434–442 Nucleic Acids Research, 1994, Vol. 22, No. 3

Illegitimate recombination in *Xenopus*: characterization of end-joined junctions

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Received September 16, 1993; Revised and Accepted December 20, 1993

ABSTRACT

When linear DNAs are injected into *Xenopus laevis* eggs, they are converted into several different kinds of recombination products. Some molecules undergo homologous recombination by a resection-annealing mechanism; some ends are precisely ligated; and some ends are joined by illegitimate means. The homologous and illegitimate products are also generated in nuclear extracts from stage VI *Xenopus* oocytes. In order to gain insight into the mechanism(s) of illegitimate end joining, we amplified, cloned and sequenced a number of junctions from eggs and from oocyte extracts. The egg junctions fell into three categories: some with no homology at the join point that may have been produced by blunt-end ligation; some based on small, but significant homologies (5 – 10 bp); and some with matches of only 1 or 2 nucleotides at the joint. Junctions made in oocyte extracts were largely of the latter type. In the extracts, formation of illegitimate joints required the addition of all four deoxyribonucleoside triphosphates and was inhibited by aphidicolin. This indicates that this process involves DNA synthesis, and mechanisms incorporating this feature are considered. The spectrum of recombination products formed in *Xenopus* eggs is very reminiscent of those produced from DNA introduced into mammalian cells.

INTRODUCTION

When linear DNA molecules are introduced into mammalian cells, they can undergo homologous recombination, end joining by ligation of blunt or complementary restriction ends, or illegitimate end joining (1). These reactions can occur among introduced molecules and/or between them and chromosomal sites. End joining normally predominates, and such events frequently obscure desired homologous interactions with chromosomal targets (1, 2). In attempts to understand the underlying mechanism(s), and motivated perhaps by a desire to minimize the effects of such events, illegitimate joining has been investigated with a variety of substrates, and a number of the resulting joints have been sequenced. Both ends without homology and ends with very small matches are joined efficiently (3). When short homologies are present, they are frequently used to set the joining register (1, 4, 5). Deletion junctions formed between chromosomal DNA sequences show this same characteristic: only very short matches between parental sequences are found at the novel joint (6). The same is true of junctions formed in extracts from human cells (7) and from *Xenopus* eggs (8, 9).

When linear DNA molecules carrying terminal direct repeats are injected into nuclei of *Xenopus* oocytes, they recombine exclusively via a homology-dependent mechanism (10). When no substantial homologies are provided, shorter, adventitious matches support a low level of recombination (11, 12). In the absence of any homology, the injected DNA is simply degraded (10, 11, 13). Partly because there are no competing recombination processes, it has been possible to make a detailed investigation of homologous recombination in oocytes and to elucidate the underlying mechanism (10, 13–16).

The situation in *Xenopus* eggs is more like that in mammalian cells, since both homologous recombination and end joining occur (17). The capacity for homologous recombination appears in mid-oogenesis and is retained in eggs (17). The capability for end joining is not present in oocytes, but develops late in the course of hormone-induced maturation to an egg, in a process that requires protein synthesis (18). The types of joints formed in egg extracts have been characterized in some detail by Pfeiffer and colleagues. They found that complementary restriction ends are ligated relatively efficiently, but paid greater attention to junctions formed between nonhomologous ends. As in mammalian cells, joining is relatively efficient, and matches of even a single base pair (bp) are utilized when they are available (8). Furthermore, DNA synthesis is required for the formation of some types of junctions in the absence of any homology (9). It has been speculated that egg extracts contain an activity that brings DNA ends into close proximity and allows such illegitimate joints to form (9).

We have also found that illegitimate end joints are formed in extracts of *Xenopus* stage VI oocyte nuclei (germinal vesicles, GVs) under some conditions (19). In this report we exploit the ease of manipulating conditions in the extracts to gain additional insights into the mechanism(s) of illegitimate recombination. We

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have determined nucleotide sequences of junctions formed in these extracts and in injected eggs and compared them to junctions described in other systems. Due to the similarities with extrachromosomal recombination in mammalian cells, continued investigation of events in *Xenopus* eggs and oocytes promises to yield lessons that are broadly applicable.

MATERIALS AND METHODS

Oocyte and egg injection

Manual and bulk GV extracts (mGV and bGV) were made from stage VI *Xenopus laevis* oocytes as described (19, 20). Equivalent results have been obtained with mGV and bGV extracts in all cases. Recombination reactions, DNA recovery, restriction enzyme digestion, and analysis by Southern blot-hybridization were also as described (19). Egg injections and DNA recovery were performed as detailed elsewhere (17). The DNAs used for PCR amplification were from a 19-hour incubation in a GV extract with 0.1 mM dNTPs, and from 3-hour incubations of 2 ng or 5 ng of DNA in unactivated or activated eggs (17).

PCR amplification of end-joined junctions

The PCR amplifications used 0.9−5 ng total DNA, 250 nM primers, 200 μM dNTPs, 10 μg gelatin, 50 mM KCl, 10 or 20 mM Tris/HCl pH 8.4, 1.5 or 2.5 mM $MgCl_2$, and 2.5 U Taq Polymerase (Perkin-Elmer Cetus) in a total volume of 100 μl. Primers were made on an Applied Biosystems DNA synthesizer and purified with an OPC-cartridge. Primer A corresponds to pBR322 sequences 199→180 (5′-GGAATGGACGATATCCCGCA-3′), including the EcoRV site. Primer B matches pBR322 sequences 1068→1085 with an extension of 5 nt on the 5′ end that creates a new XbaI site (5′-TAATCTAGATGACGACCATCAGG-3′). Thirty cycles of amplification were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) under mineral oil as follows: denaturation at 94°C for 20 sec; annealing at 50°C for 20 sec; extension at 72°C for 30 sec. Some reactions used 2 cycles with an annealing temperature of 50°C followed by 28 cycles with an annealing temperature of 55°C and extension for 10 sec. The products were extracted with phenol/chloroform/isoamyl alcohol (25:24:1 vol/vol), and water-saturated ether. The DNA was precipitated by adding 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and then collected by centrifugation. Pellets were washed with 70 % ethanol, dried, redissolved in 10 mM Tris/HCl pH 8, 1 mM EDTA and analyzed by electrophoresis on 5% polyacrylamide gels run with TBE buffer (21).

