitive to oxidation by permanganate, due to strand separation of this DNA within the relaxosome (31). In the absence of MobB, the frequency of mobilization of R1162 decreases 2 to 3 orders of magnitude. The higher transfer frequency in the presence of MobB could simply reflect stabilization of the relaxosome by the protein. We isolated mutations that resulted in unstable relaxosomes but which nevertheless permitted a high level of transfer (23). Although there were fewer complexed molecules, each appeared to be more active in nicking, which could account for the high transfer frequency. However, none of the mutations eliminated the requirement for MobB in transfer. Since mutations could be easily found that compensated for unstable relaxosomes but did not relieve the requirement for MobB, it seemed unlikely that this requirement could be explained solely by the stabilizing effect of the protein.

We have isolated and characterized a set of R1162 derivatives containing in-frame deletions in *mobB* and overlapping *mobA* (Fig. 1). The properties of these mutations indicate that stabilization of the relaxosome by MobB is genetically separable from a second role of this protein in transfer and also that a distinct region of MobA is required for MobB activity. In addition, the deletions have revealed that stabilization of the relaxosome can be enhanced by additional copies of *oriT* in the cell. We propose a model in which coupling of plasmid molecules at *oriT*, brought about by MobA and MobB, stabilizes the relaxosome.

MATERIALS AND METHODS

Strains and plasmids. The *Escherichia coli* K-12 strains used were MV10 (C600 Δ*trpE5*) (11) and JW151 (*thi endA polA1* T3^s), obtained from I. Molineux. The recipient strain in mating experiments was DF1019, a C600 derivative resistant to nalidixic acid (8). M13 derivatives were propagated in RV *lac*X42 (F′*lac*) (24), and isolated plaques formed on lawns of JM103 (19).

To construct deletion derivatives of R1162, a CG-to-GC mutation was first introduced in *mobB* at bp 4468 (Fig. 1) by oligonucleotide-directed mutagenesis (15). The mutation created an *Acc*65I site and caused a substitution of proline for alanine in MobB. A set of plasmids (Fig. 1) containing deletions that extend from the site of this mutation were constructed by first filling in the *Acc*65 site with Klenow fragment to create a unique *Sna*BI site. The *Xho*I-*Stu*I-*Xho*I linker CTCGAGGCCTCGAG was introduced at the *Sna*BI site, and deletion derivatives were generated by digestion with *Stu*I and partial digestion with *Hae*III. Only deletions rightward, as shown in Fig. 1, were obtained by this procedure, because the first *Hae*III site leftward was in a region encoding a primase essential for plasmid replication (26). The correct reading frame in the resulting plasmids, pUT1529, pUT1530, pUT1531, and pUT1532, was restored by filling in the remaining *Xho*I site, which also creates a *Pvu*I site. The plasmid pUT1562 was constructed by deletion of DNA from this site to a second *Pvu*I site at bp 4767, created by oligonucleotide mutagenesis. Plasmid pUT1533 is identical to pUT1530 except that it contains a 48-bp deletion that includes all of the *oriT*

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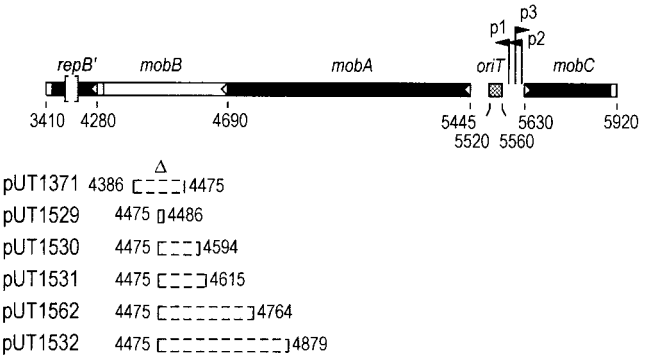

FIG. 1. Organization of genes for mobilization and of *oriT* in plasmid R1162 and locations of different in-frame deletions. The genes *mobA* and *mobB* overlap in separate reading frames. The carboxy-terminal domain of *mobA* is termed *repB'* and encodes a primase that is also translated separately (29). Initiation and termination sites for translation are indicated by open triangles and rectangles, respectively. The locations of the promoters p1 to p3 (29) are shown, with the direction of transcription from each indicated by the arrowheads. The base pair coordinates are distances from the unique *Eco*RI site in R1162.

DNA but not the adjacent promoters. The plasmid was constructed by exchanging a *Bst*1107I/*Eco*O109 fragment from another R1162 derivative containing the deletion (23).

The derivation of pUT530 (5), pUT1371, pUT1376, and pUT221 (23) has been described elsewhere. The plasmid pUT1585 was constructed by joining pUT221 and pUT1530 at their unique *Afl*III sites within R1162 DNA and then screening for spontaneous, second-site recombinants to regenerate a plasmid identical to pUT221 but with the *mobB* deletion of pUT1530. The plasmid pUT1440 consists of an 802-bp *Hpa*II-*Eco*O109 fragment of R1162 DNA (coordinates, 5135 to 5936 [Fig. 1]) cloned by replacement of the small *Cla*I-*Eco*RV fragment of pBR322 (3).

