Role of Exonucleolytic Degradation in Group I Intron Homing in Phage T4

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ABSTRACT

Homing of the phage T4 *td* intron is initiated by the intron-encoded endonuclease I-*Tev*I, which cleaves the intronless allele 23 and 25 nucleotides upstream of the intron insertion site (IS). The distance between the I-*Tev*I cleavage site (CS) and IS implicates endo- and/or exonuclease activities to resect the DNA segment between the IS and CS. Furthermore, 3′ tails must presumably be generated for strand invasion by 5′-3′ exonuclease activity. Three experimental approaches were used to probe for phage nucleases involved in homing: a comparative analysis of *in vivo* homing levels of nuclease-deficient phage, an *in vitro* assay of nuclease activity and specificity, and a coconversion analysis of flanking exon markers. It was thereby demonstrated that T4 RNase H, a 5′-3′ exonuclease, T4 DNA exonuclease A (DexA) and the exonuclease activity of T4 DNA polymerase (*43*Exo), 3′-5′ exonucleases, play a role in intron homing. The absence of these functions impacts not only homing efficiency but also the extent of degradation and flanking marker coconversion. These results underscore the critical importance of the 3′ tail in intron homing, and they provide the first direct evidence of a role for 3′ single-stranded DNA ends as intermediates in T4 recombination. Also, the involvement of RNase H, DexA, and *43*Exo in homing provides a clear example of the harnessing of functions variously involved in phage nucleic acid metabolism for intron propagation.

GROUP I intron homing occurs in all three biological kingdoms. The process involves the unidirectional movement of an intron from an intron-plus allele to a cognate intron-minus allele through a gene conversion event (reviewed by Belfort and Roberts 1997). Homing is initiated by an intron-encoded endonuclease, which makes a site-specific, double-strand break (DSB) in the intron-minus allele (Figure 1). In phage T4, intron homing occurs in the context of phage recombination-dependent DNA replication, which requires a myriad of phage replication and recombination functions. These include the strand transferase (UvsX), single-stranded DNA (ssDNA)-binding protein (gp32), DNA polymerase (gp43), helicase (gp41), DNA ligase (gp30), and a putative exonuclease complex (gp46/47) (Clyman and Belfort 1992; Mueller *et al.* 1996a). According to the models, the DSB is processed by exonucleases producing a double-strand gap with single-stranded 3′ tails for strand invasion of the homologous intron-plus allele. Repair synthesis using an intron-plus strand as a template results in intron inheritance. At least two pathways have been implicated in the homing process. These are the classic double-strand-break repair pathway (DSBR), which involves resolution of Holliday junctions leading to the generation of both crossover and noncrossover products, and the synthesis-dependent strand annealing pathway (SDSA), in which crossover products are not generated (Mueller *et al.* 1996a).

Compared with the well-defined phage replication and recombination proteins required for T4 intron homing (Kreuzer and Morrical 1994; Nossal 1994; Mueller *et al.* 1996a), the nucleases that are involved in exonucleolytic degradation of recipient strands remain obscure. I-*Tev*I, the endonuclease encoded by the T4 *td* (thymidylate synthase) gene, makes a DSB 23 and 25 nucleotides (nt) upstream of the intron insertion site (IS) in the recipient allele (Figure 1A; Bell-Pedersen *et al.* 1990). Because there is a separation between the I-*Tev*I CS and the IS, endonucleases and/or exonucleases are expected to degrade the DNA between the CS and the IS, termed the resection segment, to allow for strand invasion of exon II sequences (Figure 1A). Indeed, 100% coconversion of markers within the resection segment is observed (Bell-Pedersen *et al.* 1989). Also, 5′-3′ exonucleases are expected to generate recombinogenic 3′ tails for homologous strand invasion and subsequent repair synthesis. In a bacteriophage λ-*Escherichia coli* model system, intron homing of the *td* intron was associated with two resection activities, the λ 5′-3′ exonuclease and associated annealing function, Redαβ, and an *E. coli* 3′-5′ exonuclease, ExoIII (Clyman and Belfort 1992).

A role for both 5′-3′ and 3′-5′ exonuclease activities prompted exploration of analogous functions in T4, the more complex, natural host of the *td* intron. There are several 5′-3′ exonucleases expressed after T4 infection,

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including the putative exonuclease complex gp46/47 (Hercules and Wiberg 1971; Mickelson and Wiberg 1981), T4 RNase H (Hollingsworth and Nossal 1991), and exonucleases B and C (Shimizu and Sekiguchi 1976). The gp46/47 complex is essential for recombination in T4 and is thought to contribute substantially to processing double-stranded ends (Mosig 1998). However, there has been little biochemical evidence to support the 5′-3′ nuclease activity of any except RNase H. T4 RNase H is an early protein that degrades RNA primers in RNA-DNA hybrids and DNA in double-stranded DNA in a 5′-3′ direction (Hollingsworth and Nossal 1991). T4 RNase H has been implicated in recombinational repair, although it is not clear whether the repair defect in *rnh* mutants is directly caused by loss of exonuclease activity or by an indirect mechanism involving the persistence of RNA-DNA hybrids (Kogoma *et al.* 1993; Woodworth and Kreuzer 1996).

T4 encodes at least two 3′-5′ exonuclease activities, DNA exonuclease A (DexA) (Warner *et al.* 1972) and the proofreading exonuclease domain of T4 DNA polymerase, *43*Exo of gp43 (Lin *et al.* 1994; Wang *et al.* 1995). DexA protein, the major T4 3′-5′ DNA exonuclease induced upon phage infection, is single-strand specific and conditionally required for DNA replication (Gruber *et al.* 1988). It has been proposed that DexA acts to degrade the host genome to produce precursors for phage DNA synthesis (Warner *et al.* 1972; Gauss *et al.* 1987). Like T4 DexA, *43*Exo has single-strand-specific exonuclease activity. The 3′-5′ exonuclease function and the DNA polymerase activity reside in the same polypeptide, which is expressed early after phage infection (reviewed by Reha-Krantz and Nonay 1993).

In this study, we use molecular, biochemical, and genetic analyses to identify and characterize T4 exonucleases that participate in DNA resection required for intron homing. It was thereby demonstrated that T4 RNase H, DexA, and *43*Exo are involved in the homing process, and that the integrity of 3′ tails is a critical determinant in the intron homing process.

