In vitro cleavage of double- and single-stranded DNA by plasmid RSF1010-encoded mobilization proteins

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ABSTRACT

We have used purified RSF1010 mobilization proteins to reproduce *in vitro* a strand-specific nicking at the plasmid origin of transfer, *oriT*. In the presence of Mg^{2+} , the proteins MobA (78-kDa form of RSF1010 DNA primase), MobB, and MobC and supercoiled or linear duplex *oriT* DNA form large amounts of a cleavage complex, which is characterized by its sensitivity to protein-denaturant treatment. Upon addition of SDS to such a complex, a single strand break is generated in the DNA, and MobA is found linked to the 5' nick terminus, presumably covalently. The double-strand nicking activity of MobA requires, in addition to Mg^{2+} , the presence of MobC and is stimulated by the presence of MobB. The nick site has been shown by DNA sequencing to lie at the position cleaved *in vivo* during transfer, between nucleotides 3138/3139 in the r strand of RSF1010. We have found that MobA will also cleave DNA at sites other than *oriT* if the DNA is present in single-stranded form. Breakage in this case occurs in the absence of denaturing conditions, and after prolonged incubation, reclosure can be demonstrated.

INTRODUCTION

The 8684-base-pair (bp) plasmid RSF1010, a member of the broad-host-range IncQ group (essentially identical to R1162), is efficiently mobilized during the conjugative transfer of certain self-transmissible plasmids, notably those of the IncP-1 group. Transfer of the plasmid is linear and unidirectional (1), and requires a site on RSF1010 (*oriT*) as well as the RSF1010-encoded MobA, -B and -C proteins (2–4). The minimal *oriT* consists of no more than 38 bp of DNA, within which are located *nic*, the site where a single strand of plasmid DNA is cleaved and religated during conjugative mobilization (5). The *nic* site has been mapped by *in vivo* recombination studies to within 3 bp (5), between nucleotide positions 3138 and 3140 in the published RSF1010 sequence (6). Nicking of the plasmid DNA in the 'relaxation complex' also occurs at this location (7). An adjacent 10-bp inverted repeat (one mismatch) is thought to be the recognition site for protein(s) involved in cleavage and religation of the *oriT* DNA. Plasmids lacking the outer arm of this repeat are mobilized at about 1% of the wild-type frequency (3, 5).

The largest of the three known RSF1010 Mob proteins, A ($M_r = 77,900$), is unusual in that only its N-terminal portion is

required for *oriT* activation in the presence of an IncP-1 plasmid (4). The C-terminal half of MobA, which also exists as a separate restart product in the cell (called RepB or B'), was shown to contain a DNA primase activity that is directed toward two specific single strand initiation signals present with the plasmid origin of replication, *oriV* (8–10). Recently, the N-terminal segment of the R1162 MobA protein has been isolated as a β -galactosidase fusion protein and shown to contain site-specific, single-strand DNA endonuclease and ligaslike activities (11). Here we confirm this finding using a highly purified preparation of the intact MobA protein and demonstrate that this protein cleaves RSF1010 DNA within the transfer origin at a single, specific site when the DNA is in the supercoiled or linear duplex form. Different from the cleavage of single-stranded DNA by MobA protein, its ability to nick duplex *oriT* DNA depends on the presence of MobC protein, and protein-denaturant treatment is necessary to expose the MobA-induced single strand break. The site where RSF1010 DNA is nicked *in vitro* has been determined at the nucleotide level, and the nick site has been shown to contain a free 3'-OH end and a blocked 5'-P end. In addition, observations with a C-truncated MobA protein indicate that the MobC-dependent *oriT* nicking activity resides within the first 34% (243 amino acids) of the 78-kDa MobA polypeptide, a segment that is separated from the primase domain by at least 145 amino acids and that, as noted by Drolet et al. (7), exhibits substantial sequence similarity to proteins of the mobilization regions of plasmids pTF1 (from *Thiobacillus ferrooxidans*) and pSC101.

MATERIALS AND METHODS

Enzymes and reagents

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, terminal deoxynucleotidyl transferase, calf intestinal alkaline phosphatase, and proteinase K were from Boehringer Mannheim. Sequenase and *E. coli* SSB were from United States Biochemical Corp. The M13 reverse sequencing primer (16-mer), was from New England Biolabs. [α - 35 S]thio[dATP] (650 Ci/mmol), [α - 32 P]dideoxyATP (3000 Ci/mol), [γ - 32 P]ATP (3000 Ci/mmol), and [methyl- 3 H]thymidine (57 Ci/mmol) were from Amersham.