The plasmid used for measurement of intracellular amounts of specific mRNAs was constructed by first cloning a 2,667-bp *Sca*I-*Eco*O109 fragment, containing the entire *mob* region from R1162, into the *Eco*RV site of pBR322. The fragment and adjacent DNA were then excised by digestion with *Sal*I and *Eco*RI and cloned into pLG339 (30) by replacement of the small *Sal*I-*Eco*RI fragment. The deletion from pUT1530 was introduced by fragment exchange following digestion with *Blp*I and *Bst*1107I.

Assaying recombination between plasmids. MV10 cells containing R1162 or a derivative (see Fig. 6) were transformed with pUT1440, and colonies of cells resistant to ampicillin and streptomycin were obtained by plating. Three unrelated colonies were separately inoculated in 5 ml of broth medium containing antibiotics, and the culture was grown overnight to stationary phase. Plasmid DNA, isolated by the Qiagen miniprep procedure, was used to transform JW151. Transformants were selected by plating them on medium containing streptomycin and, separately, on medium containing ampicillin. The number of colonies in each case was determined after incubation at 37°C for approximately 48 h.

Determining relative amounts of specific mRNAs in cells. Relative amounts of specific mRNAs were determined by hybridization of total RNA, immobilized on a nitrocellulose membrane, with radiolabeled DNA probes. Total cellular RNA from 10 ml of log-phase cells was extracted by a commercially available procedure (Rneasy; Qiagen), and 3 to 5 μg of this preparation was dissolved in 25 mM $MgCl_2$ and digested at 37°C for 1 h with 10 U of DNase I (Sigma). Half of this sample was stored frozen at −70°C; the other half was further digested with 1 μg of DNase-free RNase A under the same conditions. Serial dilutions of the samples were then applied to a nitrocellulose membrane (BA85; Schleicher and Schuell) prepared according to the method of Sambrook et al. (25) in a slot blot apparatus.

Double-stranded, radiolabeled DNA probes were prepared by PCR in a reaction mixture (100 μl) that contained 100 to 200 ng of plasmid DNA; 2 mM $MgSO_4$; a 0.2 μM concentration of each primer; dGTP, dCTP, and dTTP (50 μM each); 45 μM dATP; 10 μl of [α-^{32}P]dATP (3,000 Ci/mmol; 10 μCi/μl); and 1 U of Vent DNA polymerase (New England Biolabs). Unincorporated nucleotides were removed with a spin column (Qiagen). The specific activity of the product was approximately 10^8 cpm per μg. About 0.5 μg of the probe DNA was diluted in 150 μl of H_2O, heated to 100°C for 15 min, and then chilled on ice and added to 15 to 20 ml of hybridization buffer (25) containing the immersed nitrocellulose membrane. After incubation at 42°C for 16 to 20 h, the membrane was washed once at room temperature with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and then three times with 0.2× SSC–0.1% SDS at 68°C. The membrane was dried at room temperature. The amounts of hybridized probe were quantified with a phosphorimager; the hybridized DNA was also visualized by autoradiography.

Other procedures. Bacteria were mated on semisolid medium by a standard procedure (4). The recombination frequency of M13mp9 derivatives containing two directly repeated copies of *oriT* was determined as previously described (21). DNA in whole cells and cleared lysates (12) was treated with permanganate as described by Zhang and Meyer (31) and Perwez and Meyer (23). DNA prepared from cleared lysates was also used to assay site-specific nicking at *oriT* by primer extension (23). In this assay, half of the DNA sample was digested with *Bsm*AI, which cleaves the plasmid DNA between *oriT* and the priming site. The other half was digested with *Bst*Z17, which cleaves distal to *oriT* in the direction of priming. The samples were then mixed, and the DNA was denatured and annealed to ^{32}P-, end-labeled primer. Strand extension was carried out by thermal cycling with *Taq* DNA polymerase, as previously described (23), and the sample was applied to a 0.35-mm, 8% polyacrylamide gel. The relative amounts of DNA in the bands after electrophoresis were determined with a phosphorimager. The fraction of nicked molecules was taken as the amount of DNA due to termination at the *oriT* nick site divided by the amount of DNA resulting from termination at the *Bsm*AI cleavage site.

RESULTS

Two functions of MobB in conjugal mobilization can be distinguished by mutation. The R1162 derivative pUT1371 (Fig. 1) contains a 162-bp, in-frame deletion in *mobB*, a mutation that inactivates the gene and causes at least a 100-fold decrease in the mobilization frequency of the plasmid (23) (Table 1). The mutation is complemented when MobB, encoded by the plasmid pUT221 (23), is provided in *trans* (Table 1). This indicates that the mutation only affects *mobB* and not the overlapping gene, *mobA* (Fig. 1).

