

Trans and cis requirements for intron mobility in a prokaryotic system

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Intron mobility requires cleavage of an intronless allele by an intron-encoded endonuclease, followed by transfer of the intron into the cleaved recipient. The mobile phage introns provide an opportunity to identify accessory functions involved in the intron inheritance process. To test for *trans* and *cis* requirements of mobility in *Escherichia coli*, we have exploited the *td* intron of phage T4 in both phage T4 and λ genetic backgrounds. Mobility depends on host or phage recombinase functions, RecA or UvsX, respectively. The process also requires a phage-encoded 5' $\rightarrow$ 3' exonuclease activity and associated annealing function that can be provided by phage λ . Finally, host-encoded 3' $\rightarrow$ 5' exonuclease activities are also implicated in intron inheritance. We demonstrated further that restriction enzymes could substitute for the intron-encoded endonuclease, indicating that the endonuclease does not have an essential role in recombination. Neither the precise position nor the nature of the double-strand break was critical to intron transfer. These features provide insight into the recombination pathway and are factors impacting on the spread of introns throughout natural populations

[Key Words: Phage T4; phage λ ; *td* intron endonuclease; RecA and UvsX recombinases; 5' $\rightarrow$ 3' exonuclease; 3' $\rightarrow$ 5' exonuclease]

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Mobile group I introns have been identified in a diverse range of prokaryotic and lower eukaryotic species (for review, see Dujon 1989; Lambowitz 1989; Clyman et al. 1992). Interestingly, group I introns showing a high degree of similarity appear in different locations in species that are not closely related, fueling arguments that introns crossed species lines (Michel and Dujon 1986; Lambowitz 1989). Furthermore, common features of the mobility process suggest that compatible *trans*-species mechanisms exist for acquiring and propagating mobile introns. It is therefore of interest to define precisely the recombination events that result in intron inheritance and to determine which accessory recombination and/or repair functions are required for mobility. Analogous functions would be predicted to exist in those organisms that host mobile introns, whereas the absence of such functions would be expected to present a barrier to mobile introns colonizing that biological system.

Mobility of group I introns is defined as the conversion of an intron-minus (In^-) allele to intron-plus (In^+). The process depends on a double-strand break (DSB) introduced into the intron recipient by an intron-encoded endonuclease (Dujon 1989; Lambowitz 1989; Perlman and Butow 1989; Belfort 1990). The ensuing recombination events, thought to proceed via the double-strand-break-repair (DSBR) pathway (Szostak et al. 1983), result in

unidirectional transfer of the intron to the cleaved "homing" site (Dujon et al. 1989) (Fig. 1). This pathway, as originally proposed (Szostak et al. 1983), has the DSB processed into a gap with the concomitant formation of exposed 3'-OH tails to both initiate strand invasion and serve as primers for repair synthesis. With an intron-containing donor duplex as template, this will result in transfer of the intron to the cleaved recipient.

The mobile phage introns provide an opportunity to assess the involvement of host and phage functions in intron inheritance and to probe the *cis* requirements for DSB-initiated recombination. Using the phage system, we have demonstrated that specific *td* intron sequences are not required for mobility if the intron-encoded endonuclease (I-TevI) is supplied in *trans* (Bell-Pedersen et al. 1990). The availability of well-characterized phage and host mutations in DNA recombination and repair genes allows investigation of the role of corresponding functions in the mobility event and facilitates further definition of *cis* requirements.

Three different experimental systems are used in this study. Phage λ is the model system for several analyses because it offers a number of advantages over T4: First, unlike T4, λ does not degrade host DNA, simplifying the recovery of plasmids from infected cells. Second, because of its smaller genome and unmodified DNA, λ is more amenable to genetic and in vitro manipulation. Third, λ has a simpler and better-defined recombination system

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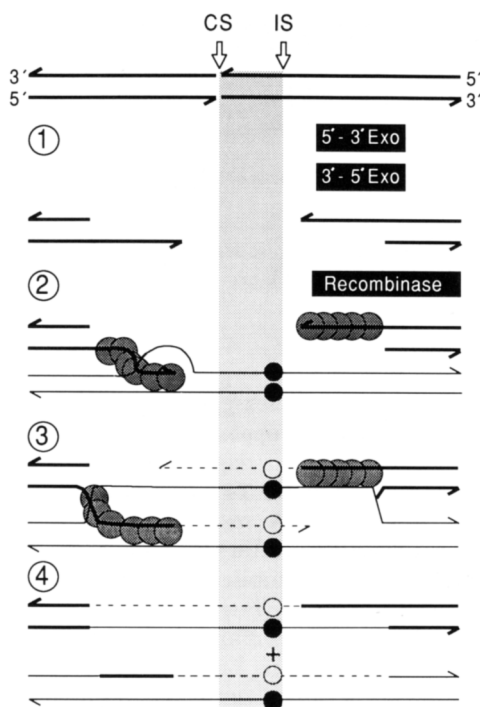


Figure 1. The DSBR pathway for intron mobility with implicated accessory functions. The model of Szostak et al. (1983) is adapted to the *td* intron, with the cleavage site (CS) upstream of the intron insertion site (IS). (Step 1) Resection of DNA ends and 3' single-stranded tail formation by requisite exonuclease activities. Vertical shading represents the requirement for gap formation. (Step 2) Binding of recombinase to 3' tails, strand invasion, and melting of the donor duplex. Recombinases RecA (*E. coli*), Bet (λ , associated with Exo), and UvsX (T4) have been implicated. Their relative roles remain speculative [see Discussion]. (Step 3) Recombinase-mediated annealing of homologous exons, D-loop formation, and repair synthesis. (Step 4) Ligation and resolution of Holliday junctions in the non-cross-over configuration. (Thick line) Recipient; (thin line) donor; (broken line) new DNA; (overlapping stippled circles) recombinase; (solid circle) original intron; (stippled circle) new intron.

than T4, and its recombination genes are clustered. Finally, unlike T4, the recombination functions of λ are dispensable for phage viability, facilitating the analysis of recombination-deficient mutants. With information gained about requirements for mobility from the λ /*Escherichia coli* system, it was possible to address specific questions utilizing T4 as the model organism. Furthermore, knowledge acquired from the λ experiments allowed us to establish an intron mobility system in uninfected cells. With this phage-free system, other requirements of the gene conversion process were tested.

Utilizing these model systems we show that transfer of the *td* intron is promoted by a combination of host and phage functions: recombinases (*E. coli* RecA or T4 UvsX), as well as 5' $\rightarrow$ 3' and 3' $\rightarrow$ 5' exonucleases. We also demonstrate that gene conversion occurs independently of I-TevI, provided that the recipient has been cleaved in sequences homologous to the intron donor.

The process also proceeds regardless of whether cleavage of the recipient generates staggered or blunt ends. That is, neither the nuclease that promotes the mobility event nor the precise nature of the DNA ends appears to impact directly on recombination. These properties are described in terms of recombination pathway and with regard to the ability of introns to invade prokaryotic genomes.

