A segment of a plasmid gene required for conjugal transfer encodes a site-specific, single-strand DNA endonuclease and ligase

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ABSTRACT

The polypeptide encoded by a segment of a gene required for the conjugal mobilization of the broad host-range plasmid R1162 has been purified as a β -galactosidase fusion protein. The hybrid protein binds specifically to a small, double-stranded DNA fragment containing the origin of transfer (*oriT*), and specifically cleaves *oriT* single-stranded DNA at the position cleaved during transfer. Only one of the two DNA strands is a substrate. A fraction of the digested DNA is resistant to λ exonuclease digestion, indicating that some molecules have protein covalently attached at the 5' end. After prolonged incubation with fusion protein, some of the cleaved molecules are religated. In vivo, M13 phage DNA containing two, directly-repeated copies of *oriT* recombine in cells containing the fusion protein. The single-stranded viral DNA forms are the probable substrates for the protein, the cleaved DNA being subsequently religated to form recombinant molecules. Cleavage of the DNA might be the reverse reaction of the ligation that normally takes place after conjugative transfer of a single, linear plasmid DNA strand.

INTRODUCTION

R1162 is a small, multicopy plasmid that is efficiently co-mobilized into diverse bacterial species during the conjugal transfer of broad host-range, self-transmissible IncP-1 group plasmids such as RK2 and R751. Transfer of R1162 DNA is polar and in a unique direction (1). M13mp19 (2) derivatives containing two, directly-repeated copies of the origin of transfer (*oriT*) undergo site-specific recombination (3). The product is a phage molecule having a single *oriT*, resulting from cleavage at a unique position within each of the two, directly-repeated *oriT* copies, deletion of the intervening DNA, and religation of the remaining fragment (3). The recombination site is at the position cleaved during conjugal transfer of the plasmid (the *nic* site) (4), but recombination takes place in the absence of both conjugation and a mobilizing plasmid (3). In addition, only the amino-terminal segment of a large polypeptide encoded within the Mob region

of R1162, and not the entire complement of *mob* genes, is required (3). We have isolated a hybrid protein consisting of this polypeptide linked to β -galactosidase. In vitro, this protein is an endonuclease, active on single-stranded DNA, that specifically cleaves one *oriT* strand at the *nic* site. The fusion protein is also able to bring about the ligation of the cleaved *oriT* DNA. These results and genetic evidence suggest that the polypeptide fragment, normally part of a larger protein, is involved in the processing of transferred, single-stranded DNA.

MATERIALS AND METHODS

Strains, plasmids and bacteriophage

The *Escherichia coli* K-12 strains used in this study are JM103 (Δ [*lac pro*] *strA thi supE endA sbcB hsdR, F' traD36 proAB lac^r lacZ* Δ M15) (5) and M182 (Δ *lacIPOZY X24 galK galU strA*) (6). The plasmid pUT966 is a pBR322 derivative that contains a segment of R1162 DNA extending between 4.8 and 6.6 kb on the R1162 map shown in Figure 1. This DNA is fused at the 4.8 kb end to a *lacZYA* fragment derived from the plasmid pMC1403 (7). The 204th codon of the large *mob* gene extending from 5.5 to 3.4 kb (Figure 1) is joined to the 8th codon of *lacZ* by means of a 12 codon linker. The bacteriophages ϕ rm19 and ϕ rm20 consist of an 81 bp EcoRI–HindIII fragment, containing *oriT*, inserted into M13mp18 and M13mp19 respectively (3). Phages ϕ rm147 and ϕ rm47 are identical to ϕ rm20 but contain the *oriT* deletion indicated in Figure 1 (4, 8, and unpublished results). ϕ rm18 is also derived from ϕ rm20, and contains a second, directly-repeated *oriT* on a 70 bp fragment inserted at the EcoRI site (3). Phages ϕ rm139 and ϕ rm151 are derivatives of ϕ rm18 that contain the deletion present in ϕ rm147 in one of the two *oriTs* (Figure 1).

