Recombination between directly repeated origins of conjugative transfer cloned in M13 bacteriophage DNA models ligation of the transferred plasmid strand

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ABSTRACT

When two, directly-repeated copies of the origin of transfer (*oriT*) of the conjugatively mobilizable, broad host-range plasmid R1162 are cloned into bacteriophage M13mp9 DNA, they undergo recombination in the presence of one of the R1162-encoded proteins required for mobilization [Meyer, R. (1989) J. Bacteriol., 171, 799–806]. Mutations in the outer arm of the inverted repeat within *oriT* inhibit this recombination. These mutations also affect a late step in transfer. We propose that recombination on the phage DNA models the processing of single-stranded DNA after entry into a recipient cell. The two, directly-repeated *oriT*s are not equivalent during the recombination reaction, because they are differently affected by the outer-arm mutations. A mutation was also isolated that reduces the specificity of the cleavage site in one of the two *oriT*s. Together, the results with the mutations suggest that phage recombinants can form only when the first cleavage occurs at one of the two *oriT*s. This is followed by the resulting free 3' end joining to the 5' end at the cleavage site of the other *oriT*.

INTRODUCTION

R1162 is a small (8.7 kb), broad host-range plasmid that is conjugatively mobilized by RK2, R751, and other plasmids of the IncP-1 group (1). Transfer of the plasmid is linear and unidirectional (2), and requires a site on R1162 (*oriT*), as well as the products of the several R1162 *mob* genes (3). The DNA of phage ϕ rm18, an M13mp19 (4) derivative containing two cloned, directly-repeated copies of *oriT* within the *lacZ*(α) segment, undergoes *oriT*-specific recombination (5), resulting in the formation of a phage molecule with a single *oriT* (Fig. 1). This recombination is easily detected, because there is translation through the recombinant *oriT*, and consequently synthesis of an active *lacZ*(α) polypeptide that results in blue plaques on medium containing X-Gal. Recombination requires the product of part of one R1162 *mob* gene, and the frequency of recombination increases when another *mob* gene is inactivated (5). The recombination occurs between base positions 30 and 32 within

the *oriT* DNA (Fig. 1) (5), which is also where *oriT* is cleaved during conjugative transfer of plasmid DNA (6). The site-specificity of phage-based *oriT* recombination, and its dependence on a segment of the *mob* genes, indicate that this process reflects some part of the mechanism of conjugation. The principal objective of the work reported here has been to identify which of the DNA processing reactions occurring during conjugative transfer are modeled by the phage recombination system.

MATERIALS AND METHODS

Bacterial strains

JM103 [Δ (*lac pro*) *strA thi supE endA sbcB hsdR* (F'-*traD36 proAB lacI^q lacZM15*)] (7) was used for propagation of M13 bacteriophage derivatives. For bacterial matings, the donor strain was MV12 (R751)(R1162) containing the test plasmid, and the recipient was MV12 Nal^r. MV12 is C600 *recA12* Δ *trpE5* (8). Plasmid R751 (IncP-1) (9) was the self-transmissible mobilizing vector, and R1162 provided the required Mob proteins.

Bacteriophage

The M13 bacteriophages in this study are derivatives of ϕ rm18 and ϕ rm20 (Fig. 1, top), which had been constructed previously by inserting DNA fragments containing *oriT* into the cloning region of M13mp19 (5). ϕ rm18 contains two, directly-repeated *oriT*s, which are referred to as *oriT*(1) and *oriT*(2). A set of ϕ rm18 derivatives with point mutations in *oriT*(1) was obtained by base-misincorporation mutagenesis (described below). A second series of phage with the identical structure, but having these mutations in *oriT*(2), was also constructed. These phages were prepared by deleting *oriT*(2) from each member of the first set by *EcoRI* digestion, followed by insertion of a *Bam*HI fragment, derived from ϕ rm18 and containing an unmutated *oriT* (Fig. 1), into the remaining *Bam*HI site. For one mutation (G to A transition at base-pair 31, Fig. 1), a phage was also constructed with the mutation in both *oriT*s, by cloning instead an identical *Bam*HI fragment but with the mutated *oriT*. In addition, a phage DNA was constructed with the base-pair 31 mutation in *oriT*(1), and two C to T transitions, at base-pairs 30 and 32, in *oriT*(2). The isolation of an *oriT* with these C to T mutations has been described previously (6). An *EcoRI*

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fragment containing these mutations was used to replace the EcoRI fragment with the unmutated *oriT*.

