Use of a transposon (Tndif) to obtain suppressing and nonsuppressing insertions of the *dif* resolvase site of *Escherichia coli*

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The *dif* locus is a RecA-independent recombination site, located in the terminus region of the chromosome of *Escherichia coli*. This site functions to reduce circular dimer chromosomes to monomers before cell division. Strains lacking this site exhibit the Dif phenotype, in which a fraction of the cells form extended filaments with abnormal nucleoids, and the SOS system is induced. We have used a transposon (Tndif), as well as linear transformation, to position *dif* in 19 locations around the chromosome. All of the suppressing insertions that we obtained were within 10 kb of the normal site, even in strains in which the normal symmetry between the origin of replication and *dif* had been altered by 200 kb. We also observed that the nonsuppressing insertions in the terminus region became suppressing if a deletion occurred that extended from the ectopic site up to or past the normal location of *dif*. We propose that *dif* is normally located at the center of converging polarities in the terminus region and that deletions that restore suppression do so by placing ectopic sites once again at the center of this polarity. Similar results and conclusions are described in this issue.

[Key Words: *E. coli*; chromosome; terminus; resolvase; *dif*]

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The *dif* locus of *Escherichia coli* is a RecA-independent recombination site that is related in sequence and function to the *cer* resolvase site of pColE1 (Blakely et al. 1991; Clerget 1991; Kuempel et al. 1991). *dif* and *cer* contain a similar core sequence, which is the binding site for the XerC and XerD resolvase proteins (Blakely et al. 1993). Similar to *cer*, *dif* appears to function as a resolvase site, which reduces circular, dimeric bacterial chromosomes to monomers before cell division. These dimers presumably arise from recombination between growing daughter chromosomes (sister chromatid exchange) in a fraction of the cells (Forro and Wertheimer 1960; Van Tubergen and Setlow 1960).

The *dif* site is located in the middle of the terminus region, directly opposite the origin of replication (*oriC*; Fig. 1). Based on the physical map of the wild-type *E. coli* chromosome, the clockwise and counterclockwise distances from *oriC* to *dif* are 2323 and 2350 kb, respectively (Kohara 1987; Rudd 1992 and pers. comm.). Consequently, *dif* is one of the last loci to be replicated during the replication cycle. This is appropriate for its function, because newly replicated *dif* sites would provide a convenient location for recombination events that

resolve dimers. These dimers must be resolved correctly or subsequent nucleoid segregation and cell division will be affected. The Dif phenotype is consistent with this model. In cultures of *dif* mutants, a fraction of the cells form extended filaments (≤ 50 μ m long) with abnormal nucleoids, and the SOS DNA repair system is induced (Blakely et al. 1991; Kuempel et al. 1991). These effects are eliminated by suppressing insertions (Tecklenburg et al. 1995). It should also be noted that the largest DNA molecules detectable by viscoelastometry in stationary-phase *dif* cells were almost twice the size observed in *dif*⁺ cells (B. Aliche and E. Uhlenhopp, pers. comm.).

We recently demonstrated that proper *dif* function could be provided by a 33-bp sequence (Tecklenburg et al. 1995) that contained the binding sites for XerC and XerD (Blakely et al. 1993). Similar results have been reported by Leslie and Sherratt (1995). Flanking DNA was not required, and the 33-bp sequence was sufficient to suppress the Dif phenotype of deletions that removed either 12 or 173 kb of terminus region DNA (Tecklenburg et al. 1995). The location of the site was important for suppression, because insertions elsewhere in the chromosome (Leslie and Sherratt 1995), or in the terminus region but 118 kb from the normal site (Tecklenburg et al. 1995), failed to suppress the Dif phenotype. However, these insertions still exhibited a high frequency of

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Figure 1. The *E. coli* chromosome, based on the physical coordinates of EcoMap6 (K. Rudd, pers. comm.; also see EcoMap5, Rudd 1992). The physical map is indicated in megabases (Mb) and starts at 0 min of the genetic map. The arrows outside the circle indicated the location and orientation of five nonsuppressing *Tndif* insertions. The origin of replication (*oriC*) is at 3952 kb (84.6 min) in a wild-type chromosome, and *dif* is directly opposite at 1599 kb (34.2 min). In strains with IN(*rrnD*–*rrnE*) 1, such as W3110, the origin is shifted and the region opposite *oriC* is at 1400 kb (30.0 min). The *Tndif* insertions are Ω(3205kb::Tndif→)3478, Ω(3321kb::Tn←dif)3477, Ω(3335kb::Tndif→)3486, Ω(3856kb::Tndif→)3485, and Ω(4519kb::Tn←dif)3479.

RecA-independent recombination with a *dif*-containing plasmid. Suppression was also obtained by an insertion, at the *dif* location, of the *loxP* site of bacteriophage P1 (Leslie and Sherratt 1995) and of *psi*, which is a *cer*-like site in pSC101 (Cornet et al. 1994). The *cer* site did not suppress the Dif phenotype (Leslie and Sherratt 1995).

The properties and interactions of *dif* sites provide a novel means to study chromosome structure and events during the completion of the replication cycle, because suppression of the Dif phenotype has only been obtained by insertion of resolvase sites at the normal *dif* location. Conversely, we also observed that an ectopic site located 118 kb from the normal *dif* site became fully functional if the DNA between it and the normal *dif* location was deleted (Tecklenburg et al. 1995). It was uncertain how the correct function of the *dif* site was influenced by its position with respect to *oriC*, as well as its requirements for flanking DNA sequences.