Purification of fusion protein

The fusion protein was followed during purification by gel electrophoresis (Figure 2), and by assaying for β -galactosidase (9). Twenty grams of cells from 6 liters of a stationary-phase culture of M182 (pUT966) were resuspended in approximately 80 ml 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM mercaptoethanol (buffer A) and lysed with a French pressure cell.

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Phenylmethylsulfonyl fluoride was immediately added to 1 mM, and the lysate centrifuged at $100,000\times g$ for 45 min. Nucleic acids were precipitated from the supernatant by the dropwise addition of 30% streptomycin sulfate to a final concentration of 0.5%, followed by stirring for 1 hr. After centrifugation at $15,000\times g$ for 20 min, over 96% of the β -galactosidase activity was found in the pellet. The precipitate was resuspended in 50 ml buffer A, forced five times through a 23G $\times$ 1 in. needle to shear the DNA, and then mixed with 20 g DEAE cellulose (Whatman). The DNA and associated protein bound to the DEAE cellulose were washed with buffer A (2 $\times$ 40 ml), and the β -galactosidase activity eluted with 0.5 M NaCl in buffer A (3 $\times$ 40 ml). The sample was then diluted to 0.4 M with additional buffer, and applied to a heparin–Sephrose column (1 $\times$ 40 cm). The column was washed with 100 ml 50 mM Tris-HCl buffer containing 0.4 M NaCl, 10 mM MgCl₂, 10 mM mercaptoethanol, and the β -galactosidase activity eluted with a 200 ml linear gradient of NaCl (0.4–1.0 M) in the same buffer. Fractions containing activity were pooled and concentrated by ultrafiltration (Diaflo membrane YM10, Amicon), then applied to a Bio-Gel A 1.5 m column (1.5 $\times$ 100 cm) and the fractions having β -galactosidase activity again pooled. The procedure gave 11 mg of protein and an overall purification (based on β -galactosidase activity) of 210-fold. The first fourteen N-terminal amino acids of the hybrid protein, determined by gas-phase amino acid sequence analysis, are AIYHLTAKTGSRSRSG, in agreement with the DNA sequence of the *mob* gene (10).

Assay for recombination at *oriT* in vivo

Recombination at *oriT* was assayed with phage ϕ rm18 (3), which contains two, directly-repeated *oriT*s cloned into M13mp19. The standard conditions for the assay are described elsewhere (3). The *oriT* insertions in ϕ rm18 interrupt the *lacZ*(α) coding sequence, and phage plaques are therefore white on lawns of JM103 in the presence of X-Gal. After recombination at both *oriT*s during propagation of the phage, molecules with a single *oriT* insertion are generated. Translation through this remaining *oriT* results in an altered *lacZ*(α) polypeptide, which can nevertheless complement the product of the *lacZ* Δ M15 gene in the host. The overall recombination frequency is enumerated by scoring the percentage of blue plaques on a lawn of JM103, after growth of the phage in the test strain.

Assay for endonuclease activity

Primer extension assay. 0.4 μ g ϕ rm20 DNA in 25 mM sodium phosphate buffer (pH 7.5), 5 mM MgCl₂, 5 mM mercaptoethanol, was mixed with 3 μ g protein in a total volume of 2.5 μ l and incubated for 10 hr at 37°C. Half of the product was further digested with 1 μ g proteinase K for 2 hr at 37°C. Each DNA sample was then hybridized with 0.8 ng of a 17-mer oligonucleotide primer (–40 primer, New England Biolabs) in 3 μ l polymerase buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 7.5 mM DTT). The primer was extended by the addition of 4 pmoles α -[³⁵S]-dATP (500 Ci/mmol), 125 pmoles each dCTP, dTTP, dGTP, and 5 units DNA polymerase I large