Results

Intron homing in the absence of T4 infection requires host RecA function

Phage λ containing the *td* intron homing site (λ *td* Δ In) can act as recipient for the *td* intron, efficiently acquiring the intron in cells carrying an intron-donor plasmid (Quirk et al. 1989). We have applied this assay, described in Figure 2, to screen hosts defective in functions involved in different DNA repair and recombination pathways. Of the mutants screened, only the RecA-deficient host had a striking effect on mobility (Fig. 2). Mutants used are described in Table 1. Intron inheritance was virtually unaffected by mutations in site-specific recombination functions, in the *fis*, *himA*, *hupA*, and *hupB* alleles (Fig. 2A), or in general recombination genes, *recJ*, *recN*, and *ruvB* (Fig. 2B). Whereas there was a modest 2- to 4-fold reduction in the *recBC* and *recF* hosts, respectively, there was a 500-fold decrease in mobility in the *recA* host. This sharp reduction in frequency of intron inheritance under *recA*⁻ conditions indicates a critical role for RecA in intron homing.

In addition to being the primary recombinase in *E. coli*, RecA is the positive activator of the SOS response. RecA cleaves LexA, the repressor of the SOS regulon, in response to DNA damage, leading to expression of DNA repair functions (Walker 1984). Because SOS is induced in cells expressing I-TevI (D. Bell-Pedersen, unpubl.), we tested whether the requirement for RecA might be the result of its role in SOS induction. When intron mobility was tested in a *lexA3* host, which produces a noncleavable LexA repressor, no difference was observed compared with the *lexA*⁺ control (Fig. 2C). These results indicate that the RecA requirement for homing is not the induction of an SOS function; rather, they implicate the role of the recombinase function of RecA. The dependence of intron mobility on a RecA-like recombinase is consistent with the requirement for exon homology in efficient homing (Quirk et al. 1989) and with the presumed need for strand invasion in the DSBR model for intron inheritance (Fig. 1).

T4 can complement the RecA requirement for intron homing—a role for UvsX

To assess whether phage T4 provides functions that complement the role of RecA, we monitored intron mobility in T4-infected cells, taking advantage of the transducing capacity of T4 (Wilson et al. 1979; Kreuzer and Alberts 1986). Intron acquisition into recipient plasmid

Table 1. Bacterial and phage strains

Organism	Strain	Relevant genotype	Source/reference
Bacteria	RJ1529	<i>fis</i> 767::Kan ^R	Johnson et al. (1988)
	RJ1003	<i>fis</i> ⁺	Johnson et al. (1988)
	RB63	<i>him</i> ⁺	Bousset and Fox (1988)
	RB182	<i>himA</i> Δ <i>Sma</i>	Bousset and Fox (1988)
	K37	<i>hup</i> ⁺	D. Friedman, pers. comm.
	K4389	<i>hupA</i>	D. Friedman, pers. comm.
	K4388	<i>hupB</i>	D. Friedman, pers. comm.
	RDK1540	<i>recN</i> 1502::Tn5 ^a	Fishel and Kolodner (1989)
	RDK1542	<i>ruvB</i> 9 ^a	Fishel and Kolodner (1989)
	JC5519	<i>recB</i> 21 <i>recC</i> 22 ^a	A.J. Clark, pers. comm.
	JC9239	<i>recF</i> 143 ^a	A.J. Clark, pers. comm.
	JC13031	<i>recJ</i> 153 ^a	A.J. Clark, pers. comm.
	JC10287	(Δ <i>recA-sr1</i>)304 ^a	R. Kolodner, pers. comm.
	RPC71	<i>recA</i> 200 ^a	R.P. Cunningham, pers. comm.
	RPC8	<i>srlC</i> ::Tn10 in KL16	R.P. Cunningham, pers. comm.
	RPC9	<i>recA</i> 56 <i>srlC</i> ::Tn10 in KL16	R.P. Cunningham, pers. comm.
	BW9109	Δ(<i>xthA-pncA</i>)90 ^a	R.P. Cunningham, pers. comm.
	SK1314	<i>xthA</i> 1 ^a	Bassett and Kushner (1984)
	SK1733	<i>recB</i> 21 <i>recC</i> 22 <i>xthA</i> 1 ^a	Bassett and Kushner (1984)
	MB2230	<i>lexA</i> 3 <i>zja</i> ::Tn10 ^a	From NO145 by P1 (N. Ossanna and D. Mount)
	AB1157	<i>rec</i> ⁺	Fishel and Kolodner (1989)
Phage	λgt11 <i>td</i> ΔIn	<i>td</i> ΔIn	Quirk et al. (1989)
	λimm21 <i>Sam</i> 7	<i>red</i> ⁺	D. Wulff, pers. comm.
	λimm21 <i>bio</i> 10 <i>Sam</i> 7	<i>red</i> ⁻	D. Wulff, Oppenheim and Court (1983)
	λcI857 <i>Sam</i> 7	<i>red</i> ⁺	lab collection
	λcI857 <i>b</i> 2141 <i>Sam</i> 7	<i>exo</i> ⁻ (<i>red</i> α ⁻¹)	Hendersen and Weil (1975)
	λcI857 <i>red</i> 113 <i>Sam</i> 7	<i>bet</i> ⁻ (<i>red</i> β ⁻)	Shulman et al. (1970)
	T4K10	<i>UvsX</i> ⁺	K. Kreuzer, Selick et al. (1988)
	T4K10 <i>UvsX</i>	<i>UvsX-am</i> 11	Kreuzer et al. (1988a)