To isolate a variety of insertions, we have constructed a *dif* transposon. This has been used to obtain insertions at many locations, both in the terminus region and elsewhere around the chromosome. All of the suppressing insertions were clustered near the normal location of *dif*, although they were isolated in derivatives of strain W3110. These strains were chosen because they contain

inversion IN(*rrnD*–*rrnE*)1 (Hill and Harnish 1981; Fig. 1) and *dif* is no longer directly opposite *oriC*. The insertions have also been used to construct additional deletions and inversions in the terminus region. We report here the results of these studies and discuss a model for our results.

Cornet et al. (this issue) describe different procedures used to study the requirements for a functioning *dif* site, and their results and conclusions are similar to those reported here.

Results

The purpose of these experiments was to identify regions in which *dif* insertions occur that are able to suppress the Dif phenotype. Most of the ectopic sites that we report here were obtained by means of a *dif* transposon (*Tndif*). This is different from *dif* insertions characterized previously by us and others (Cornet et al. 1994; Leslie and Sherratt 1995; Tecklenburg et al. 1995; see also Cornet et al., this issue). In those studies, *dif* was inserted into targeted sites by construction of appropriate plasmids, and homologous recombination was used to achieve integration. These strains were then tested to determine whether suppression occurred. By using a *dif* transposon we adopted a different approach. We planned to construct a library of random insertions. Next, an indicator would be used to identify sites that provided suppression, which could then be mapped to identify their locations.

Suppressing insertions of the *dif* transposon

The *dif* transposon that we constructed (*Tndif*; Fig. 2) contained a 231-bp sequence from the *dif* locus (Kuempel et al. 1991). Although we observed previously that a 33-bp sequence suppressed the Dif phenotype as well as the 231-bp sequence (Tecklenburg et al. 1995), we recently noted that the longer sequence gave a higher frequency of recombination between chromosomal sites, at least in some situations (P. Kuempel, unpubl.). Because we intended to use *dif* insertions to cause chromosome rearrangements and deletions, we have concentrated on insertions of the 231-bp sequence. *Tndif* was derived from pNK2859 (Kleckner et al. 1991), which encodes a Tn10 transposase with altered target specificity and consequently inserts into a wider range of sites. Expression of the transposase gene is controlled by the *P_{tac}* promoter.

Most of our library constructions and tests for suppression have been done in strains derived from W3110. We chose these strains because they contain the IN(*rrnD*–*rrnE*)1 inversion (Hill and Harnish 1981). In strains with the wild-type chromosome structure, the location of *dif* on the physical map is at 1599 kb (K. Rudd, pers. comm.), which is directly opposite *oriC* (Fig. 1). In strains with IN(*rrnD*–*rrnE*)1, the position directly opposite *oriC* is shifted 200 kb, and it occurs at 1400 kb. Our rationale for using these strains was that it would simplify determin-

Figure 2. Plasmid pHN13 and *Tndif*. The 231-bp *dif* sequence and *kan* are located between the 70-bp inserted repeats (IR). The gene for the transposase with altered target specificity is controlled by *Ptac* and is located outside the *Tndif* transposon. Only the relevant restriction enzyme sites are shown: *Bam*HI (○); *Hind*III (△); *Pst*I (●); *Kpn*I (□). The transposon contains no sites for *Eco*RI, *Eco*RV, *Bgl*I, or *Pvu*II.

ing whether suppression by ectopic *dif* sites was influenced by position opposite the origin, or whether it depended on the context of the site.

After induction of transposition in PK3471, bacteriophage P1 was used to transduce the library of *Tndif* insertions into an indicator strain. Transduction ensured that single insertions were being studied and was usually sufficient to eliminate pHN13, although 50% of the *Km*^r transductants remained Ap^r. The indicator strain PK3032 was derived from W3110, and it contained a 1.3-kb *dif* deletion ($\Delta dif::cam2771$) and a *sfiA::lac* reporter gene (Lin and Little 1988). Because *sfiA* is induced in *dif* mutants by SOS induction (Kuempel et al. 1991; Tecklenburg et al. 1995), unsuppressed colonies were red on MacConkey–lactose agar. Suppressed colonies were readily identified by their white color.

At least 15% of the *Km*^r transductants from various libraries were white on MacConkey–lactose agar, and the cells in those colonies did not exhibit the filamentation typical of *dif* mutants. We examined a number of pink colonies in an attempt to recover clones with intermediate levels of suppression and SOS induction, but restreaking invariably gave red colonies with filamen-

tous cells or white colonies with normal cells. Suppressing insertions were initially mapped by transduction to determine their location. Forty suppressed strains obtained from a series of libraries were tested, and in all of them the *Tndif* insertion was tightly linked (>90% cotransduction) to a spectinomycin resistance gene present at *dif*.

