Biol. Chem., Vol. 379, pp. 673 – 681, June 1998 · Copyright © by Walter de Gruyter & Co · Berlin · New York

Homologous Recombination and DNA-End Joining Reactions in Zygotes and Early Embryos of Zebrafish *(Danio rerio)* and *Drosophila melanogaster*

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A linear DNA with partial sequence redundancy can be recircularized in cells by either nonhomologous end joining (NEJ) or by homologous recombination (HR). We have studied the relative contributions of these processes in zygotes or early embryos of species that serve as model organisms for developmental genetics. Thus, we have microinjected a linearized plasmid substrate into zygotes of zebrafish *(Danio rerio)* or into the posterior end of *Drosophila melanogaster* early embryos before pole cell formation. Similar to the situation observed previously in *Xenopus* zygotes/early embryos, we detected a large preponderance of DNA-end joining over homologous recombination. A comparison of end-joined junctions revealed that from the three species tested, zebrafish introduced the least number of sequence distortions upon DNA-end joining, while *Drosophila* produced the largest deletions (average 14 bp) with occasional nucleotide patch insertions, reminiscent of the N nucleotides at V(D)J junctions in mammalian immune receptor genes. Double-strand gap repair by homologous sequences ('homologous recombination') involving a bimolecular reaction was readily detectable in both zebrafish and *Drosophila*. This involved specifically designed recombination substrates consisting of a mutagenized linear plasmid and DNA fragments carrying the wild-type sequence. Our results show that the basic machinery for homologous recombination is present at early developmental stages of these two genetic model organisms. However, it seems that for any experimental exploitation, such as targeted gene disruption, one would have to inhibit or bypass the overwhelming DNA-end joining activity.

Key words: Cytosine methylation / Double-strand break repair / Gene targeting by oligonucleotide / Illegitimate recombination / Microinjection / Nonhomologous recombination / PCR assay for recombination / Spermine, spermidine.

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Introduction

We have analyzed in a previous study the repair of linear DNA by either homologous recombination or by DNA-end joining in oocytes and fertilized eggs of *Xenopus laevis* (Hagmann *et al.*, 1996). While DNA repair by homologous recombination prevails in stage VI oocytes, there is a remarkable shift after fertilization to an overwhelming predominance of DNA-end joining in the repair of linear DNA (Goedecke *et al.*, 1992; Hagmann *et al.*, 1996). This shift to nonhomologous end joining may reflect a need, during early embryogenesis, for rapid repair of DNA double strand breaks at the expense of accuracy (Hagmann *et al.*, 1996). Here, we have extended this study to include early embryonic stages of two genetic model organisms, *Drosophila melanogaster* and the zebrafish *Danio rerio*. In both species, transgenic animals can be produced and efficient mutagenesis protocols exist, e.g., by P-element insertion in *Drosophila* (Rubin and Spradling, 1982). Insertional mutagenesis of zebrafish can be achieved by pseudotyped retroviral insertion (Gaiano *et al.*, 1996) and transgenic lines expressing the introduced gene in a tissue-specific fashion can be generated by microinjection (Higashijima *et al.*, 1997; Long *et al.*, 1997). However, unlike in the mouse, targeted disruption of any gene via homologous recombination has not been achieved so far in zebrafish or *Drosophila*, which was the main reason we chose them for our recombination studies. Although we observe in both species significant levels of double-strand gap repair via recombination of homologous sequences, this mechanism acts only on a minority of the introduced template molecules.

Results and Discussion

For a first series of experiments, we used the dual specificity plasmid substrate pReco-σ which had been used previously in *Xenopus*. When linearized, this plasmid can be recircularized via two alternative and mutually exclusive pathways (Figure 1A). Homologous recombination