DNA

Plasmid RSF1010 (6) was isolated as described (9). RSF-d1 is a spontaneous high-copy-number (and *mob*⁻) mutant of

RSF1010 which was isolated in this laboratory; it has the entire *oriT* sequence removed by a deletion of nucleotides (nt) 3098–3220 in RSF1010. Phages mOT18-9 n and –9 u were obtained by ligating the 155-bp *Hae*III *oriT* fragment of RSF1010 (nt 3015–3169) into the *Sma*I site of vector M13mp18. Versions n and u have the l strand and the r strand, respectively, of the cloned RSF1010 DNA contiguous with the M13 viral (+) strand. M13 phage were grown in *E. coli* JM101 (12), and the single-stranded (SS) and supercoiled (FI) DNAs were isolated by standard procedures (13). ³H-labeled RSF1010 DNA was prepared as follows. Cells of the thy⁺ strain SCS1 (14) harboring RSF1010 were grown at 37°C in 1 liter of TY (1% tryptone / 0.5% yeast extract/ 0.5% NaCl) medium containing 0.2% glucose, 20 µg/ml thiamine HCl, and 40 mM Mops·KOH, pH 7.9. At OD₅₉₀ = 0.6, 5 µCi (11 ng) [³H]thymidine plus 50 lg uridine were added per ml of culture, and aeration was continued for 3 h (OD₅₉₀ = 1.5). The labeled cells were harvested by centrifugation and plasmid DNA was prepared as described (9). The specific activity of the DNA obtained was 1.8 × 10⁵ cpm/µg.

RSF1010 mobilization proteins

The MobA, -B and -C proteins of RSF1010 were purified to >95% purity from the overproducing *E. coli* strains HB101(pVH1, pVH9), HB101(pVH1, pVH3) and HB101(pGP1-2, pOT762d1), respectively (6). A truncated MobA protein, A*₂₄₃, consisting of the first 243 amino acid residues of the 708-residue-long MobA polypeptide chain and a tail of 17 residues coded for by Tn3 sequences, was purified from IPTG-induced cells of strain HB101(pVH1, pES10). The plasmid pES10 was obtained by ligating a blunted, 1.2-kb *Pf*MI-*Pst*I fragment from pKT228 (RSF1010::Tn3) (6), containing the RSF1010 sequences 3215–3982, into the *Sma*I site of pKT223–3, a pBR322 derivative that contains the *tac* promoter (15). Analysis of the four Mob proteins preparations used in this study by SDS/polyacrylamide gel electrophoresis revealed the presence of a single polypeptide in each sample (Fig.1). The procedures for isolation of each Mob protein will be published elsewhere.

Assay for nicking of supercoiled DNA

The standard reaction mixture (20 µl) contained 20 mM Tris·HCl (pH 7.8), 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1% Brij 58, 200 ng bovine serum albumin, 20 ng MobA protein, 80 ng MobB protein, 25 ng MobC protein, and 320 ng *oriT*-containing FI DNA. After incubation at 32°C for 30 min (unless indicated otherwise), reactions were stopped by adjusting the mixture to 10 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K and continuing the incubation at 37°C for 20 min. Unless indicated, reaction mixtures were electrophoresed through a 0.8% agarose gel in TBE buffer (89 mM Tris borate/1 mM EDTA, pH 8.3) at 7V/cm for 2–3h. Gels were stained with 1 µg/ml ethidium bromide and photographed. In assays utilizing [³H]DNA, gel slices (containing DNA) were transferred to scintillation vials, covered with 0.5 ml H₂O, melted by autoclaving at 100°C for 3 min, mixed with 5 ml Beckman Ready Value scintillation fluid, and chilled. ³H radioactivity was measured, and the percent conversion of FI DNA to the respective FII derivative was calculated.

FII DNA isolation

RSF1010 and mOT18-9n FI→FII reaction products were isolated by excision from a 0.8% agarose gel. The products were treated with SDS and proteinase K as noted prior to electrophoresis. The FII DNA was recovered from gel slices by electroelution, passed over an Elutip-d column (Schleicher and Schuell), and concentrated by ethanol precipitation in the presence of 0.4 M LiCl.

End-group labeling of FII product

Gel-purified FII DNA of RSF1010 (6 lg; 1 pmol) was linearized by digestion with *Hpa*I and then denatured by adjusting the mixture to 0.2 M NaOH. After 5 min at room temperature, 0.1 volume 5 M NH₄OAc (adjusted with acetic acid to pH 4.6) was added and the DNA was precipitated with 2.5 volumes ethanol. For 5'-end labeling, half of the sample was treated with calf intestinal alkaline phosphatase followed by phenol/chloroform extraction and phosphorylation with [γ-³²P]ATP and T4 polynucleotide kinase (16). For 3'-end labeling, the other half of the sample was treated with terminal deoxynucleotidyl transferase in the presence of [α-³²P]ddATP (16). The reaction mixture (20 µl) contained a buffer of 100 mM cacodylic acid/25 mM Tris base (adjusted with KOH to pH 7.5), 1 mM CoCl₂, 0.5 mM dithiothreitol, 0.8 lM [α-³²P]ddATP (3 × 10⁶ cpm/pmol), the alkali-denatured DNA, and 8 units terminal transferase. Incubation was at 37°C for 30 min. After phenol/chloroform extraction, unreacted radioactive ddATP was removed by two cycles of ethanol precipitation in the presence of 2.5 M NH₄OAc. The labeled products were analyzed on an alkaline 0.8% agarose gel equilibrated with 30 mM NaOH/1 mM EDTA (13).