Seven of the suppressing insertions were mapped (Fig. 3) by Southern hybridization in which *dif* DNA was used as the probe. The particular restriction fragments in which an insertion had occurred could be readily identified on the physical map (Rudd 1992, and pers. comm.), because of an increased size of 1.9 kb in an overlapping set of *Eco*RI, *Eco*RV, *Bgl*I, and *Pvu*II fragments. The location and orientation could be determined from the *Pst*I and *Kpn*I digestions, which cut in the *dif* transposon (Fig. 2). An example of the full designation used for these insertions is $\Omega(1589 \text{ kb}::\leftarrow Tndif)3476$. This indicates that the insertion (Ω) of the *Tndif* transposon occurred at 1589 kb, that the *dif* sequence has the same orientation as wild type ($\leftarrow$), and that the insertion was isolated in strain PK3476. Insertions are identified in this fashion in the figure legends and in Table 1. In the text and figures,

Figure 3. Physical map of the region surrounding *dif*. The arrows indicate the locations and orientation of seven suppressing insertions mapped by Southern hybridizations. These were isolated in PK3032, in which $\Delta dif::cam2771$ extends from 1597.7 to 1599 kb. The broken arrow indicates the orientation of wild-type *dif*. The indicated restriction enzyme sites are from EcoMap6 (K. Rudd, pers. comm.). These sites are, from top to bottom, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I, and *Pvu*II. The broken lines in $\Delta cam::2771$ indicate the *Eco*RI and *Pvu*II sites in the chloramphenicol resistance gene. The full designations of the *Tndif* insertions, from left to right, are $\Omega(1589 \text{ kb}::Tn\leftarrow dif)3476$, $\Omega(1594 \text{ kb}::Tndif\rightarrow)3497$, $\Omega(1594 \text{ kb}::Tndif\rightarrow)3516$, $\Omega(1596 \text{ kb}::Tn\leftarrow dif)3518$, $\Omega(1596 \text{ kb}::Tndif\rightarrow)3504$, $\Omega(1600 \text{ kb}::Tndif\rightarrow)3517$, and $\Omega(1600 \text{ kb}::Tn\leftarrow dif)3503$.

Table 1. Genotypes of relevant bacterial strains

Strain	Genotype
PK2649	<i>trpR trpA9605 his-29 ilv arg-427 thyA deoB</i> or <i>deoC tsx</i> IN(<i>rrnD-rrnE</i>)1 Δ (<i>argF-lac</i>)205 λ <i>sfiA::lacZ</i> Ind ⁻
PK2771	<i>recB21 recC22 sbcB15 sbcC thi-1 thr-1 leuB6 lacY1 mtl-1 xyl-5 ara-14 galK2 hisG4 proA2 rpsL31 tsx-33 supE44</i> Δ <i>dif::cam2771</i>
PK3032	as PK2649, but Δ <i>dif::cam2771</i>
PK3269	as PK2649, but <i>xerC::camY17 recD::Tn10</i> Δ <i>dif::spc3269</i>
PK3270	<i>recB21 recC22 sbcB15 sbcC thi-1 thr-1 leuB6 lacY1 mtl-1 xyl-5 ara-14 galK2 hisG4 proA2 rpsL31 tsx-33 supE44</i> <i>xerC::camY17</i> Δ <i>dif::spc3269</i>
PK3321	as PK3270, but Ω (1399kb:: $\leftarrow$ <i>dif-kan</i>)3321
PK3381	as PK3270, but Ω (1399 kb:: <i>dif</i> $\rightarrow$ <i>kan</i>)3381
PK3436	as PK2649, but <i>ilv⁺ xerC⁺</i> Δ (Ω (1399kb:: $\leftarrow$ <i>dif-kan</i>)3321- <i>dif⁺</i>)3436
PK3437	as PK2649, but <i>ilv⁺ xerC⁺</i> IN(Ω (1399kb:: <i>dif</i> $\rightarrow$ <i>kan</i>)3381- <i>dif⁺</i>)3437
PK3468	IN(<i>rrnD-rrnE</i>)1 <i>lacI^Q</i> L8 Δ <i>dif::cam2771</i>
PK3471	PK3468/pHN13
PK3476	as PK3032, but Ω (1589 kb::Tn $\leftarrow$ <i>dif</i>)3476
PK3483	IN(<i>rrnD-rrnE</i>)1 <i>recD::mini-tet xerC::tmp</i>
PK3512	PK3468 <i>xerC::tmp</i> /pHN13
PK3693	as PK2649, but <i>ilv⁺</i> Δ (Ω (1399 kb:: <i>dif</i> $\rightarrow$ <i>kan</i>)3381- Ω (1632 kb::Tn <i>dif</i> $\rightarrow$)3646)3693
PK3714	as PK2649, but <i>ilv⁺</i> Δ (Ω (1596 kb::Tn <i>dif</i> $\rightarrow$)3504- Ω (1632 kb::Tn <i>dif</i> $\rightarrow$)3646)3714
PK3742	as PK2649, but <i>ilv⁺ xerC⁺</i> Δ (Ω (1487 kb::Tn $\leftarrow$ <i>dif</i>)3645- <i>dif⁺</i>)3724
PK3751	as PK2649, but <i>ilv⁺ xerC⁺</i> Δ (Ω (1487 kb::Tn $\leftarrow$ <i>dif</i>)3645- Ω (1600kb::Tn $\leftarrow$ <i>dif</i>)3503)3751

Not all Tn*dif* kan insertions are included here. The five Tn*dif* insertions outside the terminus region, which were isolated in PK3032, are described in Fig. 1. The seven suppressing insertions, which were isolated in PK3032, are described in Fig. 3. Five nonsuppressing insertions in the terminus region are shown in Fig. 4 and are described in more detail in Results. Ω (1589 kb::Tn $\leftarrow$ *dif*)3476 designates an insertion (Ω) of Tn*dif* at 1589 kb in EcoMap6 (Rudd 1993), in which $\leftarrow$ *dif* has the same orientation as $\leftarrow$ *dif⁺*, and the insertion was first isolated in PK3476.