Sequencing of end-joined junctions

PCR amplified products and pBluescript I SK^+ or KS^- vector (Stratagene) were cut with *Xba*I and *Eco*RV as recommended (Boehringer Mannheim Biochemicals). 20 ng of PCR fragments were ligated to 75 ng vector DNA using 0.5 Weiss unit of T4 DNA ligase (New England BioLabs or Boehringer Mannheim Biochemicals) in the presence of 0.5 mM ATP. The ligated DNA was used to transform competent DH5αF1′ cells, which were plated on LB plates containing 100 μg/ml Ampicillin, using X-Gal and IPTG on the agar surface. Individual white, insert-containing colonies were picked and DNA was isolated by the boiling miniprep procedure (21). Plasmid DNA was treated with RNase A followed by extraction and precipitation as above prior to restriction digestion and sequencing. Sequences were determined from denatured, double-stranded DNA by the ddNTP chain termination method using the Sequenase kit (United States Biochemicals), α-^{35}S-dATP and primer B. Reaction products were separated on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

RESULTS

End joining in injected eggs

In the experiments described here, we employed a linear DNA substrate with terminal direct repeats, pRW4 cleaved with *Xho*I (see ref. 19 and Figures 2A and 3A for diagrams of the substrate and expected recombination products). The results of injecting this substrate into activated *Xenopus* eggs are shown in Figure 1A, where the recovered DNA is analysed after *Pst*I digestion. As described elsewhere (17), one product (P, 4.36 kb) has the size expected to result from homologous recombination by resection-annealing. In addition, there are three bands with electrophoretic mobilities expected for products of head−head (h−h), head−tail (h−t) and tail−tail (t−t) end joining (17, 19, 20). The corresponding *Pst*I fragments are labeled EJ in Figure 1A. Some of these end joints resulted from ligation of the original *Xho*I ends, since digestion by that enzyme converted some EJ material to substrate-sized fragments (Figure 1A). Unactivated eggs showed much lower levels of both homologous and end-

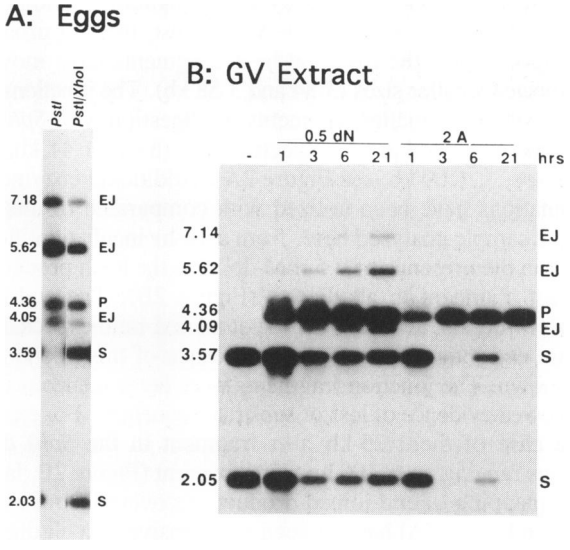

Figure 1. Recombination products from injected eggs and GV extracts. (**A**) Two ng of substrate (pRW4/*Xho*I) was injected into activated eggs, incubated for 3 hours, recovered and digested with *Pst*I or *Pst*I + *Xho*I prior to electrophoresis and Southern blot-hybridization with a pRW4 probe. Sizes of the prominent fragments are indicated on the left in kb; on the right they are identified as arising from substrate (S), products of homologous recombination (P), and products of end joining (EJ). (**B**) Time courses of recombination in manual GV extracts from stage VI oocytes in the presence of 0.5 mM of each of the four dNTPs (0.5 dN) or 2 mM ATP (2 A). A control incubation without extract is also shown (−). One ng of the recombination substrate (pRW4/*Xho*I) was incubated with the extract (equivalent to about 9 GVs) for the indicated times. The DNA was recovered and digested with *Pvu*II prior to analysis; fragment sizes and identifications are as in A. The small differences in fragment sizes between A and B reflect the locations of sites for *Pst*I and *Pvu*II (see Fig. 2A). The smears running ahead of substrate fragments correspond to partially degraded substrate molecules, while the smears running behind 4.36-kb product are annealed recombination intermediates (15).

joined products than activated eggs, but the distribution among product types was essentially the same in the two cases (17, 22).

End joining in an oocyte nuclear extract

In the presence of 2 mM ATP as the only added nucleoside triphosphate, nuclear (GV) extracts of stage VI *Xenopus* oocytes catalyze the formation exclusively of homologous recombination products, just as seen in injected oocytes (17, 19, 20). When millimolar concentrations of the four dNTPs are provided, however, the three end-joined products are also generated. These results are reproduced in Figure 1B, where the conversion of the substrate to various products is shown as a function of incubation time.

To confirm the identities of the end-joined products from the oocyte extracts, they were further mapped by digestion with various restriction enzymes. The expected structures are illustrated schematically in Figure 2A as a monomer circle (pRW4-ht, representing a h−t intramolecular junction) and an inverted dimer circle (pRW4-hh/tt, representing h−h and t−t intermolecular junctions). These maps are the simplest diagrams of all the junctions that would be present in intra- and intermolecular products.

