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

Yi-Jiun Huang, Monica M. Parker and Marlene Belfort

Molecular Genetics Program, Wadsworth Center, New York State Department of Health and School of Public Health, State University of New York, Albany, New York 12201-2002

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ABSTRACT

Homing of the phage T4 td intron is initiated by the intron-encoded endonuclease I-TevI, which cleaves the intronless allele 23 and 25 nucleotides upstream of the intron insertion site (IS). The distance between the I-TevI 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 nuclease activity 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 (43Exo), 3'-5' exonuclease activity, 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 43Exo 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 (UvX), 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-depen-
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 (Moscig 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, however, there has been little biochemical evidence to support the 5'–3' nuclease activity of any except RNase H. DNA exonuclease A (DexA) (Warner et al. 1972) and the proofreading exonuclease domain of T4 DNA polymerase, 43Exo 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, 43Exo 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 43Exo are involved in the homing process, and that the integrity of 3' tails is a critical determinant in the intron homing process.

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).

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-TevI cleavage site (CS) (Bryk et al. 1995; Mueller et al. 1995). (B) Intron homing. The intron-encoded endonuclease I-TevI cleaves the intron recipient allele 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).
The 640-bp td\Delta Ei deletion immediately upstream of the td intron was introduced into T4K10 and its exonuclease-deficient derivatives by using pAlAEi-3 (Parker et al. 1996) and the T4 insertion/substitution system (Selick et al. 1988). The td\Delta Ei 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-born dexA amber mutation was constructed by site-directed mutagenesis using the GeneEditor site-directed mutagenesis system (Promega, Madison, WI). T4K10 dexAam 43\alpha and its td\Delta Ei derivatives were constructed by marker rescue, in which T4K10 43\alpha and its td\Delta Ei derivative were crossed with the plasmid-born dexA amber mutant. The intron recipient plasmid pSUtd\DeltaIn confers chloramphenicol resistance and contains the EcoRI-EcoRI td fragment with a precise deletion of the intron (Chu et al. 1984; West et al. 1986). The BamHI site of the vector was deleted, and the td fragment was in the reverse orientation relative to plac to prevent toxic expression of I-TevI (also referred to as dt) (Claman and Belfort 1992). The recipient plasmid used in the coconversion analysis is pACYCtd\DeltaIn\N, which conferstetracycline resistance and contains four polymorphic restriction sites in each exon of the 1.4-kb td\DeltaIn fragment (MueIer et al. 1996b).

Oligonucleotides: Oligonucleotides used to measure the 5'-3' degradation of P1, 5'-TGTTTGGTTCTGATCTACA AC-3'; P2, 5'-TATTGATCGTTATTTATGATCA -3'; P3, 5'-GGCCTATTGTCCGTCAGGTG-3'; located 10, 150, and 270 bp, respectively, upstream of the CS in exon I of the td gene (MueIer et al. 1996b). The oligonucleotide used for 3'-5' degradation analysis was W606, 5'-TGATTACCTGCTCAACCCCTAA CAAA-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'-GTGTTAATTGG CGGGCCTGTCCTGTTATATGTC-3' and the intron primer MB17, 5'-TGTTCTACCTAGAGATGCTCCCG-3'.

Phage-to-plasmid homing assay: Host cells, E. coli B (Sup'), containing the recipient plasmid pSUtd\DeltaIn were grown at 37°C in TBYE (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with chloramphenicol (25 \mu g/ml) to an OD_650 of 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, concentrated DNA was prepared, and Southern hybridization was performed as described (MueIer 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 by a PhosphorImager (Molecular Dynamics) using ImageQuant software.

T4-infected cell extracts: Extracts from T4-infected cells were prepared according to MueIer et al. (1996b). E. coli B was grown to a density of 3 \times 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°C, cells were harvested by centrifugation and frozen at -80°C. Cell pellets were lysed, nucleic acids were removed by streptomycin sulfate precipitation, and proteins were harvested by ammonium sulfate fractionation exactly as described (MueIer et al. 1996b). The quality of each extract was determined by measuring I-TevI activity, in which 250 ng of Scal-linearized pBSstd\DeltaIn was incubated with 5 \mu l of cell extract at 37°C for 5 and 20 min and assayed for cleavage by agarose gel electrophoresis (BelI-Peder sen et al. 1991).

Nuclease assays: The DNA substrate for the 5'-3' degradation analysis was an I-TevI-linearized plasmid containing the td homing site (pBSstd\DeltaIn). In each reaction, 100 ng of linearized DNA was incubated with 5 \mu l of cell extract containing 350 \mu g/ml of partially purified protein (MueIer et al. 1996b) for 20 min at 37°C in JBB buffer (50 mm TrisHCl, pH 8.0, 20 mg/ml poly[dI/dC], 10 \mu g/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 (MueIer 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 polyadenylation kinase (GIBCO-BRL) according to the manufacturer's instructions. The oligonucleotide was incubated with cell extract for the indicated times at 24°C, 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 Claman and Belfort (1992). E. coli Sup cells harboring an intron-deleted recipient plasmid, pACYCtd\DeltaIn\NRS (Tet'), which carries restriction site markers in the exons, were infected with the parental phage T4K10 and its dexA, rnh, and 43\alpha derivatives. Infection was at 37°C 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°C, 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 (MueIer 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-born intronless allele (pSUtd\DeltaIn), levels of intron homing and phase DNA replication were measured by restriction and Southern hybridization analysis using an intron-specific probe. The relative ratio of intron homing product to donor phase 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 mh mutant (Figure 2B, 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 (tdwt) or with a 640-bp deletion of exon I (tdΔEI) served as intron donors to infect cells containing a plasmid-borne intron recipient (pSUtdΔIn). 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: tdwt (lanes 1 and 2) or tdΔEI (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: tdwt (lanes 1-4) and tdΔEI (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).

