Involvement of Single-Stranded Tails in Homologous Recombination of DNA Injected into Xenopus laevis Oocyte Nuclei

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Received 14 November 1990/Accepted 20 March 1991

Homologous recombination of DNA molecules injected into Xenopus laevis oocyte nuclei is extremely efficient when those molecules are linear and have overlapping homologous ends. It was previously shown that a 5’→3’ exonuclease activity in oocytes attacks injected linear DNAs and leaves them with single-stranded 3’ tails. We tested the hypothesis that such tailed molecules are early intermediates on the pathway to recombination products. Substrates with 3’ tails were made in vitro and injected into oocytes, where they recombined rapidly and efficiently. In experiments with mixed substrates, molecules with 3’ tails entered recombination intermediates and products more rapidly than did molecules with flush ends. Molecules endowed in vitro with 5’ tails also recombined efficiently in oocytes, but their rate was not faster than for flush-ended substrates. In most cases, the 5’ tails served as templates for resynthesis of the 3’ strands, regenerating duplex ends which then entered the normal recombination pathway. In oocytes from one animal, some of the 5’ tails were removed, and this was exacerbated when resynthesis was partially blocked. Analysis by two-dimensional gel electrophoresis of recombination intermediates from 5’-tailed substrates confirmed that they had acquired 3’ tails as a result of the action of the 5’→3’ exonuclease. These results demonstrate that homologous recombination in oocytes proceeds via a pathway that involves single-stranded 3’ tails. Molecular models incorporating this feature are discussed.

Homologous recombination events in many organisms appear to be initiated at the ends of DNA molecules. Viruses with linear DNA genomes have naturally occurring ends or transient linear forms that are known to be active in homologous recombination (13, 31, 36). Chromosomes may suffer double-strand breaks during replication, transcription, or repair and as a result of radiation or chemical damage (14, 32); a number of experiments have suggested that such breaks stimulate or initiate homologous recombination events (19, 20, 43). One well-understood example of homologous recombination, the interconversion of mating types in yeast cells, is initiated by a sequence-specific double-strand break at the site of conversion (37). It has recently been shown with physical methods that DNA sequences which are hotspots for gene conversion and exchange events also suffer frequent breaks in vivo (3, 38). Intentional breaks made in DNA molecules that are transfected into somatic cells stimulate homologous recombination events at the break sites in diverse species, including prokaryotes, fungi, and cultured cells from both plants and mammals (16, 24, 40, 43, 44).

Double-strand breaks are thought to elevate levels of homologous recombination in their vicinity by serving as substrates for cellular or viral enzymatic activities that act at molecular ends. In Escherichia coli, duplex ends are used as entry sites for the RecBCD enzyme, which travels along DNA and promotes initiation, or possibly resolution, of recombination events (35, 41). Other genes affecting recombination in E. coli and some bacteriophages encode strand-specific exonucleases that digest duplex DNA molecules and leave protruding single-stranded tails (9, 27). DNA molecules with single-stranded tails or displaced single strands have long been invoked to explain the initiation of homologous recombination events (17, 24, 30, 40). Conclusive evidence for the role of single strands has been elusive, since most information about molecular mechanisms has been inferred from analyzing the ultimate products of recombination, not the intermediates themselves. There is, however, strong evidence (both genetic and physical) that molecules with protruding 3’ ends are used in recombination in some E. coli pathways, in bacteriophages, and in Saccharomyces cerevisiae (13, 21, 23, 31, 39, 42, 46).

We have been studying recombination events between DNA molecules injected into Xenopus oocytes, in which both inter- and intramolecular events occur efficiently (4, 28). We have shown that only linear molecules are competent to recombine, that homologous ends are by far the most efficient substrates for recombination, and that linear DNA is degraded by a 5’→3’ exonuclease activity in oocyte nuclei (4, 28, 29). The natural suggestion was that the single-stranded 3’ tails left by this nuclease were important intermediates in the recombination process (29). We have tested this hypothesis by making 3’ tails on plasmid substrates in vitro and monitoring the fate of these molecules after injection. We show here that the 3’ tails were stable during incubation in oocytes and that the rate of recombination of the tailed molecules was accelerated relative to that of flush-ended molecules. Molecules with 5’ tails were also efficiently recombined in oocytes but were first converted to flush-ended duplexes by resynthesis of the missing strand or (more rarely) by removal of the 5’ tail. We believe the data best support a nonconservative, single-strand annealing mechanism, which has been proposed to explain recombination of DNA molecules transfected into mammalian somatic cells (24) and some types of recombination events in bacteria (7, 39).