Representative digests are shown in Figure 2B. The three end-joined products in each case are marked with stars. In the *Pvu*II digest, these fragments have sizes of 7.14 kb (h−h), 5.62 kb (h−t) and 4.09 kb (t−t). The intensities of these bands were not measurably reduced by co-digestion with *Xho*I, indicating that the products were not formed by simple ligation of the original restriction ends. In the *Pvu*II + *Sca*I digest, the t−t product is unchanged, while the h−t and h−h fragments have moved to the expected smaller sizes (3.84 and 3.58 kb). The junctions were isolated on even smaller fragments by digestion with *Sph*I, and again they appeared at the expected sizes (h−h, 1.41 kb; h−t, 1.25 kb, t−t, 1.09 kb; see Figure 2A). Additional enzymes and combinations have been utilized with comparable results (22).

In the sample analysed here, from a 17-hr incubation in a GV extract in the presence of 0.5 mM dNTPs, the h−h product was the least abundant in all digests (Figure 2B). The end-joined products were not always observed at a 1:2:1 ratio expected from random end interactions. The significance of this phenomenon is unknown. The junction fragments were heterogeneous in size and showed evidence of loss of substrate sequences. For example, in the case of the 1.25-kb h−t fragment in the *Sph*I digest, deletions ranging up to 100 bp were apparent (Figure 2B, lane 4).

The mapping of end-joined products recovered from injected eggs (see Figure 1A) has not been as extensive, but all digestion results were consistent with this identification.

Amplification and cloning of junction fragments

To characterize further the end-joined products, we designed a PCR scheme to amplify specifically a small region including the h−t junctions (Figure 3A). DNA samples recovered from various recombination reactions were cleaved with *Pvu*II. PCR primers A and B correspond to sequences near the ends of the original terminal duplication, as shown. This scheme allowed cloning and sequence analysis of individual junctions from a mixture of DNAs which share sequencing primer sites. The h−h and t−t junctions were not recovered because they contain inverted repeats, which are inefficiently amplified. Unrecombined substrate and homologous products were not amplified because the primer pairs were divergent. While only h−t products were analysed, we expect the nature of h−h and t−t junctions to be very similar.

Products of the PCR amplifications are shown in Figure 3B. No DNA was amplified from a negative control reaction containing a mixture of pRW4/*Xho*I + *Pvu*II and pBR322/*Pvu*II, which correspond to substrate and homologous recombination product cleaved with *Pvu*II (lane 1). A positive control, pRW4/*Pvu*II (which has the same structure as a precisely ligated h−t junction), is also shown (lane 2). Inclusion of material that carries through the extraction procedure from uninjected eggs did not alter the product amplified from this positive control sample (lane 3). Both of these samples resulted in the amplification of the expected 391-bp product. PCR products from joints made in GV extracts were heterogeneous and smaller than the control fragment (lane 4). This was expected, since direct analysis of junction-containing fragments showed the presence of deletions (Figure 2B).

Amplification of several different samples recovered from eggs (activated and unactivated eggs injected with 2 or 5 ng DNA) yielded a band at 391 bp, plus a broad collection of fragments with slower mobilities (Figure 3B, lanes 5 and 6). These heterogeneous species were determined to be heteroduplexes between strands derived from two individual products with different junctions (22). We presume that they formed by renaturation of product strands in the PCR once product

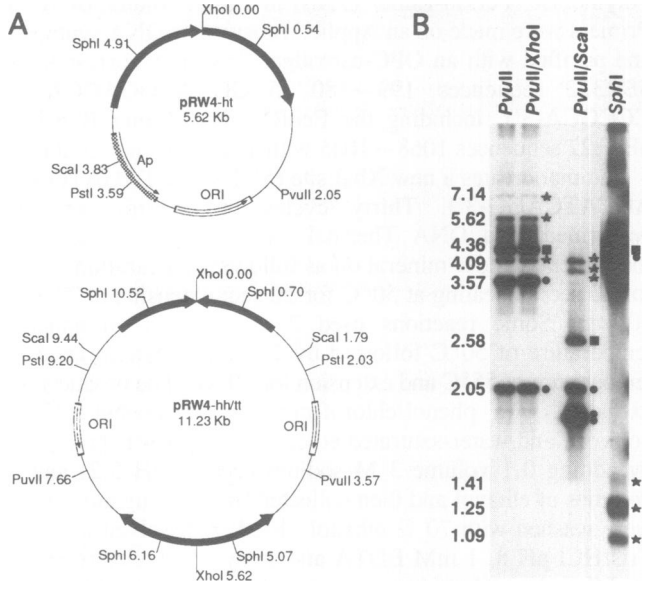

Figure 2. Mapping the products of end joining. (**A**) Diagrams of expected products. Sites for restriction enzymes used in mapping experiments are shown, with their distances (in kb) from the *Xho*I site that represents the ends of the linear substrate. Heavy black arrows denote the 1246-bp direct repeats. The β-lactamase gene (Ap) and plasmid replication origin region (ORI) are indicated (Ap is omitted from the dimer map for clarity). The pRW4-ht plasmid shows the disposition of sites that would result from head-tail joining (equivalent to uncut pRW4). The inverted dimer, pRW4-hh/tt, shows expected products of both head−head and tail−tail joining. In practice, many larger, intermolecular products are formed, but the important features of their restriction maps are all portrayed in these diagrams. The location of the *Xho*I site is shown, although it is often deleted in the end-joining process. (**B**) Digests of products formed in the GV extract. DNA recovered after incubation in a bulk GV extract with 0.5 mM dNTPs was digested with various restriction enzymes and analysed as in Figure 1. The positions of expected fragments from end-joined products are indicated with stars; homologous recombination products are denoted by squares; and substrate fragments are labeled with circles. Some common bands are overexposed so that the end-joined bands may be seen clearly. See the text for interpretations.