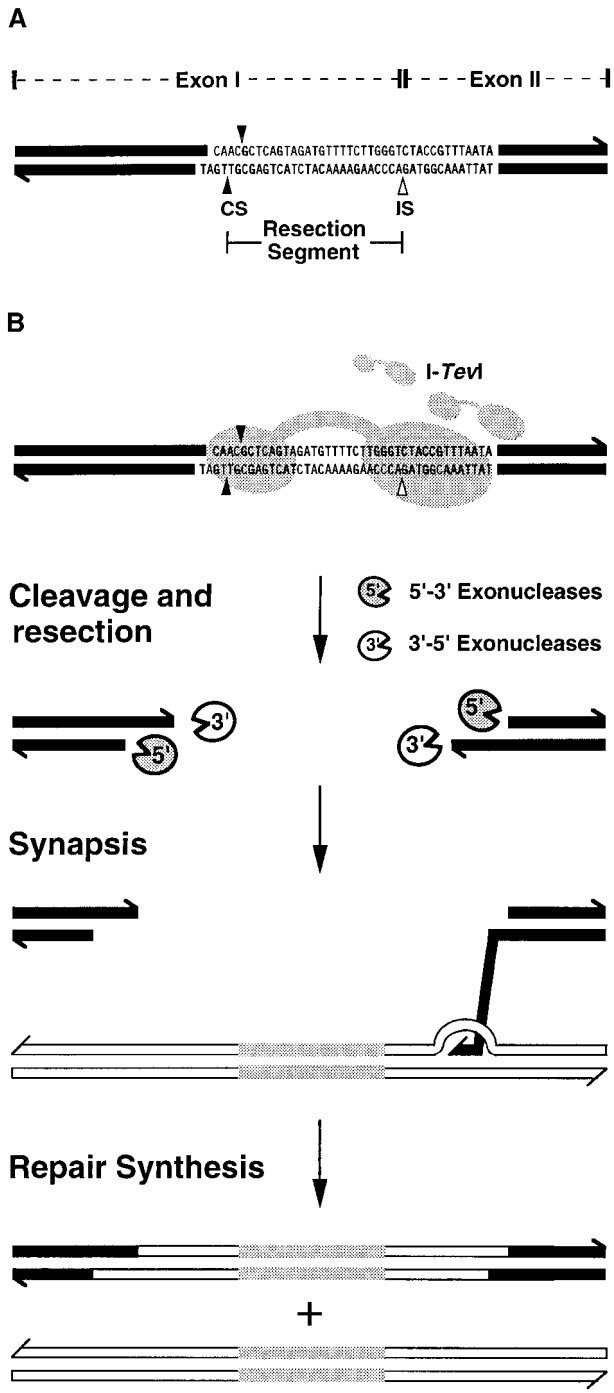

Figure 1.—Intron homing in bacteriophage T4. (A) Homing site of the *td* intron. The homing site (individual nucleotides) comprises the endonuclease-binding site, which overlaps the *td* intron insertion site (IS) and the I-*Tev*I cleavage site (CS) (Bryk *et al.* 1995; Mueller *et al.* 1995). (B) Intron homing. The intron-encoded endonuclease I-*Tev*I cleaves the intron recipient allelle at the CS, which is 23 and 25 nt upstream of the IS. Resection is presumed to require both 3′-5′ and 5′-3′ degradation to remove DNA sequences between the CS and the IS, and it produces recombinogenic 3′ tails for strand invasion. Synapsis involves the invasion of the homologous intron-plus allele by the 3′ tails. Repair synthesis results in two intron-containing alleles (Mueller *et al.* 1996a).

MATERIALS AND METHODS

Strains and plasmids: All phage used in this work are derivatives of T4K10, which is deficient in DenA and DenB endonucleases, protecting plasmid DNA from degradation during phage infection, and two additional amber mutations in genes *38* and *51*; these were used to prevent phage proliferation in a suppressor-minus (Sup°) host (Selick *et al.* 1988). The exonuclease mutations were introduced into the T4K10 background by marker rescue, in which T4K10 was used to infect cells containing the mutant gene on a plasmid. T4K10 *dexA* carries an insertion in the DexA coding region (Gauss *et al.* 1987). T4K10 *rnh* has a deletion from nt 10 to 777 of the RNase H coding region (Woodworth and Kreuzer 1996). T4K10 *43exo* has a single-base-pair mutation that causes an Asp-to-Ala change at residue 219 (D219A) (Frey *et al.* 1993).

The 640-bp *td*ΔEI deletion immediately upstream of the *td* intron was introduced into T4K10 and its exonuclease-deficient derivatives by using pAIΔEI-3 (Parker *et al.* 1996) and the T4 insertion/substitution system (Selick *et al.* 1988). The *td*ΔEI derivatives have 127 bp of homology remaining to exon I of the recipient allele. Mutations were confirmed by either the polymerase chain reaction (PCR) or DNA sequencing. The plasmid-borne *dexA* amber mutation was constructed by site-directed mutagenesis using the GeneEditor site-directed mutagenesis system (Promega, Madison, WI). T4K10 *dexAam 43exo* and its *td*ΔEI derivatives were constructed by marker rescue, in which T4K10 *43exo* and its *td*ΔEI derivative were crossed with the plasmid-borne *dexA* amber mutant. The intron recipient plasmid pSU*td*ΔIn confers chloramphenicol resistance and contains the *Eco*RI-*Eco*RI *td* fragment with a precise deletion of the intron (Chu *et al.* 1984; West *et al.* 1986). The *Bam*HI site of the vector was deleted, and the *td* fragment was in the reverse orientation relative to p*lac* to prevent toxic expression of I-*Tev*I (also referred to as *dt*) (Clyman and Belfort 1992). The recipient plasmid used in the coconversion analysis is pACYC*td*ΔIn▽RS, which confers tetracycline resistance and contains four polymorphic restriction sites in each exon of the 1.4-kb *td*ΔIn fragment (Mueller *et al.* 1996b).

Oligonucleotides: Oligonucleotides used to measure the 5′-3′ degradation were P1, 5′-TGGATTTGCAGTGGTATCA AC-3′; P2, 5′-TATTGATCGTATTAAAAAACTGCC-3′; and P3, 5′-GGCAAAACAGTCTGGGATG-3′, located 10, 150, and 270 bp, respectively, upstream of the CS in exon I of the *td* gene (Mueller *et al.* 1996b). The oligonucleotide used for 3′-5′ degradation analysis was W606, 5′-TGATACCACTGCAAAT CCAAA-3′, located 10 nt upstream of the CS. PCR primers used to amplify intron-plus transductants for coconversion analysis were the exon I primer W340, 5′-GTGTAATTGG CGGGCCTGCTCTGTTATATGC-3′; and the intron primer MB17, 5′-TGTCTACTAGAGAGGTTCCCCG-3′.

Phage-to-plasmid homing assay: Host cells, *E. coli* B (Sup°), containing the recipient plasmid pSU*td*ΔIn were grown at 37° in TBYE (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with chloramphenicol (25 μg/ml) to an OD_{650} = 0.2. Cells were harvested by centrifugation, concentrated 10-fold, and infected with phage at a multiplicity of infection (m.o.i.) of 4. After 30 min of infection, cells were harvested, DNA was prepared, and Southern hybridization was performed as described (Mueller *et al.* 1996a). The 734-bp intron-specific probe was generated by PCR, gel purified, and labeled by random priming according to the manufacturer's directions (GIBCO-BRL, Gaithersburg, MD). Southern blots were analyzed on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

T4-infected cell extracts: Extracts from T4-infected cells were prepared according to Mueller *et al.* (1996b). *E. coli* B was grown to a density of 3×10^8 cells/ml and infected with T4K10 or its exonuclease-deficient derivatives at an m.o.i. of 6. After 17 min at 37°, cells were harvested by centrifugation and frozen at −80°. Cell pellets were lysed, nucleic acids were removed by streptomycin sulfate precipitation, and proteins were harvested by ammonium sulfate fractionation exactly as described (Mueller *et al.* 1996b). The quality of each extract was determined by measuring I-*Tev*I activity, in which 250 ng of *Sca*I-linearized pBS*td*ΔIn was incubated with 5 μl of cell extract at 37° for 5 and 20 min and assayed for cleavage by agarose gel electrophoresis (Bell-Pedersen *et al.* 1991).

Nuclease assays: The DNA substrate for the 5′-3′ degradation analysis was an I-*Tev*I-linearized plasmid containing the *td* homing site (pBS*td*ΔIn). In each reaction, 100 ng of linearized DNA was incubated with 5 μl of cell extract containing 350 μg/ml of partially purified protein (Mueller *et al.* 1996b) for 20 min at 37° in JBB buffer (50 mm Tris-HCl, pH 8.0, 20 mg/ml poly(dI/dC), 10 μg/ml BSA) supplemented with 2 mm $MgCl_2$. The reactions were stopped at various times by phenol extraction followed by ethanol precipitation. DNA preparation and dot-blot hybridization were performed as described previously (Mueller *et al.* 1996b), and the results were analyzed on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

For the 3′-5′ degradation analysis, oligonucleotide W606 was 5′ labeled with polynucleotide kinase (GIBCO-BRL) according to the manufacturer's instructions. The oligonucleotide was incubated with cell extract for the indicated times at 24°, and the reaction was stopped by phenol extraction and ethanol precipitation. Products were separated on 10% acrylamide-urea gels, and the results were quantitated as described above.