Figure 1. Simplified map of R1162, showing the region of the plasmid containing the genes for mobilization (Mob), and the location of the origin of conjugative transfer (*oriT*). Also shown is the location of the large *mob* gene, and the segment of this gene present in the gene fusion constructed for this work. The arrows indicate the direction of transcription. The sequence of the 38 base-pair *oriT* (4), and the deletions in *oriT* examined in this study, are depicted below. The derivatives of ϕ rm20 and, ϕ rm18 containing each deletion are designated at the right. Su^r, Sm^r: region encoding resistance to sulfonamides and streptomycin, respectively; *oriV*: origin of replication; *nic*: site within *oriT* cleaved during transfer. Plasmid coordinates are in kilobase-pairs of DNA.

fragment, with incubation at 42°C for 30 min. A chase solution containing 250 pmoles each dNTP and an additional unit of enzyme was then added, and incubation continued for 30 min. The labeled product was separated on an 8% polyacrylamide sequencing gel (11) and visualized by autoradiography.

Assay for cleavage of template strand

0.2 μ g ϕ rm20 viral DNA and 1.0 μ g of a 30-mer complementary oligonucleotide were mixed together in 20 μ l 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂. The oligonucleotide spans the single Bsu36I site in ϕ rm20, located 245 bases from the *oriT_{nic}* site on the 5' side. The DNAs were incubated 8 hrs at 37°C with 10 units Bsu36I restriction endonuclease, and then end-labeled at 37°C for 60 min in 30 μ l polymerase buffer (above) also containing 7 pmoles each dTTP, dGTP, and α -³²P-dATP (3000 Ci/mmol), and 5 units DNA polymerase I large fragment. The DNA was resuspended in 10 μ l 100 mM Tris-HCl (pH 7.5) and incubated with 2.5 μ g (1 μ l) fusion protein at 37°C for 15 hrs. Some samples were further treated with 1 μ g proteinase K at 37°C for 1 hr. Also, some samples were resuspended in 10 μ l 67 mM glycine-KOH (pH 9.4), 2.5 mM MgCl₂, 50 μ g/ml bovine serum albumin, and digested with λ exonuclease (8 units) at 37°C for 1 hr. All samples were boiled and analyzed on an 8% polyacrylamide sequencing gel.

Figure 2. Purification of fusion protein followed by SDS-polyacrylamide gel electrophoresis. (A) cell extract (100 μ g), (B) 0.5% streptomycin sulfate precipitate (25 μ g), (C) eluant from DEAE cellulose (15 μ g), (D) pooled active fractions from heparin-Sepharose CL-6B column (7 μ g), (E) pooled active fractions from BioGel A-1.5m column (5 μ g). Molecular weight markers (F) are β -galactosidase (116 kdal), phosphorylase B (97.4 kdal), bovine serum albumin (66 kdal) and egg albumin (45 kdal). Gel (7.5% polyacrylamide, 0.2% bis-acrylamide) was prepared according to Laemmli (20).

Assay for ligation of DNA

Phage ϕ rm18 DNA was end-labeled at the Bsu36I site as described above, except that 1.5 μ g DNA and 1.0 μ g oligonucleotide were annealed in a final volume of 30 μ l. The DNA was subsequently annealed with 1.2 μ g of a second 30-mer oligonucleotide, spanning the single NarI site in ϕ rm18, and digested overnight with 10 units NarI restriction endonuclease. 0.9 μ g of this DNA were then precipitated, re-dissolved in 10 μ l 100 mM Tris-HCl (pH 7.5), and incubated with 5 μ g fusion protein. Samples were taken at various times and analyzed by 8% polyacrylamide gel electrophoresis.