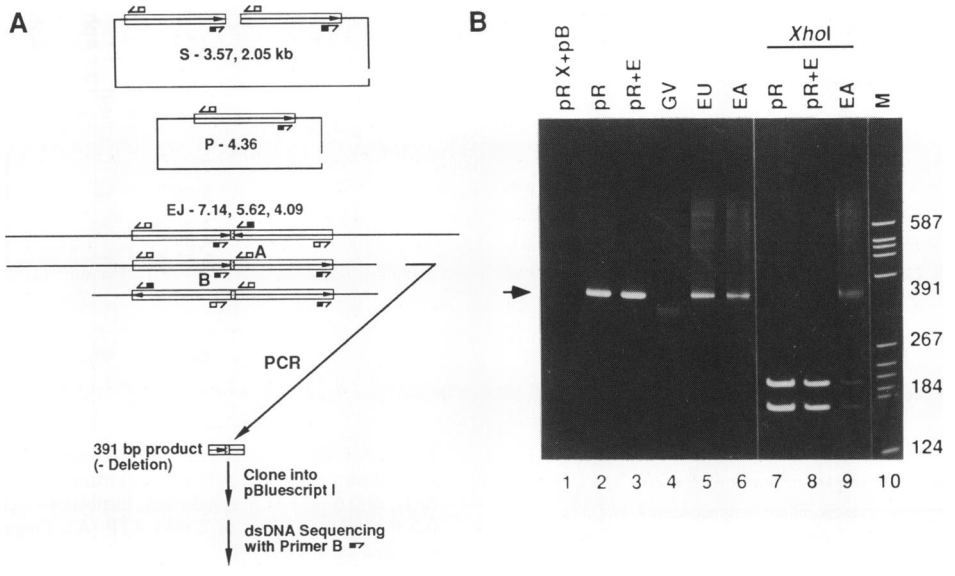

Figure 3. PCR amplification of head-tail junctions. (**A**) Scheme for amplification and cloning. Maps are shown for *Pvu*II-digested substrate (S), homologous recombination product (P) and the three end-joined products (EJ), and their sizes are given in kb. Boxes with arrowheads indicate the 1246-bp duplications; thin lines represent nonduplicated pRW4 sequences. Locations and orientations of the PCR primers are shown as boxes (primer A open, primer B filled) with half arrowheads indicating 3′ ends. Only the head-tail junction should be amplified, resulting in a product of 391 bp, minus any sequences lost upon formation of the end joint (indicated by a stippled box at the junction). The PCR products were cloned between the *Xba*I and *Eco*RV sites of pBluescript I. (**B**) Electrophoretic analysis of PCR products. Reactions were run with DNAs recovered from GV extracts (incubated with dNTPs) (lane 4) and from injected eggs (lanes 5, 6, and 9). The negative control (lane 1) represents amplification from a mixture of pRW4/*Xho*I + *Pvu*II and pBR322/*Pvu*II, which are equivalent to substrate and homologous product DNAs. The positive control template (lanes 2 and 7) was pRW4/*Pvu*II. A control reaction, which included contaminants from one uninjected egg (after extractions as described in the Materials and Methods) added to pRW4/*Pvu*II prior to amplification, is also shown (lanes 3 and 8). Some samples were digested with *Xho*I prior to analysis (lanes 7–9). Samples were fractionated by electrophoresis in a 5% polyacrylamide gel, and stained with ethidium bromide. The sizes of DNA markers (pBR322/*Hae*III) are shown in bp.

Table 1. Junctions formed in GV extracts

Clone	Sequence of junction	Bp deleted: Left	Right	Match
a	CATGGAGCTTTAA	18	11	3
b	CCTCCCCTAACGC	42	45	1
c, p	CCCGCGTTTAATG	37	14	2
d	CCGCGTTTTATCA	37	27	1
e, h	TCCCCGCGATAAG	39	5	3
f	GCGTTGCAGTCAG	35	50	2
g	TCCCCGCTTTAAT	40	12	2
i	GCGTTGCTTTAAT	35	12	2
j	GCGTCG/GCTTTA	31	12	0
k	TGTCTGCTTTAAT	48	12	2
l	TGGATTGCAGTCA	74	50	1
m	GCGTCGCTTTAAT	30	12	2
n	TGTAGGCTAACGC	69	44	2
o	GCGTTGCACCGTG	35	58	2

Sequences of individual cloned junctions are shown. Homologous sequences at the junctions are double underlined. In the case of the one junction showing no underlying homology (j), the break between parental sequences is indicated by a slash. For each clone, the number of base pairs deleted from each substrate end is given, as is the total number of matched nucleotides at the junction.

concentrations became high and primers were depleted. This artifact was not evident in the PCR products from the GV extract sample (lane 4) because amplification was less extensive and product concentrations were never sufficiently high. The amplified junctions were tested for sensitivity to *Xho*I cleavage. As shown in Figure 3B, all of the material from positive control reactions using pRW4 was susceptible to *Xho*I digestion (lanes 7 and 8). Some of the material recovered from injected eggs was also cleaved (lane 9), whereas all of the DNA amplified from the GV extract was resistant to *Xho*I (22; not shown here), consistent with direct Southern blot analysis (Figures 1 and 2).