Coconversion analysis: Plasmid transduction was performed as described by Clyman and Belfort (1992). *E. coli* Sup^+ cells harboring an intron-deleted recipient plasmid, pACYC*td*-ΔIn▽RS (Tet^r), which carries restriction site markers in the exons, were infected with the parental phage T4K10 and its *dexA*, *rnh*, and *43exo* derivatives. Infection was at 37° for 2 hr at an m.o.i. of 3. Phage progeny were adsorbed onto *E. coli* B, a Sup° host, for 20 min at 24°, and cells were plated onto tetracycline-containing plates. Single-intron-containing transductants were identified by PCR with exon I- and intron-specific primers. The loss of restriction sites in the intron-containing recombinants was determined by restriction enzyme digestion of the PCR products followed by agarose gel analysis. Coconversion frequency of each restriction marker was determined by the loss of the polymorphic restriction sites of individual transductants (Mueller *et al.* 1996b).

RESULTS

Identification of T4 "homing exonucleases": Exonucleases required either to generate the hypothetical 3′-recombinogenic tails for strand invasion in intron homing or to degrade the resection segment were examined by genetic analysis. The assay employs phage with different exonuclease deficiencies to serve as *td* intron donors, with the intronless *td* recipient allele carried on a plasmid (Figure 2A, left). After infecting *E. coli* cells containing a plasmid-borne intronless allele (pSU*td*-ΔIn), levels of intron homing and phage DNA replication were measured by restriction and Southern hybridization analysis using an intron-specific probe. The relative ratio of intron homing product to donor phage DNA in each infection was used as a measure of intron homing in the different genetic backgrounds (Figure 2, B and C; Table 1).

The efficiency of intron homing was first determined 30 min after phage infection with "wild-type" T4K10 phage and a mutant T4K10 derivative deficient in the 5′-3′ exonuclease RNase H. In the assay, the efficiency of intron homing decreased to 46% of wild-type levels in the *rnh* mutant (Figure 2B, *cf.* lanes 1 and 2; Table 1). However, in considering the active role of RNase H in DNA replication, as well as the intertwining of replication and homing, caution is required in interpreting these reduced levels of intron homing (see below).

Next, the two major 3′-5′ exonucleases, DexA and

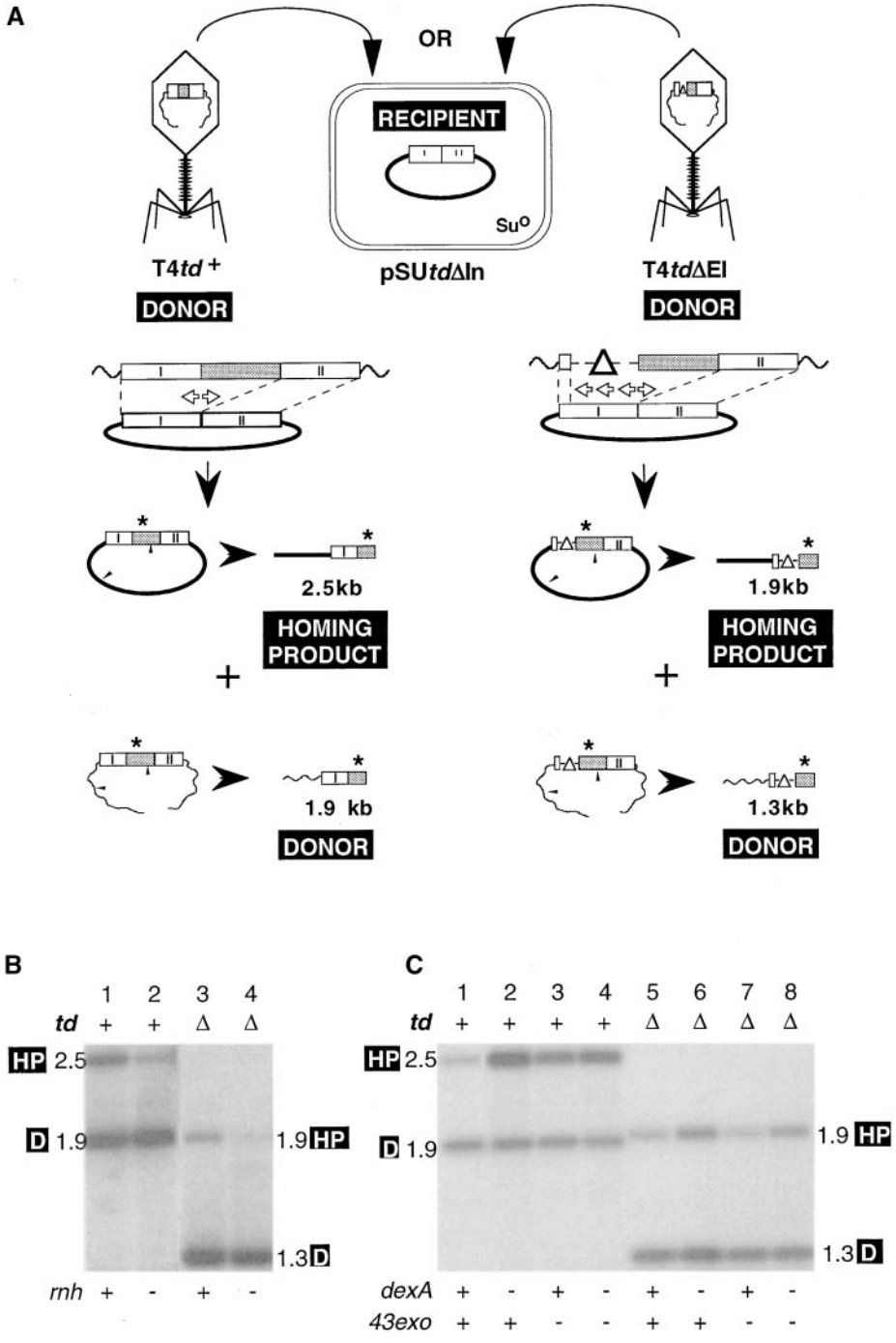

Figure 2.—*In vivo* homing. (A) Schematic of assay. Intron-plus derivatives of T4K10 with wild-type *td* exons (*td*$^+$) or with a 640-bp deletion of exon I (*td*ΔEI) served as intron donors to infect cells containing a plasmid-borne intron recipient (pSU*td*ΔIn). The intron donor and homing product can be distinguished by the length of restriction fragment hybridized with an intron-specific probe (asterisk). Exons, white; intron, gray; white arrows, degradation; Δ, 640-bp deletion in *td* exon I. (B) Analysis of *rnh* mutant. Donor allele: *td*$^+$ (lanes 1 and 2) or *td*ΔEI (lanes 3 and 4). Phage exonuclease genotype: wild type (lanes 1 and 3) or *rnh* (lanes 2 and 4). (C) Analysis of 3′-5′ exonuclease mutants. Donor allele: *td*$^+$ (lanes 1–4) and *td*ΔEI (lanes 5–8). Phage exonuclease genotype: wild type (lanes 1 and 5), *dexA* (lanes 2 and 6), *43exo* (lanes 3 and 7), and *dexA 43exo* (lanes 4 and 8).