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MATERIALS AND METHODS

Oocytes and injection procedures. Methods used for obtaining and culturing oocytes and for injecting and recovering DNA were described previously (29).

Preparation and analysis of plasmid DNA. The plasmids used as recombination substrates were pRW4 and pHRS3. pRW4 is a derivative of pRDK41 (11); it consists of pBR322 with a tandem repeat of the tetracycline resistance (Tet) gene separated by a unique XhoI site (29). Cleavage of pRW4 with XhoI produces a linear molecule having direct repeats of 1,246 bp at the ends. pHRS3 is identical to pRW4 except for two restriction sites at unique points that flank the tandem repeats. pHRS3 has a KpnI linker inserted into the SspI site (see Fig. 2) and an XhoI linker inserted into the StyI site. M13mp19 replicative form I (RFI) DNA was used as a nonhomologous, nonrecombining competitor DNA in some experiments.

Preparation of plasmid and M13 RFI DNAs, preparative and analytical restriction digests, hybridization methods, and probe preparation have been described (29). Both oligonucleotide (oligo) and random-primed (12) probes were hybridized to membranes by the method of Church and Gilbert (8). Two of the oligo probes were complementary to each other and were identical to pBR322 sequences 63 to 78 (oligo 1; 5'-GCTAACGGATCAGGC-3') and 78 to 63 (oligo 2; 5'-GCTCGACTGCTTCG-3'), near the N terminus of the Tet gene. Oligo 13 (5'-CGTCCGGTGCATGGAGCC-3') corresponds to pBR322 sequences 1241 to 1259, at the other end of the Tet gene. Oligo hybridizations were all done at 42°C; random-primed probes were hybridized at 60 to 65°C.

Preparation of tailed molecules. Linear molecules with 3' tails were prepared with T7 gene 6 exonuclease (U.S. Biochemical Corp., Cleveland, Ohio), and molecules with 5' tails were prepared with exonuclease III (ExoIII; New England BioLabs, Beverly, Mass.), as described in detail previously (29). In all experiments, the single-stranded tails were in the range of 300 to 500 nucleotides (nt), which is not sufficient to expose homologous sequences in the 1,246-bp overlap of the recombination substrates. The lengths of the tails and the synchrony of degradation were assessed by analysis of SI-treated molecules in neutral agarose gels and by determination of the lengths of individual strands in alkaline agarose gels. In the samples used here, >99% of the molecules were tailed. We occasionally observed aberrant electrophoretic behavior of molecules with single-stranded tails (e.g., Fig. 3). This was independent of extensive sequence homology and in some cases was minimized by heating the samples prior to injection or analysis.

Preparation of recessed 3' ends blocked with dideoxynucleotides. ExoIII-treated pRW4 was incubated with Klenow polymerase in the presence of all four dideoxynucleotides for 10 min at room temperature. The 50-μl reaction mixture contained 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 25 μM each of the dideoxynucleoside triphosphates (ddNTPs), and 10 U of Klenow polymerase. The reaction was quenched by addition of EDTA to 25 mM. To check the efficacy of the block to resynthesis, a portion of the reaction mixture was removed prior to quenching and diluted into a reaction mixture with the same buffer components containing [α-32P]dCTP and the three other dNTPs at 50 μM each. A similar reaction mixture with unblocked ExoIII-treated pRW4 and appropriately diluted ddNTPs was run as a control. The ddNTPs in these reactions were sufficiently diluted to allow synthesis from recessed 3' ends that had not been blocked. The amount of incorporated label was estimated by thin-layer chromatography of the products on polyethyleneimine-cellulose in 0.4 M NH₄HCO₃. The substrate used in the experiment shown in Fig. 6 was substantially blocked with the control but allowed a small amount of resynthesis by Klenow polymerase.

Two-dimensional (2-D) agarose gel electrophoresis. The method for 2-D gel electrophoresis has been described (10, 29).

RESULTS

Injection of molecules with 3' tails made in vitro. To test the idea that 3' tails are involved in homologous recombination in Xenopus oocytes (29), we first established that such molecules were capable of recombination and did not rapidly lose the tails by nuclease degradation. 3' tails of 300 to 350 nt were made on XhoI-linearized pRW4 with T7 gene 6 exonuclease, and these molecules were injected into oocytes in parallel with flush-ended pRW4 at equal concentrations. (The flush ends in these experiments were, in fact, 4-nt 5' tails created by digestion with XhoI. We have shown, however, that ends generated by restriction enzymes, whether they are flush or have 3' or 5' tails, are digested indistinguishably by the 5'→3' exonuclease [29]. The demonstration later in this report that 3' tails are rapidly made flush in oocytes provides additional justification for calling XhoI ends flush in this context.) Oocyte nuclei were recovered by manual dissection after various times of incubation, and the injected DNA was purified. Recovered DNA was analyzed by digestion with PvuII, which yields characteristic fragments from unrecombined substrate (3,560 and 2,050 bp) and recombinant product (4,363 bp). The presence of the tails was monitored with oligo probes complementary to sequences near the tip of the 2,050-bp substrate fragment (Fig. 1).