Two further derivatives, similar in structure to ϕ rm18, were constructed that contained a deletion of base-pairs 1 through 8 in one of the *oriT*s (Fig. 1). The isolation of this deletion has been described elsewhere (6). A bacteriophage containing an unmutated *oriT*(1), and *oriT*(2) with the deletion, was prepared by linking a HindIII–EcoRI fragment containing the deleted *oriT* (6) with the small HindIII–EcoRI fragment of pBR322, and then cloning the product into the EcoRI site of ϕ rm20. Recombination results in a phage having a single, full-size *oriT* and forming blue plaques. We also wanted to measure the recombination frequency of phage DNA containing the deletion in *oriT*(1), and an

unmutated *oriT*(2). In this case, recombination would result in phage DNA with a single *oriT* retaining the deletion. Because of this, the reading frame would be shifted, and recombinant phage would produce white plaques and would therefore not be detected. In order to correct for this, we introduced a single base deletion at the PstI site, to restore the reading frame required for blue plaques. This deletion was generated by partial digestion of PstI-cleaved DNA with mung bean nuclease.

Assay for bacteriophage recombination

The assay for *oriT*-specific recombination in M13 derivatives has been described elsewhere (5). In brief, a culture of JM103(pUT208), 2×10^6 cells/ml in $2 \times$ YT medium (10), was

Figure 1. (top) Structure of ϕ rm18 and ϕ rm20 DNA in region of cloned *oriT*s. The phage ϕ rm20 is identical to the molecules formed by *oriT*-specific recombination of ϕ rm18. The 5.52 and 5.56 kb endpoints of *oriT* refer to its location on the R1162 map (5). Restriction sites are BamHI (B), HindIII (H), PstI (P), EcoRI (R) and SalI (S). (below) Base sequence of *oriT* DNA, showing the locations of the mutations discussed in the text. The inverted repeat within the sequence is indicated by the horizontal arrows, and the cleavage site by the bracket. The table at the right gives the phage recombination frequencies for ϕ rm18 derivatives containing the indicated mutations.

infected with test phage by transferring part of an isolated plaque with a fine needle. The plasmid pUT208 is a pBR322 derivative with a cloned segment of R1162 DNA encoding all the R1162 mob proteins necessary for the recombination reaction (5). After 5 hrs of growth at 37°C, the progeny phage were diluted, mixed with JM103 cells (without plasmid), X-Gal, IPTG, and soft agar, and poured onto a 2×YT plate to form a lawn. The percentage of blue plaques was determined after overnight incubation at 37°C. The data are the average of two independent experiments, each with two isolated plaques.

Assays for frequency of conjugative mobilization and conjugation-dependent plasmid recombination at *oriT*

These assays have also been previously described (6). Plasmids containing the test *oriT* fragment were constructed by cloning

the *oriT* DNA, as a HindIII-EcoRI fragment, into pBR322. The frequency of mobilization was measured as the number of transjugant colonies per potential donor cell (donor:recipient cell ratio 1:10). Matings were carried out for 90–100 min on solid medium. Plasmids were also constructed that contain two, directly-repeated copies of *oriT*, flanking DNA encoding kanamycin-resistance (Km^r) (2), and having the general structure shown in Fig. 2. These are also derivatives of pBR322, and were constructed as described elsewhere (6). A donor strain containing one of these plasmids (as well as R1162 and the mobilizing vector R751) was mated with MV12 Nal^r , and transjugant colonies selected for carbenicillin-resistance (Cb^r). These were then screened for loss of Km^r , resulting from cleavage within each *oriT* during transfer, and deletion of the intervening DNA.

Figure 2. (top) Structures of plasmids containing two directly-repeated *oriT*s flanking a gene for kanamycin-resistance (Km^r). The *oriT*s are represented by the thick segments, with the arrows indicating the previously determined (2) direction of transfer. Shading designates a mutated *oriT*. (below) The locations of mutations tested for their effect on transfer frequency, and on conjugation-dependent *oriT* recombination when present in molecules having the structures at the top of the figure.