Δ (Ω (1487kb::Tn $\leftarrow$ *dif*)3645- Ω (1600 kb::Tn $\leftarrow$ *dif*)3503)3751 designates Δ 3751, formed by XerC-dependent recombination between *dif* insertions at 1487 and 1600 kb, which were arranged as direct repeats. Strain construction for isolation of deletions from double *dif* strains is described in Materials and methods.

insertions are simply identified by the kilobase coordinates of the insertion site.

We have demonstrated that the high frequency of suppressing insertions (~15%) in our libraries was attributable to the increased growth rate provided by these insertions. For example, we have constructed libraries in a *xerC::tmp* mutant (PK3512), in which insertions near *dif* should provide no growth advantage. On transduction to the indicator strain PK3032, only 1% (2 of 180) of the transductants exhibited the suppressed phenotype. These suppressing insertions were tightly linked to *dif*. We have also conducted reconstruction experiments using mixed cultures of *xerC⁺* strains containing suppressing and nonsuppressing insertions. Suppressing insertions were enriched ~20-fold after bacterial growth and transduction.

Nonsuppressing insertions of the dif-transposon

Although libraries constructed in *xerC⁺* strains were enriched for suppressing insertions, the majority of insertions (~85%) were not suppressing. Five insertions of this type have been mapped (Fig. 1). Southern hybridization was once again used to determine the sizes of the chromosomal restriction fragments in which insertion had occurred, and the possible regions of insertion were then identified on the physical map (K. Rudd, pers.

comm.). These sites were subsequently confirmed by cotransduction with closely linked Tn10 insertions (Singer et al. 1989).

We have also used the *dif* transposon to isolate additional insertions that were in the terminus region but that were not at the *dif* site and were nonsuppressing. These libraries were made in a *xerC* mutant, to avoid enrichment of suppressing insertions, and transductions was made to strains that contained various Tn10 insertions to simplify identification of insertions in particular regions. The insertions at 1487, 1491, 1625, and 1632 kb were obtained in this fashion (Fig. 4B).

An insertion at 1539 kb was isolated by a somewhat different procedure. The library was constructed in a strain that contained a 155-kb *dif* deletion, Δ 2035 (Fig. 4A). This deletion was formed by homologous recombination between Tn10 insertions at 1543 and 1698 kb; a Tn10 consequently remained at the site of the deletion. Cotransduction of Tc^r and Km^r was then used to identify insertions that were near the ends of the deletion. The original isolate of the insertion at 1539 kb also contained Δ 2035, and this strain was suppressed for the *Dif* phenotype. Transduction was then used to separate the insertion from the deletion. The insertion at 1539 kb was not suppressing when combined with smaller *dif* deletions, such as Δ *dif::cam2771* or Δ *dif::spc3269* (Fig. 4A).

The insertions described in the preceding sections were all isolated in derivatives of W3110, which contains

Figure 4. Map of the terminus region. (A) *dif* deletions used in these studies. (B) Physical coordinates in the terminus region, showing relevant Tn10 insertions and loci. The location and orientation of nine Tndif insertions, none of which suppress the Dif phenotype, are indicated by arrows. The physical coordinates are indicated by chevrons every 20 kb, which also indicate the proposed polarity of the terminus region. (C) Deletions formed by recombination between *dif* sites. All of these suppress the Dif phenotype. (D) Diagram indicating how the inversion of the terminus region in PK3437 shifts the center of this polarity to 1399 kb. The complete designations for the *dif* insertions, from left to right, are as follows: $\Omega(1399\text{kb}::\leftarrow dif-kan)3321$, $\Omega(1399\text{kb}::dif\rightarrow kan)3381$, $\Omega(1481\text{kb}::\leftarrow dif-spc)3185$, $\Omega(1481\text{kb}::dif\rightarrow spc)3186$, $\Omega(1487\text{kb}::Tn\leftarrow dif)3645$, $\Omega(1491\text{kb}::Tn\leftarrow dif)3581$, $\Omega(1539\text{kb}::Tn\leftarrow dif)3533$, $\Omega(1625\text{kb}::Tn\leftarrow dif)3642$, and $\Omega(1632\text{kb}::Tndif\rightarrow)3646$.

IN[*rrnD-rrnE*]1. Comparable results with respect to suppression or nonsuppression have been obtained when these insertions were tested in strains with a wild-type chromosome structure.

Targeted insertions at 1399 kb

All of the suppressing insertions obtained in strains related to W3110 mapped near the normal *dif* site at 1599 kb, and none were opposite *oriC*, near 1400 kb. It seemed likely that insertions in this region did not suppress, and consequently they were not present among the suppressed clones picked for further characterization. It was also possible, however, that the transposon did not insert into this region or that these suppressing insertions were not apparent on the MacConkey–lactose plates. As an additional test of that location, we have constructed insertions of the 231-bp sequence in plasmids and used linear transformation to insert the *dif* sequence at 1399 kb. Both orientations of *dif* have been isolated, and for the insertions shown in Figure 4B, *kan* and *dif* are oriented with respect to each other as shown in Figure 2. An additional set of insertions was obtained at 1399 kb, in which *dif* and *kan* were oriented toward each other (P.

Kuempel, unpubl.). None of these insertions suppressed the Dif phenotype.

Recombination at *dif* sites

We reported previously that *dif* sites in the terminus region, whether suppressing or nonsuppressing, recombined in a RecA-independent fashion with a *dif*-containing plasmid (pPK706) at a frequency of $\sim 2 \times 10^{-2}$ (Tecklenburg et al. 1995). A similar frequency was observed for the two insertions at 1399 kb (Fig. 4B), which are the most distant, nonsuppressing sites that we have isolated in the terminus region. Sites located outside the terminus region, at 3321, 3856, and 4519 kb (Fig. 1) also recombined with pPK706, although the frequency was $\sim 10^{-3}$ (P. Kuempel, unpubl.).