Mapping of the nick site

The nucleotide sequence of the 3' end at the nick was determined as follows. Gel-purified FII DNA of mOT18-9n (2 pmol) was linearized by digestion with *Hind*III and end-labeled at the phosphatase-sensitive 5' ends with [γ-³²P]ATP and T4 polynucleotide kinase (16). After labeling, the DNA was cut with *Eco*RI, and the 206-bp *Eco*RI-*Hind*III fragment (Fig.7) was isolated by 5% polyacrylamide gel electrophoresis followed by electroelution and ethanol precipitation. The ³²P-end-labeled DNA fragment was then subjected to base-specific chemical cleavage reactions as described by Maxam and Gilbert (16). To map the 5' end of the nicked strand, a primer extension assay was performed on mOT18-9n FII DNA which had been denatured by incubation with 0.2 M NaOH for 5 min. Approx. 0.5 pmol of the denatured DNA and 1 pmol of the M13 reverse (16-mer) primer were mixed in a 10-µl reaction volume containing 40 mM Tris·HCl (pH 7.5), 10 mM MgCl₂, and 50 mM NaCl. The mixture was heated at 60°C for 5 min, then cooled slowly (30 min) to room temperature, and the products were extended with Sequenase in the presence of [³⁵S]dATP. Labeling and extension-termination reaction conditions were as described (17), except that ddNTPs were omitted from the termination mixture. Size markers were generated by performing dideoxy sequencing reactions (17) on alkali-denatured mOT18-9n FI DNA using the same primer and hybridization condition. The products of DNA sequencing reactions were electrophoresed at 70°C through a 6% polyacrylamide gel containing 8 M urea and a buffer of 135 mM Tris borate/ 2.5 mM EDTA, pH 8.8, using an LKB 2010 MacroPhor apparatus.

Assay for cleavage of SS DNA

The standard reaction mixture (20 μ l) contained 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 2.5 mM $MnCl_2$, 0.1 mM EDTA, 15% (v/v) glycerol, 0.8 μ g MobA protein, and 1 μ g mOT18-9u circular SS DNA. Incubations were at 37°C for the times indicated. Reactions were stopped by adding EDTA, SDS and proteinase K to 10 mM, 0.1% and 0.2 mg/ml, respectively, and continuing the incubation at 37°C for an additional 30 min. The mixtures were then made 0.01% in bromophenol blue and electrophoresed through a 1.4% agarose gel in TBE buffer at 9V/cm for 1.5 h. Gels were stained with 2 μ g/ml ethidium bromide and photographed.

RESULTS

Reversible nicking of plasmid RSF1010 DNA by purified RSF1010 Mob proteins

Incubation of RSF1010 DNA with a mixture of the MobA, -B and -C proteins in the presence of Mg^{2+} , followed by addition of SDS and proteinase K digestion, resulted in the conversion of the supercoiled (FI) DNA to an open circular form, FII (Fig. 2). This apparent nicking activity of the ensemble of the three known RSF1010 Mob proteins, however, is quite different from the classical DNA endonucleases. Firstly, as will be documented below, the nicking reaction is specific for DNA containing the RSF1010 *oriT* sequence and results in the linking of protein to the 5' end of the broken strand. Secondly, the formation of the FII species quickly increased with time but appeared to cease after about 30 min of incubation at 32°C at which time typically 80–85% of the input FI DNA was recovered as FII (Fig. 2, lanes 1–6); the further addition of Mob proteins at this point and continuation of incubation for another 30 min did not significantly increase the final yield of FII product (lane 7). Thirdly, similar to the SDS (or alkali)-cleavable complexes formed between eukaryotic type-I topoisomerases and DNA (18, 19), high-salt treatment of the reaction mixture after the initial incubation but prior to the addition of SDS rapidly

reversed the nicking reaction. Thus, after just 5 min of exposure of the preincubated mixture to 0.6 M NaCl, the proportion of nicked DNA returned to essentially the background level seen for the untreated DNA (lanes 8–10). However, when the salt (0.6 M NaCl) was added simultaneously with an excess of EDTA over Mg^{2+} , no decrease in the proportion of nicked DNA was evident even after incubations up to 30 min at 32°C (lanes 11–13) or up to 16 h at 4°C (not shown). In another experiment (data not shown), it was found that shifting the incubation temperature from 32°C to 50°C also rapidly reversed the nicking reaction. Again, the temperature-induced 'back reaction' was totally dependent on the presence of free Mg^{2+} ions in the assay mixture and resulted in the regeneration of FI DNA that was indistinguishable from the original plasmid DNA with respect to its superhelicity. This simple salt- or temperature-induced reversal of the reaction and its blockage by excess EDTA suggested that the cleavage of FI DNA by the RSF1010 Mob proteins was the result of protein-denaturant treatment which trapped a putative intermediate of a Mg^{2+} -dependent strand breakage reaction and a Mg^{2+} -dependent resealing reaction. The fact that virtually all of the nicked form was converted back to the supertwisted form and not to the relaxed (FI') form further suggested that the two ends of the putatively broken DNA strand were held tightly by protein at all times so that no loss in superhelical tension resulted. An alternative possibility, considered in reactions with topoisomerases (e.g., ref. 20), is that the association of protein with DNA is noncovalent and the breakage of the phosphodiester bond was induced by protein denaturation. However, cleavage of single-stranded DNA by the MobA protein has been detected without the use of protein denaturants (see below).