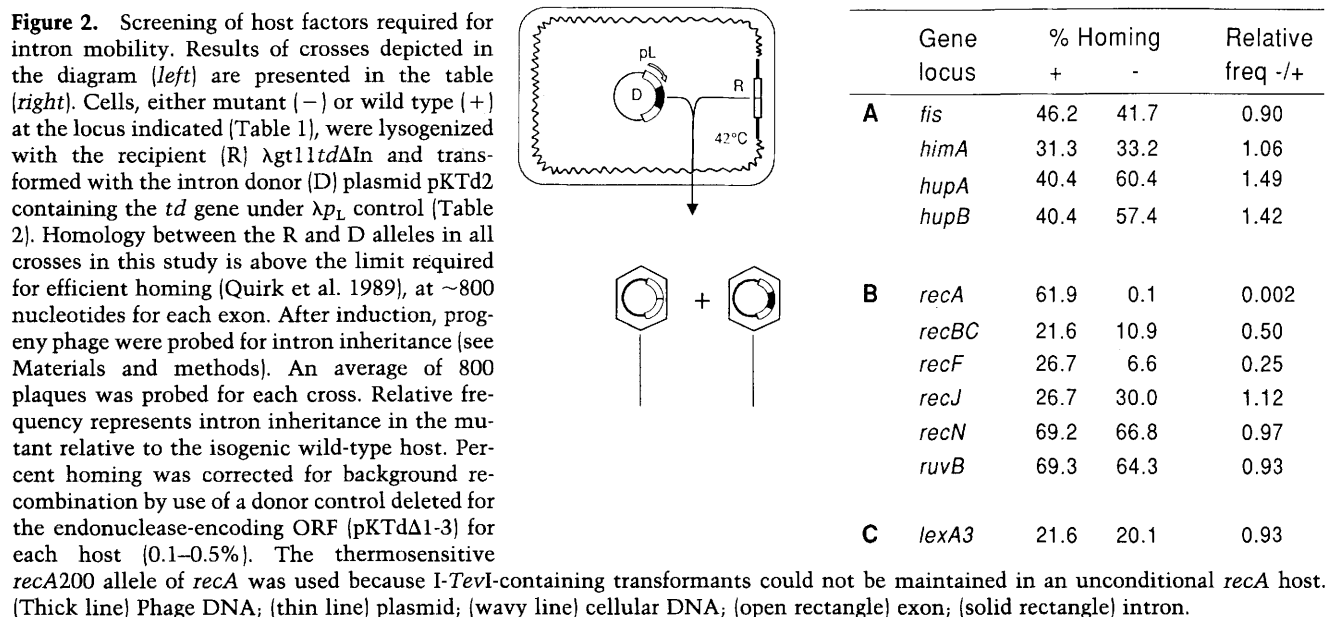
^aIn AB1157 background, *thr*-1 *leu*B6 Δ(*gpt-proA*)2 *his*G4 *arg*E3 *thi*-1 *ara*-14 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L31 *tsx*-33 *sup*E44 (*rac*).

DNA packaged by T4 was probed in transductants that inherited the plasmid from defective progeny phage (Fig. 3A,B). Plasmids are described in Table 2. Efficient intron homing was observed in both *recA*⁺ and *recA*⁻ cells (Fig. 3B, crosses 1 and 2, respectively). However, when cleavage was prevented by using a recipient with a 10-bp insert at the intron insertion site (*td*ΔIn10r), homing was abolished (Fig. 3B, cross 3), indicating that recombination is absolutely dependent on a DSB. Over four trials, the average intron inheritance frequency among the transductants was 50.7% ± 1.7%, 42.5% ± 1.3%, and 0.2% ± 0.6% for crosses 1, 2, and 3, respectively (Fig. 3B). Restriction analysis of plasmid DNA from In⁺ transductants showed the pattern of DNA fragments diagnostic of insertion of the *td* intron into its homing site (Fig. 3C). These results demonstrate that T4-mediated intron mobility can occur in the absence of host RecA function and that T4 functions satisfy the requirement for recombinase activity predicted by the DSB model.

The T4-encoded *UvsX* gene product is a recombinase that is functionally similar to RecA (Yonesaki et al. 1985; Formosa and Alberts 1986) and might thus have a role in T4-mediated intron homing. To test for a *UvsX* requirement in the mobility reaction, we performed Southern analysis on DNA isolated from T4*UvsX*-infected cells and probed for intron acquisition by recipient

plasmids (Fig. 4). A dependence on *UvsX* was reflected in the appearance of intron-containing recombinant plasmids, after *EcoRV* digestion, when the infecting phage was *UvsX*⁺ (Fig. 4, lanes 1,3,8) but not when the phage was *UvsX*⁻ (Fig. 4, lanes 2,4). This was true irrespective of whether cells were *recA*⁺ (Fig. 4, lanes 1,2,8) or *recA*⁻ (Fig. 4, lanes 3,4). This latter result is consistent with the finding that RecA is inactive during T4 infection, rendering general recombination in infected cells dependent on the *UvsX* function (Yonesaki and Minagawa 1988). The intron-containing bands were not evident when the recipient contained a disrupted homing site (Fig. 4, lane 5), indicating that this *UvsX*-dependent recombination event requires a DSB. Furthermore, the only band that disappeared upon digestion with *Hha*I, which cleaves only unmodified (i.e., unreplicated) DNA, is the recipient (pSU*td*ΔIn-r) band (Fig. 4, lane 8; more evident on darker exposure). This result suggests that homing took place into replicating DNA or that homing occurred into DNA that subsequently replicated.

Because plasmids that have homology to T4 replicate in a *UvsX*-dependent manner (Kreuzer et al. 1988b), it was important to distinguish between a *UvsX* requirement for homing versus replication. We therefore used recipient plasmids containing a T4 tertiary replication origin, which renders T4-driven replication of plasmids



independent of UvsX (Kreuzer et al. 1988a). Again, intron inheritance was dependent on UvsX function (Fig. 5). After digestion with *SspI* (which was used in this experiment because it cleaves T4-modified DNA more completely than does *EcoRV*), the intron-containing band appeared only when infecting phage were *UvsX*⁺ (Fig. 5, lanes 1,3), regardless of whether cells were *recA*⁺ (Fig. 5, lanes 1,2) or *recA*[–] (Fig. 5, lanes 3,4). Thus, in a system in which plasmid replication is independent of UvsX, intron transfer still requires UvsX function, arguing that the UvsX requirement for mobility reflects a direct role for this protein in recombination.

λ -Encoded factors required for intron mobility

Uninfected *E. coli* appears unable to support intron mobility even in *recA*⁺ cells. Whereas the *td* intron is capable of homing from a plasmid donor to T4td Δ In or λ td Δ In, intron transfer is undetectable from a plasmid donor to a plasmid recipient (Quirk et al. 1989; Fig. 6, cross 1). To test the involvement of phage functions in mobility, we again resorted to the λ system, for reasons indicated in the introductory section. We thus probed plasmid-to-plasmid intron homing in the presence of a wild-type λ helper phage. Recombination-proficient λ promoted efficient (18.7–19.7%) intron homing (Fig. 6, crosses 2 and 5). Consistent with a stimulation of DSB-dependent recombination by the λ helper, recombination dropped to background levels (<0.5%) when the donor plasmid was deleted in the intron open reading frame (ORF), which encodes the homing site-specific endonuclease required for mobility (Quirk et al. 1989; Belfort 1990; Clyman et al. 1992) (Fig. 6, cross 3). Likewise, when ORF⁺ transformants were infected with λ bio10, in which the phage recombination region is deleted, intron mobility was eliminated (Fig. 6, cross 4), indicating that

λ recombination functions are necessary for intron homing in this system.