The nonsuppressing sites in the terminus region could also recombine with other *dif* sites, both suppressing and nonsuppressing. As we have reported previously, this recombination forms deletions or inversions, it is XerC-dependent, and it can generate hybrid, functional sites (Tecklenburg et al. 1995). With the more extensive set of ectopic sites reported here, it has been possible to determine what is required for a fully functional site.

Examples of some of these rearrangements are provided by studies done with the insertions at 1399 kb and *dif*⁺. A "double-*dif*" strain, containing *dif*⁺ (1599 kb) and a *dif* insertion at 1399 kb as direct repeats, was stable as a *xerC* mutant. Once transduced to *xerC*⁺, however, the intervening 200 kb of DNA was rapidly lost through recombination between the *dif* sequences, and strains with the resulting deletion (Δ 3436; Fig. 4C) were suppressed. Southern hybridizations demonstrated that strains with this deletion contained the expected fusion fragment (Fig. 5). A variation of this double-*dif* strain was constructed that contained *dif*⁺ and an insertion at 1399 kb as inverted repeats. Both orientations of the intervening DNA were present in *xerC*⁺ cells grown for 50 gen-

erations (Fig. 5). Because the intensities of the new *dif*-containing fragments due to the inversion were less than those of the original fragments, this indicates that the inverted orientation of this 200-kb region was present at a lower frequency than the normal orientation. Previously, we described another strain, that inverted the 118-kb region between *dif*⁺ and an insertion at 1481 kb (Tecklenburg et al. 1995). In that strain, both orientations were present at equal frequency.

The insertions have been used to construct a number of other deletions (Fig. 4C). Δ 3693 was formed by XerC-dependent recombination between *dif* insertions at 1399 and 1632 kb. Neither of these insertions was suppressing by itself, but strains containing this 233-kb deletion were suppressed. Several other deletions, which also suppressed the Dif phenotype, have been formed by this procedure. Δ 3742 was formed by recombination between *dif*⁺ and an insertion at 1487 kb, and Δ 3751 was formed by recombination between insertions at 1487 and 1600 kb. Finally, Δ 3714 was formed by recombination between insertions at 1596 and 1632 kb.

Figure 5. Southern hybridization of double *dif* strains and their progeny. DNA from *xerC*⁻ and *xerC*⁺ strains was digested with *Bgl*I (B) and *Kpn*I (K) and probed with the 33-bp *dif* sequence. Deletion: PK3394 is *xerC dif*⁺ and contains an insertion at 1399 kb [Ω (1399kb::←*dif*-*kan*)3321]. The insertion at 1399 kb is in a 4.4-kb *Bgl*I and a 3.8-kb *Kpn*I fragment, and *dif*⁺ is in an 11-kb *Bgl*I and a 5.7-kb *Kpn*I fragment. In PK3436, the *xerC*⁺ derivative of PK3394, recombination between the *dif* sequences produced a 200-kb deletion. The *dif* fusion fragment is in a 7.4-kb *Bgl*I and a 5.3-kb *Kpn*I fragment. Inversion: PK3395 is *xerC dif*⁺ and contains an insertion at 1399 kb [Ω (1399kb::*dif*→*kan*)3381]. The insertion at 1399 kb is in a 4.4-kb *Bgl*I and a 2.1-kb *Kpn*I fragment, and *dif*⁺ is in an 11-kb *Bgl*I and a 5.7-kb *Kpn*I fragment. In PK3437, the *xerC*⁺ derivative of PK3395, inversions are apparent in cells grown for 50 generations. The original *dif* sites were present, as well as two new *dif*-containing fragments resulting from the inversion. The new *dif* site at 1399 kb is in a 6.8-kb *Bgl*I and a 2.6-kb *Kpn*I fragment. The new *dif* site at 1599 kb is in an 8.3-kb *Bgl*I and a 5.1-kb *Kpn*I fragment.

Discussion

The experiments reported here were conducted to obtain further information about the requirements of a functional *dif* site. Most of these insertions were obtained by means of a *dif* transposon (Tndif), although some were obtained by linear transformation at a specific site. Based on the properties of these insertions, as well as results reported previously by us and others, the requirements for a suppressing site are as follows.

1. Insertion of a 33-bp sequence is sufficient to suppress the Dif phenotype (Leslie and Sherratt 1995; Tecklenburg et al. 1995), although the 231-bp site described here gives higher frequencies of recombination between chromosomal sites.
2. Both orientations of the *dif* sequence are functional for suppressing the Dif phenotype (Fig. 3; Kuempel et al. 1991; Leslie and Sherratt 1995).
3. Other resolvase sites can suppress the Dif phenotype, when placed at the site of a *dif* deletion. These include the *loxP* site of P1 (Leslie and Sherratt 1995) and the *psi* site of pSC101 (Cornet et al. 1994). The *cer* site of pColE1 did not suppress the phenotype (Leslie and Sherratt 1995).
4. Specific flanking sequences are not required for activity; we have observed suppression through insertion of the 33-bp *dif* sequence at the site of a deletion that removed 173 kb from the terminus region (Tecklenburg et al. 1995).
5. Suppressing sites are confined to a small region that surrounds the normal *dif* site (Fig. 3). Using a procedure that enriched for suppressing insertions of Tndif, the most distant insertion that we isolated was 10 kb on the counterclockwise side of the normal site. The closest nonsuppressing insertions were located 60 kb to the counterclockwise side and 26 kb to the clockwise side (Fig. 4B).