The requirements for this reversible nicking reaction have been characterized using 3H -labeled RSF1010 DNA as a substrate to quantitate the extent of FI cleavage. The nicking reaction was dependent on the presence of both the MobA and MobC proteins

Figure 1. Polyacrylamide gel analysis of purified RSF1010 Mob proteins. Proteins (≈ 4 μ g each) were denatured, reduced, and electrophoresed in a 20% polyacrylamide/0.11% methylene-bisacrylamide gel containing 0.1% SDS. Protein bands were stained with Coomassie blue R-250. Lanes: 1, MobA (molecular mass predicted from the DNA sequence, 77.9 kDa); 2, MobB (15.1 kDa); 3, MobC (10.9 kDa); 4, MobA*₂₄₃ (28.6 kDa); S, protein size standards, with sizes given in kDa.

Figure 2. Reversible nicking of RSF1010 DNA by the MobA, -B, and -C proteins. Standard reaction mixtures with supercoiled RSF1010 DNA as substrate were incubated at 32°C for various times. All reactions were terminated by adjusting the mixture to 10 mM EDTA/0.5% SDS and proteinase K at 500 μ g/ml. After 20 min at 37°C, the mixtures were made 5% in Ficoll and 0.01% in bromophenol blue and loaded onto a 0.8% agarose gel in 40 mM Tris acetate/1 mM EDTA, pH 7.9. Electrophoresis was carried out in the presence of 0.5 mg/ml ethidium bromide at 4.5 V/cm for 4 h. Lanes: 1, unreacted RSF1010; 2–6, reaction mixture incubated for 5, 10, 20, 30, and 60 min, respectively; 7, at 30 min the mixture received an additional 20 ng MobA, 80 ng MobB, and 25 ng MobC and incubation was continued for another 30 min; 8–10, at 30 min the mixture was adjusted to 0.6 M NaCl by adding 2 μ l 5 M NaCl and incubation was continued for another 1, 5, and 30 min, respectively; 11–13, same as samples 8–10, except that the preincubated mixture was adjusted to 0.6 M NaCl/10 mM EDTA; 14, a mixture of relaxed (by calf thymus topoisomerase I treatment) and linear RSF1010 DNA (by *EcoRI* digestion). Positions of the supercoiled (I), relaxed (I'), nicked (II), and linear (III) plasmid forms are indicated.

Table 1. Protein requirements of the complete system

Exp.	Protein omitted	FI DNA nicked
A	None	% of input 85
	MobA	<3
	MobB	56
	MobC	<3
B*	None	61
	MobB	38
	MobC	<3

DNA cleavage reactions were performed and quantitated as described in *Materials and Methods* using [³H]RSF1010 DNA as substrate.

*MobA*₂₄₃ (8 ng) was used in place of the intact MobA protein.

Figure 3. Titration of the amount of each Mob protein required for maximal nicking activity. Assays with [³H]RSF1010 DNA as substrate were as described in *Materials and Methods*, except that increasing amounts of the specified Mob protein were added to standard reaction mixtures lacking only that particular protein.

Figure 4. Optimal ionic conditions for the formation of a cleavable Mob protein-DNA complex. Assays with [³H]RSF1010 DNA as substrate were carried out under variations of the standard condition. A, effect of varying the NaCl concentration; B, effect of varying the MgCl₂ concentration; C, pH optimum in 20 mM Tris/Cl (○) or 20 mM Bis Tris Propane/Cl (●).

(Table 1). Protein MobB stimulated cleavage but was not absolutely required. Interestingly, a truncated MobA protein, A*₂₄₃, containing only the amino acid residues 1–243 of the 708-aa MobA polypeptide, was also active, consistent with the previous *in vivo* and *in vitro* observations (4, 11). As observed with the intact protein, MobA*₂₄₃ required MobC for activity and was stimulated by the presence of MobB.

When the concentration of any of the Mob proteins in the reaction mixture was increased (keeping the concentrations of the other two proteins constant), the amount of FI DNA converted to FII increased to a maximum value (Fig. 3). At near saturation (80% FI to FII conversion), the ratio of MobA, -C and -B protein molecules to RSF1010 molecules was 1.8:1, 17:1 and 64:1,

Figure 5. Specificity of the nicking reaction to *oriT*. Various supercoiled DNAs (0.32 l_γ) were incubated with (+) or without (–) the MobA, -B and -C proteins under standard conditions as described under *Materials and Methods*. Lanes: 1 and 2, RSF1010; 3 and 4, RSF-d1; 5 and 6, pBR322; 7 and 8, M13 mp18; 9 and 10, mOT18-9n. The lower and upper bands represent FI and FII DNA, respectively.

respectively. This estimation of the number of protein molecules/DNA molecule is based on the assumption that all protein molecules in our Mob preparations were active and that the active species of each protein is a monomer. Our preliminary physical characterization of the three proteins, however, indicates that at least MobB and MobC are oligomeric proteins.