We generated additional in-frame *mobB* deletions, starting at bp 4475, near the middle of the gene and extending toward the N-terminal coding end (Fig. 1). Three of the resulting plasmids, pUT1529, pUT1530, and pUT1531, contain dele-

TABLE 1. Effect of internal in-frame deletions in *mobA* and *mobB* on plasmid mobilization frequency and on generation time of the host

Plasmid	Mobilization frequency[a] with:		Generation time of host[b] (min) with:	
	pACYC184 (−MobB)	pUT221 (+MobB)	pACYC184 (−MobB)	pUT221 (+MobB)
R1162	4.3×10^{-3} (1.0)	3.4×10^{-3} (1.0)	35 ± 3	33 ± 3
pUT1371	3.7×10^{-5} (0.009)	2.0×10^{-3} (0.6)	81 ± 3	40 ± 8
pUT1529	6.9×10^{-4} (0.2)	4.4×10^{-3} (1.3)	135 ± 10	35 ± 12
pUT1530	1.3×10^{-3} (0.3)	3.6×10^{-3} (1.1)	69 ± 3	38 ± 4
pUT1531	2.3×10^{-3} (0.5)	5.1×10^{-3} (1.5)	115 ± 5	71 ± 1
pUT1562	6.6×10^{-6} (0.002)	5.0×10^{-5} (0.01)	124 ± 4	115 ± 5
pUT1532	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	102 ± 3	119 ± 1
pUT1533	NT	NT	81 ± 3	78 ± 5
pUT1376	3.3×10^{-6}	4.3×10^{-3}	121 ± 11	159 ± 9

[a] Average of two experiments (range, ±25% or less), shown as transconjugants per donor. The mobilization frequency relative to that for R1162 is given in parentheses. NT, not tested.

[b] Average and range of two experiments.

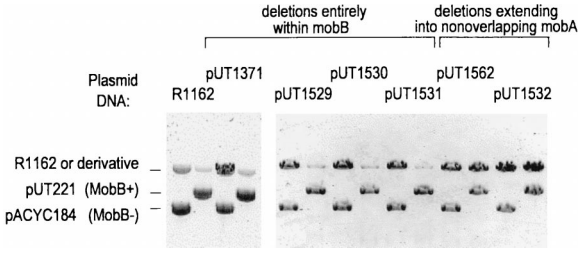

FIG. 2. DNA yields following alkaline extraction (17) for R1162 and derivatives containing deletions in *mobB*. The cells also contained pACYC184 (6) or pUT221, a pACYC184 derivative containing *mobB* (23). Plasmid DNAs were linearized by digestion with *Eco*RI and displayed by 0.8% agarose gel electrophoresis.

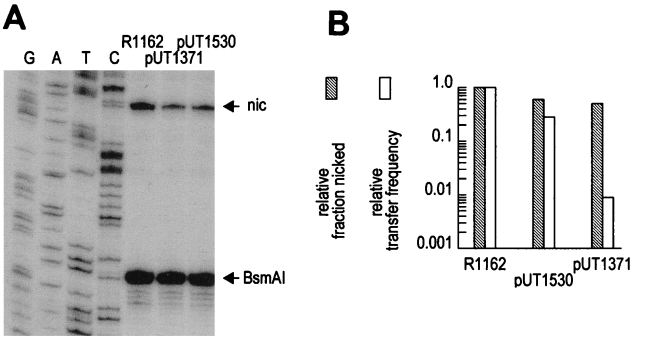

FIG. 3. (A) Proportion of plasmid DNA molecules nicked at *oriT* for R1162, pUT1371, and pUT1530. The bands on the polyacrylamide gel reflect primer extension with termination at the nick site of *oriT* (nic) and on an equal amount of template with termination at a *Bsm*AI site proximal to the primer. (B) Relative transfer frequency (obtained from Table 1) and relative fraction of nicked molecules (by quantitative analysis of the bands in the gel), with values set at 1.0 for R1162.

tions of 12, 120, and 141 bp, respectively, that are entirely within *mobB*. These deletions, as well as the one in pUT1371, were all complemented to the same level by pUT221 (Table 1). However, in the absence of complementation, pUT1529, pUT1530, and pUT1531 were each mobilized at a substantially higher frequency than pUT1371. This higher rate of transfer was observed even for pUT1531, the plasmid that contained a deletion removing about one-third of *mobB*.

The mutation in pUT1371 results not only in a low rate of transfer but also in relaxosomes that are unstable. There is a smaller proportion of nicked molecules in the cell and, as a consequence, an increased yield of supercoiled DNA compared to those with R1162 following alkaline extraction (23) (Fig. 2). Like the low frequency of transfer, this instability was also reversed by providing MobB in *trans* (Fig. 2). In the same assay, plasmid DNA yields for pUT1529, pUT1530, and pUT1531 were also greater than the yield of R1162, indicating that the relaxosomes of these plasmids were likewise unstable (Fig. 2), and again, the yield was reduced when full-length MobB was present in the cell.