Figure 1 shows the results of this experiment. In Fig. 1A, the filter was hybridized with a whole plasmid probe, which allowed visualization of all substrate and product sequences. The samples from the two substrates were run in pairs, the tailed molecules on the right at each time point. In the un injected samples, fragments from the tailed molecules had a mobility faster than that of equivalent flush-ended fragments. Both tailed and untailed substrates recombined efficiently in the oocytes, as evidenced by conversion to the 4,363-bp band, but the molecules with tails had a greater ratio of product to substrate at short times of incubation. After 4 h most or all the DNA recovered from oocytes injected with tailed substrate had recombined, while the flush-ended substrate had produced considerably less than 50% product. In addition, at early times DNA from oocytes injected with tailed molecules had prominent smears of hybridization that migrated slower than the product. These smears represent intermediates in recombination (28, 29, 29a; see below).

Figures 1B and C show the same filter hybridized to oligo probes located near the XhoI end of the 2,050-bp fragment. The probe also hybridized to the 3,560- and 4,363-bp fragments at internal sites (Fig. 1C). Oligo 2 is complementary to the strand of the 2,050-bp fragment which ends 5'; it failed to hybridize to the molecules with 3' tails, as expected. After incubation, no evidence of resynthesis of this strand was seen. Oligo 1, complementary to the 3'-ending strand on the 2,050-bp fragment, hybridized to both the tailed and flush-ended molecules. The input tailed molecules increased in mobility on incubation in the oocytes but continued to
hybridize to this probe. These observations indicated that 3' tails remained intact during incubation, that new 5'-ending strands were not synthesized, and that 5'→3' degradation continued on the tagged molecules prior to their entry into recombination products.

We observed only a small amount of degradation of the flush-ended molecules during the course of recombination in this experiment (Fig. 1). Even after 4 h of incubation, no degraded unrecombined molecules were seen at these exposures. The ratio of degraded to intact unrecombined substrate molecules varies considerably in oocytes from different animals, as does the rate of recombination (28). In contrast, molecules with tails underwent further visible degradation during the course of the experiment. It may be that recessed 5' termini have a greater affinity for the exonuclease than do flush ends, so that degradation by a limited number of nuclease molecules is more evident with this substrate. In most oocytes (see, for example, Fig. 3, 5, and 7) degradation of flush ends is quite obvious.

Competition between flush-ended and tailed molecules. Several experiments with separately injected tagged and flush-ended substrates suggested that tagged molecules have a greater rate of recombination. Since recoveries vary among different samples (Fig. 1, 4-h time points) and since the concentration of molecules delivered per oocyte has an influence on the rate of recombination (28), we designed an experiment that allowed direct comparison of the rates of recombination of tagged and untailed molecules. Two equivalent but distinguishable substrates were injected into the same oocytes under conditions in which intramolecular recombination events predominate (28).

The two plasmids were pRW4 and a marked derivative of it, pHR3; they differ in restriction sites flanking the tail. The products of recombination (shown as monomer circles) can be distinguished by digestion with PstI (which produces monomer lines from all products of recombination) in combination with either KpnI or SpII, as shown. The sizes of the digestion products of either substrate are shown to the left (for unrecombined substrate) or right (recombinant product) of the pairs of digests. The small SpII-SpII fragments of either substrate were run on the same analytical gels and are not indicated here. Parentheses around the fragment sizes for the tailed substrate indicate that only one strand has this length, while the other has been reseated. This is reflected in more rapid gel mobilities of these fragments (see Fig. 3).
different mixtures was recovered and incubated for various times. Recombination was measured by digesting the recovered DNA with PstI. The time course of appearance of recombination products (4,363-bp band) was similar to those seen earlier and was nearly complete by 4.5 h.