Optimal ionic conditions in the reaction mixture were found to be 25–125 mM NaCl, 3–15 mM MgCl₂, and pH 7.8 (Fig. 4). The nicking reaction was strongly inhibited by monovalent salts above 0.2 M, with Na⁺, K⁺, Li⁺, or NH₄⁺ having equivalent effects. No activity was detected in the absence of added MgCl₂, and excess EDTA was an inhibitor. Mn²⁺, Ca²⁺ and Ba²⁺ could substitute for Mg²⁺ at an optimal efficiency of 72, 54 and 47%, respectively (at 1 mM MnCl₂, 5 mM CaCl₂, and 10 mM Ba (OAc)₂). The temperature optimum for cleavage was around 32°C; at 20°C and 45°C, the final yield of FII product was 62 and 31%, respectively, of that at 32°C. Cofactors like (d)ATP, cAMP, NAD, or S-adenosylmethionine (up to 1 mM each) did not influence the reaction (data not shown).

Substrate specificity of the RSF1010 Mob proteins

The MobA, B, C-mediated nicking reaction was absolutely specific for DNA substrates containing the RSF1010 transfer origin (Fig. 5). For example, plasmid RSF-d1, a spontaneous deletion mutant of RSF1010 that lacks the entire *oriT* sequence (nt 3098–3220), was not attacked by the three Mob proteins, nor was plasmid pBR322 or phage M13 FI DNA. On the other hand, nicking was readily observed with mOT18-9n FI, an M13 mp18-based *in vitro* recombinant carrying the 155-bp *Hae*III fragment of RSF1010. The 38 bp minimal *oriT* sequence has been shown previously to be located within this DNA fragment (2, 5).

Analysis of the site of cleavage

Since the RSF1010 origin of transfer was required for cleavage of FI DNA by the MobA, -B and -C proteins, we tested the ability of these proteins to nick the duplex within the origin. Supercoiled RSF1010 was incubated with a mixture of the three proteins and after quenching the reaction with SDS and digestion with proteinase K, the FII product was purified and linearized by digestion with *Hpa*I or *Xmn*I. Analysis using alkaline agarose gel electrophoresis revealed the presence of three discrete bands

Figure 6. Structure analysis of the FII product. The RSF1010 FI→FII product was isolated as described in *Materials and Methods*. (A) Samples of 1 μ g were digested with *XmnI* (lane 3) or *HpaI* (lane 4), denatured in 0.1M NaOH, and subjected to electrophoresis on an alkaline 0.8% agarose gel. Products were visualized by ethidium bromide staining. Lanes 1 and 2 contain unreacted RSF1010, cut with *XmnI* or *HpaI*, respectively. The migration of size markers (1-kb ladder, BRL) is indicated at the left. (B) The *HpaI*-cut FII DNA was labeled at its 3' (lane 1) or 5' (lane 2) ends with 32 P prior to electrophoretic analysis. The products were run on a separate alkaline agarose gel and autoradiographed. (C) Structure of RSF1010 arising upon cleavage of the r strand of the DNA within *oriT* and linearization by *HpaI*. Distances are in kilobases (kb).

for both the *XmnI*- and *HpaI*-cut product DNA (Fig. 6A, lanes 3 and 4). The top band corresponds to full-length RSF1010 single strands. The two smaller bands, which are not detected in the restriction digests of unreacted RSF1010 (lanes 1 and 2), migrate at approx. 1.1 and 7.6 kb in the case of the *XmnI*-cut DNA and at 3.1 and 5.7 kb in the case of the *HpaI*-cut DNA. These correspond to the lengths of DNA separating the *oriT* site from the respective restriction site (Fig. 6C) and indicate that the single strand break *in vitro* has occurred at or near this site.

To examine the nature of the 3' and 5' ends at the nick, samples of the *HpaI*-cut DNA were end-labeled prior to alkaline gel electrophoresis. Either the 5' termini were labeled by polynucleotide kinase and [γ - 32 P]ATP (after dephosphorylation with calf intestine alkaline phosphatase) or the 3' termini were labeled by terminal transferase and [α - 32 P]ddATP. After electrophoresis, the 32 P-labeled products were visualized by autoradiography. As shown in Fig. 6B, lanes 1 and 2, single-stranded fragments of 3.1 and 5.7 kb were obtained with the 3'-end labeled product, while the 5'-end labeled product gave rise only to the larger of the two fragments. These results showed that the nick site can be labeled at its 3' end but not at its 5' end. We concluded from the above data that the RSF1010 Mob proteins cleave the bottom (r) strand of the DNA in the *oriT* region and that this cleavage generates a free 3'-OH but a blocked 5'-phosphoryl end.