If the effect of MobB on transfer is solely through stabilizing the relaxosome, then the different *mobB* deletions should have approximately commensurate effects on the proportion of molecules nicked at *oriT* and on the frequency of mobilization. We measured the fraction of molecules nicked at *oriT* for pUT1371, pUT1530, and R1162. Plasmid DNA in cleared lysates (12) was treated with SDS and phenol to disrupt the relaxosome, and the proportion of molecules specifically nicked within *oriT* was determined by primer extension and measurement of radioactivity in DNA bands after gel electrophoresis (Fig. 3A). The proportion of nicked molecules was 0.23 for R1162; this decreased to 0.14 for pUT1530 and 0.12 for pUT1371 (Fig. 3B). Thus, although the deletions in pUT1530 and pUT1371 affected the frequency of plasmid mobilization to different extents (Table 1 and Fig. 3B), they had similar effects on nicking. Moreover, the decrease in transfer frequency and the proportion of nicked molecules were similar for pUT1530, but transfer of pUT1371 was lower than could be accounted for by the level of nicked molecules. We conclude that mutations in *mobB* can differentially affect the frequency of transfer and the stability of the relaxosome. Mutations within the N-terminal half of MobB destabilize the relaxosome, and this probably accounts in large part for the small but detectable decrease in the transfer frequency of these plasmids (Table 1). The C-terminal region of MobB, conserved in these deletions and inactivated in pUT1371, is important for transfer in a second way, unconnected with relaxosome stability.

A region of *mobA* is required for activity of MobB. We also isolated and characterized two plasmids that contained in-frame deletions extending further from bp 4475, across the beginning of *mobB* and into *mobA* (pUT1532 and pUT1562 [Fig. 1]). Because DNA required for initiation of translation of *mobB* was deleted from these plasmids, we did not expect them to be mobilized at high frequency by R751. The mobilization frequency of pUT1562 was low and similar to that of pUT1371, and conjugal transfer of pUT1532 was not detected (Table 1). However, unlike the plasmids having deletions entirely within *mobB*, pUT1532 and pUT1562 could not be complemented for transfer by MobB in *trans* (Table 1). In addition, the high yield of pUT1562 and pUT1532 DNA following alkaline extraction was unaffected when MobB was in the cell (Fig. 2).

The MobA proteins encoded by pUT1532 and pUT1562 could simply fail to bind to *oriT* DNA. To test this possibility, we took advantage of the facts that this binding locally disrupts the DNA duplex and the resulting unpaired pyrimidines can be detected by their increased sensitivity to permanganate (31). Strand separation still occurs in the absence of MobB, although because MobB stabilizes the relaxosomes, sensitivity to permanganate is enhanced when this protein is present in the cell (23). We compared the permanganate sensitivity of the *oriT* DNA in the relaxosomes of pUT1530, pUT1562, and pUT1532. In each case, a cleared lysate of plasmid-containing cells was prepared and exposed to permanganate, and the oxidized bases on the negative strand, the one not transferred, were mapped by primer extension. As reported elsewhere for R1162 (31), three adjacent thymine residues in the *oriT* DNA of pUT1530 were sensitive to permanganate, and this sensitivity was enhanced by MobB (Fig. 4A, lanes a and b). The sensitive bases are located at bp 25, 26, and 27 in the *oriT* base sequence shown at the top of the figure. These bases were unreactive in the *oriT* of pUT1532 (Fig. 4A, lanes e and f), indicating that the MobA encoded by this plasmid binds poorly to *oriT* DNA or is unstable, thus accounting for the low frequency of mobilization of the plasmid. In contrast, although pUT1562 was mobilized poorly, the bases in the *oriT* DNA of pUT1562 were still sensitive to oxidation (Fig. 4A, lanes c and d). The sensitivity was about half that detected in the *oriT* DNA of pUT1530. However, unlike the relaxosome of pUT1530 (or pUT1371 [23]), permanganate sensitivity was not increased when MobB was present in the lysate (Fig. 4A, lane d). Thus, for both stabilization of the relaxosome and mobilization of the plasmid, the region of MobA affected by the deletion in pUT1562 is required for MobB to be active.

MobA not only interacts with duplex DNA in the relaxosome but also binds the single *oriT* DNA strand normally

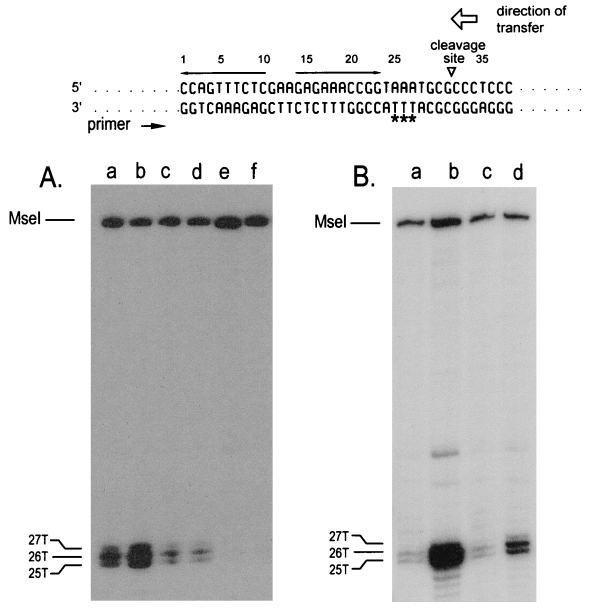

FIG. 4. (A) Permanganate-sensitive bases on the unnicked *oriT* DNA strand for pUT1530 (lanes a and b), pUT1562 (lanes c and d), and pUT1532 (lanes e and f). The locations of the bases were determined from a sequencing ladder (not shown) generated with the same template and primer. Plasmid DNAs in cleared lysates were treated with permanganate prior to primer extension by PCR (31, 32). The cells also contained either pACYC184 (lanes a, c, and e) or pUT221 (lanes b, d, and f). (B) Permanganate sensitivity of the unnicked *oriT* DNA strand for pUT1530 isolated from cells also containing pBR322 (lane c) or pUT530 (lane d). Whole cells were exposed to permanganate prior to extraction of the DNA (31). For comparison, the permanganate sensitivity of pUT1530 DNA isolated from cells treated under identical conditions but containing pACYC184 (lane a) or pUT221 (lane b) is also shown. The base sequence of *oriT* is given at the top of the figure; the permanganate-sensitive bases are identified by asterisks. The inverted repeat (opposing arrows), the site cleaved by MobA in the top (transferred) strand, and the direction of transfer are also indicated.