Gel retardation assay

The RFI DNAs of phages ϕ rm20, ϕ rm47 and ϕ rm147 were digested with HindIII and EcoRI, and the small *oriT* fragments, purified by gel electrophoresis, end-labeled as described above. As a control, a 63 bp M13mp18 (2) HindIII fragment (bp 5375–5438) was also purified and end-labeled. The sizes of the *oriT* DNA fragments were 81 bp (ϕ rm20), 80 bp (ϕ rm47) and 64 bp (ϕ rm147). 0.2 ng DNA and 0–45 ng protein were mixed in 13 μ l buffer containing 20 mM HEPES, pH 8, 250 mM NaCl,

Table 1. Recombination of ϕ rm18 in JM103 strains containing cloned DNA

DNA cloned in pBR322:	Recombination frequency (% blue plaques):
1. R1162 DNA (4.8–6.6 kb)	17, 23
2. R1162 DNA (4.8–6.6 kb) fused to <i>lacZYA</i>	22, 22
3. <i>lacZYA</i>	<0.1, <0.3

Results of two independent experiments.

Figure 3. Gel retardation assay with purified hybrid protein and HindIII-EcoRI *oriT* DNA fragments derived from (A) ϕ rm20, (C) ϕ rm147 and (D) ϕ rm47. The DNA in Panel B is a 63 bp control DNA fragment from M13mp18. DNA (0.2 ng) was mixed with (a) 0, (b) 0.6, (c) 1.5, (d) 3, (e) 9, (f) 18, (g) 30 and (h) 45 ng protein (protein:DNA molar ratios 0:1, 1:1, 2.5:1, 5:1, 15:1, 30:1, 50:1, 75:1).

20 mM MgCl₂, 5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, and 37% glycerol. The mixture was incubated at 37°C for 10 min before being applied to an 8% polyacrylamide gel.

RESULTS

Mob hybrid protein binds specifically to double-stranded DNA containing *oriT*

We constructed a plasmid with the 4.8–6.6 kb segment of R1162 joined to *lacZYA* DNA. The resulting molecule encodes a fusion

protein consisting of the amino-terminal segment of a polypeptide, encoded by part of the Mob gene that extends from 5.5 to 3.4 kb (Figure 1) (10, 12), linked at the carboxy-terminal end to β -galactosidase. The Mob polypeptide segment is required for recombination between the directly-repeated *oriT*s in ϕ rm18, an M13mp19 derivative (3). The hybrid Mob- β -galactosidase protein also supports ϕ rm18 recombination in vivo, and at the same frequency as the unfused protein (Table 1, lines 1, 2). In cells containing pBR322 with only the *lacZYA* portion (pMC1403), recombination is at the background level (line 3).

Figure 4. Primer extension assay with M13mp19, ϕ rm19 and ϕ rm20 viral DNA templates that have been cleaved with Mob fusion protein. Half of each sample was further digested with proteinase K (+); the other half was untreated (-). For each template, dideoxy-terminated extensions from the same primer are included as markers. The arrows indicate the location of the *nic* site in ϕ rm19 and ϕ rm20.

We purified the hybrid protein and studied its behavior with both single- and double-stranded DNA. Protein was incubated with a radioactive, 81 bp *oriT* DNA fragment, and the mixture then examined for retarded migration during polyacrylamide gel electrophoresis (Figure 3A, lanes a–h). As the ratio of protein to DNA was increased, a larger proportion of the radioactivity remained in the well of the gel, presumably because the complex of DNA with the large protein could not enter the gel matrix. In contrast, when the protein was mixed with a similarly-sized (63 bp) fragment lacking *oriT*, the DNA continued to enter the gel, and there was little radioactivity in any of the wells (Figure 3B, lanes a–h). Two mutated *oriT*s, each deleted for one arm of the inverted repeat, were also tested for binding to protein.

In each case, binding to the protein was still evident, although it was reduced compared to the normal *oriT* (Figures 3C and 3D). However, we were unable to demonstrate particular nicking or cleavage at *oriT*: incubation of the protein with the RFI DNAs of ϕ rm18 (two *oriT*s), ϕ rm20 (one *oriT*), or the parental vector M13mp19 resulted in equal amounts of non-specific nicking (data not shown), with conversion of RFI to the open-circular form.