Table 1. Base sequence at *oriT* cleavage site after recombination of parental DNA containing a G to A mutation in *oriT* at position 31.

Mutation and corresponding sequence at cleavage site in parental molecule:		Number of recombinant phage containing indicated sequence at <i>oriT</i> :	
<hr/>			
oriT(1):	oriT(2):		
Phage parental DNA (Fig. 1)			
1. (31A) <u>GCAC</u>	(wt) GCGC	8 <u>GCAC</u>	4 GCGC
2. (31A) <u>GCAC</u>	(30,32T) <u>GTGT</u>	5 <u>GCAT</u>	3 <u>GTGT</u>
3. (wt) <u>GCGC</u>	(31A) <u>GCA</u>	0 <u>GCAC</u>	12 GCGC
<hr/>			
Plasmid parental DNA (Fig. 2)			
4. (wt) GCGC	(31A) <u>GCAC</u>	0 <u>GCAC</u>	8 GCGC
5. (31A) <u>GCAC</u>	(wt) GCGC	8 <u>GCAC</u>	0 GCGC

Mutated bases are underlined in each case.

Base misincorporation mutagenesis

oriT DNA was mutagenized by misincorporation of α S-dTTP into a gapped substrate by DNA polymerase I large fragment (11). The procedure was designed to avoid the purification of gapped molecules (11) and to enrich for the mutagenized product. Approximately 18 μ g of ϕ rm18 DNA was first digested with *Sal*I, which cleaves near *oriT* (Fig. 1). T was added to the 3'-recessed ends, by resuspending the DNA in 89 μ l buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 7.5 mM DTT), heating at 65°C for 10 min, cooling rapidly to room temperature, and then adding 5 units DNA polymerase large fragment and dTTP to 500 μ M and incubating for 3 hr. The DNA was resuspended in 300 μ l 55 mM Tris-HCl (pH 8.0), 75 mM NaCl, 5 mM MgCl₂, 10 mM DTT, and digested with exonuclease III (100 units) at 37°C. After 30 sec, 50 μ l samples (approx. 3 μ g DNA) were taken at

Figure 3. Base sequence of *oriT*s for phage DNAs containing 31A mutation in either *oriT*(1) or *oriT*(2), and, for each of these parental molecules, the base sequences of the hybrid *oriT*s after recombination.

15 sec intervals, and the protein extracted with phenol. Half the DNA in each sample was resuspended in 20 μ l 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and mixed with excess (20 μ g) uracil-containing phage DNA (12) in 20 μ l of the same buffer. The solution was boiled for 3 min, cooled slowly to room temp, then resuspended in mutagenesis buffer (130 mM HEPES-NaOH, pH

7.6, 0.2 mM $MnCl_2$, 2 mM mercaptoethanol, 0.1 mg/ml BSA). Misincorporation was carried out overnight at 25°C by adding 5 units polymerase I large fragment and α -S dTTP to 0.2mM. The DNA was resuspended in 100 μ l buffer containing 20 mM HEPES (pH 8), 12 mM $MgCl_2$, 2 mM DTT, 1.25 mM each dNTP, 1.25 mM ATP, 5 units polymerase I fragment and 200

Figure 4. Base sequence of *oriT*s for phage DNA containing 31A mutation in *oriT(1)* and 30,32T mutation in *oriT(2)*, and of hybrid *oriT*s after recombination.

units T4 DNA ligase, and incubated for 3 hr at room temp. DNA samples were used directly for transformation of JM103 without further treatment.

The addition of T to the SalI-digested DNA minimizes transformation by unmutagenized molecules that had escaped the brief exonuclease treatment, because the resulting strands fail to form a ligatable substrate after hybridization with each other, or with excess U-containing DNA, due to the additional T at the 3' position. In addition, the U-containing template favors recovery of the mutagenized strand (12).