Recombination of 5' tailed molecules. It was of interest to know whether molecules with 5' tails would also show accelerated kinetics of recombination. Conceivably, only 3' tails are normally created in oocytes, due to the specificity of the dominant nuclease, but tails of either polarity might be capable of participating in recombination. An example of an experiment with molecules having 5' tails (and recessed 3' termini) is shown in Fig. 4. Oocytes were injected with ExoIII-treated pHRS3 having 5' tails of 400 to 450 nt and

FIG. 3. Competition between 3' tailed and flush-ended substrates. Equimolar amounts of XhoI-linearized pHRS3 and pH4W were mixed with a 3:1 mass excess of Clal-digested M13mp19 RFI DNA and injected into oocytes at a total concentration of 10 ng per oocyte. Four different mixtures were made in which the recombination substrates were either flush ended (F) or endowed with 3' tails (T) prior to injection, as indicated in the top two lines. The oocytes were incubated for the times given; un injected samples are shown in the leftmost lanes. DNA was recovered and digested with enzyme mixtures K or S (Fig. 2). Electrophoresis was in a 1% agarose gel which was transferred and hybridized with a random-primed pBR322 probe. The 4,363- and 3,804-bp bands diagnostic of recombination products are marked to the right. In K digests, the 4,363-bp band is from pHW4 products and the 3,804-bp band is from pHRS3 products; this is reversed in the S digests (see Fig. 2). The second set of lanes for each time point corresponds to the situation diagrammed explicitly in Fig. 2, i.e., flush-ended pHRS3 plus 3' tailed pH4W.

analytical digests showed only recombinant fragments diagnostic for products from pH4W (i.e., K produced only the 4,363-bp band and S produced only the 3,804-bp band; Fig. 2). The characteristic smears of intermediates were also seen, but they associated only with the products from the tailed substrate. After 150 min in the oocytes, products and intermediates from both substrates appeared, but bands derived from the tailed substrate clearly predominated. After 24 h almost all of the recovered DNA was recombinant, and the products were present in a 50:50 ratio. This demonstrated that the input amounts of the substrates were equal, that both substrates were fully capable of recombining, and thus that the ratio of products at early times was due to a difference in the rates of recombination between the tailed and untailed substrates.

The kinetics of recombination in the other three mixtures completely support the assertions made for the sample just described. When the tailed and flush-ended substrates were reversed, the appearance of products specific for the two substrates also reversed. When both substrates were tailed, both products appeared at early times and in equivalent amounts. When neither substrate had tails, the kinetics of product formation was slower than for tailed molecules, but products from the two substrates appeared in equal amounts. This result shows that providing 3' tails accelerates the kinetics of recombination and implies that the strand-specific exonuclease activity in oocytes gives rise to molecules which enter the pathway of homologous recombination.

FIG. 4. (A to C) Processing of 5'-tailed substrates. ExoIII-treated linear pH4W having 450-nt 5' tails was mixed with an equal mass of PstI-digested M13 RFI DNA and injected into oocytes (10 ng per oocyte). DNA recovered after various times of incubation was analyzed by digestion with PstI. The filter was hybridized sequentially with a whole pBR322 probe and two oligo probes (D). Lanes: M, standard DNAs (XhoI-digested pH4W [5,610 bp], linear pBR322 [4,363 bp], and unrecombined pH4W XhoI-PstI fragments [3,585 and 2,025 bp]; 3'; un injected pH4W with 5' tails; 5'; un injected pH4W with 5' tails. Remaining lanes have injected 5'-tailed DNA incubated in oocytes for the times indicated. The un injected tailed DNAs show the specificity of the oligo probes, which hybridize near the XhoI tip of the 3,585-bp fragment. (D) Portions of the 3'-ending strands (half-arrowheads) removed by ExoIII are shown as dashed lines. Oligos are shown beside the strand whose sequence they share, and thus they hybridize to the opposite strand.
In un.injected samples, fragments from the tailed substrate (Fig. 4, lanes 5'T) were of faster mobility than flush-ended controls (lanes M). After as little as 10 min of incubation in oocytes, many of the injected molecules had the same mobility as flush-ended controls, implying that the missing strands were resynthesized.

Resynthesis was confirmed by hybridizing the same filter to oligo probes located near the xhol end of the 3,585-bp fragment (Fig. 4B and C). This fragment did not hybridize to oligo 1 in the un.injected sample (lanes 5'T), but some hybridization was restored after incubation in the oocytes (lanes 10’ to 60’). Not all molecules experienced resynthesis to the point of complementarity to oligo 1, since the hybridization signal from the 3,585-bp fragment was weaker than that from the 2,050-bp fragment (compare the relative intensities of the fragments with the oligo and the whole pBR322 probe). The fact that the signal was also lower with oligo 2 implies that some nucleotides were removed from the 5’ tail on some molecules before resynthesis was complete. These probes hybridize to sequences that are 35 nt from the original molecular ends defined by the xhol site.