Because the λ Red recombination pathway is stimulated by double-strand cuts within regions of homology, resulting in gene conversion events (Thaler et al. 1987a), we examined mutations in the *red* genes (deleted in λ bio10) to determine whether these are the phage functions that act to promote mobility. We probed a role for Exo, the *red α* -encoded 5' $\rightarrow$ 3' exonuclease that processively degrades double-stranded DNA, and Bet, the *red β* -encoded Exo-associated annealing activity, in intron inheritance. Infection of plasmid-containing cells with phage defective in either *red α* or *red β* resulted in a sharp decrease in intron mobility (Fig. 6, crosses 6 and 7), supporting a role for Exo and Bet in mobility. This requirement for Red functions mirrors that for homology-dependent double-strand gap repair in *E. coli* (Takahashi and Kobayashi 1990), supporting a straightforward DSB mechanism for mobility in favor of an intron-specific recombination pathway. Corroborative data are provided by Eddy and Gold (1992) with an artificial mobile intron constructed with the *EcoRI* endonuclease and methylase genes. Thus, the *EcoRI* intron could be mobilized at levels comparable to authentic mobile introns upon induction of λ Red functions.

Probing the roles of 5' $\rightarrow$ 3' exonuclease and annealing functions

Because mobility did not drop to background levels with the *red* mutants (Fig. 6, cf. cross 4 with 6 and 7), it was unclear whether Exo and Bet are sufficient to promote intron transfer. We therefore developed a three-plasmid mobility system using the cloned λ Red genes (Fig. 7A). In these experiments the Cam^R recipient plasmid is cleaved in vitro with I-TevI and then transformed into cells car-

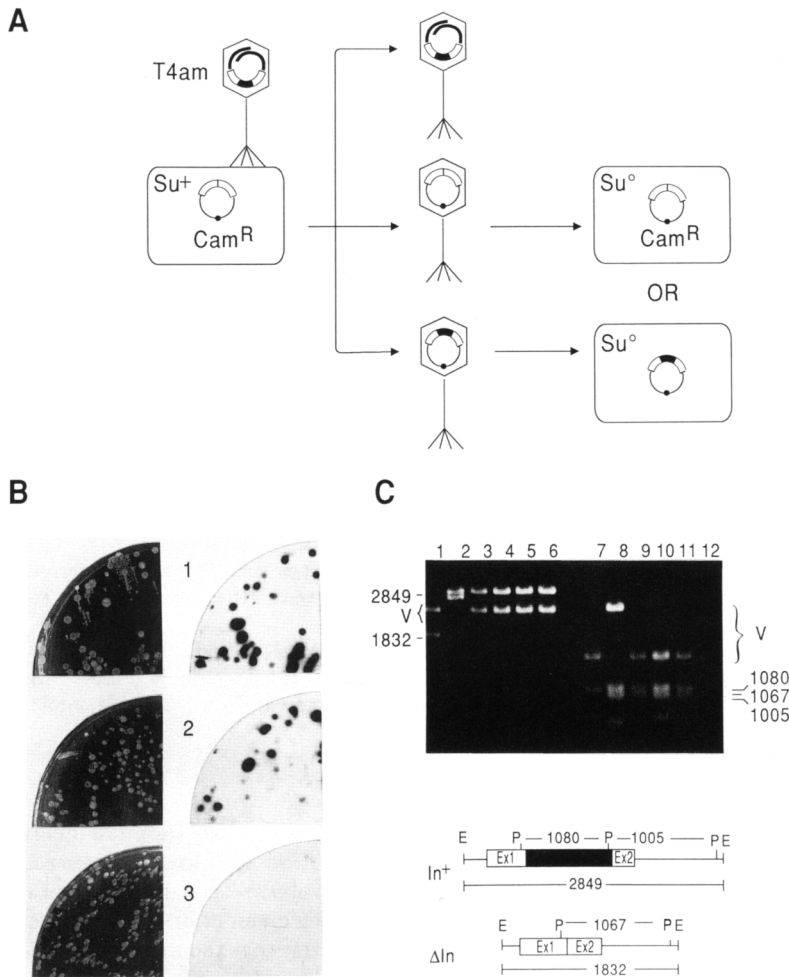


Figure 3. T4-mediated intron mobility in a *recA* host. (A) Schematic representation of the T4 transduction assay for intron mobility. *Cam*^R transductants arising from delivery of plasmid DNA by T4-transducing particles were selected on *Su*⁰ cells, which cannot support growth of defective intron donor phage T4K10. (Thick line) Phage DNA; (thin line) plasmid DNA; (open rectangle) exon; (solid rectangle) intron. (B) Transduction assay. Transductants (left) from crosses in AB1157(*recA*⁺)/pSUTdIn-r (1), JC10287(*recA*[−])/pSUTdIn-r (2), or AB1157(*recA*⁺)/pSUTdIn10r (3) were lifted onto filters and probed with an intron-specific oligonucleotide (right). (C) Restriction analysis of transductants. The agarose gel shows *Eco*RI (lanes 1–6) and *Pvu*II (lanes 7–12) digests of pSUTdIn-r DNA (lanes 1,7), pUCtdIn⁺ (lanes 2,8), and plasmid DNA isolated from transductants hybridizing to the intron probe (lanes 3–6,9–12). (V) Vector bands. (Below) An *Eco*RI (E) and *Pvu*II (P) restriction map of *tdIn*⁺ and *tdIn* inserts.

rying the *Kan*^R intron donor and Red functions on independent plasmids. In the presence of both *red* genes, but not *red* α or *red* β alone, an ~40-fold stimulation in the number of *Cam*^R transformants was observed over *red*[−] controls (Fig. 7B). Faithful intron inheritance was verified in different ways. First, the recipient was linearized with *Nco*I, which cleaves outside the region of *td* homology. In this case, no stimulation of intron transfer was observed, indicating that homing is dependent on a DSB in the region of homology between donor and recipient (Fig. 7B). Second, isolation of DNA from the *Cam*^R transformants and retransformation always resulted in *Kan*^R and *Cam*^R coinheritance, indicating that repair of the DSB in the recipient plasmid is associated with acquisition of the *Kan*^R intron (data not shown). Third, to provide physical verification that intron homing had occurred, *Cam*^R transformants were analyzed by polymerase chain reaction (PCR). By use of exon primers bordering the intron insertion site, all transformants tested produced the expected intron-carrying fragment (Fig. 7D, lanes 2–6). A transformant from a control experiment using uncleaved recipient plasmid generated the expected intronless PCR product (Fig. 7D, lane 1). Taken together, these results argue strongly that Exo and

Bet are both necessary and sufficient to mediate DSB-dependent intron transfer in *E. coli*.

Role for a 3' → 5' exonuclease in gap formation

The dependence of intron homing on a 5' → 3' exonuclease suggests the means for generating 3' tails as intermediates in recombination (Fig. 1). However, given the eccentric nature of the DSB, the 3' end of the top strand must also be degraded. Resection must extend beyond the intron-insertion site for repair synthesis to result in intron inheritance by DSBR. The gapping functions are likely to be cellular, because intron transfer occurs in the absence of phage infection when Red functions are expressed. Although RecBC and RecJ are candidate nucleases (Smith 1989), homing was only reduced by 50% in a *recBC* genetic background and not at all in a *recJ* background (Fig. 2).