RESULTS

Mutations in the outer arm of the *oriT* inverted repeat affect phage recombination frequency

We have shown previously that mutations at base-pairs 30 and 32 of *oriT* (Fig. 1) map at the cleavage site, and reduce the frequency of conjugative transfer (6). However, when these mutations (designated 30,32T) are present in one or both of the directly repeated *oriT*s of ϕ rm18, they do not affect *oriT*-specific recombination frequency, which is approximately 8–10% (5 and Fig. 1, line 1). We therefore isolated additional mutations in *oriT*(1) of ϕ rm18 (Fig. 1), and tested these for their effect on the recombination frequency of the phage DNA. The results with several of these mutations are shown in Fig. 1, lines 2–8. Deletion of the outer arm of the inverted repeat resulted in a decreased recombination frequency (line 2). Point mutations within this arm, at base-pairs 6, 7 and 8, also reduced the frequency of recombination (lines 3–5), whereas mutations at base-pairs 10 and 11 had no effect (lines 6–7). In all cases, the mutations in *oriT*(1) resulted in a recombination frequency that was still above the background level (approximately 0.1%). When the mutations were moved to *oriT*(2), recombination frequencies were more severely reduced (Fig. 1). We conclude that the outer arm is involved in the recombination reaction. In addition, the magnitude of the effect of these mutations depends on whether they are located in *oriT*(1) or *oriT*(2). Thus, the two *oriT*s are not equivalent in the recombination reaction.

Cleavage of an *oriT* containing a mutation at the recombination site can occur at more than one position

We also isolated a mutation within *oriT* at base-pair 31. This mutation (designated 31A) reduced the recombination frequency when located in *oriT*(1) but, unlike the mutations in the outer arm of the inverted repeat, resulted in a greater frequency of recombination when placed in *oriT*(2) (Fig. 1, line 8). We thought that this new mutation would allow us to define more precisely the location of cleavage, located between base-pairs 30 and 31 or 31 and 32 (5). We sequenced the *oriT* DNA from 12 independent recombinants, derived from a parental phage with the 31A mutation in *oriT*(1) and with an unmutated *oriT*(2). If the DNA is cleaved between bases 30 and 31, then the mutated base will not be present in the recombinant *oriT*s, whereas the A will be retained if the DNA is cleaved between bases 31 and 32. The results (Table 1, line 1 and Fig. 3) show that the A was retained in some recombinant *oriT*s, but not in others, so that evidently there could be cleavage at more than one position. Either there was cleavage between bases 30 and 31, and between 31 and 32, or cleavage had become more non-specific, due to the 31A mutation. To distinguish between these possibilities, we constructed a phage with the 31A mutation in *oriT*(1), and the 30,32T mutations in *oriT*(2), and again sequenced independent

recombinant molecules. As before, the 31A mutation was retained in the majority of the recombinant DNAs, but not in all of them (Table 1, line 2, and Fig. 4). The recombinants retaining the 31A mutation had the sequence GCAT at the cleavage site, indicating that cleavage had occurred between bases 31 and 32. The remaining recombinants had the sequence GTGT. In these cases, recombination occurred not between bases 30 and 31, but to the left of base 30 as drawn in Fig. 1. We conclude that the 31A mutation causes an indeterminacy in the cleavage site position.

We also examined the recombinants derived from phage having the 31A mutation in *oriT*(2), and the normal sequence in *oriT*(1). In this case, no variation in cleavage site was detected (Table 1, line 3). All the recombinant molecules contained the sequence GCGC at the *oriT* cleavage site (Fig. 3), indicating that cleavage had occurred between base-pairs 31 and 32.

The indeterminacy that occurs when the 31A mutation is in *oriT*(1) raises the possibility that in this case cleavage can occur at a different location in each *oriT*. This event cannot be both frequent and general, because then recombinant phage giving blue plaques would be obtained that contained *oriT*s with in-phase deletions or insertions, and these have not been observed. However, non-identical cleavages might occur rarely, resulting most often in frameshift mutations in the recombinant *oriT*. The phage would form white plaques, and the frameshifts would therefore not be detected in the recombination assay. We looked for recombinant *oriT*s containing a -1 frameshift. A derivative of ϕ rm18 was constructed that contained the 31A mutation in *oriT*(1), and a $+4$ insertion at the EcoRI site between *oriT*(2) and the *lacZ*(α) coding segment (by filling in the recessed ends). Recombinants arising from cleavage at the same position within each *oriT* would no longer form blue plaques, due to the frameshifting mutation at the EcoRI site. If a -1 frameshift occurred during the formation of the recombinant *oriT*, then proper phase would be restored in the *lacZ*(α) coding region and blue plaques would be obtained. Approximately 40,000 plaques were screened from four independent recombination experiments, and no blue plaques consisting of phage with a recombinant *oriT* were obtained. Thus, the indeterminacy occurs solely at the first cleavage, at a position which then determines the site of recombination.