transferred during conjugation and can both cleave this DNA at the site nicked in the relaxosome and ligate the ends (1, 27). This property is reflected in vivo by site-specific recombination between *oriT*s cloned in M13 single-stranded DNA, when MobA is provided in the cell (21). The reaction can be monitored by infecting plasmid-containing cells with M13mp9 phages (20) having two directly repeated copies of *oriT* cloned in the *lacZ*(α) cloning region. These phages form white plaques on medium containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). After recombination between the two *oriT*s, the phages form blue plaques, due to translation through the remaining *oriT* and production of an altered but active α-complementing fragment. We tested pUT1562, as well as pUT1531, for the ability to support *oriT* recombination in M13. In both cases, the recombination frequency of the M13 derivative with two directly repeated *oriT*s was 4% in cells of RV and above the background level of 0.3% in Rec^+ cells. These frequencies were unchanged when MobB was present in the cell. Thus, the activity of MobA on single-stranded DNA was unaffected by either MobB or the region of MobA needed for the activity of this protein.

Unstable relaxosomes cause long generation times. The generation time of MV10 (R1162) cells in broth was approximately 35 min (Table 1). However, cells containing R1162 derivatives with deletions in *mobB* had substantially longer generation times (Table 1) and formed long filaments (not shown). This was true for pUT1371, in which *mobB* is completely inactivated, and also for pUT1529, pUT1530, and pUT1531, which are still mobilized at high frequency. For each of these plasmids, MobB in *trans* restored the characteristics of normal growth (Table 1). The plasmids pUT1562 and pUT1532 also caused long generation times, but in these cases the effect was not complemented by MobB (Table 1). These observations suggested that it was the unstable relaxosomes, resulting from the deletion of either the N-terminal region of MobB or the region of MobA required for MobB activity, that caused the poor growth.

MobA and MobC proteins act together as repressors at *oriT* (9) and regulate the activity of the adjacent promoters p1, p2, and p3 (29) (Fig. 1). Destabilization of the relaxosome probably decreases the level of repression, resulting in greater synthesis of plasmid proteins, which in turn stresses the cell and causes slower growth. Several other observations are consistent with this interpretation. The plasmid pUT1533 is identical to pUT1530 but contains a deletion that removes *oriT* but not the adjacent promoters. In contrast to pUT1530, this plasmid resulted in poor growth, whether or not pUT221 was also present (Table 1). Thus, MobB cannot be acting simply as an antidote by binding to MobA and reducing the toxicity of this or some other protein. In addition, *oriT* must be present in its normal location. The plasmid pUT1376 contains the *mobB* deletion present in pUT1371, but *oriT* is at a new position distant from the promoters p1 to p3. Although the *oriT* in this plasmid was active in mobilization, the cells had a long generation time, whether or not pUT221 was present (Table 1). Finally, point mutations restoring good growth were isolated by serially culturing cells containing pUT1530 in broth. Four independent point mutations were identified, and in each case these mapped in the promoters adjacent to *oriT*.

Derepression of transcription by the Δ*mobB* mutation in pUT1530 was confirmed by hybridization of radiolabeled probes to total cellular RNA. In order to avoid changes in copy number due to changes in transcription from the promoters adjacent to *oriT*, the *mob* DNA from pUT1530 was first cloned into the vector pLG339 (30), which has a replicon derived from pSC101. Transcription was measured in cells containing the resulting plasmid (pUT1596 [Fig. 5]) and also either pUT221 ($MobB^+$) or pACYC184 (no MobB present). Two radiolabeled probes were used, one hybridizing to part of the *mobA* transcript initiated from p1 and p3, and thus regulated at *oriT*, and another, as an internal control, hybridizing to transcripts of the kanamycin resistance (*kan*) gene of the vector (Fig. 5). The results (Fig. 5A and B) show that MobB in the cell did not affect transcription of the *kan* gene but reduced the amount of *mobA* transcript in the cell. We conclude that when MobB stabilizes the relaxosome, it also increases repression of transcription at *oriT*.

Multiple copies of *oriT* in *trans* suppress poor cell growth and stabilize relaxosomes in strains containing certain defective MobB proteins. The plasmid pUT530 (5) consists of a copy of the R1162 *oriT* cloned into the vector pBR322. When this plasmid was introduced into cells containing pUT1529 or pUT530, the resulting strains became healthy and grew with short generation times (Table 2). This effect depended on the cloned *oriT* in pUT530, because suppression by pBR322 was not observed (data not shown). Thus, copies of *oriT* in *trans* suppressed the poor growth phenotype caused by the *mobB* deletion in pUT1530. A copy of *oriT* in pUT1530 was also required, since the poor growth of cells containing pUT1533, a derivative of pUT1530 lacking *oriT*, was unaffected by pUT530 (Table 2).