DNA sequencing experiments were performed to determine the exact position of the nick on the RSF1010 map. The DNA substrate used in these experiments was mOT18-9n FI. The FII

Figure 7. Determination of the nick site. (A) 5'-end mapping. The mOT18-9n FI→FII product, isolated from an agarose gel, was subjected to primer extension analysis as described under *Materials and Methods*. Reactions were initiated at the M13 reverse (16-mer) primer hybridizing 25 to 40 bp to the left of the cloned RSF1010 *oriT* DNA as denoted on the bottom of the figure. Lane a shows the 'run-off' product. The adjacent four lanes show the products of dideoxy sequencing reactions used as size markers. The relevant sequences of the newly synthesized l strand are shown on the right. The nick (bar) is within the complementary (r) strand. Arrows mark the inner arms of the 10-bp (1 mismatch) and 11-bp (2 mismatches) inverted repeats present in the nick site region (6). (B) 5'-end mapping. An aliquot of the FII DNA was linearized with *HindIII*, end-labeled at the 5' positions of the restriction site, and then cut with *EcoRI*. The resulting 206-bp fragment was isolated and subjected to Maxam-Gilbert chemical sequencing (lanes G, G+A, C+T, and C). Numbering of the sequence shown on the right corresponds to the published RSF1010 sequence (6).

product, predicted to carry the single strand discontinuity on its non-viral (–) strand (see Fig. 7C), was treated with proteinase K and then isolated by agarose gel electrophoresis. To map the 5' end of the nick site, the M13 reverse sequencing primer was used for priming complementary (+) strand synthesis by modified T7 DNA polymerase (Sequenase). The primer extension product was found to terminate after the G at nt 3138 in the l strand of RSF1010 (Fig. 7A). In another experiment, the gel-isolated FII product was cut with *HindIII*, treated with alkaline phosphatase, and labeled at the 5' ends with 32 P using polynucleotide kinase. The DNA was then cut with a second enzyme, *EcoRI*, to separate the two labeled ends. The resulting 206-bp *EcoRI*-*HindIII* fragment, including the nick site, was isolated and sequenced by the chemical degradation method (16). The result (Fig. 7B) identifies nt 3139 (G) of the RSF1010 r strand as the 3' side of the nick. Thus, no nucleotides were removed from the site of the nick by the MobA, B, C-mediated DNA cleavage. The DNA sequence around the nick site can potentially form two hairpin

structures with the nick occurring within the 8-nucleotide-long spacer sequence (6; Fig. 7A).

MobA is the DNA-nicking enzyme

During analysis of the requirements for the formation of a cleavable Mob protein-DNA complex, we noticed that linear RSF1010 also supported the complex formation. The protein and divalent cation requirements for the cleavage of linear DNA were identical to those for reactions carried out with supercoiled DNA, except that increased levels of MobC were needed in order to observe efficient nicking of the linear duplex. In experiments performed at a saturating level of MobC (protein/DNA molar ratio = 85:1), up to 50% of the linears were found, on examination in an alkaline agarose gel, to be uniquely nicked within *oriT* (data not shown).

The following experiment was performed to determine which of the two required Mob proteins, A or C, becomes linked to the DNA in the cleavage reaction. 5'-end-labeled *Bst*XI fragments of RSF1010 were incubated with MobB, MobC and either MobA or MobA*₂₄₃, and SDS was then added. The resulting products were analyzed with or without protease treatment on a protein denaturing SDS-agarose gel (Fig. 8). The nick site is contained within the *Bst*XI fragment C (nt 2899–3589). Comparison of the products before (lanes 2 and 3) and after (lanes 4 and 5) proteinase K treatment reveals that a substantial fraction of the fragment C molecules is retarded in their gel mobility, consistent with protein being tightly (presumably covalently) linked to the cleavage product. Molecules cleaved using MobA*₂₄₃ (28.6 kDa) in place of the intact protein (77.9 kDa) show a much smaller shift in gel mobility (compare lanes 2 and 3), thus indicating that this protein, and not MobC, is linked to the DNA in the cleavage complex. Since a free 3'-OH group and a blocked 5' end is found at the nick (Fig. 6B), it is most likely that MobA is linked to the 5'-phosphoryl end of the cleaved DNA strand.

Cleavage and religation of single-stranded DNA by MobA protein

The viral SS DNA of mOT18-9u (*oriT*⁺; r strand) was incubated with purified MobA protein and subsequently analyzed by electrophoresis on a native agarose gel. To remove any bound protein, the reaction products were treated with SDS and proteinase K prior to loading. With increasing incubation time, the circular SS DNA was first converted to the linear form and then to numerous smaller pieces (Fig. 9A, lanes 1–6). The size of the fragments was influenced by the ratio of protein to substrate present in the reaction mixture. With an increased level of DNA, at a constant amount of MobA, an increase in the average size of the fragments was observed (Fig. 9B). Prolonged incubation (> 3 h) resulted in little if any additional cleavage of the DNA.

These results suggested that MobA protein acts stoichiometrically, whereby it cleaves a phosphodiester bond and binds to the DNA. Furthermore, the resolution of small discrete DNA bands upon agarose gel electrophoresis indicated that multiple sites exist at which the enzyme preferentially breaks the DNA. Similar cleavage patterns were obtained with the SS DNAs of mOT18-9n (*oriT*⁺; l strand) and M13 mp18, the *oriT*-free vector (not shown).