Mutations affect conjugation-dependent recombination at *oriT*

The deletion mutation in the outer arm of the inverted repeat reduced the frequency of plasmid mobilization by more than 10-fold (Fig. 2, compare lines 1 and 6) (6). The point mutations also reduced the transfer frequency, although to a lesser extent. The deletion mutation had also been tested previously for its effect on recombination between two, directly-repeated *oriT*s, cloned in each of two orientations in plasmid DNA (Fig. 2, top) (2). This recombination, which is strictly conjugation-dependent (6), is presumably the consequence of cleavage at each *oriT* during generation of the linear transfer intermediate, followed by ligation of the cleaved end of one *oriT* to the complementary end of the other. This results in excision of the segment of DNA, encoding kanamycin-resistance, that is located between the two *oriT*s, and thus, the frequency of recombination can be measured as the fraction of kanamycin-sensitive transconjugants. The deletion mutation reduced the recombination frequency only when it is located in *oriT*(2) (2, and line 1, Fig. 2). The point mutations at base-pairs 6, 7 and 8 had an identical, although less marked, position effect (lines 2–4).

Figure 5. Hypothetical sequence of events during recombination of directly-repeated *oriT*s on M13 single-stranded DNA. In this model, one *oriT* is first cleaved, and the resulting free 3' end then interacts with the second *oriT* to form a hybrid recombinant. At the left, *oriT*(1) is cleaved first, resulting in a hybrid phage. If *oriT*(2) is initially cleaved (right), a small, circular intermediate is instead formed, and a recombinant phage DNA is not created.

The 31A mutation reduced the frequency of conjugation-dependent recombination, whether it was in position 1 or 2 (Fig. 2, line 5). We tested whether the indeterminacy in cleavage, observed for phage DNA recombination, was also present during transfer. The hybrid *oriT*s from independently-generated recombinant plasmids, generated from molecules with structures I and II in Fig. 2 and containing the 31A mutation, were cloned and sequenced. When the 31A mutation was in *oriT*(2), it was absent from all the recombinant *oriT*s, but when it was in *oriT*(1), all the recombinant *oriT*s retained the mutation (Table 1, lines 4,5). These results indicate that no cleavages had occurred to the left of the 31,32 cleavage position (that is, towards the 5.52 kb *oriT* endpoint) during conjugative transfer. Thus, there was no cleavage indeterminacy comparable to that for *oriT* recombination on phage DNA.

DISCUSSION

Our results with the *oriT* mutations help to define the molecular events occurring during phage-based *oriT* recombination, and their likely relationship to the mechanism of conjugative DNA transfer. Assuming that transfer of R1162 is broadly similar to the transfer of F, then two distinct DNA processing reactions at *oriT* must occur. The first of these is nicking of the *oriT* plasmid DNA in the donor cell, and the second is ligation of the transferred, single DNA strand in the recipient. We previously showed that the 30,32T mutations, and the deletion of *oriT* base-pairs 1–10, both reduce the frequency of conjugative transfer (6). However, the 30,32A mutation affects the conjugative recombination of molecules with structure I (Fig. 2), but not those with structure II, whereas the deletion mutation acts in the

opposite manner (2 and Fig. 2). Plasmid recombinants must contain the Rep-Cb^r DNA fragment, and these are generated simply during linear transfer only if the first cleavage is at *oriT*(2) (Fig. 2), and the second at *oriT*(1). The results with the mutations are therefore understandable if the 30,32T mutation specifically affects the initial nicking of *oriT* DNA in a donor cell, prior to transfer, while the deletion mutation affects subsequent ligation of the transferred, single-stranded DNA in the recipient (2).