Because the amplified products were heterogeneous, we cloned individual examples for sequence determination. The clones of GV extract products all carried inserts smaller than 391 bp and

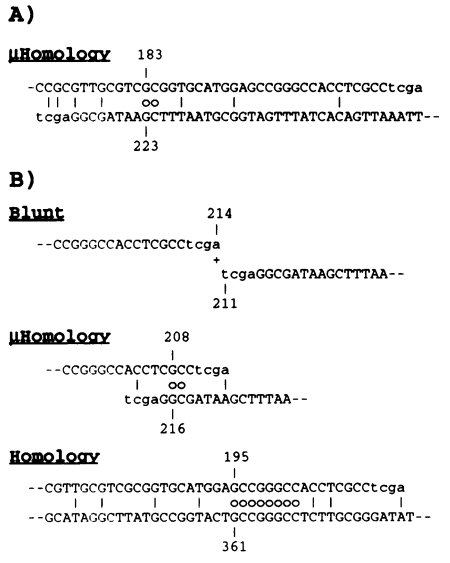

Figure 4. Alignments of parental sequences that produced the junctions in examples of end joints from GV extracts (A) and from injected eggs (B). The crossover point is indicated by open circles (when there was homology at the junction), or a cross (when no homology between parental sequences was present). Additional matches outside the crossover point are indicated with vertical lines. In each case, the top sequence represents the strand ending 3′ at the left of the *Xho*I cut (arrowhead in Figures 2A and 3A), and the lower sequence is that of the 5′ ending strand from the right of the cut. Lower case letters show the 5′ tails of the substrate produced by *Xho*I cleavage; these 4 nucleotides were initially present on the lower strand, but would have to have been restored by DNA synthesis on the upper strand. Positions are numbered relative to the *Xba*I site of primer B (see Figure 3). The joining events illustrated correspond to GV extract junction m (Table 1) and egg junctions D, L and P (Table 3), from top to bottom.

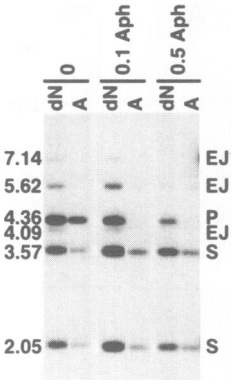

Figure 5. Effects of aphidicolin on recombination in GV extracts. Reactions like those shown in Figure 1B were performed in the presence of no (0), 0.1 mM (0.1), or 0.5 mM (0.5) aphidicolin. Incubations were for 22 hours and included 0.5 mM dNTPs (dN), or 2 mM ATP (A). Fragment sizes and identities are indicated as in Figure 1.

were resistant to *Xho*I, as expected. The sizes of the inserts corresponded to deletions ranging from tens to a little over 100 bp. In the case of the egg junctions (which generally did not contain large deletions), several different types of clones were obtained. About 14% produced mixtures of two plasmids, presumably resulting from transformation with heteroduplex inserts. 23% of the plasmids contained full-length inserts that were sensitive to *Xho*I (*Xho*I^s); 77% carried *Xho*I resistant (*Xho*I^r) inserts, most of which were close to full-length. Again, these observations correspond well to the direct analyses of the egg recombination products and PCR products.

Nucleotide sequences of cloned junctions from GV extracts

Sixteen inserts representing junctions formed in GV extracts were sequenced. All showed simple crossovers from sequences near one substrate end to sequences near the other, and all contained substantial deletions of parental sequences. Alignments of parental sequences around the junction are shown for one example in Figure 4A. The sequences at all 16 junctions are presented in Table 1. In all cases, nucleotides were deleted from both of the original *Xho*I-generated substrate ends. With only one exception (j, which showed no homology), there was a very small match (1−3 nt) at the crossover point. In neighboring regions, there was no significant homology. The matched nucleotides at the junctions were frequently GC-rich. In fact, the match was GC in 7 of the 16 cases and XGC or GCX in another 3 cases (Table 1). While the joints were quite variable, their locations relative to the original molecular ends clustered to some extent: 10 fell in the range of 30−42 bp from the left end and 7 were located 11−14 bp from the right end. The 5 examples that deleted 12 bp from the right end all joined to different sequences 30−48 bp from the left end.

Characteristics of these GV extract junctions are collected in Table 2. The average deletion was approximately 60 bp, and examples as large as 121 bp were found, in good agreement with the direct analysis (Figure 2B). Although short matches were typically found at the crossover point, it does not appear that end joining in the GV extract is homology-driven. In essentially all cases, there were sequences very near the observed crossover that presented better matches of 4−5 contiguous bp, but these were not used.

Nucleotide sequences of cloned junctions from injected eggs

Sequences of selected junctions formed in eggs were also determined. Analysis of two *Xho*I^s inserts confirmed that they were identical to the corresponding region of pRW4 and thus resulted from precise ligation of the *Xho*I termini. Twenty *Xho*I^r junction sequences fell into three categories. Examples are shown in Figure 4B, and all the junctions are presented in Table 3. Seven showed no homology between parental sequences at the crossover point. Two of these apparently resulted from blunt-end joining of filled-in *Xho*I ends, since all nucleotides of both 5′ overhangs were present in the products (Blunt in Figure 4B; D, H in Table 3). The others in this category also joined sequences very near both ends and may have resulted from blunt-end ligation after very limited loss of terminal nucleotides.