Plasmid pUT530 did not suppress the poor growth of cells containing pUT1371, in contrast to those containing pUT1529

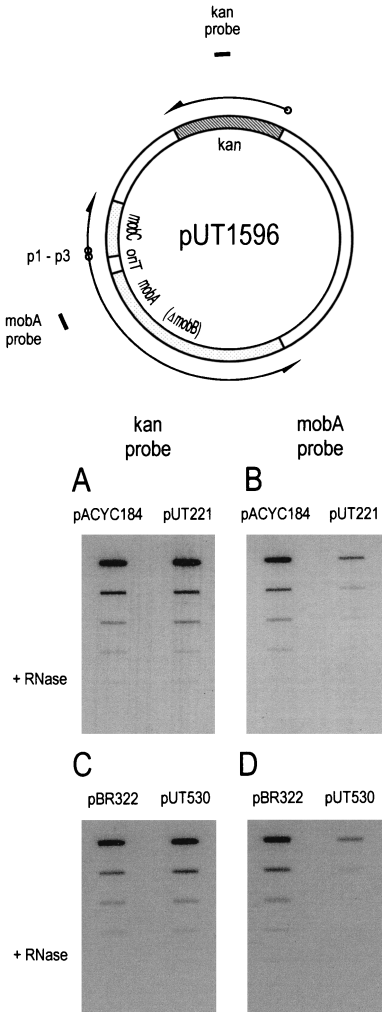

FIG. 5. Hybridization of radiolabeled DNA probes to RNA immobilized on a nitrocellulose membrane. An undiluted, DNase I-treated sample and 1:10, 1:20, and 1:40 dilutions were applied in each column of slots. In the bottom slot of each column, three times the amount of undiluted sample was digested with RNase A prior to binding to the membrane. At the top is the reporter plasmid with the approximate locations of the probes for the *kan* and *mobA* transcripts.

and pUT1530 (Table 2). This could mean that suppression required MobB and that the necessary activity was conserved on the MobB fragments encoded by pUT1529 and pUT1530 but not pUT1371. To test this, we constructed the plasmid pUT1585, analogous to pUT221 but with the *mobB* deletion of pUT1530. When cells contained this plasmid as well as pUT1371, the introduction of pUT530 resulted in healthy cells (Table 2). There was no suppression when the cells contained the parental vector pACYC184 instead of pUT1585 (Table 2). In contrast, pUT530 was nonsuppressing when introduced into cells that contained pUT1585, but with pUT1562 instead of pUT1371 (Table 2). Thus, suppression required the region of MobB conserved in pUT1530 (and pUT1529) and also the domain of MobA required for MobB activity.

Cells containing pUT1530 grow poorly because the R1162 promoters adjacent to *oriT* are partially derepressed; the cells become healthy when full repression is restored by providing MobB in *trans* to stabilize the relaxosomes. Do copies of *oriT* in *trans* suppress poor growth by a similar mechanism? We asked first whether pUT530, like MobB, increased the permanganate sensitivity of the *oriT* DNA in pUT1530. In order to minimize disruption of any macromolecular complexes in the cytoplasm, we treated whole cells rather than cleared lysates with permanganate (31). Sensitive bases on the nonnicked strand were again identified by primer extension. As before, full-length MobB, encoded by pUT221, enhanced strand separation within *oriT* (Fig. 4B, lanes a and b). In addition, we found that copies of *oriT* cloned in pUT530 likewise increased the permanganate sensitivity of the *oriT* DNA of pUT1530 (Fig. 4B, lanes c and d).

Extra copies of *oriT* also repressed transcription initiated from the promoters adjacent to *oriT*. As before with MobB, we measured the relative amounts of the *kan* and *mobA* transcripts for plasmid pUT1596, but this time in cells containing either extra copies of *oriT* (pUT530) or only the vector pBR322. The results (Fig. 5C and D) indicate that although pUT530 had no effect on the amount of *kan* transcript, it repressed transcription of *mobA*. Moreover, the levels of repression, determined by comparing the relative amounts of bound radioactive probe at the same dilution of RNA, were similar for MobB and *oriT*. We estimate that the amount of *mobA* transcript decreased about 12-fold when MobB was present and 5- to 6-fold in the presence of the additional copies of *oriT*.

The relaxosome is recombinogenic at *oriT* by a mechanism that requires MobB. Additional copies of *oriT* in the cell can act in *trans*, in a way that depends on MobA and MobB, to repress transcription from neighboring promoters. If repression came about by a direct interaction between plasmid molecules, then it might increase the frequency of their recombination. To test this, we used the plasmid pUT1440, which is similar to pUT530 but contains a larger, 802-bp fragment of cloned R1162 *mob* DNA. This DNA includes *oriT* and adjacent parts of *mobA* and *mobC*. The plasmid was introduced by transformation into MV10 cells containing R1162 or a derivative, and the DNA was then extracted and used to transform JW151 (*polA1*) for resistance to ampicillin and, separately, for resistance to streptomycin. Because the replicon of pUT1440 is inactive in JW151, ampicillin-resistant colonies were primarily the result of recombination that had taken place between pUT1440 and R1162 prior to extraction of the DNA. The ratio of ampicillin-resistant transformants to streptomycin-resistant transformants was thus a measure of the recombination frequency in MV10.