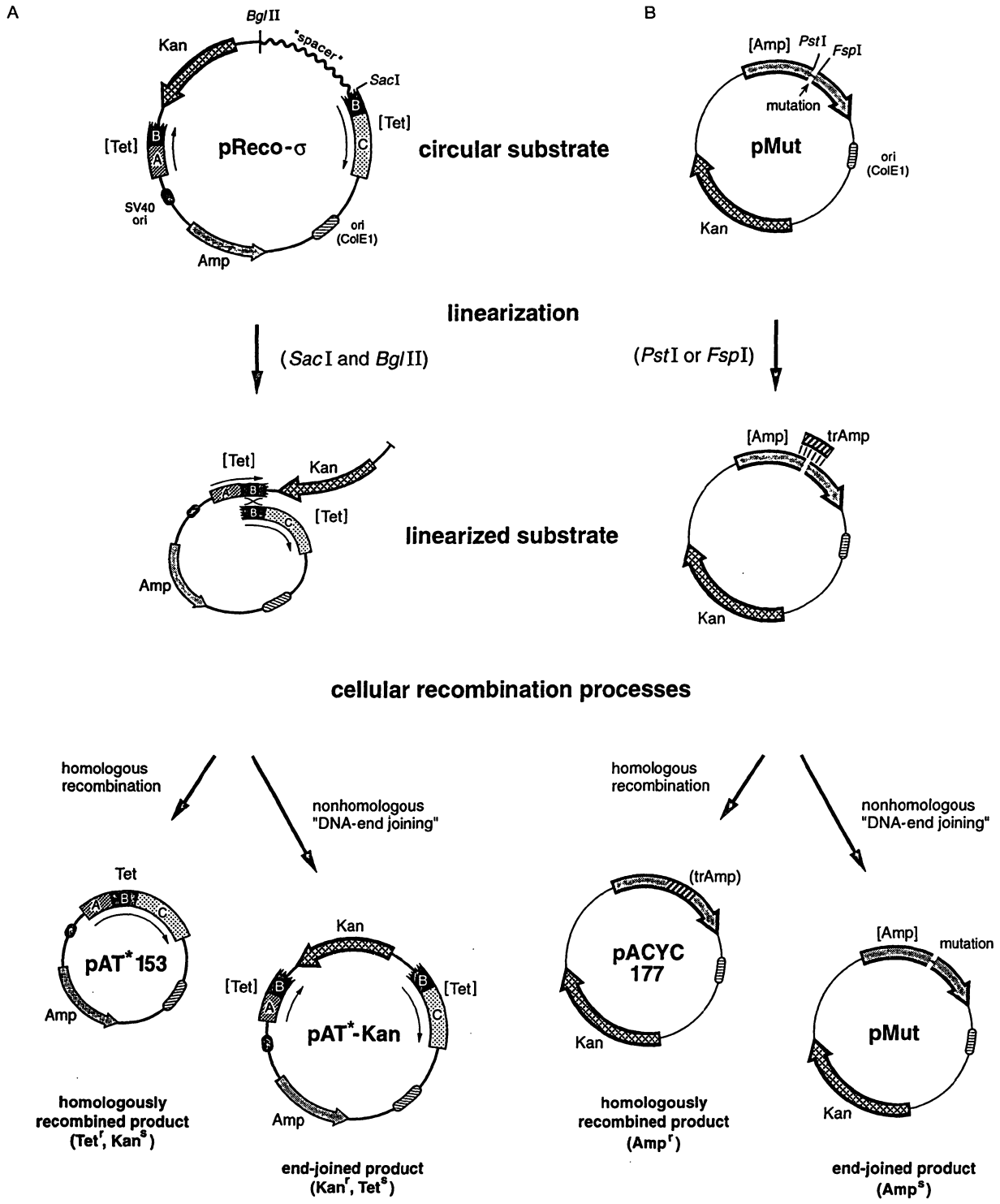


Fig. 1 Schematic Representation of the Plasmids Used.
(A) The pReco-σ assay. The circular form of the recombination vector pReco-σ (named after the 'sigma' shape of its recombining form) contains a disrupted Tet^r-gene ([Tet] AB and BC) with a partial internal duplication (B box). The inserted DNA consists mainly of a functional Kan^r-gene. Digestion of pReco-σ with *SacI*/*BglII* results in the linearized recombination substrate. After introduction into cells, the linearized vector can be recircularized by either homologous recombination or nonhomologous DNA-end joining, resulting in plasmids conferring Tet-resistance (pAT*153) or Kan-resistance (pAT*-Kan), respectively (Hagmann *et al.*, 1996).
(B) The pMut-trAmp assay, a bimolecular reaction most suitable for measuring double strand gap repair via homologous recombination. The circular form of the recombination vector pMut contains a mutagenized non-functional Amp-resistance gene (for details, see Materials and Methods). Upon linearization with *FspI* or *PstI*, the vector is coinjected into cells together with a 478 bp DNA fragment of a truncated wild-type Amp^r gene (trAmp). Besides the double-stranded trAmp fragment, single-stranded wild-type oligonucleotides were also used (see Materials and Methods). Amp-resistance can be restored via homologous recombination between pMut and trAmp which results in the original plasmid, pACYC 177. Alternatively, the linear vector can be end-joined, resulting in a plasmid which only confers resistance to kanamycin.

between two internally duplicated stretches of DNA within the tetracycline (Tet)-resistance gene yields a functional Tet^r gene, which is detected in a bacterial transformation assay. On the other hand, nonhomologous DNA-end joining results in kanamycin (Kan)-resistance. These features allow the simultaneous detection of both types of recombination events. To quantify and compare the efficiencies of the two recombination processes under various assay conditions, the DNA recovery of each sample was monitored by the number of chloramphenicol (Cm)-resistant colonies due to the coinjected pCm184 circular reference plasmid (Hagmann *et al.*, 1996). As an alternative to bacterial transformation with recombination products, for some experiments we designed a novel PCR-based detection method. In this case, recombination products were analyzed with various pairs of primers located in either the Tet- or the Kan-resistance gene. Although it is only semi-quantitative, the PCR-based detection is somewhat faster and more sensitive than the electroporation procedure. This alternative assay confirmed previous results from *Xenopus* oocytes and early embryos (data not shown). In the second part of our analysis, we have specifically looked for double strand gap repair via homologous sequences, using a bimolecular substrate reaction (Figure 1B). (This mechanism hitherto will be simply referred to as homologous recombination, to avoid confusion with DNA-end joining also addressed in this report).