**Kinetics of flush-ended versus 5’tailed substrates.** We measured the relative kinetics of recombination of 5’tailed and flush-ended molecules by coinjecting marked substrates as done previously for 3’tailed molecules (Fig. 5). Three equimolar mixtures of plasmids were used; they consisted of (i) pHRS3 and pRW4 both with flush ends, (ii) pHRS3 having 400-nt 3’ tails and flush-ended pRW4, and (iii) pHRS3 having 450-nt 5’ tails and flush-ended pRW4. A 3:1 mass excess of M13mp19 RFI DNA cut with Clal was also included to drive recombination toward intramolecular events (28). As before, these mixtures were injected into groups of oocytes, and DNA was recovered after various times of incubation. The relative levels of recombinant product from either plasmid were measured by digestion of the recovered DNA with enzyme mixtures S and K (see Fig. 2).

As shown in Fig. 5, the mixture containing molecules with 3’ tails showed the same relative kinetics of recombination as previously described (Fig. 3). Products derived from the 3’tailed molecules were the first to arise and accumulated more rapidly than did products from either the flush-ended or 5’tailed species. As late as 60 min after injection, there were no products from flush-ended or 5’tailed molecules.

After 3 h, there appeared to be more products from 5’tailed than from flush-ended substrates, although this may have been due to an inequality in the input DNA (Fig. 5D). Clearly the acceleration was not as great as with 5’ tails, since no products from 3’tailed substrates were seen at 20 or 60 min. As before, the resected 3’ strands were resynthesized on a large fraction of the molecules as early as 20 min after injection. Unlike the 2,025- and 1,460-bp fragments from 3’tailed molecules, those starting with 5’ tails regained the mobility of full-length fragments (Fig. 5A).

**5’ tail removal.** The failure of 5’ tails to accelerate recombination may have been due to the rapid resynthesis of the complementary strand rather than to incompetence of the 5’ tails per se. We hoped to stabilize the 5’ tails by blocking resynthesis and thus to determine whether persistent 5’ tails would accelerate recombination as did 3’ tails. The block to resynthesis was not complete, but this substrate revealed an alternative pathway for processing of 5’ tails. The results suggest that these tails can be removed if resynthesis does not occur.

pRW4 was endowed with 500-nt 5’ tails by treatment with ExoIII. An aliquot was incubated with Klenow polymerase and ddNTPs as described in Materials and Methods. Both samples were mixed with flush-ended pHRS3 and a 3:1 mass excess of Clal-cut M13 RFI DNA and injected into oocytes. Recombination products from the two templates were distinguished as before. In contrast to the experiment shown in Fig. 5, products from the 5’tailed molecules began to appear earlier than those from flush-ended molecules (Fig. 6). This is shown by the presence of the 4,363-bp band in the K lanes at early time points. However, in these same samples we saw evidence that, in addition to some resynthesis, the 5’ tails were being removed from some of the injected molecules.

The alternative fates of 5’ tails are seen most clearly in the case of the shorter end fragment from the tailed substrate. In S digests, this fragment had the mobility indicated by spot c in Fig. 6. After only 15 min in oocytes, little material was left at this mobility, and it was replaced by bands at 1,460 bp, corresponding to resynthesis of the resected strand, and at 1,000 bp (marked with an asterisk), which is exactly the size expected if the 500-nt single-stranded tail were removed. Oligo probes have been used to confirm the identity of this
latter fragment and the corresponding one (3,050 bp) from the other end (data not shown).

The removal of the 5' tail at early times after injection is probably sufficient to explain the apparent acceleration of recombination of the tail substrates in this experiment. We have observed that flush-ended molecules with shorter homologous overlaps recombine faster in oocytes (10a). This result is expected according to the single-strand annealing model of recombination (see Discussion), since the amount of 5'→3' degradation necessary to expose complementary sequences is reduced when the overlap is smaller. Removal of the 5' tails in the experiment shown in Fig. 6 shortens the overlap from 1,250 bp to 750 bp (if one tail is removed) or 250 bp (if both tails are removed).