Mob hybrid protein cleaves single-stranded DNA at the *oriT* *nic* site

The phages ϕ rm19 and ϕ rm20, derivatives of M13mp18 and M13mp19 (2) respectively, contain *oriT* cloned in different orientations. We incubated the single-stranded viral DNAs from

Figure 5. Cleavage of template strand at *oriT* and attachment of protein to 5' end of cleaved site. Figure 5A: Polyacrylamide gel electrophoresis of (a) *Hinf*I-digested and end-labeled pBR322 marker DNA, denatured prior to electrophoresis, (b) ϕ rm20 viral DNA, end-labelled at *Bsu*36I site, and (c) end-labeled ϕ rm20 DNA digested with fusion protein. Figure 5B: Samples analyzed as for 5A, but region of gel containing digestion products enlarged to show detail. (a) end-labeled ϕ rm20 viral DNA cleaved with fusion protein, (b) same as (a), but further digested with proteinase K, (c) same as (a), but further digested with λ exonuclease, (d) cleaved DNA digested with λ exonuclease followed by proteinase K. Markers (e) are as in 5A. Fragment sizes are in bases of DNA.

these phages, and from the vector M13mp19, with the hybrid protein. We then determined the positions of cleavage by a primer extension assay (Figure 4). Synthesis from the primer was terminated specifically in the case of ϕ rm20. In addition, the termination site was at *nic*, the location within *oriT* that is cleaved during phage recombination and conjugative transfer (3, 4). There was no specific cleavage of the other *oriT* strand, present in ϕ rm19 viral DNA (Figure 4). The particular endpoint for ϕ rm20 in the primer extension was not due to a pre-existing cleavage at *nic* in the template DNA, since this DNA was also used for the accompanying DNA sequencing with the primer, shown in the same figure, and in this case no specific termination at *nic* was observed.

A DNA strand of defined length was synthesized with the ϕ rm20 DNA template in the primer extension assay, whether

or not the template DNA was first treated with proteinase K after incubation with the hybrid protein (Figure 4). However, it remained possible that the template DNA was not cleaved, but that instead synthesis was terminated by a bound, proteinase K-resistant protein fragment. To show directly that the template strand was cleaved, we first hybridized ϕ rm20 DNA with a 30-mer oligonucleotide spanning the single *Bsu*36I site, located 245 bases away from the *oriT* cleavage site. The hybridized DNA was then digested with the restriction endonuclease *Bsu*36I, and the product end-labeled by filling in the recessed 3' ends. This labeled DNA was treated with the hybrid protein, boiled to dissociate the oligomer, and subjected to electrophoresis on an 8% polyacrylamide sequencing gel. After treatment with protein, a single labeled band of about 250 bases appeared in the gel (Figure 5A, lane c), indicating that the hybrid protein cleaves

Figure 6. Digestion of ϕ rm18 viral DNA with fusion protein. DNA was cleaved at the *Bsu*36I site and end-labeled (A), further cleaved at the *Nar*I site (B), and then digested with fusion protein for (C) 24, (D) 48 and (E) 72 hrs at 37°C. Marker as in Figure 5A.

the *oriT* DNA strand. (In this experiment, the other labeled DNA strand is part of the hybridizing oligonucleotide, and is too small to appear in the gel). We conclude that the hybrid protein is a strand- and site-specific endonuclease that cleaves within *oriT* at *nic*.

Single-stranded, cleaved *oriT* DNA is ligated by fusion protein

Phage ϕ rm18 DNA, containing two directly-repeated copies of *oriT*, is a substrate for *oriT*-specific recombination in vivo. The product is a phage DNA containing a single *oriT* copy (3). We asked whether such recombinant molecules could be generated in vitro with the purified fusion protein. Single-stranded ϕ rm18 viral DNA was end-labeled at the Bsu36I site, as described above. The product was then hybridized to an oligonucleotide spanning the single NarI site, located 607 bases away from the Bsu36I site on the other side of the two *oriT*s (Figure 6). The DNA was incubated with NarI, and for different times with fusion protein, and the products separated by polyacrylamide gel electrophoresis. Fragments of 315 and 245 bases were generated (Figure 6, lanes C–E), the expected size for molecules singly-cleaved at *oriT*(1) or *oriT*(2). Several other, fainter bands were also present, which

we believe are due to secondary cleavage sites for the protein. In addition, after prolonged incubation with fusion protein, a fragment of 537 bases was obtained. When digestion with the fusion protein was omitted, only a single large fragment, the result of NarI digestion of the labeled DNA, was observed (lane B).