*43*Exo, were examined. The polymerase mutant *43exo* carries an amino acid substitution, D219A, resulting in deficiency in the 3′-5′ exonuclease while leaving the DNA polymerization activity intact (Frey *et al.* 1993; Reha-Krantz and Nonay 1993). In contrast to the T4 *rnh* infection, the levels of intron homing in T4 *dexA*, T4 *43exo*, and T4 *dexA 43exo* infections were consistently higher than that of the wild type (Figure 2C, lanes 1–4; Table 1). The homing efficiency was *ca.* 3.4-fold elevated with T4 *dexA*, and 1.6- and 1.9-fold elevated with T4 *43exo*, and the T4 *dexA 43exo* double mutant, respectively. To verify these results, a *dexA* amber mutant was used to infect *E. coli* B Sup° and *E. coli* B-*supE* suppressor-plus (Sup$^+$) hosts. The homing efficiency of the T4 *dexA* amber mutant was reproducibly twofold higher in the Sup° than in the Sup$^+$ host (data not shown). The higher levels of intron homing were attributed to the persistence of 3′-recombinogenic ends in the exonuclease-deficient backgrounds.

Because *43*Exo and DexA are the two major T4 3′-5′ exonucleases (Warner *et al.* 1972), and because exonuclease activity is expected to be required for removal of the resection segment, it seems paradoxical that the T4 *dexA 43exo* double mutant supported a higher level

TABLE 1

Summary data of intron homing

td exons:[a]	*td*$^+$			*td*ΔEI			Ratio:
Exonuclease[b]	Homing efficiency[c]	*n*	*P*[d]	Homing efficiency[c]	*n*	*P*	*td*Δ:*td*$^+$[e]
Wild type	0.76 ± 0.32 (1.00)	6	—	0.51 ± 0.22 (1.00)	5	—	0.67
RNase H	0.35 ± 0.18 (0.46)	4	0.014	0.11 ± 0.05 (0.22)	4	0.027	0.31
DexA	2.60 ± 1.16 (3.42)	6	0.029	0.86 ± 0.47 (1.67)	6	0.072	0.33
*43*Exo	1.25 ± 0.53 (1.64)	6	0.005	0.56 ± 0.32 (1.10)	7	0.086	0.45
DexA *43*Exo	1.44 ± 0.42 (1.89)	7	0.001	0.69 ± 0.09 (1.35)	6	0.008	0.48

[a] The intron donor phage has either wild-type exons (*td*$^+$) or an exon I deletion (*td*ΔEI) of 640 bp in the sequences immediately flanking the intron (Figure 2).
[b] The nuclease deficiency of the donor phage.
[c] The ratio of bands representing the intron homing product to the phage donor DNA on Southern blots (Figure 2). This ratio is presented as the mean with standard deviations for *n* independent experiments. Numbers in parentheses are normalized to the wild-type phage background, which in all cases is T4K10.
[d] Probability value resulting from paired *t*-test analysis.
[e] The *td*ΔEI:*td* ratio is derived from the means of the independent experiments.

of intron homing than the wild type (Figure 2C, lane 4; Table 1). While this observation implies that lengthy 3′ tails promote intron homing, it implicates a role for additional nucleases in the degradation of the resection segment. This function may be performed by additional 3′-5′ exonucleases or by endonucleases, with *E. coli* exonucleases I, III, and V, encoded by *sbcB*, *xthA*, and *recBCD*, respectively, as candidates. We assayed homing of a T4 *dexA* mutant in *E. coli* 3′-5′ exonuclease mutants *sbcB*, *xthA*, and *recBC*. High levels of homing were found for each single-mutant host as well as in an *xthA recBC* double mutant. In addition, high levels of homing were observed for a T4 *dexA 43exo* double mutant in an *sbcB* host (data not shown). These results suggest that, besides T4 DexA, *43*Exo, and the *E. coli* exonucleases, there are other yet-undefined exo- and/or endonuclease activities involved in the resection of recipient DNA during intron homing.

Homing exonucleases are required to degrade heterologous sequences: To increase the requirement for exonucleolytic degradation, a 640-bp deletion was made immediately flanking the *td* intron in exon I of the phage donors (Figure 2A, right), introducing a 640-bp sequence heterology between intron donor and recipient. This modified donor provided a more sensitive assay for the role of exonuclease function because heterologous sequences must presumably be degraded in the recipient to the point of sequence homology for efficient intron homing. Furthermore, the role of a phage function in replication (*e.g.*, RNase H) becomes less of a consideration when comparing wild-type with exon-deleted donors in an otherwise identical phage genetic background. Exon I was selected for deletion to avoid complexities introduced by persistent binding of I-*Tev*I to product sequences downstream of the cleavage site (Mueller *et al.* 1996b).

Exon-deleted phage donors with additional mutations in the *rnh*, *dexA*, and *43exo* genes were tested for the effect of the exonuclease mutations on intron homing, *i.e.*, on degradation of heterologous sequences (Table 1; Figure 2B, lanes 3 and 4; Figure 2C, lanes 5–8). Because phage donors carry the *td* exon I deletion (*td*ΔEI), the sizes of restriction fragments of both phage donor and recombinant DNA in each infection are 640 bp shorter than that of wild type (compare the 1.3- and 1.9-kb fragments to the 1.9- and 2.5-kb fragments in Figure 2, A–C). Homing efficiency in each infection was compared between the wild-type phage and those with the *td*ΔEI deletion, with and without exonuclease mutations (Figure 2, B and C; Table 1). Parental phage with *td*ΔEI exons supported homing ~67% as efficiently as *td*$^+$ phage (Table 1, *td*Δ:*td*$^+$ ratio), a decrease consistent with homing levels attained when exon homology is reduced (Parker *et al.* 1999). In contrast, in the T4 *rnh* infection, the homing efficiency with the *td*ΔEI donor was reduced to ~31% of that of the *td*$^+$ donor. Thus, homing appears to be more highly dependent on RNase H in the presence of extensive heterology, when the demands on 5′-3′ degradation are presumably greater.

Similarly, in T4 *dexA*, T4 *43exo*, and T4 *dexA 43exo* infections, there was a drop in homing efficiency to between one-half and one-third of the *td*$^+$ donor in the equivalent T4 exonuclease-deficient background, with *td*ΔEI/*td*$^+$ ratios of 33, 45, and 48%, respectively. The significantly decreased levels of intron homing with *td*ΔEI relative to *td*$^+$ donors in the absence of DexA and *43*Exo indicate a role for these 3′-5′ exonucleases in removing flanking heterology during intron homing.