Both the loss of single-stranded tails and the acceleration of recombination are even more evident in the substrates in which resynthesis of the 3' strand was partially blocked by prior incorporation of dideoxynucleotides (Fig. 6B). The fact that some resynthesis occurs indicates that the dideoxynucleotides are removed from some molecules; this has been observed by others as well (22). Apparently there is a competition between nuclease and polymerase for processing of the injected molecular ends. Most of the ends seem to be either completely resynthesized (yielding the 1,470-bp band) or completely removed (giving the 1,000-bp band). Most batches of oocytes favored resynthesis over degradation; those used in the experiment in Fig. 6 had unusually potent nuclease activity. In the absence of extensive tail degradation (e.g., Fig. 5), we have not observed accelerated recombination of molecules having 5' tails.

Recombination intermediates from 5' tailed substrates. It seemed probable that molecules that were injected with 5' tails would recombine by the same mechanism as flush-ended molecules after resynthesis of the resected strand or removal of the tail. This mechanism involves 5'→3' degradation and formation of annealed intermediates. Evidence for such degradation of substrate ends was clear in the experiment shown in Fig. 5 but less so in the experiments shown in Fig. 4 and 6. This reflects variations among batches of oocytes in the potency of their exonuclease activity (28). To get more detailed information on the route to recombination products taken by 5' tailed substrates, we characterized the process by 2-D gel electrophoresis.

As mentioned above, at intermediate times after injection, we see both completed recombination products and a slower smear upon electrophoresis that we attribute to recombination intermediates. Our analysis of these intermediates is described in more detail elsewhere (28, 29a). Using oligo probes with 2-D gels of PvuII-digested recovered DNA (Fig. 7), we have shown the following. (i) Completed products and undergraded substrate fragments run on a diagonal described by duplex molecules with strands of the same length (spots a, b, c, in Fig. 7). (ii) Strands from partly degraded substrate fragments flare away from the diagonal; the 3' strands are essentially full length, while the 5' strands are degraded (29). (iii) The intermediates, which trail behind products in the first dimension, dissociate in alkali into species that run rapidly in the second dimension, indicating that they were held together by noncovalent joints. (iv) The strands from the intermediates migrate as four diffuse spots, each corresponding to a different strand; as in the substrate fragments, the 3' strands (I and III) are essentially intact, while the 5' strands (II and IV) have been shortened. This provides further evidence that the 3' tails produced in oocytes are on the pathway to recombination products.

We argued above that 5' tailed substrates rapidly become flush ended in oocytes, by either degradation or resynthesis of the tail, prior to recombination. If so, intermediates from 5' tailed substrates should have the same appearance in 2-D
The 2,050-bp fragment from...

FIG. 8. Analysis of intermediates after injection of 5'-tailed substrate. Linear pRW4 with 500-nt 5' tails (made with ExoIII) was injected and recovered from oocytes after 50 or 150 min of incubation. DNAs from these two samples were mixed, digested with PvuII, and run in a 2-D agarose gel as described for Fig. 7. The gel was transferred to a nylon filter and hybridized sequentially with the probes indicated beneath each panel. The marker DNAs at the left of each panel were run in the first dimension only and included un.injected 5'-tailed substrate.

The 2,050-bp fragment from uninjectected DNA, shown by horizontal arrows in the left-hand lanes of panels B and C, hybridized to oligo 2 but not oligo 1 because the 3'-ending strand was removed by ExoIII (the approximate amount of degradation is shown with dashes in panel D). After injection, this fragment increased to the size expected from flush-end molecules (2,050 bp; small arrowheads) and hybridized to both probes. Large arrowheads in panels B and C show strands from the 3,560-bp fragment of unrecombined DNA. Strands I to IV are shown in panel D and marked with arrows in spots derived from intermediates in panels A to C. The vertical arrows in panels B and C show the strands which hybridize to the probes (compare with Fig. 7). In panel D, half-arrowheads denote 3' ends; 5' ends are plain. Oligos are shown beside the strand to which they are identical; they hybridize to the opposite strand.

gel as those from untailed substrates. This was found to be the case (Fig. 8). (In the oocytes used in this experiment, resynthesis of the resected strand was rapid, and no tail removal was observed.) Using a whole pBR322 probe, four signals are seen from the intermediates. Oligo probes 1 and 2 established that the identities of these signals were the same as for the flush-ended substrate. For example, oligo 1 hybridized to an internal region of degraded strand II (5' terminated) and to resynthesized and undegraded strand III (3' terminated). With this probe, the degradation of strand II is particularly evident, as smears from both substrate fragments (large arrowhead in Fig. 8C) and from intermediates (arrow II). The 2,050-bp fragments from uninjectected DNA did not hybridize to oligo 1 (arrow to left lane in Fig. 8C), showing that the 3'-ending strands in the intermediates arose by resynthesis. It can also be seen in Fig. 8 that the substrate fragments have partially degraded 5' strands and full-length 3' strands, as expected for resynthesized substrates subsequently acted on by the 5'--3' exonuclease in the oocytes.