To further examine the involvement of 3' → 5' exonuclease activity, we tested the effects of exonuclease-deficient mutants in the gene encoding ExoIII (*xthA*) and we retested ExoV (*recBC*). In a modification of the experiment presented in Figure 2, with the recipient phage introduced by infection rather than by induction, we ob-

Table 2. *Plasmids*

Name	Relevant features/use	Replicon ^a	Drug ^b	Source/reference
pKTD2	ORF ⁺ , <i>td</i> driven by λ pL	ColE1	Amp	Quirk et al. (1989)
pKTD Δ 1-3	ORF ⁻ , <i>td</i> driven by λ pL	ColE1	Amp	Quirk et al. (1989)
pUC <i>tdIn</i> ⁺ r ^c	<i>tdIn</i> ⁺ , ^d in pUC19	ColE1	Amp	this work
pSU <i>tdIn</i> -r ^c	<i>tdIn</i> ^d in pSU8 Δ BamHI ^e	pA15	Cam	this work
pSU <i>tdIn</i> 10r ^c	<i>tdIn</i> with 10-nucleotide polylinker, in pSU8 Δ BamHI	pA15	Cam	this work
pSUT4or <i>tdIn</i> -r ^{c,f}	derivative of pSU <i>tdIn</i> -r with T4 replication origin	pA15	Kan	this work
pCL <i>td</i> ::Kan ^R	intron donor, Kan ^R gene in place of <i>td</i> intron	pSC101	Kan Spc	Bell-Pedersen et al. (1990); this work
pBSt <i>td</i> ::Kan ^R	intron donor, Kan ^R gene in place of <i>td</i> intron	ColE1	Kan Amp	Bell-Pedersen et al. (1990); this work
pCL1920	vector for intron or <i>red</i> donor	pSC101	Spc	Lerner and Inouye (1990)
pMC7	vector for <i>red</i> functions	ColE1	Tet	Fenton and Poteete (1984)
pBSKS	vector for intron donor	ColE1	Amp	Stratagene
pJKKm ^f	ColE1 "exclusion" plasmid	ColE1	Kan	Kirschman and Cramer (1988)

^aPlasmids having any of the three different replication origins are compatible.

^bDrug resistances are as follows: (Amp) ampicillin; (Cam) chloramphenicol; (Tet) tetracycline; (Kan) kanamycin; (Spc) spectinomycin.

^cThe r designation indicates that the *td* gene is in antitranscriptional orientation relative to *plac* to prevent lethal expression of I-TevI.

^dEcoRI fragment containing *td* gene (Chu et al. 1984).

^eDeletion of the *Bam*HI site in recipient plasmids facilitates selection against intron-donor plasmids that contain this site.

^fThis plasmid is deleted for the 375-bp *Eco*RI–*Hind*III interval at the 5' end of the *td* fragment and has the T4 gene 34 tertiary origin on a 404-bp *Eco*RI–*Eco*RV fragment from pKK061 in the *Hpa*I–*Eco*RI interval at the 3' end of the *td* fragment (Chu et al. 1984; Kreuzer and Alberts 1986).

served a consistent, allele-dependent reduction in *td* intron mobility in the *xthA* mutants. There was an overall two- to eightfold decrease in mobility (Table 3, cf. crosses 3 and 4 with cross 1), with the deletion mutant causing a consistent seven- to eightfold drop in mobility (Table 3, cross 4). Whereas the *recBC* mutant again exhibited a twofold reduction in homing in this assay (Table 3, cross 5; cf. Fig. 2), mobility was completely abolished in an *xthA-recBC* double mutant (Table 3, cross 6). Although this result must be interpreted with caution given the potential instability of plasmids in an *xthA-recBC* background (Bassett and Kushner 1984), these data suggest that mobility is eliminated in the absence of both nucleases. Furthermore, although the results imply that ExoIII and ExoV have redundant functions in the homing process, ExoIII seems to be the more critical activity.

Homing appears independent of I-TevI and of the nature of the DSB

The ability of the *td* intron to be transferred into a recipient cleaved in vitro with I-TevI suggests that the nuclease is not involved in recombination per se (Fig. 7). However, these experiments do not rule out the possibility that I-TevI forms a stable complex with the cleaved recipient and is cotransported into the host cells with the DNA. To address this issue we wished to cleave the target DNA with unrelated restriction enzymes. We also wished to ascertain the effect of differences in the nature of the cleaved ends on homing. We therefore utilized a homing-site variant in which three restriction

enzyme sites (*Bam*HI, *Sma*I, and *Kpn*I) were engineered precisely at the intron-insertion site. In this construct, *tdIn*10, the 10-nucleotide polylinker completely inhibits cleavage by I-TevI (Bell-Pedersen et al. 1991). The results in Figure 7C indicate first that recombination is as efficient when the DSB is made with restriction enzymes as with I-TevI, further arguing against a required role for I-TevI in recombination. Second, intron inheritance was efficient whether the ends were blunt (*Sma*I) or recessed in the 5' (*Kpn*I) or 3' (*Bam*HI) directions, indicating that mobility occurs independently of the nature of the DSB. These latter results are consistent with the requirement for both 5' → 3' and 3' → 5' exonucleases, activities that are necessary to process the DNA ends into effective recombination substrates.

Discussion

With the *td* intron of phage T4 as the model system, we have implicated several host- and phage-encoded recombination functions in the intron homing process. These include both recombinases and nucleases, whose activities are consistent with the generation of predicted intermediates in the DSB pathway for intron inheritance (Fig. 1).

The role of RecA recombinase can be readily understood in terms of the homology requirement of mobility (Quirk et al. 1989). Although both RecA and Bet promote annealing of complementary strands, only RecA is capable of directing strand invasion (Kmiec and Holloman 1981; Muniyappa and Radding 1986). We speculate that the dual requirement for both annealing functions in