***In vitro* exonuclease activities on natural and artificial substrates:** Because of the rapid degradation of DNA after T4 infection and the difficulty of monitoring this process *in vivo*, the ability of RNase H, DexA, and *43*Exo to effect DNA resection was examined *in vitro* (Mueller *et al.* 1996b). Extracts prepared 17 min after infection of cells with either wild-type T4K10 or exonuclease-mutant phage were compared (Figure 3). The 5′-3′ activity was

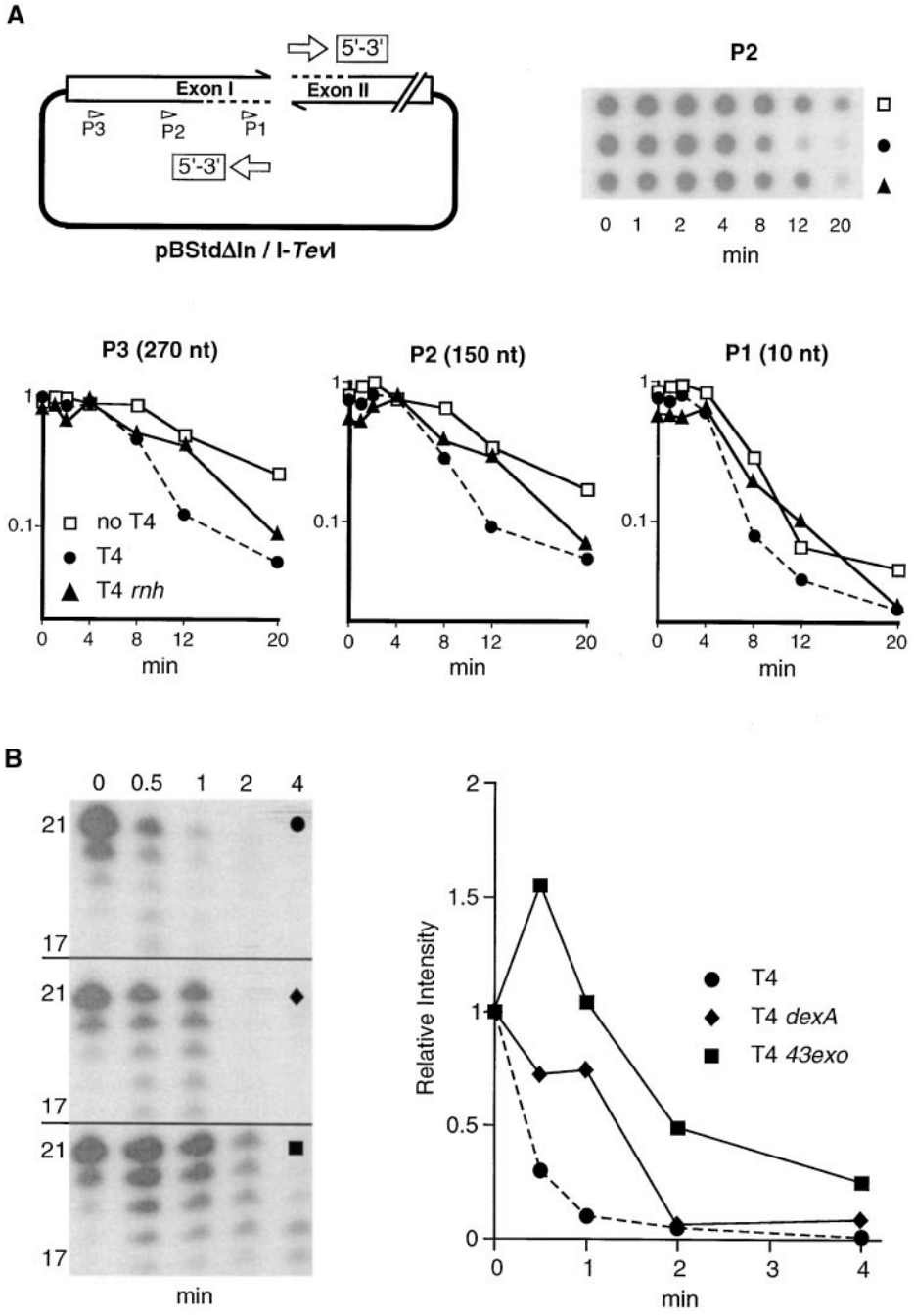

Figure 3.—*In vitro* detection of exonucleolytic degradation. (A) 5′-3′ degradation. Top left, schematic of the linear DNA substrate, pBS*td*ΔIn cleaved with I-*Tev*I. Probes P1–P3, complementary to the strand of exon I with a 5′ end, were used to monitor 5′-3′ degradation. Top right, representive dot-blot analysis using P2 as probe. Extracts prepared from uninfected (□), T4K10-phage-infected (●), and T4K10 *rnh*-infected cells (▲) were incubated with pBS*td*ΔIn linearized with I-*Tev*I for 20 min and analyzed as described in materials and methods. Blots were quantified with a PhosphorImager, and the results were plotted below for P1–P3. (B) 3′-5′ degradation. Extracts prepared from cells infected with T4K10 (●), T4K10 *dexA* (◆), and T4K10 *43exo* (■) were incubated with a 5′-labeled, 21-nt substrate, W606, to measure 3′-5′ exonucleolytic degradation. Total products ranging from 17 to 21 nt were quantified on a PhosphorImager and plotted. The trends of both 5′-3′ and 3′-5′ degradation analyses were reproducible over at least three independent experiments.

measured by the degradation of an I-*Tev*I-linearized intron recipient plasmid (pBS*td*ΔIn / I-*Tev*I) in a quantitative dot-blot assay using oligonucleotide probes complementary to the strand undergoing degradation in *td* exon I (Figure 3A; Mueller *et al.* 1996b). The probes hybridize 10 nt (P1), 150 nt (P2), and 270 nt (P3) from the CS. While in all extracts there was less degradation for probes that are further upstream of the CS (Figure 3, *cf.* P1 with P2 and P3), extracts from T4 *rnh*-infected cells reproducibly displayed a reduced level of 5′-3′ exonucleolytic degradation compared with extracts from wild-type infection. Probing of the complementary strand indicated that the 3′ strand was not degraded by the rnh^+ extract, verifying that 5′-3′ degradation was specifically measured in the assay (data not shown). The *in vitro* dot-blot analysis therefore supports the role of RNase H in the process of 5′-3′ degradation with homing-site-specific substrates.

Because the 3′-5′ exonucleases of interest act on single-stranded substrates, the above assay was inappropriate, and the *in vitro* nuclease activities of DexA and *43*Exo were examined on a 5′-labeled oligonucleotide substrate (Figure 3B). The 3′-5′ degradation of a 21-mer oligonucleotide that represents the sequence immediately upstream of the CS was monitored after incubation with extracts from phage-infected cells. The reaction

products were purified and subjected to denaturing gel analysis. Because the oligonucleotide is 5′ labeled, only the intact and 3′-5′-degraded substrates retain radioactivity. Degradation was compared from nt 21 down to nt 17, as no degradation products accumulated in this window. It is thus clear that less degradation occurred when incubating the labeled single-stranded substrates with extracts from T4 *dexA*- and T4 *43exo*-infected cells than with extracts prepared from wild-type infections. In contrast to the predominant effect of the *dexA* mutation in the *in vivo* homing assay (Figure 2; Table 1), extracts prepared from T4 *43exo*-infected cells consistently showed a greater deficiency in degradation of the oligonucleotide substrate (Figure 3B). The paradoxical results between the two experiments will be discussed.

Limited coconversion of flanking markers in exonuclease mutants: The combined action of the exonucleases at the DSB creates a gap in the recipient molecule (Figure 1B). This gap necessitates the acquisition of exon markers from the donor DNA during the repair process. To complement the *in vitro* analysis of exonucleolytic degradation, an *in vivo* genetic analysis of coconversion of flanking exon markers in wild-type and nuclease-deficient phage was performed. The coconversion assay takes advantage of the T4 phage's ability to package plasmid DNA after infection (Wilson *et al.* 1979; Kreuzer and Alberts 1986; Clyman and Belfort 1992; Mueller *et al.* 1996b). T4K10, with amber mutations in two packaging functions, served as an intron donor to infect cells carrying a plasmid-borne intron recipient (pACYC*td*ΔIn▽RS; Mueller *et al.* 1996b). The recipient plasmid carries four polymorphic restriction sites that are 21, 112, 360, and 535 bp upstream of the CS in *td* exon I (markers a–d in Figure 4; Mueller *et al.* 1996b). After infection, phage particles were adsorbed onto a Sup° *E. coli* B host, and the plasmid-containing transductants were selected on tetracycline-containing medium. Intron-plus recombinants were screened by PCR, and the loss of markers a–d was monitored by restriction enzyme analysis of the exon-I-containing PCR products of a single homing event from each independent infection (Figure 4A). The percentage of events that showed coconversion at each site was plotted for the wild-type T4K10 and three nuclease-defective variants (Figure 4B). The ratio of coconversion at sites a–d for each mutant relative to the wild type was also plotted (Figure 4C).