The recombination frequency between pUT1440 and R1162 was 0.004, and this frequency was little changed when pUT1530 instead of R1162 was in the cell (Fig. 6). In contrast, recombination between pUT1440 and either pUT1371 or

TABLE 2. Effect of cloned copies of *oriT* on inhibition of cell growth by R1162 derivatives

R1162 derivative present	Generation time (min) of strain containing complementing plasmid[a]	
	None	pUT530
pUT1530	80 ± 4	35 ± 4
pUT1529	82 ± 6	40 ± 2
pUT1371	81 ± 3	95 ± 5
pUT1533	78 ± 3	74 ± 4
pUT1371 + pUT1585	NT	35 ± 4
pUT1371 + pACYC184	NT	87 ± 3
pUT1562 + pUT1585	NT	91 ± 6
pUT1562 + pACYC184	NT	104 ± 4

[a] Average and range for two experiments. NT, not tested.

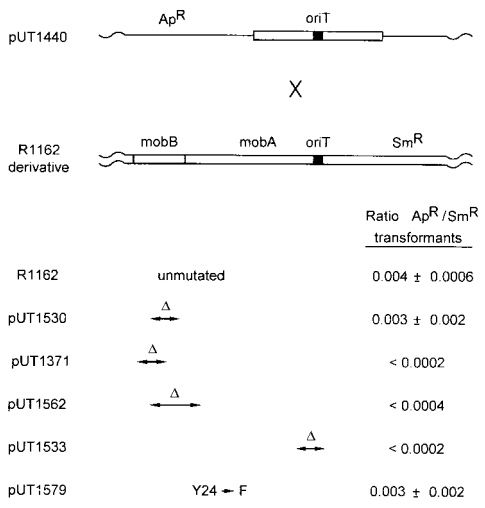

FIG. 6. Recombination frequency between pUT1440, a derivative of pBR322 containing a cloned fragment of R1162 *mob* DNA that includes *oriT*, and R1162 or a derivative. In each case, the result is the average and standard deviation of three experiments.

pUT1562, plasmids which do not encode an active MobB, was undetectable under our assay conditions. In addition, no recombination was observed when *oriT* was deleted from pUT1530 (pUT1533 [Fig. 6]). These results suggested that MobB promotes an interaction between plasmid DNA molecules at *oriT* and that this interaction influences the rate of recombination. However, it was also possible that a greater overall level of nicking in the relaxosomes of R1162 and pUT1530 was resulting in a substrate more favorable for recombination. The plasmid pUT1579 is identical to R1162 but contains a mutation causing a substitution of phenylalanine for tyrosine-24, the active nucleophile in strand cleavage (27). The protein forms a normal complex as assayed by sensitivity of the DNA to permanganate, but nicking is undetectable in vivo (33). Nevertheless, R1162 and pUT1579 recombined with pUT1440 at essentially the same frequency (Fig. 6). We conclude that the relaxosome enhances plasmid recombination by a mechanism that does not depend on nicking at *oriT*.

DISCUSSION

We show here that two effects of MobB, stabilization of the relaxosome and enhancement of the frequency of transfer, can be genetically distinguished by mutations within the gene. The *mobB* deletions in pUT1529, pUT1530, and pUT1531 destabilize the relaxosome but have only a small effect on the frequency of mobilization (Table 1). These deletions map in the region of the gene encoding the amino-terminal half of the protein.

Deletion of the region of *mobA* adjacent to *mobB* results in a relaxosome that is no longer responsive to MobB, and as a result mobilization decreases to the level observed when MobB is effectively absent (pUT1562 [Table 1]). However, this deletion has a smaller effect on the DNA-processing reactions carried out by MobA: cleavage and ligation of single-stranded DNA, as monitored by phage recombination, or the separation of DNA strands within the relaxosome. We interpret these results to mean that the region of *mobA* adjacent to *mobB* encodes a domain of the protein required for recognition of MobB. When this region is absent, MobA becomes blind to the presence of MobB, and the frequency of mobilization becomes similar to that of pUT1371. R1162 and pSC101, a plasmid which is unrelated to R1162 overall, have *oriT*s with very similar base sequences (16). In addition, pSC101 encodes a mobilization protein with tracts of amino acids identical to those in MobA. The similarities between the two proteins map at the amino-terminal end of MobA, throughout the DNA-binding region, but do not extend into the domain required for recognition of MobB. Interestingly, there is no protein encoded by pSC101 that can be identified as MobB-like on the basis of sequence similarities. Thus, MobB and a cognate site within MobA might represent a particular adaptation within the IncQ plasmid group.