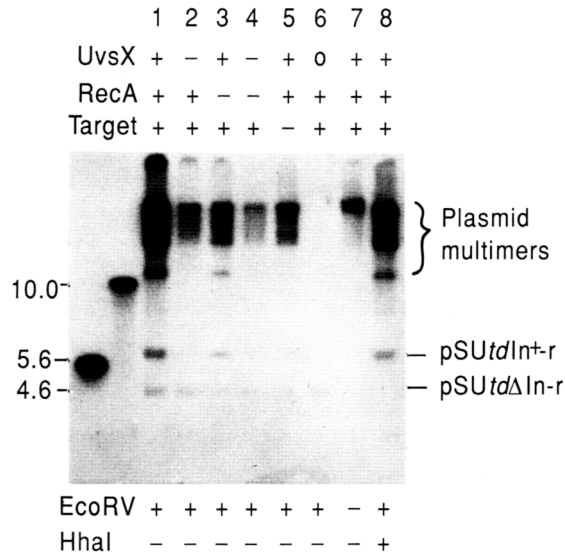


Figure 4. T4 UvsX-dependent intron transfer. Intron transfer was monitored from T4 into recipient plasmid pSUTdIn-r with an intact homing site (lanes 1–4, 6–8; target +) or into pSUTdΔIn-r with a disrupted homing site (lane 5; target –). The intron donor was T4K10 (lanes 1, 3, 5, 7, 8; UvsX +) or T4K10UvsX-*am*11 (lanes 2, 4; UvsX –). DNA in lane 6 was from uninfected cells (UvsX 0). Cells were either RPC8 (lanes 1, 2, 5–8; RecA +) or RPC9 (lanes 3, 4; RecA –). Total DNA [undigested (lane 7); digested with *EcoRV* (lanes 1–6); digested with *EcoRV* and *HhaI* (lane 8)] was separated on a 0.7% agarose gel, blotted onto a nitrocellulose filter (Schleicher & Schuell), and probed with a 0.9-kb intron-specific probe. This probe gave a weak signal with the intron recipient (pSUTdΔIn-r), which was present at very high concentration on the stained gel. *EcoRV* cleaves both modified DNA (T4 DNA, or plasmid replicated in the presence of T4) and unmodified DNA (unreplicated plasmid), whereas *HhaI* cleaves only unmodified DNA. Plasmid multimers are replication products that appear on the blot as the result of partial *EcoRV* activity on modified DNA. The two left-most lanes contain size markers. The bands corresponding to the intron recipient and the recombinant are labeled at right.

these experiments resides in the molecular association of Bet with Exo (Muniyappa and Radding 1986), resulting in direct delivery of Bet to the single-stranded tails. Bet may then serve to protect the single strands from nucleolytic cleavage or stimulate joint molecule formation by RecA (Muniyappa and Radding 1986).

Takahashi and Kobayashi (1990) reported a RecA requirement for Red-dependent gap repair in a plasmid carrying inverted repeats. Although these and our results demonstrate a requirement for RecA recombinase, previous work has shown that Red-mediated recombination between phage molecules (Thaler et al. 1987b) or between λ and a plasmid carrying homologous DNA (Berg 1971; Thaler et al. 1987a) is RecA independent except under conditions of limited DNA replication. We can reconcile this difference by conjecturing that λ recombination is not strictly analogous to Red-mediated intron homing or that intron mobility in this system mimics replication-deficient conditions during λ recombination.

The alternative explanation, that the observed RecA requirement arises from the protection of DSBs (Register and Griffith 1986) generated by I-TevI cleavage rather than from recombinase activity, seems unattractive in view of the requirement for UvsX recombinase function in T4 infection.

The UvsX protein of T4 has both annealing and synaptic activities (Yonesaki et al. 1985; Formosa and Alberts 1986). Predictably, therefore, T4UvsX⁺-infected cells are able to mobilize the *td* intron even in a *recA*[–] background (Figs. 3–5). Interestingly, intron homing is not observed during infection by T4UvsX[–] phage even in a *recA*⁺ host (Figs. 4 and 5). This result is consistent with previous findings that RecA is inactive in T4-infected cells (Yonesaki and Minagawa 1988) and further supports the role of UvsX in intron homing. The ability of UvsX to assume the role of RecA in intron mobility mirrors its ability to drive T4 general recombination in the absence of RecA (Mortelmans and Friedberg 1972).

The dependence of intron homing on a 5' → 3' nuclease activity (in this case, λ Exo) suggests that 3' tails are requisite intermediates in recombination. The existence of long 3' tails generated during DSBR has been demonstrated recently in yeast (Sun et al. 1991). These tails are essential for break-dependent meiotic recombination. Whereas gap formation in yeast is neither required by the model nor suggested by experiments (Sun et al. 1991), gapping is indicated for *td* intron mobility by the DSBR pathway. This requirement arises from the eccentric DSB generated by I-TevI and the need to resect the top strand beyond the intron insertion site to achieve conversion of the intron by DSBR. A role for a 3' → 5' exonuclease activity at the DSB is supported by reduced homing in *xthA* mutants deficient in ExoIII (Table 3). Although ExoIII is a multifunctional enzyme, with AP

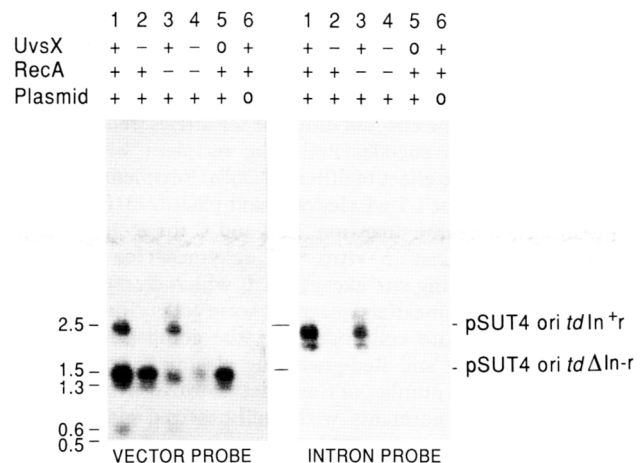
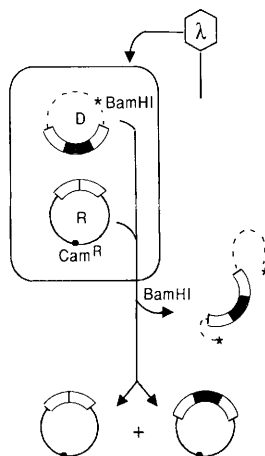


Figure 5. A direct role for UvsX in intron mobility. The experiment was performed as in Fig. 4, except that the recipient pSUT4ori Δ In-r contained a T4 replication origin (lanes 1–5). Lanes are labeled +/– as in Fig. 4; (0) controls with either no phage (lane 5) or no plasmid (lane 6). DNA was digested with *SspI* and probed with “vector” (pSUT4ori Δ In-r) or intron probes.

Figure 6. Plasmid-to-plasmid homing with λ helper. Cells with intron-donor (D, pUCtdIn⁺r, Amp^R) and recipient (R, pSUTdIn-r, Cam^R) plasmids were infected in log phase with λ helper. Plasmid DNA was extracted and digested with BamHI to select against the donor, and Cam^R-Amp^S transformants were probed for the intron. An ORF⁻ control donor (td Δ 1-3) was used in all crosses; the background recombination was <0.5% as for cross 3. Each experiment was repeated twice, with <10% variation, except for the λ red113 cross, where homing ranged between 1% and 4.2%. Similar results were obtained with λ prophage induction. Cross 2 is isogenic with crosses 3 and 4, and cross 5 with crosses 6 and 7 (Table 1).