To test whether MobA will cleave SS DNA spontaneously, the products were loaded directly onto the gel without prior SDS and protease treatment (Fig. 9A, lane 7). The products migrated in the gel as somewhat diffuse bands faster than the input

Figure 8. Protein MobA caused mobility shift of the cleavage product. Reaction mixtures (20 μ l) containing 0.3 μ g (9,000 cpm) 5' ³²P-labeled *Bst*XI-digested RSF1010 DNA, 80 ng MobB, 80 ng MobC, and either 20 ng MobA (lanes 2 and 4) or 8 ng MobA*₂₄₃ (lanes 3 and 5) were incubated at 32°C for 1 h. All reactions were terminated by adding 2 μ l 100 mM EDTA/5% SDS. Samples in lanes 4 and 5 were further treated with proteinase K (0.5 mg/ml) at 37°C for 20 min. The mixtures were then electrophoresed at 7 V/cm for 2.5 h through a 1% agarose gel in TBE buffer containing 0.1% SDS. The gel was dried and autoradiographed. Lane 1 is a DNA control (no proteins). Arrows indicate the positions of the DNA-MobA and DNA-MobA*₂₄₃ complexes that result from nicking of the *oriT*-containing fragment C (690 bp). The minor retardation band seen in lane 2 may represent a proteolytic product of the DNA-MobA complex.

Figure 9. Cleavage of single-stranded by MobA protein. Assays with mOT18-9u viral SS DNA as substrate were as described in *Materials and Methods*, except for the modifications noted. (A) Time course. Lane 1, unreacted mOT18-9u; lanes 2–6, reaction mixtures incubated for 10, 30, 60, 120, and 180 min, respectively; lane 7, same as lane 6, except that the mixture was not treated with SDS and proteinase K prior to electrophoresis. (B) Effect of increasing DNA concentrations. Lane 1, unreacted mOT18-9u; lanes 2–5, reactions (20 μ l each) performed in the presence of 0.5, 1.0, 2.0, and 4.0 μ g of mOT18-9u SS DNA, respectively. The amount of DNA applied to each slot was 1 μ g. (C) Effect of various divalent cations. Assay mixtures contained either no added metal ion (lane 1), 5 mM MgCl₂ (lane 2), 5 mM Ba(OAc)₂ (lane 3), 5 mM CaCl₂ (lane 4), or 2.5 mM MnCl₂ (lane 5). Incubations in B and C were for 3 h.

mOT18-9u DNA, suggesting that the cleavage had occurred during the incubation and was not the result of protein-denaturant treatment. This is contrast to observations with supercoiled or linear duplex *oriT* DNA, where nicked molecules were only detected after terminating the reaction using a denaturing agent or condition (e.g., 0.5% SDS, 1 M NaOH, phenol extraction, incubation with proteinase K, or heating at 95°C in the presence of EDTA).

When reaction products similar to those analyzed in Fig. 9A were sized on an SDS-agarose gel, virtually all of the DNA fragments in samples which had not been treated with protease

Figure 10. DNA circle formation from MobA-treated single-stranded DNA. mOT18-9u SS DNA was incubated with MobA protein for 3 h under standard conditions as described under *Materials and Methods*. Following treatment with proteinase K, phenol/chloroform extraction, and ethanol precipitation, the DNA was mounted on grids for EM analysis as described (9). Circular DNA molecules were randomly selected, photographed, and measured. (A) shows a histogram of size distribution of circular SS DNA (154 molecules) and a picture of selected DNA circles present in the reaction prior to cleavage by MobA protein. (B) shows a histogram of size distribution of circular SS DNA (152 molecules) and a picture of selected DNA circles (arrows) seen in the EM after incubating mOT18-9u with MobA protein. (Bar = 1kb.)

migrated as discrete bands slightly slower than their deproteinized counterparts, indicating that they contained tightly bound protein. This was confirmed by end-labeling experiments performed essentially as described in *Materials and Methods*. The fragments could be labeled at their 3' ends but not at their 5' ends (results not presented).

The reactions in the experiments of Fig. 9A and B were performed in the presence of Mn^{2+} , a metal ion which is known to alter the specificity of many enzymes that interact with DNA. Substituting Mg^{2+} , Ba^{2+} , or Ca^{2+} for Mn^{2+} ion, resulted in less overall breakage of SS DNA but the general pattern of cleavage appeared to remain the same except for changes in the relative intensities of certain bands (Fig. 9C). Increasing the salt concentration resulted in a decreased amount of breakage at all sites, without preferential loss of the weak sites; no activity could be detected at NaCl concentrations above 0.6M. The presence of *E. coli* SSB protein inhibited the cleavage at some of the MobA sites but not at others (data not shown).

To examine whether MobA is also capable of bringing about the ligation of cleaved SS DNA, the products formed from mOT18-9u circular SS DNA during a 3-h incubation in the presence of Mn^{2+} were deproteinized and then observed by electron microscopy (Fig. 10). As expected, most of the products were linear SS DNA fragments of different lengths. A small fraction ($\approx 5\%$) of the product molecules, however, appeared as covalently closed SS circles, heterogeneous in size and shorter than unit-length mOT18-9 DNA. These circles were most likely formed by the intramolecular joining of MobA-generated linear fragments, each of which may contain an active enzyme intermediate tightly bound to the 5'-P end.