DISCUSSION

In earlier work, we found that linear DNA molecules injected into Xenopus oocyte nuclei were acted on by a 5'--3' exonuclease (29). We hypothesized that the single-stranded 5' tails thus generated were important for subsequent recombination of injected DNAs when sequences homologous to the tails were available. In the present studies, we have shown that preparing substrates with 3' tails prior to injection accelerates recombination. This was demonstrated by direct comparison with flush-ended substrates injected into the same oocytes (Fig. 3). This finding provides direct support for the hypothesis that 3'-tailed molecules are genuine intermediates in oocyte recombination. The characterization of later intermediates in recombination (Fig. 7 and 8; 28, 29a) is also consistent with this hypothesis.

Recombination substrates with single-stranded 5' tails were also injected into oocytes. The usual fate of such molecules was to have the resected strand rapidly resynthesized, as we had shown previously with linear DNAs not capable of recombining (29). Furthermore, oocytes are known to support repair-type DNA synthesis, although (unlike eggs) they are not capable of true DNA replication (15). In other experiments, some of the 5' tails were removed (Fig. 6; 28), and this was most significant when resynthesis was partially blocked by incorporation of dideoxynucleotides at the recessed 3' termini. In most oocytes, resynthesis predominates over end removal, but some terminal nucleotides may be lost in all cases (Fig. 4). It is conceivable that the nuclease that removes single-stranded 5' tails is the same 5'--3' exonuclease (29) that creates 3' tails from flush ends.

Conversion of 5' tails to flush ends, whether by resynthesis or by degradation, occurred more rapidly than recombination. The resulting flush-ended molecules became substrates for the 5'--3' exonuclease and entered the normal recombination pathway, involving 3' tails. This was shown most clearly by characterization of apparent intermediates in the recombination of injected 5'-tailed substrates. These intermediates, like those from flush-ended substrates, have essentially full-length 3' strands and shortened 5' strands (Fig. 8). This processing of 5' tails and the absence of evidence for their existence in vivo (29) make it extremely unlikely that they play a role in oocyte recombination.
Single-stranded 3' tails have been postulated to be on the pathway to recombination products in a number of systems, and in some cases the evidence for their existence is strong. The involvement of a 5'→3' exonuclease or single-stranded 3' tails has been demonstrated in the bacteriophage λ red pathway (36), the *E. coli* RecE pathway (39), the bacteriophage T4 UvsXY pathway (13), and in at least some RAD52-dependent gene conversion events in yeast cells (23, 46). Models explaining how 3' tails initiate the formation of recombination intermediates are illustrated in Fig. 9. We consider them in the context of the recombination of linear pRW4 in oocytes.

Single-stranded tails might invade homologous sequences in a region of duplex (Fig. 9A). Branch migration in such a structure would create a Holliday junction, which could be resolved by cleaving and religating pairs of strands. The bacterial RecA protein is capable of catalyzing the invasion step (47), and activities from a number of organisms have been shown to be capable of resolving synthetic Holliday junctions (45). A variation on this model is that the invading 3' end could serve as a primer for DNA synthesis, thus generating recombinants by a break-and-copy mechanism, as in bacteriophage T4 (31) (Fig. 9B).

An alternative to models requiring invasion by the 3' tail is the annealing model (Fig. 9C). Here the tail is lengthened by continued action of the 5'→3' exonuclease until homologous sequences are exposed as single strands in both parents. Annealing occurs, 5'→3' degradation continues to the point where all duplicated sequences are eliminated (or the 3' tail is removed), and then DNA ligase seals the remaining nicks. The annealing may be facilitated by strand-exchange proteins, and there may be some short tracks of DNA synthesis during covalent closure of the joints. Another possibility is that the homologous joints are initially held together by DNA triplex structures (18) until they are further degraded to allow annealing. It is also possible that single strands are exposed by helicase activity, thus allowing annealing without prior nuclease degradation (44). In this scenario, 3'-ending strands could still pair, and 5'-ending strands could either pair or become substrates for 5'→3' exonuclease activity. Our data best support the annealing model for recombination in oocytes. This conclusion is based largely on our analysis of recombination intermediates, which is described in greater detail elsewhere (29a). Two key features can be seen, however, in the data in this report. First, intermediates formed a continuous smear behind completed products in gel electrophoresis (Fig. 1, 3, and 5), as would be expected for linear duplexes with internal protruding single strands of different lengths. In fact, we have constructed molecules with this structure in vitro and shown that they have this electrophoretic behavior (28a). Second, the strands in the intermediates had the sizes predicted by the annealing model (Fig. 7 and 8): the 3' ends did not appear to have been extended as predicted by the break-and-copy model, and the 5' strands were resected below a critical size before they appeared in the intermediates. This latter observation makes the helicase/annealing model (44) less attractive, since longer 5'-ending strands would be expected in intermediates in that case.