For each infecting phage, exon coconversion showed a bias, with markers close to the CS being inherited at higher frequency (Figure 4B). The polarity is attributed to exonuclease progression along the DNA. Although the transduction level in T4 *rnh* infections dropped to 5–10% of wild-type infections (data not shown), sufficient recombinants were generated for analysis (n = 61). Coconversion frequencies of markers that were within 112 bp of the CS in T4 *rnh* infections did not deviate appreciably from those of the wild-type phage (Figure 4, B and C, markers a and b). However, differences between the wild-type and *rnh* phage infections became clear for the distal markers c and d, which are located 360 and 535 bp upstream of the CS. The coconversion frequencies in the *rnh* infection were 66 and 30% of the wild-type level for markers that are 360 and 535 bp upstream of the CS, respectively, indicating the *in vivo* effect of RNase H in the exonucleolytic process.

In T4 *dexA* and T4 *43exo* mutant infections, there was less coconversion for all markers examined along *td* exon I. Compared with the wild-type infection, the coconversion frequency was ~80% of wild-type levels in marker a, 21 bp upstream to the CS (Figure 4, B and C). For marker d, which is 535 bp away from the CS, the coconversion frequency in the T4 *dexA* infection dropped to 39% of the wild-type level, whereas that in the T4 *43exo* infection dropped to 24% of the wild type (Figure 4C). The difference between the *rnh* infection and the *dexA* and *43exo* infections suggests that DexA and *43*Exo process DNA along the entire coconversion tract, whereas RNase H is more involved in extensive exonucleolytic degradation, as discussed below. The reduced levels of coconversion by the *rnh* and *dexA* mutants most likely reflect deficiencies in exonucleolytic degradation. Furthermore, the limited coconversion of flanking markers suggests that, in addition to their role in intron homing, RNase H, DexA, and *43*Exo influence the inheritance of donor sequences in the flanking exons.

DISCUSSION

T4 exonucleases RNase H, DexA, and *43*Exo were examined by molecular, biochemical, and genetic analyses for a role in intron homing. T4 gp46/47 was not studied because homing is abolished in its absence (Mueller *et al.* 1996a). The altered levels of intron inheritance in T4 *rnh*, T4 *dexA*, and T4 *43exo* infections compared with the wild-type phage infection implicate the 5′-3′ exonuclease T4 RNase H and the two major T4 3′-5′ exonucleases, DexA and *43*Exo, in intron homing (Figure 1). The role of these exonucleases in the degradation of heterologous sequence between donor and recipient was also implied (Figure 2 and Table 1). *In vitro* assays corroborated the 5′-3′ exonuclease deficiency in T4 *rnh*-infected cell extracts, as well as the 3′-5′ exonuclease deficiency in T4 *dexA*- and T4 *43exo*-infected cell extracts (Figure 3), consistent with limited coconversion of flanking markers in T4 *rnh*, *dexA*, and *43exo* mutant infections (Figures 3 and 4). These results support the roles of RNase H, DexA, and 43Exo in both intron homing and coinheritance of flanking exon sequences.

The 3′ tail as a major determinant of intron homing efficiency: Interestingly, while infection with T4 *rnh* resulted in decreased levels of intron homing, elevated

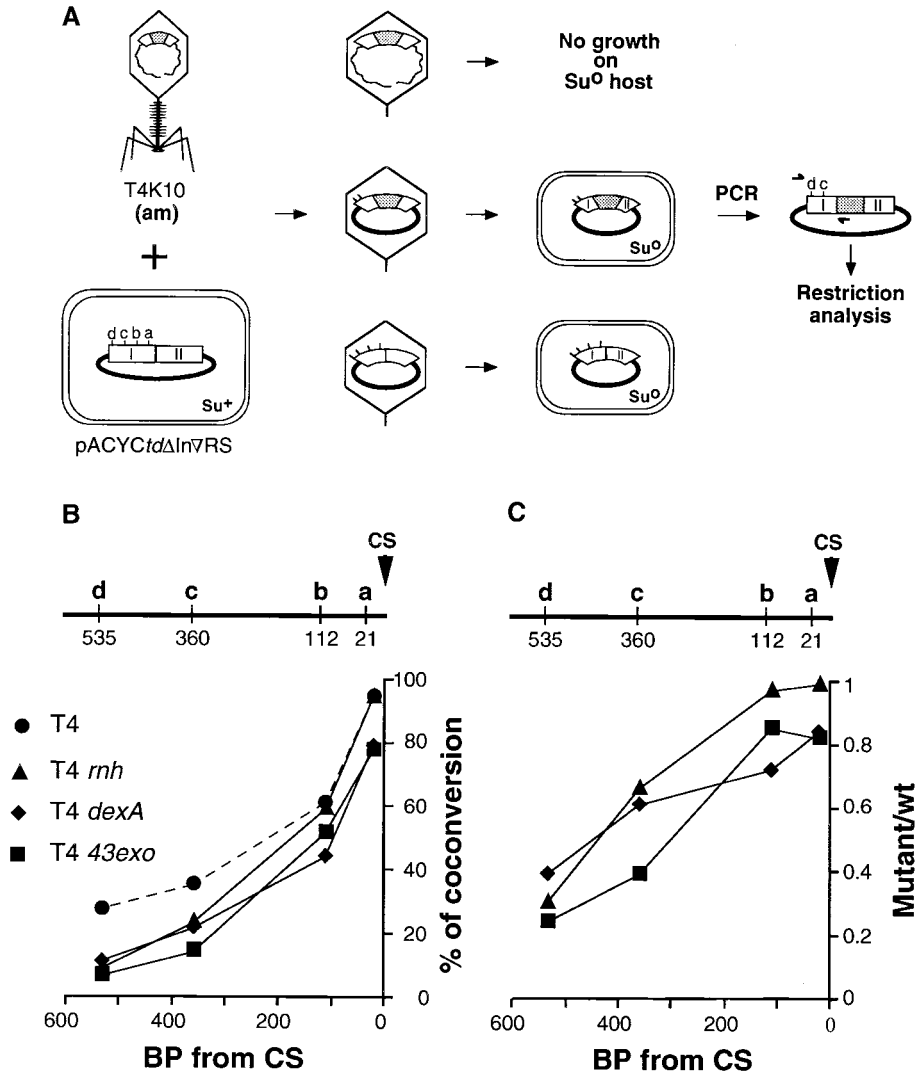

Figure 4.—Coconversion of flanking markers in intron homing. (A) Schematic of transduction assay. T4 phage serves as a transducing agent to infect Sup^+ cells containing a plasmid-borne intron recipient (pACYC-*td*ΔInV RS), which carries four polymorphic restriction markers on exon I (a–d). After infection, only transductants are able to grow on a Sup° host. The coconversion frequency of each marker was measured by the loss of restriction enzyme sites in the recombinant plasmid from individual homing events by a PCR-based restriction analysis. The restriction enzyme sites in exon I are as follows: a, *Spe*I; b, *Nar*I; c, *Bss*HII; d, *Nae*I, located 21, 112, 360, and 535 bp, respectively, upstream of the CS. (B) Quantitative analysis of individual conversion events in T4 wild-type (●, 140 events), *rnh* (▲, 61 events), *dexA* (◆, 66 events) and *43exo* (■, 66 events) infection. In a χ^2 test, all differences from wild type are significant ($P < 0.05$), except for markers a and b in the *rnh* infections. (C) Coconversion ratio of mutant to wild-type phage for each marker examined. (▲) T4*rnh*:T4; (◆) T4*dexA*:T4; (■) T4*43exo*:T4.