The second category (n = 6) showed microhomologies (1−3 bp) at the crossover point, like the junctions formed in GV extracts (μHomology in Figure 4B). Sequences quite close to the original molecular ends were joined in these cases (Table 3). The remaining seven junctions were characterized by more extensive matches at and in the vicinity of the crossover point (Homology in Figure 4B). These homologies were still quite short, 5/6 to 10/12 bp, but were reminiscent of matches observed at rare junctions produced in injected oocytes in the absence of longer homologies (11, 12). We attribute these to the homology-dependent, resection-annealing mechanism. While some of these homologous egg junctions resulted in small deletions, they also included all of the longest deletions recovered (J, P and R in Table 3). Properties of the three categories of egg junctions are

Table 2. Characteristics of sequenced *Xho*I[r] end joints[a]

	GV Extract	Injected Egg Blunt	μHomol	Homol
Number	16	7	6	7
Change[b]				
Mean	−62 ± 27	−5 ± 8	−18 ± 15	−80 ± 60
Range	−28/−121	+4/−18	−5/−40	−16/−166
Homology[c]				
Mean	1.9 ± 0.8	0	2.0 ± 0.9	3.7 ± 2.0 (7.0/8.9)
Range	0−3	0	1−3	2−8 (5/6−10/12)

[a]Based on sequences like those presented in Figure 4. See Materials and methods for sources of the DNAs amplified in each specific case. The distinctions between junctions formed by blunt-end ligation (Blunt), by use of microhomologies (μHomol), or longer homologies (Homologous) are discussed in the text.
[b]Number of bp added (+) or deleted (−) compared to pRW4.
[c]Number of matched bp at the junctions. The quality of the homologies are also indicated parenthetically for the homologous products. For example, 10/12 represents 10 matches out of 12 consecutive nucleotides.

Table 3. Junctions formed in eggs

Clone	Sequence of junction	Bp deleted: Left	Right	Match	Class
A	TCGCCT/ATAAGC	3	8	0	Blunt
C	CCGGGC/ATAAGC	13	8	0	Blunt
D, H	CCTCGA/TCGAGG	0	0	0	Blunt
M	ACCTCG/TCGAGG	6	0	0	Blunt
O	GCCTCG/GCTTTA	1	12	0	Blunt
Q	CACCTC/ATAAGC	7	8	0	Blunt
B, E	CGCCTCGATAAGC	0	6	3	μHom
G	CGGTGCAGCTTTA	24	11	1	μHom
L	ACCTCGCGATAAG	5	5	2	μHom
N	GCGTCGCTTTAAT	30	12	2	μHom
S	CTCGCCTTAATGC	3	15	1	μHom
F	GCATGGAGGCGAT	19	2	3 (6/8)	Hom
I	GCGTCGAGGCGAT	31	0	3 (6/8)	Hom
J	GTCGAGGCGATAA	124	0	2 (8/10)	Hom
K	GCGTCGCAGTCAG	30	49	3 (5/6)	Hom
P	AGCCGGGCCTCTT	12	150	8 (10/12)	Hom
R	GATTT-ATGCGGTA	112	18	4 (8/10)	Hom
T	AGCCG-GGCGATA	13	4	3 (6/8)	Hom

Sequences of individual cloned junctions are shown and classified as described in the text (μHom = microhomology at the junction; Hom = longer homology). For blunt junctions, the break between parental sequences is indicated by a slash. In the other cases, homologous sequences at the junctions are double underlined; and for the homologous products, additional matches adjacent to the crossover are single underlined. A hyphen indicates that a gap must be inserted on one strand to extend the homology. For each clone, the number of base pairs deleted from each substrate end is given. The total number of matched nucleotides at the junction is reported; numbers in parentheses show longer, imperfect matches for the junctions classified as Hom.

Table 4. Recombination through oogenesis[a]

	Stage I−II	Stage III	Stage IV−VI	GV+ATP[b]	GV+dNTPs[b]	Eggs
Homologous[c]	−	+	++	++	++	++
Precise Ligation[d]	+	+	−	−	−	+
Blunt Ligation[e]	?	?	−	−	−	+
μHomologies[f]	−	−	−	−	+	+

[a]These results were derived from pRW4/*Xho*I substrate only and differences with other substrates are possible. The question marks (?) indicate that the capacity to ligate blunt ends has not been tested in stage I and III oocytes. The number of plus signs roughly indicates the activity for that type of recombination: little or no activity (−), 5−20 % of substrate processed (+), or greater than 50 % of substrate processed (++).
[b]GV refers to Stage VI GV extracts in the presence of the indicated NTPs.
[c]Homologous recombination includes events based on the long homology in pRW4 (1246 bp) and the shorter imperfect homologies used by eggs (5/6 to 10/12).
[d]Precise ligation is defined as ligation of the original substrate ends with no loss or addition of nts.
[e]Blunt ligation includes end-joined products formed by fill-in DNA synthesis prior to blunt-end ligation and those junctions without a sequence match (proposed to be joined by the same mechanism). A low level of blunt-ended substrate DNA (pBR322/*Sca*I) was ligated in stage II GV extracts (22), but these are not included here.
[f]Microhomologies are very short matches (1−3 bp) at the join point. Injected stage VI oocytes have been observed to ligate a small fraction of GC-rich restriction ends (28), but these are not included here.

summarized in Table 2. It should be emphasized that the best of the μHomology matches and the worst of the Homologous ones are not vastly different, and the distinction between these categories is somewhat arbitrary.

It is clear that junctions made in eggs usually involved joining of sequences nearer to one or both molecular ends than junctions generated in the GV extracts (compare Tables 1 and 3). In the extracts, all joining events entailed deletion of nucleotides from both parental ends, while 11 of the 20 egg junctions included one or more nucleotides from the *Xho*I 5′ tail from at least one end. Both in GV extracts and in eggs, some sites at each parental end were found at multiple junctions, but only rarely in combination with the same sequence partner from the other end. Only two pairs of GV extract joints (c = p, e = h) and two of egg joints (D = H, B = E) were recovered in duplicate.