Cross	Helper	<i>red</i>		ORF	Total colonies	Intron plus	% Homing
		α	β				
1	None	-	-	+	208	1	<0.5
2	λ^+	+	+	+	326	61	18.7
3	λ^+	+	+	-	260	1	<0.5
4	λ bio10	-	-	+	311	0	<0.5
5	λ^+	+	+	+	148	29	19.7
6	λ b2141	-	+	+	487	8	1.6
7	λ red113	+	-	+	423	18	4.2

endonuclease, 3'-phosphatase, and RNase H activities (Linn 1985), it seems logical that the observed reduction in homing efficiency is attributable to its 3' $\rightarrow$ 5' exonuclease activity. Furthermore, RecBC nuclease appears to provide a redundant exonuclease activity. Although *recBC-xthA* double mutants cannot support intron homing (Table 3), an auxiliary role for other *E. coli* 3' $\rightarrow$ 5' exonucleases (Kornberg and Baker 1991) remains a possibility.

Further support for gap formation preceding 3'-tail invasion is provided by 100% coconversion of markers between the *td* intron-insertion site and the cleavage site (Bell-Pedersen et al. 1989, 1990). Moreover, the observation that intron transfer is independent of the nature of the DSB (3' or 5' extensions or blunt ends; Fig. 7), par-

ticularly given that the cut occurs within the short stretch of heterology created by the linker insertion in *td* Δ 10, suggests that 3' $\rightarrow$ 5' exonucleolytic activity plays a general role in this recombination pathway in *E. coli*, rather than being confined to meeting the specific needs of *td* intron homing.

The requirement for gap formation in *td* intron transfer suggests that different molecular events may account for the observed coconversion of flanking markers during DSB in the *E. coli*/phage system and in yeast. In the absence of gap formation in the eukaryotic system, coconversion would result from mismatch repair of heteroduplex DNA. In contrast, when gap formation occurs, as in the prokaryotic system, coconversion would have to result, at least in part, directly from gap repair.

Figure 7. Three-plasmid mobility system. (A) Schematic of the system. The recipient plasmid (R) was cleaved in vitro and delivered to cells containing the Kan^R donor plasmid (D) and the third plasmid supplying Red functions. Arrows represent exon primers used for PCR analysis. (B) The effect of different *red* alleles (*red* α encodes Exo; *red* β encodes Bet). The recipient was pSUTdIn-r. (C) The effect of different DSBs. Recipients were pSUTdIn-r for I-TevI cleavage and pSUTdIn10r for restriction enzyme digestion. In B and C the recipients were linearized in vitro. All enzymes cleave within the *td* homing site except *Nco*I, which cleaves vector sequences. Linearized plasmids were gel purified and transformed into cells harboring the compatible Kan^R intron donor and Red-producing plasmids. Percent homing is the number of Cam^R transformants relative to total transformants with nonlinearized plasmids. Plasmids were extracted from transformants and retransformed to confirm coinheritance of Kan^R and Cam^R markers. (D) Verification of intron inheritance of Cam^R transformants by PCR. Exon-specific primers (see A) are predicted to generate products of 195 and 1505 bp for In⁻ and In⁺ alleles, respectively. PCR templates were the recipient plasmid (lane 1) and five Cam^R transformants (lanes 2–6). Lanes 7 and 8 contain DNA size markers.

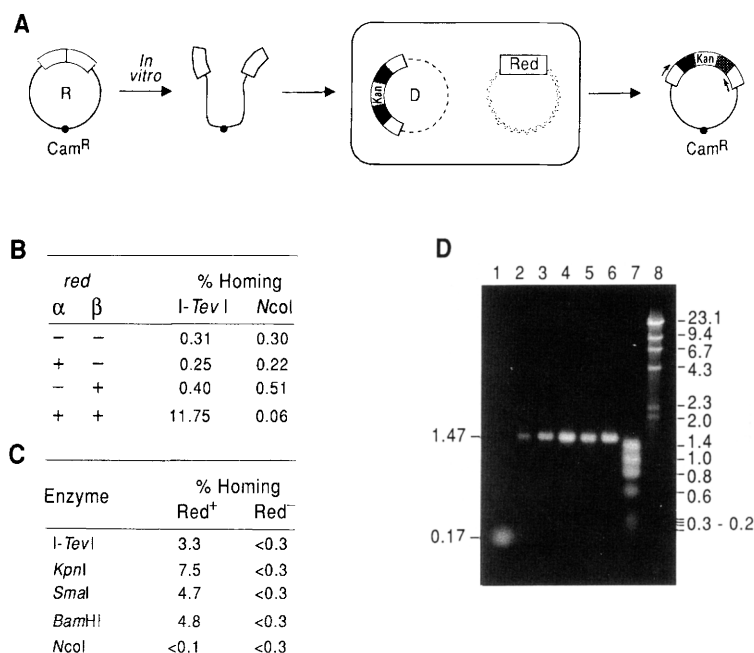


Table 3. A role for *ExoIII* in intron homing

Cross	Genotype		Strain	ORF	Plaques scored	In ⁺ plaques	In ⁺ (%) ^a
	<i>recBC</i>	<i>xthA</i>					
1	+	+	AB1157	+	650	155	23.8
2	+	+	AB1157	—	469	8	1.7
3	+	— ^b	SK1314	+	913	93	10.2
4	+	— ^c	BW9109	+	226	7	3.1
5	—	+	JC5519	+	517	50	9.7
6	—	— ^b	SK1733	+	999	1	0.1

^aPercent In⁺ was determined as in Fig. 2, except that the recipient phage was introduced by infection at 37°C in this experiment. Each cross was repeated at least twice.

^bThe *xthA1* allele is present in these strains.

^cThe $\Delta xthA$ allele is present in this strain.

All of seven characterized endonucleases encoded by mobile introns in prokaryotes and eukaryotes generate DSBs with 3' extensions 2–4 nucleotides in length (for review, see Clyman et al. 1992). In contrast, endonuclease I-*Tev*III, encoded by the *nrdB* intron of T-even phage RB3, cleaves the In[−] target to yield 5' extensions (Eddy and Gold 1991). Interestingly, attempts to demonstrate mobility of this intron have failed, suggesting a possible link between the nature of the cleaved DNA ends and the ability to undergo recombination. However, the results presented here demonstrate efficient intron inheritance whether the DSB has 3' or 5' extensions or blunt ends, arguing against a correlation between a 3' extension and the ability to undergo recombination. Furthermore, intron conversion occurred readily regardless of whether the DSB was precisely at the intron insertion site or displaced from the site, as long as cleavage occurred within stretches of homology between donor and recipient. These experiments suggest that neither the nature of the DSB nor its precise location within homologous sequences is a major determinant in the mobility process.

The three-plasmid mobility experiment, in which the recipient was cleaved in vitro with I-*Tev*I or with restriction enzymes, indicates that I-*Tev*I is not required for recombination beyond generating the DSB (Fig. 7). Similarly in the case of the artificial intron constructed by Eddy and Gold (1992), *Eco*RI was able to serve as the mobilizing nuclease. This situation is in contrast to that with classical transposases, which cleave their DNA substrates and are subsequently involved in the formation and resolution of recombination intermediates (Berg and Howe 1989). These variations parallel differences in the *cis* requirements of these different classes of mobile elements: Transposition requires the transposase to act at specific sequences at the transposon termini to promote DNA cleavage and strand exchange (Berg and Howe 1989), whereas intron homing occurs independently of any intron sequences (Bell-Pedersen et al. 1990), requiring only a DSB and exon homology.