43Exo, 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 43Exo 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
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 sbB, xthA, and xthB, respectively, as candidates. We assayed homing of a T4 ΔexA mutant in E. coli 3'-5' exonuclease mutants sbB, xthA, and xthB. High levels of homing were found for each single-mutant host as well as in an xthA xthB double mutant. In addition, high levels of homing were observed for a T4 ΔexA 43exo double mutant in an sbB host (data not shown). These results suggest that, besides T4 DexA, 43Exo, 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 3' 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-TevI 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 3' deletion (ΔEI), the sizes of restriction fragments of both phage donor and recombinant DNA in each infection are 640 bp shorter than those of wild type (compare the 1.3- and 1.9-kb fragments to the 1.9- and 2.5-kb fragments in Figure 2A-C). Homing efficiency in each infection was compared between the wild-type phage and those with the ΔEI deletion, with and without exonuclease mutations (Figure 2, B and C; Table 1). Parental phage with ΔEI exons supported homing ~67% as efficiently as 3' deletion (Table 1, ΔEI/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 ΔEI donor was reduced to ~31% of that of the Δtd 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 ΔexA, T4 43exo, and T4 ΔexA 43exo infections, there was a drop in homing efficiency to between one-half and one-third of the Δtd donor in the equivalent T4 exonuclease-deficient background, with ΔEI/td + ratios of 33, 45, and 48%, respectively. The significantly decreased levels of intron homing with ΔEI relative to Δtd donors in the absence of DexA and 43Exo 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 43Exo 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

### Summary data of intron homing

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<th>Exonuclease</th>
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<td>0.008</td>
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* The intron donor phage has either wild-type exons (td +) or an exon I deletion (ΔEI) of 640 bp in the sequences immediately flanking the intron (Figure 2).

* The nuclease deficiency of the donor phage.

* 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.

* Probability value resulting from paired t-test analysis.

* The ΔEI/td ratio is derived from the means of the independent experiments.

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In vitro detection of exonucleolytic degradation. (A) 5'→3' degradation. Top left, schematic of the linear DNA substrate, pBSΔln cleaved with I-Tev. Probes P1-P3, complementary to the strand of exon I with a 5' end, were used to monitor 5'→3' degradation. Top right, representative dot blot analysis using P2 as probe. Extracts prepared from uninfected (■), T4K10-phage-infected (●), and T4K10 rnh-infected cells (▲) were incubated with pBSΔln linearized with I-Tev 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.

Figure 3—In vitro detection of exonucleolytic degradation. (A) 5'→3' degradation. Top left, schematic of the linear DNA substrate, pBSΔln cleaved with I-Tev. Probes P1-P3, complementary to the strand of exon I with a 5' end, were used to monitor 5'→3' degradation. Top right, representative dot blot analysis using P2 as probe. Extracts prepared from uninfected (■), T4K10-phage-infected (●), and T4K10 rnh-infected cells (▲) were incubated with pBSΔln linearized with I-Tev 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.

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measured by the degradation of an I-Tev-linearized intron recipient plasmid (pBSΔln/I-Tev) in a quantitative dot blot assay using oligonucleotide probes complementary to the strand undergoing degradation in td exon I (Figure 3A; Müller 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 mh-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 mh+ 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 43Exo 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 vitro analysis of exonuclease 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 the inheritance of donor sequences in the flanking intron recipient (pACYC amber mutations in two packaging functions, served as the inheritance of donor sequences in the flanking, respectively, indicating the in vivo effect of RNase H in the exonuclease process.

In T4 dexA and T4 43exo mutant infections, there was less coconversion for all markers examined along ttd 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 43Exo process DNA along the entire coconversion tract, whereas RNase H is more involved in extensive exonuclease degradation, as discussed below. The reduced levels of coconversion by the rnh and dexA mutants most likely reflect deficiencies in exonuclease degradation. Furthermore, the limited coconversion of flanking markers suggests that, in addition to their role in intron homing, RNase H, DexA, and 43Exo influence the inheritance of donor sequences in the flanking exons.

**Discussion**

T4 exonucleases RNase H, DexA, and 43Exo 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' exonuclease, DexA and 43Exo, 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
homing levels were observed with T4 dεxA 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 dεxA 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 dεxA 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 synopsis, 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 dεxA has a more dramatic effect on the homing efficiency than mutation of 43exo (Table 1, td+ and tdΔEI donors), in vitro kinetic analysis indicates that 43Exo 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.

Our results suggest that 43Exo 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 processing, and it is likely that they are involved in removal of the resection segment, hence the reduced 3' ssDNA tails produced during T4 replication (Gauss et al. 1987; Gruber et al. 1988), whereas the degradation tract of 43Exo is very short, limited to removal of a misincorporated nucleotide at the 3' end of newly synthesized DNA (Hershfeld 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 tdA: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 43Exo contribute to 3'-end processing, and it is likely that they are involved in removal of the resection segment, hence the reduced tdA: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, non-homologies 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 43Exo 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' exonuclease 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 eccentrical 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 Redx 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 Denninger 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 (Prakash 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 multifunctional 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 Musser et al. 1996).

Likewise, the 3'-5' exonuclease activities DexA and 43Exo 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 43Exo activity serving a proofreading function (Rehakrantz 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 (Hao 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 43Exo 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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