6. Nonsuppressing insertions, both in the terminus region as well as in the rest of the chromosome, are still functional for certain types of recombination. For example, they readily recombined with a *dif* site on a plasmid (Leslie and Sherratt 1995; Tecklenburg et al. 1995).
7. It is not necessary that suppressing sites be located opposite the origin of replication. In strains with IN-*(rrnD-rrnE)1*, which shifts the region opposite *oriC* by 200–1400 kb on the physical map (Fig. 1), all suppressing insertions were still located near the normal location of *dif* at 1599 kb (Fig. 3). It should also be noted that the region at 1400 kb was also near a terminator site (*TerA*, 1350 kb), which is comparable with *dif*'s normal location with respect to *TerC* (1617 kb). Insertions directed to a site at 1399 kb did not suppress the Dif phenotype (Fig. 4B).

In spite of these specific requirements for sites that can suppress the Dif phenotype, deletions can apparently convert any terminus region—a nonsuppressing site into suppressing site. In general, removal of DNA between a nonfunctional site and the normal position of *dif* restored full function to the ectopic site. We have observed the following patterns.

1. One set of deletions removed DNA between *dif*⁺ (1599 kb) and nonsuppressing sites located to the left of *dif*. Examples shown in Figure 4C are $\Delta 3436$ formed by recombination between *dif*⁺ and an insertion at 1399 kb, and $\Delta 3742$, formed by recombination between *dif*⁺ and an insertion at 1487 kb. A similar suppressing deletion was formed between *dif*⁺ and an insertion at 1491 kb (data not shown). All of these deletions can be considered to start at the right-hand side of the ectopic site and extend past *dif*⁺ to the adjacent *hip* gene at 1599 kb.
2. In another set of deletions that generated suppressing sites, the deletions extend several kilobases past *dif*⁺. An example shown in Figure 4C is $\Delta 3751$, formed between insertions at 1487 and 1600 kb. A comparable deletion was formed between insertions at 1491 and 1600 kb (data not shown). Also, we previously described $\Delta 3546$, formed between insertions at 1481 and 1602 kb (Tecklenburg et al. 1995). Although all of these deletions suppressed the Dif phenotype, strains harboring these deletions sometimes produced a few filamentous cells.
3. A suppressing site can also be generated by a deletion to the right of *dif*. $\Delta 3714$ was formed by recombination between insertions at 1596 and 1632 kb (Fig. 4C).
4. $\Delta 3693$ (Fig. 4C) removed 233 kb of DNA between nonsuppressing sites at 1399 and 1632 kb. Neither site, by itself, suppressed the Dif phenotype, presumably because of some property of the region between the ectopic insertion and the normal location of *dif*. This region was removed by the 233-kb deletion, and the cells appeared completely normal.
5. Perhaps the most informative insertion is that located at 1539 kb (Fig. 4B). This insertion is nonsuppressing, but it becomes suppressing when combined with

$\Delta 2035$ (Fig. 4A), which extends to within 4 kb of the insertion. However, the insertion at 1539 kb is not suppressing when combined with smaller deletions that removed DNA to the left of *dif*⁺, such as $\Delta dif::spc3269$ (Fig. 4A). Moreover, although $\Delta 2035$ restored suppression to the insertion at 1539 kb, it had no effect on more distant *dif* insertions at 1481 and 1399 kb. Apparently, a deletion must extend near an ectopic *dif* site to remove whatever inhibits proper function of the sites. As a test of this, we have used Tn10, situated in $\Delta 2035$, to isolate Tn10-derived deletions (Bochner et al. 1980) in a strain that also contained an insertion at 1481 kb. Approximately 1% of these derivatives had a suppressed phenotype. These deletions have not been mapped, but it is likely that they extend farther to the left and end near the ectopic *dif* insertion, removing sites that blocked its full function.

What model can be proposed to explain this pattern of sites and deletions required for proper *dif* function? We propose that there is a polarity in the terminus region and that two regions of opposite polarity converge at *dif*. This is illustrated by the arrows indicating the physical coordinates for the map in Figure 4B. This could be a continuation of polarities observed previously in other parts of the chromosome. For example, the majority of Chi sites (Burland et al. 1993) and transcription units (Brewer 1990) are oriented toward the terminus in the halves of the chromosome replicated in clockwise and counterclockwise directions. However, the polarity could also be caused by presently uncharacterized elements, or by sites that are only present in the terminus region. Based on earlier studies on inversions (Rebollo et al. 1988) and more recent studies on hyperrecombination (Louarn et al. 1994), Louarn and co-workers have also proposed that the terminus is flanked by polar regions. It is likely that these different procedures have all identified the same underlying polarity.