DISCUSSION

A site- and strand-specific nick, introduced into the origin of transfer of plasmid RSF1010/R1162, is thought to initiate unidirectional DNA transfer during bacterial conjugation (1, 5). Here, we have reproduced this nicking at *oriT* using purified RSF1010 mobilization proteins in an *in vitro* reaction. The reaction is absolutely dependent on two Mob proteins, A (M_r 77,900 form of RSF1010 primase) and C, and as for the *in vivo*-formed ColE1, ColE2 and RP4/RK2 plasmid relaxation complexes extensively studied previously (21–24), protein denaturant or protease treatment is necessary to expose the enzyme-induced single strand break. After induction of relaxation with SDS, the MobA protein or an N-terminal fragment of it remains attached to the DNA at the site of nick, implying that it is the catalytically active component of the RSF1010 *oriT* nickase. Since DNA cleavage by MobA generates a free 3'-OH end and a blocked 5' end, we infer that the protein is joined to the 5' nick terminus by a high-energy phosphate bond. DNA sequencing has revealed the position of the phosphodiester bond interrupted *in vitro* in the sequence 5' GTGCG¹CCC 3' between nucleotides 3138/3139 on the RSF1010 DNA sequence map (6). This is at the location (*nic*; 3138–3140) where, according to the recombination studies of Brasch and Meyer (5), *oriT* nicking and religation occurs *in vivo* during transfer.

The MobA/C-mediated nicking reaction is protein concentration dependent but, at saturating levels of the two proteins and under optimal ionic conditions, only 50–56% of the input substrate DNA is converted to the nicked species in a 30-min reaction. When the third known RSF1010 Mob protein, B, is also present, the final yield of nicked product increases to maximally 85%. Whether this MobB stimulation reflects an interaction of the protein with MobA or MobC or is due to an effect on DNA structure is not clear at present. Our preliminary studies of the purified protein (subunit M_r = 15,100) indicate that it binds weakly and nonspecifically to double-stranded DNA (unpublished results).

The nicking reaction *in vitro* is absolutely dependent on the presence of a divalent cation, either Mg^{2+} , Mn^{2+} , Ca^{2+} or Ba^{2+} , and is reversible with respect to salt and temperature. Thus, the specifically nicked DNA that accumulates during the reaction carried out in low salt/ Mg^{2+} buffer rapidly disappears if prior to SDS-treatment the salt concentration is increased to 0.6M NaCl or the incubation temperature is raised to 50°C. Addition of excess EDTA to the preincubated reaction mixture, however, blocks this salt- or temperature-induced reverse reaction. Following reversal, the DNA product remains supertwisted, with no detectable change in the superhelical density. Taken together, these observations are most consistent with the DNA being nicked prior to the time of quenching, with MobA possibly acting as a protein linker maintaining to topological state of the DNA through covalent and noncovalent interactions. This structure resembles the transient intermediate in the nicking-closing reaction of type-I DNA topoisomerases (18, 20).

The efficient *in vitro* cleavage of supercoiled RSF1010 DNA requires a nearly 20-fold molar excess of MobC protein monomers (M_r = 10,900) compared to only 2 MobA protein monomers per plasmid molecule. An even larger molar excess of MobC (85-fold) is necessary to saturate the reaction on linearized RSF1010 DNA, indicating that the protein has a structural role. Large protein-*oriT* complexes can be seen in the electron microscope after incubation of the MobA and MobC

proteins with an *oriT*-containing plasmid DNA (unpublished). The size of this complex exceeds that of the MobA-*oriT* complex, which is hardly detectable by electron microscopy but which can be readily demonstrated using gel retardation assays (unpublished; ref. 11). Protein MobC alone fails to form any observable *oriT*-specific complex with duplex DNA (supercoiled or linear) and is presumably brought into position through its interaction with MobA bound to *oriT* (unpublished). MobC binding at the *oriT* site may result in a localized destabilization of the DNA helix, allowing the MobA protein to interact with its single-stranded recognition sequence which is then nicked in a reversible way. This view is favored by the fact that MobC protein is no longer required for DNA cleavage by MobA protein when the substrate is present in single-stranded form.

Studies on the interaction between MobA protein and single-stranded DNA have revealed that the breakage and the religation reactions are separable, similar to the interactions between eukaryotic topoisomerase I enzymes and single-stranded DNA (25, 26). The *in vitro* cleavage of single-stranded DNA described here (Fig. 9) is site-specific, but the sequence of 38 nucleotides at the RSF1010 transfer origin is clearly not required for specific recognition by the MobA protein since M13 viral DNA that lacks the *oriT* sequence is attacked by the enzyme, even when coated with *E. coli* SSB protein (data not shown). However, it has been shown earlier for a C-truncated form of the R1162 MobA protein that it is capable of recognizing and cleaving at the single-stranded origin of transfer, both *in vitro* and in an M13 phage-based *in vivo* recombination system (11, 27). Different from the reaction with duplex *oriT* DNA, no protein-denaturant treatment is necessary to expose the MobA-induced breaks in single stranded DNA. After cleavage, MobA is linked to the 5' end of the fragment generated and retains its activity for the rejoining reaction. This two-step reaction may reflect the mechanism of the enzyme on double-stranded DNA. We have not yet investigated whether MobA as a covalently bound protein is capable of catalyzing additional cleavage (and religation) of DNA. The gene-A protein of bacteriophage ϕ X174 functions as a site- and strand-specific breaking and rejoining enzyme during DNA replication and catalyzes a reaction of this nature (reviewed in ref. 28).

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