These studies open the way for determining how T4 satisfies all of the requirements for mobilizing its introns. It remains unclear which T4 functions substitute

for the λ Red system and provide the necessary 5' → 3' nuclease and associated annealing activities, although the products of genes 46 and 47, which form a nuclease complex, are likely candidates. Regardless, T4 will have had to participate in the acquisition of its complement of mobile introns beyond providing UvsX because, clearly, wild-type *E. coli* is incapable of supplying all requisite functions. In this regard, it is noteworthy that no mobile introns have yet been reported in uninfected eubacterial cells. This raises the possibility that the spread of introns throughout bacterial populations is impeded by the inability of these cells to potentiate efficient double-strand gap repair except under specific genetic conditions (Smith 1989). Mobile introns would therefore be confined to phages that have the recombinational accessories to incorporate these elements into DSBs within their genomes. Likewise, transfer of mobile introns across species lines (Dujon 1989; Lambowitz 1989) would depend on the requirements for recombination being met by the new host organism.

Materials and methods

Strains and plasmids

Bacterial and phage strains are listed in Table 1, and plasmids are listed in Table 2.

Screening host mutants for intron mobility

The intron recipient λ gt11td Δ In (Quirk et al. 1989) has a thermolabile repressor and the *Sam*100 mutation, preventing cell lysis in nonsuppressing hosts. Lysogens containing donor plasmid were grown at 30°C in TB YET (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 50 μ g/ml of thymine) with ampicillin (200 μ g/ml) to 2×10^8 cells/ml. All cultures were thermoinduced for 15 min at 44°C and transferred to 37°C for 4–6 hr with shaking. Cultures were lysed with chloroform, and lysates were harvested by centrifugation and titered on a *supF* host (Ymel). Plaque lifts and hybridization with an intron-specific probe were as described previously (Quirk et al. 1989). An aliquot of each culture was removed immediately before induction and in experiments where mobility was not observed; for example, in a *recA200* host, plasmid-borne ORF expression was tested by

transforming N99(λ CI857) and monitoring intron mobility into T2L (Quirk et al. 1989).

For testing intron homing by phage infection, the intron donor was pUCtdIn⁺r. Transformants (5×10^8 cells) were plated in top agar on TB plates, and 2- μ l dilutions of λ tdInS⁺, a spontaneous S⁺ revertant of λ gt11tdIn, were spotted on the bacterial lawn and incubated overnight at 37°C. Clearings from the highest dilution that produced confluent lysis were scraped into TM (10 mM Tris at pH 7.4, 10 mM MgCl₂) saturated with chloroform. Intron inheritance by progeny phage was tested as described above.

T4 transduction

T4-mediated plasmid transduction was performed as described by Kreuzer and Alberts (1986), except that stationary-phase cultures of *E. coli* B were used as the Su⁰ transduction host (Fig. 3). Su⁺ transformants containing the intron recipient plasmid pSUTdIn-r (Cam^R, Table 2) were infected with T4K10, which has amber mutations in the capsid-processing proteins 38 and 51 and mutations in *denA* and *denB* to prevent plasmid degradation (Selick et al. 1988). Alternatively, plasmid pSUTdIn10r with a disrupted homing site was used. Progeny phage were then infected into a Su⁰ host so that only cells that inherited transduced plasmid DNA from defective phage would grow on chloramphenicol plates. Cam^R transductants were then lifted onto filters and probed with an intron-specific oligonucleotide (Quirk et al. 1989).

Southern analysis of intron transfer from T4 to a plasmid recipient

Cells containing a tdIn recipient plasmid were grown in TBYES to an OD₆₅₀ of 0.2 and infected with T4K10 derivatives at an m.o.i. of 4. After incubation at 37°C for 1 hr with aeration, total DNA was extracted according to Kreuzer et al. (1988a), except that DNA was concentrated on a spin column ultrafilter (Centricon-100) instead of being dialyzed. DNA yields were ~250 ng/ μ l in a total volume of 50–100 μ l. DNAs (0.5–1 μ g) were restricted and subjected to Southern hybridization. The probes were a random primer-labeled 0.9-kb *AccI*-*PvuII* intron fragment (for map, see Chu et al. 1984) or the intronless recombinant pSUT4oritdIn-r.

Plasmid-to-plasmid homing with phage λ helper

C600 (*thi*, *thr*, *leu*, *lacY*, *tonA*, *supE44*, F⁻) cells were transformed with intron recipient pSUTdIn-r and intron donor pUCtdIn⁺r. The unique *Bam*HI site in the recipient was removed by linearization with *Bam*HI and Klenow fill-in. Overnight cultures were diluted 1 : 50 into fresh TBYES media containing ampicillin (200 μ g/ml) and chloramphenicol (25 μ g/ml) and grown to 2×10^8 cells/ml. Aliquots of 5 ml were harvested by centrifugation, resuspended in 0.2–0.5 ml of TM containing 1×10^9 λ Sam7 derivatives (Table 1), and incubated for 15 min at 30°C to allow phage adsorption. The infected cells were added to 10 ml of fresh TBYES and incubated with shaking for 4–5 hr at 38°C. Aliquots were removed, and plasmid DNA was isolated, while the remaining culture was lysed with CHCl₃ and titered to monitor for successful infection (generally $>10^9$ phage/ml). DNAs were treated with *Bam*HI before transformation to inactivate intron donor DNA for transformation. The retransformation host RRI harbored a ColE1 replicon (pJKKmf⁻) to further block cotransformation of the intron donor plasmid. To confirm that Cam^R transformants did not coinhered the pUCtd donor, transformants were either plated in parallel onto

ampicillin plates or replica-plated to check for Amp^S colonies. Cam^R transformants were probed for intron inheritance as described previously (Quirk et al. 1989).

Red-dependent three-plasmid mobility system

The intron recipient plasmid was linearized in vitro by *I*-TevI in the case of pSUTdIn-r or by a restriction enzyme in the case of pSUTdIn10r, which contains a 10-nucleotide (5'-ACCCGG-GATC-3') restriction site polylinker (*Kpn*I, *Sma*I, *Bam*HI) at the intron-insertion site. Linearized plasmids were gel purified, and equal amounts were transformed (Inoue et al. 1990) into cells harboring the compatible Kan^R-marked intron donor and Red-producing plasmids. IPTG (100 μ g/ml) was added at an OD₆₀₀ of 0.4 to induce the Red functions before making cells competent. The intron donor was either on pCL1920 (pCLtd::Kan^R), with Red functions supplied from pMC7 (Fenton and Poteete 1984), or on pBSKS (pBSStd::Kan^R), with Red functions supplied from pCL1920 (Lerner and Inoue 1990).

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