One way to visualize the role of polarity is to imagine that it is related to the processing of newly synthesized DNA into nucleoids. Sites that supercoil DNA into separate domains (Løbner-Olesen and Kuempel 1992), for example, might function in a sequential, polar fashion (Fig. 6). Whatever the nature of this polarity, we propose that the signals and processing that occur in clockwise and counterclockwise directions converge at *dif* but do not cross it. By being where these polarities converge, *dif* is in a location that facilitates its function as a resolvase site. Location in this region could possibly increase the frequency or the duration of interactions at *dif* sites in the sister chromosomes. Our present data indicate that this region has a maximum size of 85 kb, but it is probably considerably smaller. If shifted outside this region, an ectopic site would be surrounded by processing sites of the same polarity, which would place it in a region that impeded proper interaction. Deletions that extend from the ectopic *dif* site up to or past the normal location of *dif* would place the site once again in the center of the converging polarities (see Fig. 6). A deletion that ren-

Figure 6. Model for polarity in the terminus region and how this affects *dif* function. (I) A dimeric chromosome with region c positioned for interaction. The dimer is shown with no nucleoid structure. (II) The processing of daughter chromosomes into nucleoid domains proceeds in a sequential, polar fashion and is centered at region c. Regions b, c, and d have not yet been processed. (III) Processing from both sides has proceeded up to region c. If *dif* is in region c, it is positioned for proper function. If *dif* is in another region, such as region a, the sites cannot interact properly. (IV) If a deletion starts near region a and ends at or after c, polarity would now be centered at a, and *dif* located in this region would be positioned for proper function.

dered one site functional, such as the effect of $\Delta 2035$ on the insertion at 1539 kb, would not be effective for a more distant site, because that site would still be surrounded by processing sites of the same polarity.

It should be noted that occurrence of functional, ectopic sites indicates that at least with respect to *dif* function, there is nothing specific about the region at *dif*. Provided that the new site is at a region where the polarities converge, the ectopic site will be functional. Deletions in the terminus region can be readily tolerated. Insertions and inversions (see below) also would not affect *dif* function, provided they maintained this polarity around *dif*.

It is usually assumed that the function of the *dif* locus is to resolve dimeric chromosomes. It might have other functions, however, such as providing the center about which nucleoid processing occurs, regardless of whether sister chromatid exchange has occurred. A possible model for this has been proposed by Sherratt and co-

workers (Sherratt et al. 1995), in which a stable Holliday structure forms at *dif* shortly after its replication, and resolution does not occur until nucleoid processing is almost complete. Daughter chromosomes joined at *dif* and polar processing sites flanking that junction could be important for the proper formation of daughter nucleoids.

Although processing into nucleoids must follow replication, the processes do not need to be tightly linked. As proposed by Louarn et al. (1994), the restructuring of newly formed DNA follows its own pattern and occurs even if replication has deviated from the standard pattern. This uncoupling would allow proper processing to occur, even in situations of asymmetric replication in which forks did not meet at *dif*. This could occur in cells in which the two forks traveled at different rates, or in cells with rearranged chromosomes such as W3110. The Tus/Ter system would ensure that the replication patterns did not become too asymmetric (Hill 1992).

The properties of these polar sites are presently unknown. As described by Cornet et al. (this issue), a site or sites are present in the region near 1617 kb. As we have described above, there are probably many such sites throughout the terminus region.

An apparent contradiction to the above polarity model is strains with inversions of part of the terminus region. Previously, we reported that in cells in which the region between 1481 and 1599 kb was inverting, there was no preferred orientation (Tecklenburg et al. 1995). Figure 5 shows that cells can also tolerate an inversion of 200 kb of the terminus region, between 1399 and 1599 kb, although in this case the wild-type orientation was preferred. Figure 4D shows that, when this region is inverted, the right-hand *dif* site in PK3437 would be flanked by regions that had the same processing polarity. This seems to violate the polarity model described above. It should be noted, however, that the left-hand *dif* site at 1399 kb would now be surrounded by sites of converging polarity, and this could be the functional site when the region is inverted. This raises a number of testable questions concerning recombination at suppressing and nonsuppressing, ectopic *dif* sites. As one approach for answering some of these questions, we have recently developed a physical assay for monitoring recombination between chromosomal *dif* sites of these types.

Materials and methods

Bacterial strains and media

Bacterial strains are described in Table 1. LB medium (Miller 1992) was used for most liquid cultures, and plates contained LB medium with 1.5% agar (Difco). MacConkey agar base (Difco) was supplemented with 1.0% lactose. Phage were grown on LB agar plates in a 2.5-ml top-agar layer of LB medium with 0.5% agar. Antibiotic concentrations ($\mu\text{g/ml}$) were as follows: Kanamycin (Km), 100; tetracycline (Tc), 20; ampicillin (Ap) 100; chloramphenicol (Cm) 25; spectinomycin (Sp) 100; trimethoprim (Tp) 10.

Construction of *dif* deletions

The construction of $\Delta dif::spc3269$ was similar to that of $\Delta dif::kan2336$ (Kuempel et al. 1991), except that a 1.7-kb fragment that contained the spectinomycin resistance gene (*spc*) of pHP45- Ω (Prentki and Krisch 1984) was inserted at the site of the 12-kb deletion in pBS12 to give pOL123. $\Delta dif::spc3269$ was then crossed into the chromosome by linear transformation to obtain PK3269. Construction of $\Delta dif::cam2771$ started with pPK999, which is a pUC19 derivative that contains a 7-kb *Pst*I fragment (chromosome coordinates 1594–1601 kb). The chloramphenicol resistance gene (*cam*) was isolated from pACYC184 as a 1.3-kb *Hae*II fragment. The ends were filled in, and the fragment was inserted between the *Eco*RV and *Cla*I sites that correspond to chromosome coordinates 1597.7 and 1599 kb to give pLim1000. $\Delta dif::cam2771$ was then crossed into the chromosome by linear transformation to obtain PK2771.