homing levels were observed with T4 *dexA* and T4 *43exo* (Figure 2, B and C). These results indicate the importance of 5′-3′ nucleases, including RNase H, in generating 3′ tails for invasion of the donor allele. The elevation of homing when 3′-5′ degradation was reduced in T4 *dexA* and T4 *43exo* backgrounds suggests that the persistence of 3′ recombinogenic ends to invade the donor alleles boosts subsequent repair synthesis (Figure 5, B and C). Further support was derived from a genetic assay in which homing was depressed by reductions in exon length. When there was only 25 bp of homology flanking the break, homing was greatly elevated in a *dexA* mutant compared to wild type, suggesting that degradation of the 3′ tail by DexA leads to reduced homing efficiency (Parker *et al.* 1999). By virtue of the 3′ tail influencing the efficiency of synapsis, the 3′-5′ exonucleases are major determinants in intron homing. All models of T4 recombination predict the involvement of 3′ ssDNA ends (Mosig 1998). Indeed, the stimulatory effect on homing in the absence 3′-5′ exonuclease activities provides the first direct evidence for 3′ ssDNA ends as intermediates in T4 recombination. This is consistent with evidence in favor of ssDNA tails with 3′ polarity being critical recombination intermediates, provided by the demonstration of lengthy 3′ tails (>1 kb) associated with the λ Red recombination pathway (Hill *et al.* 1997). Furthermore, in yeast, meiotic DSB repair is blocked in *rad50S* mutants in which the 3′ tails do not form (Sun *et al.* 1991). We propose that the more stable and lengthy the 3′ tail, the more successful the search for homology and, when found, the more efficient the synaptic complex and heteroduplex formation (Figure 5C).

There are several noteworthy aspects of the 3′-5′ exonucleases. First, although mutation of *dexA* has a more dramatic effect on the homing efficiency than mutation of *43exo* (Table 1, td^+ and *td*ΔEI donors), *in vitro* kinetic analysis indicates that *43*Exo is more active over very short distances (Figure 3B). This paradox may reflect differences in substrate affinity, processivity, and/or kinetics of degradation. Indeed, the rate of degradation by DexA can vary significantly depending on the se-

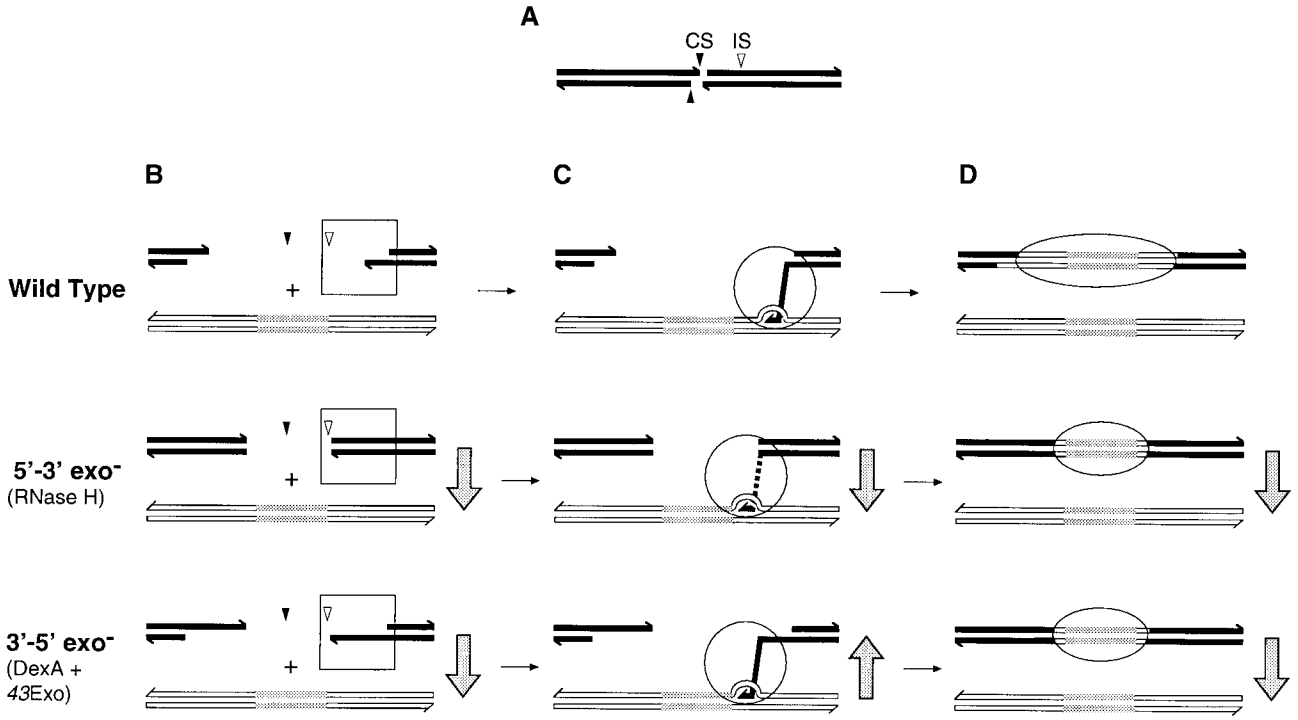

Figure 5.—Relationship of nuclease activity to homing efficiency and coconversion. (A) Cleavage of homing site. (B) Degradation, (C) strand invasion, and (D) coconversion for the wild-type and mutant infections. ⇓ or ⇑, decrease or increase, respectively, relative to wild type. (B) Squares highlight processed ends. 5′-3′ exonuclease deficiency eliminates 3′ tails while simultaneously blocking the activity of the single-strand-specific 3′-5′ nucleases; 3′-5′ exonuclease deficiency results in extended 3′ tails. (C) Circles highlight synaptic complexes. Strand invasion, proposed to correlate with homing efficiency (H.E.), is inhibited with 5′-3′ exonuclease deficiency and stimulated with 3′-5′ exonuclease deficiency in direct relationship to the length of the 3′ tail. (D) Ovals highlight coconversion tracts. These are limited by both 5′-3′ and 3′-5′ exonuclease deficiencies.

quence of the DNA substrate (Gruber *et al.* 1988). Our results suggest that *43*Exo has a higher affinity for a ssDNA end and/or more rapid kinetics than DexA, but lower processivity, acting only over a very short distance before releasing. Support for the model comes from the role each nuclease plays during the phage life cycle. DexA is thought to degrade the host DNA as well as the long 3′ ssDNA tails produced during T4 replication (Gauss *et al.* 1987; Gruber *et al.* 1988), whereas the degradation tract of *43*Exo is very short, limited to removal of a misincorporated nucleotide at the 3′ end of newly synthesized DNA (Hershfield and Nossal 1972; Huang and Lehman 1972).