Which of these two *oriT* DNA processing reactions is modeled in the phage recombination system? Our results indicate that phage recombination at *oriT* models the second reaction, the ligation of incoming single-stranded DNA. The results here show that mutations in the outer arm of the inverted repeat affect phage recombination, as well as conjugative recombination for plasmids with structure II. We propose therefore that phage recombination takes place on the viral strand that is generated during replication. This strand would then be recognized by the DNA processing system as the single-stranded intermediate in conjugation. Since there must be site-specific cleavage at *oriT*, the ligation of incoming transferred DNA must be potentially reversible. This may be a normally occurring feature of the reaction (for example, if transferred DNA were generated by a rolling-circle mechanism), or might be a reverse reaction that is normally suppressed during transfer. Our conclusion is also strengthened by the previous observation that the 30,32T mutations do not affect the frequency of phage recombination (5), which is expected if only nicking of double-stranded DNA is impaired.

Because the mutations in the outer arm, and the 31A mutation, have different effects on recombination depending on whether they are in *oriT*(1) or *oriT*(2), the two *oriT*s must be functionally distinct in the reaction. Some clues about how the *oriT*s are different can be derived from the behavior of the 31A mutation. When located in *oriT*(1), the 31A mutation causes a cleavage site indeterminacy that is not present when the mutation is in *oriT*(2) (Table 1). If the cleavages at the *oriT*s are consecutive, occurring first at one origin and then the other, the indeterminacy would have to be in the *oriT* that is cleaved first in order for it to be detected in the recombinant. This is true because if the indeterminacy were generated in the second *oriT*, then many of the blue plaque recombinants would contain in-phase deletions and insertions, and this is not observed. In addition, we do not detect any -1 frameshifting mutations in the recombinant *oriT*s (Results). The fact that the cleavage indeterminacy is observed only when the 31A mutation is in *oriT*(1) suggests that the initial cleavage must occur at this site to generate a detectable recombinant. We therefore imagine that recombination occurs according to the steps shown in Fig. 5, left, ft, ft, with initial cleavage at *oriT*(1) to generate a free 3' end. This then interacts with *oriT*(2), resulting in a second cleavage at this *oriT* and subsequent ligation of the 3' end of *oriT*(1) to the resulting 5' end of *oriT*(2). Presumably, the first cleavage could also occur at *oriT*(2), but the resulting ligation would generate a small circular intermediate that would not be detected in the phage assay. A reasonable possibility is that the reaction proceeds by means of a protein attached to the 3' end of the first cleavage site, although there is no evidence for this at present.

In conjugative recombination, the 31A-induced indeterminacy in the *oriT* cleavage site is not observed. In order for a recombinant molecule to be formed, plasmid DNA must first be nicked at *oriT*(2) in the donor cell. Our results indicate that this nicking, in contrast to cleavage of *oriT* on phage DNA, is not affected by the 31A mutation. For molecules with structure I,

Fig. 2, cleavage site indeterminacy is therefore not observed, because the mutation does not affect nicking at *oriT*(2), and *oriT*(1) is normal. For molecules with structure II, the initial nick is at the normal *oriT*, and the second cleavage occurs at the *oriT* with the 31A mutation. However, as indicated by the phage results, the indeterminacy is introduced at the first cleavage, and therefore none is observed for the plasmid recombinants.

One additional property of the 31A mutation that needs to be explained is why the recombination frequency is reduced when the mutation is in *oriT*(1), elevated when it is in *oriT*(2), and intermediate when it is in both locations (Fig. 1, line 8). We have proposed that in order to generate a detectable recombinant phage, the initial cleavage must occur at *oriT*(1) (Fig. 5). Thus, when the 31A mutation is located in *oriT*(1), cleavage at this origin, and consequently the number of phage recombinants, is reduced. When present in *oriT*(2), the mutation suppresses initial cleavage at this site. Cleavage at this position does not result in recombinant phage, but instead removes the DNA from the pool of molecules that can form such phage. If this cleavage is inhibited by the mutation, a larger number of molecules remain available to form recombinant phage, and the phage recombination frequency is increased. If the mutation is in both *oriT*s, then the effect at each position is opposing, and an intermediate frequency of recombination is observed.

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