A fragment of approximately 540 bases would be generated following cleavage at *oriT*(1) and *oriT*(2), deletion of the small intervening fragment, and ligation of the remaining DNA. Our suspicion that this DNA is ligation product was strengthened by the observation that it exactly co-migrated with Bsu36I + NarI cleaved ϕ rm20 viral DNA, identical in base composition and size to the ligated DNA (not shown). In order to confirm that this DNA is composed of formerly non-contiguous segments, we used DNA from the phages ϕ rm139 and ϕ rm151, ϕ rm18 derivatives. Phage ϕ rm151 has a deletion of the outer arm of the inverted repeat in *oriT*(1); ϕ rm139 has an identical deletion in *oriT*(2) (Figure 1). However, because of the presence of different linking fragments, the deletion in *oriT*(1) (ϕ rm151) results in a net deletion of 17 bases, whereas the deletion in *oriT*(2) (ϕ rm139) results in a net insertion of 25 bases. As a result, after digestion of the NarI–Bsu36I fragments with fusion protein, identical

Figure 7. Digestion of viral DNAs with fusion protein. Lanes A, D, G: DNA end-labeled at Bsu36I site, B, E, H: DNA further cleaved at the NarI site, C, F, I: DNA cleaved at Bsu36I and NarI sites and incubated with fusion protein for 48 hrs. Marker as in Figure 5A.

fragments of 315 bases are generated following cleavage of ϕ rm18 and ϕ rm151 at *oriT*(1), whereas the corresponding fragment for ϕ rm139 is larger, due to the 25-base increase in the size of *oriT*(2) (Figure 7, lanes C, F, I). Cleavage at *oriT*(2) would result in identically-sized fragments for all three phage DNAs, and this is also seen in Figure 7. In contrast, the presumptive ligation product of ϕ rm151 is smaller than that of ϕ rm18, whereas the ϕ rm18 and ϕ rm139 bands are the same size. This is the result predicted for ligated DNA: the ligation product of ϕ rm151 would be smaller because the deletion in *oriT*(1) would still be present, resulting in a 17-base decrease in total length. In contrast, the deletion in *oriT*(2) would be missing in the ligated DNA, since the DNA between the two *oriT* *nic* sites is eliminated, and the product would therefore be the same size as that derived from ϕ rm18. Alternative explanations, for example anomalous cleavages of phage DNA by fusion protein, cannot simply account for these sizes.

Hybrid protein is covalently linked to the 5' end of the cleaved *oriT*

End-labeled ϕ rm20 DNA was incubated with fusion protein, and then further digested with proteinase K. Two labeled species were generated, one migrating with the DNA formed prior to proteinase K treatment, and the other migrating slightly less rapidly (Figure 5B, lane b). We supposed that the slower band is derived from DNA that remains covalently joined to the fusion protein. In the absence of proteinase K digestion, this protein-DNA covalent complex would not enter the gel, due to the attachment of the large fusion protein. After treatment, however, the DNA would migrate more rapidly, its slightly lower mobility in comparison to the other band due to one or more remaining, proteinase K-resistant amino acids. The results following digestion with λ exonuclease support this interpretation. This enzyme digests DNA specifically from the 5' end, and is active on single- as well as double-stranded substrates (13). The DNA fragment generated by the Mob fusion protein was sensitive to the exonuclease (Figure 5B, lane c). In contrast, if the DNA was first treated with λ exonuclease and subsequently with proteinase K, the more slowly migrating fragment became predominant (lane d). This would be expected if the 5' end of the DNA in this fragment was protected from λ exonuclease digestion by covalently bound protein.