As implied above, it seems possible that the oocyte exonuclease and DNA ligase are sufficient to generate recombination products from substrates with homologous overlapping ends. The λ exonuclease has been shown (7) to continue degradation on structures like those proposed as annealed intermediates in the annealing pathway (Fig. 9C). This enzyme stops abruptly once the complementary strand has been assimilated, leaving a ligatable nick (6). While λ exonuclease is highly processive and is not capable of initiating degradation at a simple recessed 5' end (5), the *Xenopus* exonuclease has this capability (Fig. 1; 29).

Whatever the ultimate mechanism of recombination, ques-

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FIG. 9. Models of recombination utilizing 3' tails. A, B, and C are three models of homologous recombination that have appeared in the literature which utilize ends of linear molecules degraded by strand-specific exonucleases (7, 13, 24, 31, 32, 40). The models are shown as intramolecular events occurring within *Xhol*-linearized pRW4. Equivalent intermolecular events also occur between overlapping ends on different molecules. (A) A 3' tail invades a homologous duplex and forms a Holliday junction (17). The heteroduplex is extended by branch migration and could isomerize or be resolved in a number of different ways (17, 21, 40, 42). (B) An invading 3' tail is used as a primer for displacement synthesis (dashed line); recombination could be completed by several different types of resolution (13, 31, 32, 40). (C) 5'→3' strand-specific exonuclease degradation on overlapping duplexes creates complementary single strands that anneal to form a heteroduplex intermediate (7, 24). The formation of the intermediates in model A does not necessarily require the invading strand to be of a certain polarity, and models A and B do not require the displaced strand in the invaded duplex to be near an end as drawn here. Conventions are as in previous diagrams.
tions arise regarding the normal functions of the exonuclease activity and of this recombination process in frogs. For several reasons, we suspect that the exonuclease is stored, like most components of the oocyte, for use in embryogenesis. Since the first events after fertilization require very rapid DNA synthesis, it seems reasonable to postulate that the enzyme is involved in some aspect of embryonic DNA metabolism, be it DNA replication, repair, or recombination. In this and in other systems in which exogenous DNA is introduced into cells, it is premature to conclude that activities catalyzing homologous recombination events among exogenous DNA molecules necessarily serve the same purpose for endogenous chromosomes. With this in mind, the processing of exogenous DNA in oocytes is still interesting from both biological and practical viewpoints.

The nonconservative annealing mode of recombination of exogenous DNA that we imagine to occur in oocytes appears similar, if not identical, to recombination studied in a plethora of mitotic cell types. Essentially the same annealing model, with some variations, has been repeatedly proposed to explain recombination events occurring in cultured animal and plant cells by numerous independent groups (1, 2, 24, 34, 44). Nonconservative events also predominate in some circumstances in yeast cells (33). If, as our data suggest, the 5'→3' exonuclease plays a central role in this type of recombination, it may be a ubiquitous component of eukaryotic DNA metabolism that has generally escaped attention. Interestingly, a similar activity purified from rabbit liver was described about 20 years ago (26) and was postulated to be involved in the repair of UV-induced damage (25).

Based on the studies cited above, the annealing pathway of homologous recombination appears to be functional in a number of higher eukaryotes. If so, a full understanding of its mechanism and of the activities which catalyze individual steps could aid in devising more efficient strategies for genetic manipulation of these organisms. The relatively immense capacity of oocytes to support this mode of homologous recombination makes them an exceptional system for in vivo studies of recombination mechanisms, as well as a source for the purification of the activities involved.

ACKNOWLEDGMENTS

We thank members of this laboratory for helpful discussions and ideas. We also thank Susan Rosenberg for stimulating discussions. We are grateful to Jim Haber, Tim Formosa, Geneviève Pont-Kingdon, Chris Lehman, and Renée Dawson for useful comments on the manuscript.

This work was supported by grant DMB-8718227 from the National Science Foundation to D.C.; E.M. was supported in part by Public Health Service predoctoral training grant 5-T32-GM07464.

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