Construction of *xerC::tmp*

pMU575, which contains a trimethoprim resistance gene (*tmp*), was provided by J. Pittard (University of Melbourne, Australia). The *tmp* gene was removed as a 1.6-kb *Sph*I fragment and cloned into pUC4-Kiss (Pharmacia) that had been digested with *Sph*I. This replaced the *kan* gene with the *tmp* gene. *tmp* was then isolated from this plasmid (pWS1) in a 1.6-kb *Eco*RI fragment on which the ends had been filled in. pUC18-*xerC* contained *xerC* in an 8.3-kb *Eco*RI fragment, and after digestion with *Bsa*BI, *tmp* was inserted at that site in *xerC* to give pWSII. *xerC::tmp* was then inserted into the chromosome by linear transformation to obtain PK3483.

Construction of *Tndif*

The 231-bp *dif* fragment corresponds to positions 1926–2157 kb in the sequence of Black et al. (1991). This fragment, with *Hind*III and *Bam*HI ends, was isolated from a derivative of pMAK705 (Kuempel et al. 1991) and inserted into pUC18 DNA digested previously with *Hind*III and *Bam*HI. This construct was then digested with *Bam*HI, and the Tn903 kanamycin resistance gene (*kan*; Pharmacia) was inserted as a 1.3-kb *Bam*HI fragment. The *dif kan* fragment was then isolated from HN5 as a *Pvu*II-*Eco*RI fragment. After filling in the ends, this was inserted into a 4.8-kb fragment obtained from pNK2859 (Kleckner et al. 1991) by digesting with *Bam*HI and filling in the ends. The product (pHN13) contains the *dif kan* genes inserted between the 70-bp inverted repeats. Figure 2 shows the relevant restriction enzyme sites in pHN13. The polarity arrow for *dif* indicates the sequence 5'→3' of Black et al. (1991).

Construction of insertions at 1399 kb

pJPB61 was provided by J.P. Bouche (CNRS, Toulouse, France), and it contained a 4.5-kb *Hind*III-*Bam*HI fragment (chromosome coordinates 1396–1401 kb) inserted in pBR325. The *dif kan* fragment was isolated as a *Pvu*II-*Eco*RI fragment from pHN5. The ends were filled in and the fragment was inserted into the *Eco*RV site of pJPB61 in both orientations to give pHN7 and pHN8. In the chromosome, this *Eco*RV site is located at 1399 kb. pHN7 and pHN8 were linearized by digestion with *Eco*RI and transformed into PK3270. $\Omega(1399 \text{ kb}::\leftarrow dif-kan)3321$ was derived from pHN7 and $\Omega(1399 \text{ kb}::dif\rightarrow kan)3381$ was derived from pHN8.

Transposon libraries

Single colonies of strains containing pHN13 (PK3471 or PK3512) were inoculated into LB medium containing 10^{-3} M

isopropyl β -D-thiogalactoside (IPTG) and 100 μ g/ml of kanamycin and grown to stationary phase. Bacteriophage P1 were then grown on these bacteria in a LB top-agar layer (Miller 1992). The bacteriophage were harvested after 16 hr of growth, and a number of separate libraries were constructed to avoid sibs. The *Tndif* insertions were then transduced into PK3032 or derivatives described in Results. To test for suppression, we first selected *Km*^r transductants on LB agar plates containing 100 μ M/ml of kanamycin, restreaked once, and then streaked the cells on MacConkey-lactose plates. In *lac* mutants containing the *sfiaA::lacZ* reporter gene, colonies expressing the *Dif* phenotype were red, because of expression of the *sfiaA* gene when SOS was induced (Kuempel et al. 1991). The cells in these colonies were filamentous. Suppressed colonies were white and contained normal-length cells.

Southern hybridizations

Total genomic DNA for hybridization was isolated by an SDS-lysozyme method (Ausubel et al. 1987). DNA was digested with the appropriate restriction enzymes, separated by electrophoresis in 0.7% agarose gels, and transferred to Zeta-Probe membranes (Bio-Rad), using standard procedures. After transfer, the membranes were baked for 1 hr at 80°C. Hybridization was conducted for 16 hr at 55°C. The sequence of the 33-bp *dif* oligonucleotide, which was labeled with [γ -³²P]ATP by phosphorylation with bacteriophage T4 polynucleotide kinase, has been described (Kuempel et al. 1991). After autoradiography, the fragment sizes were compared with the physical map of the relevant region (Rudd 1992, and pers. comm.) to determine the insertion sites. Digestions with *Eco*RI, *Eco*RV, *Bgl*II, and *Pvu*II were used to identify fragments in which an insertion had occurred. Digestions with *Kpn*I and *Pst*I, which cut in the *Tndif* transposon (Fig. 2), were used to determine the orientation of the insertions.

Recombination between *dif* sites

A number of deletions were formed by *XerC*-dependent recombination between two *dif* sites in the same orientation. These double *dif* strains were constructed in an *ilv xerC::cam* or *ilv xerC::tmp* background. If one of the *dif* sites was *dif*⁺, incorporation of the second site could be obtained by selecting for *Km*^r (for *dif kan* insertions) or *Sp*^r (for *dif spc* insertions) transductants. If *dif kan* was to be present at both sites, incorporation of the second site was obtained by selecting for a closely linked marker conferring a different antibiotic resistance, such as a *Tn10* insertion. Once both *dif* sites were present, the strain was transduced to *ilv*⁺, and *xerC*⁺ cotransductants were identified by loss of *Cm*^r or *Tp*^r. Deletions were initially identified by loss of markers between the two *dif* sites, and they were subsequently confirmed by Southern hybridizations. Inversions were formed by the same procedure, except *dif* sites in inverted orientation were used.

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Use of a transposon (Tndif) to obtain suppressing and nonsuppressing insertions of the dif resolvase site of *Escherichia coli*.

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