Effect of inhibitors on end joining in GV extracts

Because end joining in the GV extract occurs only when all four dNTPs are added, we sought to test further the involvement of DNA synthesis in the reaction, using inhibitors of specific DNA polymerases. Dideoxynucleoside triphosphates (ddNTPs) inhibit polymerase β, but not polymerases α, δ or ε (23). The presence of ddNTPs partially inhibits repair-type DNA synthesis in oocyte extracts (24). In the presence of 0.5 mM of each of the normal dNTPs, addition of 1.4 mM ddATP did not appreciably block end-joining in the extract (25). ddNTPs supported homologous recombination but not end-joining when they were added alone or in combination with individual NTPs (25). Thus, ddNTPs can provide the hydrolyzable high-energy phosphodiester bond required for homologous recombination (19), but they neither serve as precursors for DNA synthesis nor do they interfere when precursors are present. These results suggest that DNA polymerase β does not play a central role in the end-joining reaction or in homologous recombination.

Aphidicolin is an effective inhibitor of DNA polymerases α, δ and ε (23); and it completely blocks the synthesis of a complementary strand on a single-stranded template in GV extracts (20, 24). Addition of 0.5 mM aphidicolin to extracts along with 0.5 mM dNTPs substantially reduced both end joining and homologous recombination (Figure 5). Aphidicolin also reduced the amount of homologous product in the presence of ATP alone (where no end joints were formed) (Figure 5), suggesting that the resection-annealing process also depends to some extent on DNA synthesis. The reduced recovery of DNA in the presence of aphidicolin (Figure 5) and other polymerase inhibitors (22) may reflect degradation by the oocyte exonuclease at internal nicks in the absence of repair synthesis, as well as a requirement for synthesis to form homologous and illegitimate joints.

DISCUSSION

Recombination activities in eggs and oocytes

We have examined the recombination products generated from linear substrate DNA in *Xenopus* eggs and in oocyte nuclear extracts. In addition to utilizing long terminal repeats for homologous recombination, both systems catalyzed the formation of end joints.

The sequences of end junctions formed in eggs suggest that they are produced by four different mechanisms. First, the complementary *Xho*I ends on the substrate are simply religated; this must occur prior to significant degradation of the 5′ tails

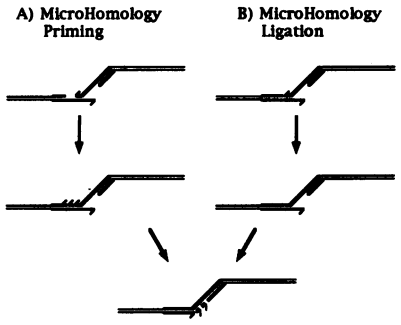

Figure 6. Models for end joining by the use of microhomologies. Half arrowheads indicate 3′ ends; 5′ ends are plain. Multiple short segments with half arrowheads represent gap filling by DNA synthesis. See text for explanatory details.

by the known 5′→3′ exonuclease. Second, blunt ends are joined; some blunt ends are created by filling in the XhoI ends, some by degrading a short distance (< 10 bp) from the original ends prior to joining. Third, short, but significant, homologies (5−10 bp) near the ends are used to support homologous recombination; similar junctions have been observed in injected oocytes when longer homologies are absent or blocked (11, 12). Fourth, isolated microhomologies (1−3 bp) are used to set the register for joining; this is very reminiscent of junctions formed in mammalian cells and in *Xenopus* egg extracts between short, single-stranded tails having the same polarity but noncomplementary sequences (3, 8). In GV extracts from stage VI oocytes, essentially all end joints formed were produced by this latter mechanism, involving microhomologies (1−3 bp).

Combining this information with the results of our earlier studies (17, 19), we can summarize the recombination capabilities that can be assayed by injection or extract incubation at various stages of oogenesis (Table 4). Early stage oocytes are capable only of ligating restriction ends. By stage III, a capacity for homologous recombination by the resection-annealing mechanism begins to appear, and it continues to accumulate up to stage VI. As assayed by direct nuclear injection, oocytes never show any ability to support illegitimate end joining. Ovulated eggs catalyze homologous recombination, but also support three types of end joining that require little or no sequence homology.

In the presence of ATP, GV extracts from stage VI oocytes catalyze only homologous recombination by the resection-annealing pathway, just as seen in injections of the same stage oocytes (17, 19). With millimolar levels of added dNTPs, however, the GV extracts also display end-joining capability (19), (see Figure 1B). As noted above, the sequences of these junctions showed that they were based on microhomologies. The dependence of this style of end joining on added dNTPs and the observed inhibition by aphidicolin suggest that extensive DNA synthesis may be required as part of the process.

Why do microhomology based end-joining events occur in GV extracts, but not in injected oocytes? One possibility is that homologous recombination is more efficient in intact oocytes and competes successfully with end joining. This is supported by the observation of reduced resection-annealing activity in GV extracts (17), and may be correlated with loss or dilution of oocyte components. A second possibility is that NTP levels in whole oocytes favor homologous recombination; the measured (μM) levels of dNTPs in oocytes (26) are inadequate to support end

joining in GV extracts (19). Third, there may be cytoplasmic contamination in the GV extracts by activities that encourage end joining (see below).

Mechanisms of illegitimate recombination

In Figure 6 we offer two models to account for the formation of end joints based on microhomologies. In both cases we envision 5′→3′ resection, leaving single-stranded 3′ tails (13). In A, the terminus of one such tail makes transient contact with one or a few complementary nucleotides in another tail. If DNA polymerase finds and uses the bound 3′ end as a primer before the duplex dissociates, it will extend the complementary region by synthesis, thereby stabilizing the association between the tails. The gap on the other strand would be filled similarly by DNA synthesis after removal of unpaired nucleotides at the 3′ end, but this could be accomplished more leisurely because the structure is held together by the first joint. The alternative in B postulates the initial, transient association of a 3′ terminus adjacent to a recessed 5′ end and fixation by ligation. Completion of the junction would proceed by gap filling, as in A.