Relaxosomes stabilized by MobB cause maximal repression of transcription from the promoters adjacent to *oriT*. When the relaxosomes are unstable, partial derepression presumably leads to cells that form filaments and grow with long generation times. Since derepression affects the expression of not only the *mob* genes but also the replication genes downstream from *mobA* (9), it is not clear whether overexpression of a particular gene or overall plasmid gene expression is the basis for the poor growth. The nicking domain of MobA is not solely responsible, since a deletion that eliminates this region does not restore normal growth (pUT1532 [Table 1]). In any case, the effect of derepression is probably amplified by an increase in plasmid copy number, due to the increase in expression of the replication genes (10, 13).

The destabilizing effect of the *mobB* mutations in pUT1529 and pUT1530 could be overcome by additional copies of *oriT* in *trans*. This suppression required the remaining fragment of MobB as well as MobA protein with its recognition domain for MobB. An increased frequency of recombination between *oriT*-containing plasmid molecules showed identical requirements. From these observations, we propose that MobA and MobB link the *oriT*s on different plasmid molecules by means of a protein bridge and that this structure is required for stable relaxosomes and, consequently, for optimal repression of plasmid promoters and healthy growth of cells (Fig. 7A). As a result, inactivation of MobB (in the plasmid pUT1371) results in both a low level of transfer and high levels of transcription, with the cells growing poorly (Fig. 7B). The same effects are observed when the region of *mobA* adjacent to *mobB* is deleted (Fig. 7C), because MobB is then no longer able to recognize MobA. The mutations in pUT1529, pUT1530, and pUT1531 partially impair the ability of MobB to stabilize the relaxosomes, so that transcription is high and cells again grow poorly (Fig. 7D). However, an increased dosage of *oriT*, provided by pUT530, partially offsets this instability by driving the *oriT* interaction toward the coupled complex, thereby decreasing gene repression (Fig. 7E).

These molecular interactions proposed in Fig. 7 are reminiscent of the coupling or "handcuffing" which is thought to be part of the mechanism of replication control for plasmids RK2 and R6K and phage P1 (2, 18, 22). In these cases, excess iterons bind the plasmid-specific initiation protein, freezing it at the origin of replication and preventing a new round of replication. For R1162, control of replication would be more indirect. Copy number is determined by the level in the cell of the plasmid-specific replication protein RepC (10, 13), and enhanced repression at *oriT* reduces the overall level of transcription through the gene for this protein (9).

Although lacking the amino-terminal region, the MobB proteins encoded by pUT1529, pUT1530, and pUT1531 are still very active for transfer. MobB might also be required for the R1162 relaxosome to recognize the conjugal apparatus of the mobilizing, self-transmissible plasmid. MobB could act as a bridge, or it could modify the conformation of MobA to better

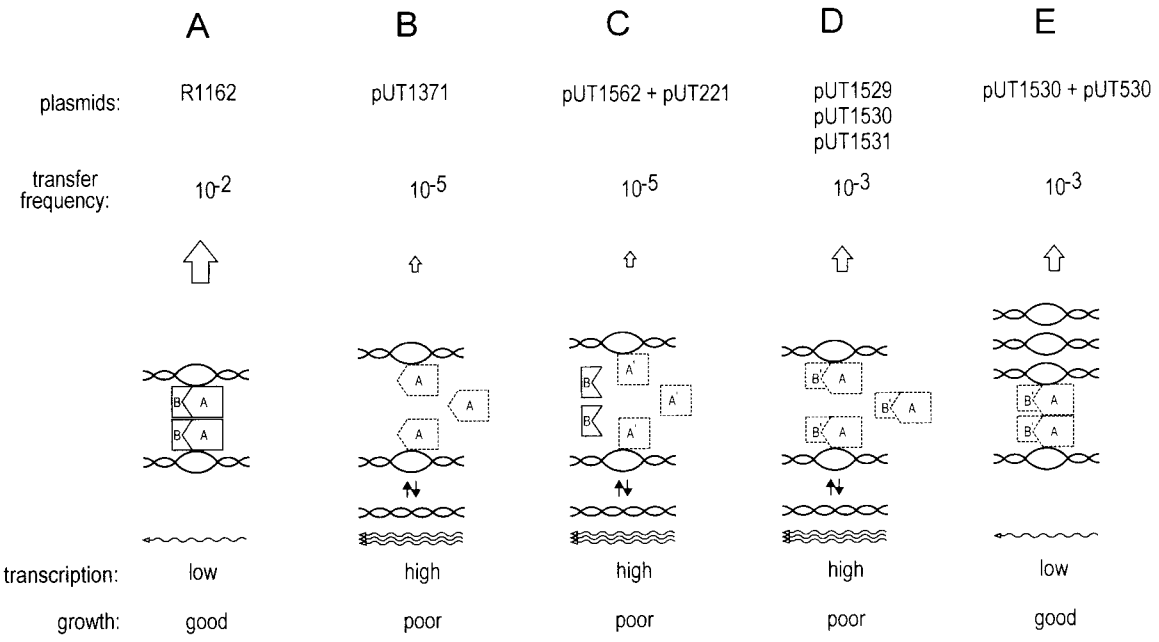

FIG. 7. Model describing the interactions of MobA and MobB with *oriT* DNA and the effects of these interactions on transfer, transcription and cell growth.

fit the conjugal machinery. Thus, MobB could mediate interactions between relaxosomes and also between the relaxosome and a docking site for mobilization. Experiments to obtain physical evidence for these interactions are now under way.

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