Second, the efficiency of homing of the T4 *dexA 43exo* double mutant is intermediate between that of the two single mutants, rather than being additive, suggesting that the two nucleases are acting on the same recombination pathway. We presume that the severe replication deficiency of the double mutant prevents homing from attaining the level produced by the *dexA* single mutant. Regrettably, further study of the T4 *dexA 43exo* double mutant by coconversion analysis and *in vitro* degradation is hampered by insufficient phage production. Third, when heterologous sequences need to be resected, homing efficiencies of the 3′-5′ exonuclease mutants are less elevated relative to the wild type, as reflected in lower *td*Δ:*td*$^+$ ratios for the exonuclease mutants than for the wild type (Table 1). Removal of the resection segment is requisite to homing, both in principle (Figure 1) and as evidenced by 100% coconversion of markers residing within the segment (Bell-Pedersen *et al.* 1989). The data suggest that DexA and *43*Exo contribute to 3′-end processing, and it is likely that they are involved in removal of the resection segment, hence the reduced *td*Δ:*td*$^+$ ratios for the *dexA* and *43exo* mutants relative to parental phage. These results imply that a balance is struck between the requirement for resection to the point of homology and maintaining adequate 3′ tails, both of which are required for successful strand invasion.

In contrast to the 5′-3′ exonuclease requirement, the requirement for 3′-5′ exonucleolytic degradation is not obvious in many recombination systems. In *Saccharomyces cerevisiae*, efficient recombination was observed without 3′-5′ exonucleolytic degradation (Sun *et al.* 1991). Furthermore, in a Xenopus oocyte system, nonhomologies at the ends of an otherwise efficient substrate greatly reduced recombination, suggesting the absence of any activity that can efficiently remove 3′ tails (Jeong-Yu and Carroll 1992). In contrast, in *S. cerevisiae*, where 3′-homologous tails are stable (Sun *et al.* 1991), elaborate complexes exist for removing 3′-

nonhomologous tails. Whereas repair endonucleases RAD1 and RAD10, mismatch repair proteins MSH2 and MSH3, and helicase SRS2 are all required to remove lengthy heterologies, the 3′-5′ proofreading activity of DNA polymerase δ suffices to remove 3′-nonhomologous tails shorter than 30 nt (Paques and Haber 1997).

Homing efficiencies and coconversion frequencies are not directly related: With T4 *rnh* as the intron donor, both homing efficiency and coconversion frequency are reduced (Figures 2B, 4 and 5, B and D). The reduction in 5′-3′ exonuclease activity is suspected to lead to a shortening of the 3′ tails, resulting in a reduction in strand invasion and, therefore, homing efficiency (Figure 5C). Because T4 RNase H removes RNA from RNA-DNA hybrids, we cannot rule out that the reduced homing levels are an indirect effect of the absence of this function (Woodworth and Kreuzer 1996). The replication impairment in *rnh* mutant phage may also adversely affect homing levels. However, the coconversion analysis argues in favor of a direct role for the 5′-3′ DNA exonuclease activity of RNase H. The reduced 5′-3′ activity in the RNase H mutant (Figure 5, B and D) would be expected to also result in diminished 3′-5′ resection because both DexA and *43*Exo are single-strand-specific exonucleases, leading to shorter coconversion tracts. Interestingly, the reduction in coconversion in the RNase H mutant is seen only at distal markers. This suggests that other nucleases or helicases are also involved in generating 3′ tails of sufficient length for strand invasion, with gp46/47 as a likely candidate for providing this function.

In contrast, mutation of 3′-5′ exonuclease activities results in an increase in homing efficiency, presumably because of preservation of the 3′ tails (Figure 5, B and C). However, defective 3′-5′ exonucleases also result in a reduction in coconversion tracts relative to the wild-type phage (Figure 5D). Limited coconversion likely results from strand invasion by the extended 3′ tail occurring close to the intron. Likewise, the lengthy, noninvading 3′ tail would limit coconversion of exon markers at the other end of the intron (Figure 5C). This is particularly the case, because the SDSA pathway, in which the recipient rather than the donor serves as a template for repair of the noninvading strand, plays a major role in intron homing (Mueller *et al.* 1996a).

The requirement for multiple nucleases in intron homing and DSB repair: Considering the eccentric cleavage of T4 homing endonucleases, along with detectable or even elevated levels of intron homing in T4 *rnh*, T4 *dexA*, and T4 *43exo* mutant infections in various exonuclease-deficient host backgrounds, one must conclude that there are yet-to-be identified endo- and/or exonuclease activities. In a λ-based homing system, it was also found that the level of intron homing decreased substantially in the absence of the major λ 5′-3′ exonuclease Redα and its associated annealing function (Clyman and Belfort 1992; Parker *et al.* 1996). However, the occurrence of residual homing in a *red*$^-$ background was indicative of multiple factors contributing to 5′-3′ degradation. Similarly, multiple exonucleases have been implicated in recombination and repair in *E. coli* and *Salmonella typhimurium* (Kushner *et al.* 1974; Miesel and Roth 1996; Razavy *et al.* 1996; Viswanathan and Lovett 1998), as well as in *S. cerevisiae* nuclear and mitochondrial systems (Sun *et al.* 1991; Huang and Symington 1993; Morishima *et al.* 1993; Zassenhaus and Denniger 1994; Fiorentini *et al.* 1997).

T4 gp46 and gp47, which form a putative exonuclease complex, are essential for both recombination-dependent replication and intron homing (George and Kreuzer 1996; Mueller *et al.* 1996a). Although modest deficiency in 5′-3′ exonucleolytic degradation was observed in the absence of gp46/47 in our *in vitro* dot-blot assay (data not shown), it is difficult to interpret the role of this protein complex in the absence of any detectable homing *in vivo* (Mueller *et al.* 1996a). Whereas genetic evidence supports a role for gp46/47 in generating ssDNA during recombination (Prashad and Hosoda 1972; Cunningham and Berger 1977, 1978), and the complex has been associated with 5′-3′ exonuclease activity (Mickelson and Wiberg 1981), *in vitro* biochemical evidence has not been forthcoming. Double-stranded ends do become stable in the absence of gp46/47, but whether this is owing to its own nuclease activity or another yet-unidentified function of the complex that ultimately leads to degradation is not yet clear (Mosig 1998). Further biochemical characterization of gp46/47 is therefore required before its role in homing can be determined.

Multiple roles of the T4 homing exonucleases: The 5′-3′ exonuclease activity of T4 RNase H is important in removing the short RNA primers from the DNA replication fork for discontinuous, lagging-strand DNA synthesis. Also, the activity of RNase H on double-stranded DNA suggests a role in other aspects of nucleic acid metabolism (Woodworth and Kreuzer 1996). Further supporting its mutlifunctional nature, T4 RNase H shares sequence homology with a group of proteins involved in recombination and repair, such as phage T7 gene 6 exonuclease, phage T5 D15 exonuclease, and the N-terminal 5′-3′ exonuclease domain of *E. coli* DNA polymerase I (reviewed by Mueser *et al.* 1996).

Likewise, the 3′-5′ exonucleases DexA and *43*Exo have interesting cellular functions. While DexA degrades host DNA to generate precursors for DNA synthesis, gp43 is the polymerase that replicates T4 phage DNA, with the *43*Exo activity serving a proofreading function (Reha-Krantz 1994). While DexA bears no obvious similarity to other proteins in the DNA database, T4 DNA polymerase, with DNA synthesis and 3′-5′ exonuclease activities in a single polypeptide, shares sequence homology with family B DNA polymerases from other phages, viruses, yeast, and humans (Ho and Braithwaite 1991). The efficiency of intron homing

appears to be determined by a balance between these multifunctional 5′-3′ and 3′-5′ nuclease activities. Whereas RNase H stimulates intron homing and DexA and *43*Exo depress the process, all three functions extend exon coconversion tracts and, therefore, influence the genetic consequences of an intron homing event. Thus, mobile group I phage introns, by virtue of their invasiveness and their ability to harness functions involved in the life cycle of their phage host, ensure their own propagation as selfish DNA elements.

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