In their study of joints formed in human cell extracts, Thacker *et al.* (7) proposed a mechanism in which interactions between microhomologies precede end trimming, repair synthesis and ligation. We prefer models in which a transient joint is stabilized initially by primed synthesis or ligation (Figure 6). This is because duplexes as short as 1−3 bp are unlikely to be stable for significant periods of time to allow the required removal of single-stranded tails. Furthermore, we always found that there were better matches that were not used in the vicinity of the microhomologies that were used to support joining. This suggests that the interaction between strands is not primarily homology-driven.

In eggs, recombination via these microhomologies incorporates sequences quite close to both molecular ends in the final products. This indicates that there is little degradation of either 5′ or 3′ ends prior to joining. If we are correct in attributing the initial contact to 3′ termini (Figure 6A and B), the longer deletions produced in GV extracts suggest the presence of an activity that removes nucleotides from 3′ ends. This could be either a 3′→5′ exonuclease or a single-strand endonuclease that works after some resection by the known 5′→3′ exonuclease. Since the loss of 3′ ends is very slow in injected oocytes (13, 14, 27), the activity in the extracts may be the result of low-level cytoplasmic contamination, although by one measure contamination was undetectable (20).

We also found that homologous recombination in GV extracts was partially sensitive to inhibitors of DNA synthesis, although there was no requirement for added dNTPs. In the simplest version of the resection-annealing mechanism (15), there is no *a priori* necessity for DNA synthesis. It may be, however, that gaps are often formed beyond the ends of the homologous overlap before the strands are ligated. In the substrates we use, there are a few nonhomologous nucleotides on both 3′ ends that must be removed prior to ligation; there is no reason to believe that the 5′→3′ exonuclease stops when the end of the homology is reached (27); and we have direct evidence that gaps are formed when a substrate with a relatively short overlap (300 bp) is injected into oocytes (28). DNA synthesis would be required to fill such gaps in order for stable, covalently-closed products to be formed. Any nicks elsewhere on these plasmids could also be extended into gaps by the oocyte exonuclease requiring subsequent repair synthesis by DNA polymerase. We do not know which, if any, of these possibilities contributes to the apparent requirement for DNA synthesis for homologous recombination in extracts. It is also formally possible that the effects of the inhibitors were indirect.

The efficiency of end joining is quite high in injected eggs, comparable to that of homologous recombination. In GV extracts, the overall rate of substrate processing is lower, but the yield of product increases with increasing substrate concentration (20). The higher efficiency in eggs is probably due to higher concentrations of the necessary activities, but it may also reflect the participation of an end-binding or end-alignment factor that is not present in oocytes (9). Activation of such a factor could explain the appearance of all types of end joining processes upon egg maturation (18). It could promote or stabilize end associations in the absence of homology and in the presence of short matches, including those long enough to be classed as essentially homologous crossovers (5−10 bp). In oocytes, which hypothetically lack this agent, short homologies do not compete well with long overlaps, and they lead to recombination products only when the favored use of long homologies is prevented (11, 12).

Simple ligation, blunt-end joining and recombination through microhomologies have all been documented in egg extracts (8), just as we observe in injected eggs. Inhibition of the joining of blunt ends to 3′ tails by the presence of a nonextendable nucleotide on the 3′ blunt end has been taken as evidence for alignment of these ends to allow priming of DNA synthesis using the 3′ tail as template (9). An alternative is that a single, untemplated nucleotide is occasionally added to blunt ends and that this provides a 1-nt single-stranded tail that can set the register for joining to a 3′ tail, similarly to the use of microhomologies. This could also be the origin of the blunt joints we recovered from eggs, where the blunt end would have been created by fill-in or by degradation and the 3′ tail by 5′→3′ exonuclease action.

Concluding remarks

As noted previously, the spectrum of end joints produced in *Xenopus* eggs and egg extracts is very similar to that observed with linear DNAs introduced into mammalian cells (1). In both situations, we imagine that end-joining capabilities are present to repair double-strand breaks in chromosomal DNA, which, if left unrepaired, would lead to loss of whole chromosomes or large segments thereof. In support of this notion, sequences of deletion junctions in mammalian cells suggest the participation of microhomologies in the joining step; this includes deletions induced by γ-irradiation, which is known to stimulate double-strand breaks (6). Continued characterization of the end joining mechanisms in *Xenopus* may contribute to an understanding of the processing of double-strand breaks in many organisms.

In mammalian cells, as in *Xenopus* eggs, nonconservative homologous recombination of extrachromosomal DNAs is relatively efficient, but it competes with nonhomologous end joining (1). Conceivably, specific inhibition of end joining could increase the recovery of homologous products. The studies in eggs and egg extracts suggest that interference with a hypothetical end-binding activity (9) or temporary inhibition of DNA synthesis might achieve this distinction. Although there are probably additional mechanistic limitations to homologous exchanges with chromosomal sites (15, 29), reducing end joining has the potential to improve the efficiency of targeted genome alterations.

ACKNOWLEDGMENTS

The authors are extremely grateful to Dave Mahaffey for providing eggs, to Larry Wangh for advice on his egg hardening procedure, and to Robert Schackmann for expert synthesis of oligonucleotides. Thanks also go to members of this lab and to Glenn Herrick for helpful discussions. The manuscript was improved by comments of David Segal, Geneviève Pont-Kingdon, and Janet Lindsley. This work was supported by grants from the National Science Foundation (DCB-8718227 and MCB-9019139). C.W.L. was supported in part by a predoctoral training grant in genetics from the National Institutes of Health (US Public Health Service Grant 5-T32-GM07464).

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