DISCUSSION

The amino-terminal segment of a large protein encoded within the R1162 Mob region promotes strand- and site-specific cleavage within *oriT*, as well as religation of the cleaved DNA. In addition, this protein is required for recombination in vivo between directly-repeated copies of *oriT* propagated by M13mp19. In both cases, *oriT* DNA is cleaved at the *nic* site. How are these activities related to the mechanism of R1162 transfer during conjugation? Genetic evidence indicates that the phage recombination models the recircularization of plasmid DNA after transfer into a recipient cell: mutations in *oriT* that affect phage-based *oriT* recombination also affect a late step in DNA processing during transfer, presumably ligation of the transferred DNA (8). We believe that during phage recombination in vivo, both cleavage and ligation of single-stranded *oriT* DNA are carried out by the Mob polypeptide fragment. We have shown that this polypeptide cleaves single-stranded DNA at the proper position within *oriT*, but that only one of the two *oriT* strands is sensitive. This is

understandable if transfer of R1162, like that of F and several other Gram-negative plasmids, is strand-specific (14). The transfer of R1162 is polar in the direction shown in Figure 1 (1). The strand specificity of the Mob polypeptide would then mean that R1162 DNA is transferred in the 5' to 3' direction, which is also the polarity of transfer of F (15).

A fraction of cleaved DNA molecules appear to have protein covalently attached to the 5' end of the cleavage site. It is not clear whether this form of the cleaved DNA is the most significant in terms of the DNA processing that takes place during conjugation. Prior to transfer, plasmid DNA is nicked in the donor cell, and it is possible that Mob protein is normally attached to the 5' end at this time. The protein-DNA complex would then be transferred to the recipient, where it would be the substrate for subsequent ligation, with resulting circularization of the DNA molecule. This view is favored by the fact that relaxation complex-induced nicking of ColE1 DNA, a reaction that models the nicking occurring prior to plasmid mobilization, results in covalent attachment of protein to the DNA (16). In the case examined here, the protein-free DNA might arise aberrantly, possibly because part of the Mob protein is missing. Alternatively, protein-free, cleaved DNA might be a normal intermediate in conjugation, with Mob protein fragment, with its large β -galactosidase tag, showing slower dissociation. It has been pointed out that the DNAs of single-stranded phage undergo processing steps during replication that are similar to those occurring during plasmid conjugation (17), with both the site-specific nicking of a supercoiled form, and ligation of single-stranded DNA. Interestingly, in some cases replicative intermediates of these phage involve covalently-bound protein (ϕ X174), whereas in other cases there is no covalent linkage (M13, fd) (18).

The ligation of viral DNA proceeds very slowly under our conditions, and further experiments will be necessary to optimize this reaction before it can be characterized in detail. Part of the difficulty might be that the product is itself a substrate for cleavage, so that large amounts do not therefore accumulate. In this connection, it is noteworthy that the recombination frequency of phage in cells encoding the *mob* polypeptide fragment is greater than in cells containing the intact *mob* gene (3). After the conjugal transfer of plasmid DNA, the transferred strand must be ligated, and re-cleavage would presumably be deleterious. Thus, for the normal protein the cleavage activity might be suppressed, whereas it could be unmasked in the polypeptide fragment.

It is also possible that cleavage of single-stranded *oriT* DNA plays a role in the mechanism of conjugative transfer, and is not simply the reverse of the ligation normally occurring after entry of the DNA strand into the recipient cell. If transfer were accompanied by rolling circle replication in the donor, so that the transferred strand were extended at the 3' end, then cleavage would be necessary to terminate transfer. Thus, the ϕ X174 *cisA* protein is a strand- and site-specific endonuclease both in vivo and in vitro (19). It is also possible that although DNA synthesis is not normally initiated by a rolling circle mechanism, some adventitious synthesis by this mechanism does occur. The endonucleolytic activity could then function as part of a correction system, insuring that unit-length molecules are available to be circularized.

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