Fertilized eggs of zebrafish were microinjected with 50 and 200 pg of linearized pReco-σ plasmid DNA per egg (Figure 2). The experiments in zebrafish were performed both in the presence (Figure 2A and B) or absence (Figure 2C) of spermine/spermidine. These two related polycationic substances are frequently coinjected with DNA to generate transgenic mice and, more recently, also trans-

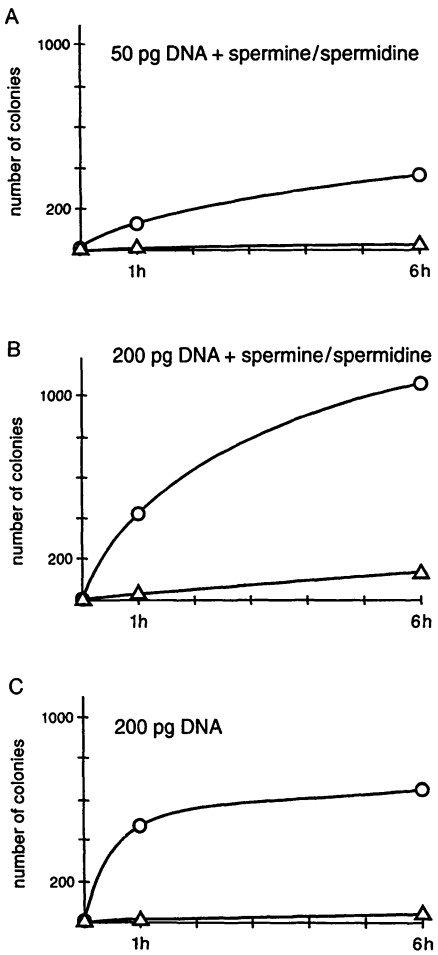


Fig. 2 Zebrafish Zygotes Display Efficient DNA-End Joining which Is Further Boosted by Spermine/Spermidine.
Two different concentrations of linearized pReco-σ plasmid [50 ng/μl (A) and 200 ng/μl (B) and (C)] were injected into fertilized eggs of zebrafish either in the presence [(A) and (B)] or absence [(C)] of 30 μM spermine/70 μM spermidine. The number of recovered Kan^r-colonies (circles) was approximately proportional to the amount of injected DNA [(A) and (B)]. When comparing (B) and (C), spermine/spermidine appears to enhance DNA-end joining (circles) and also probably homologous recombination (triangles, representing Tet^r-colonies).

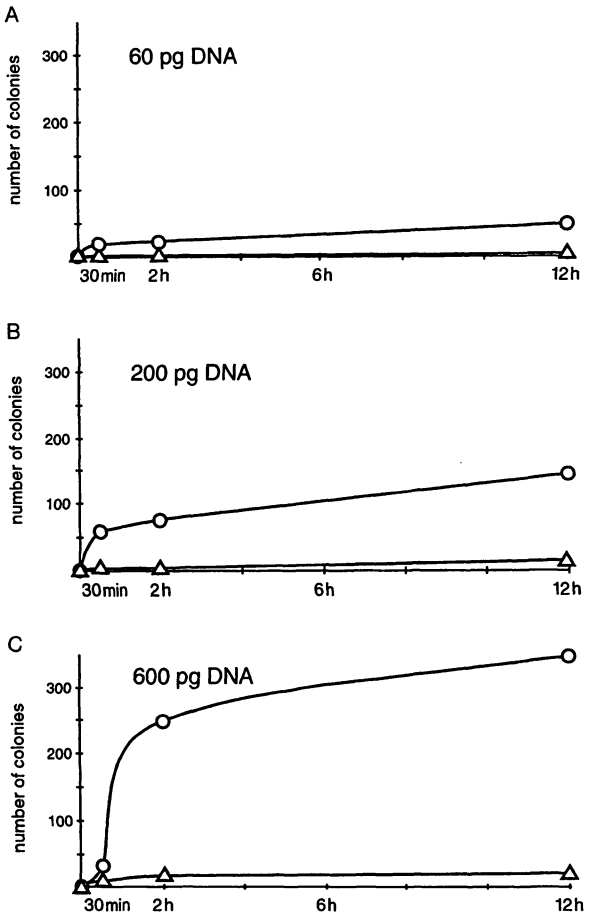
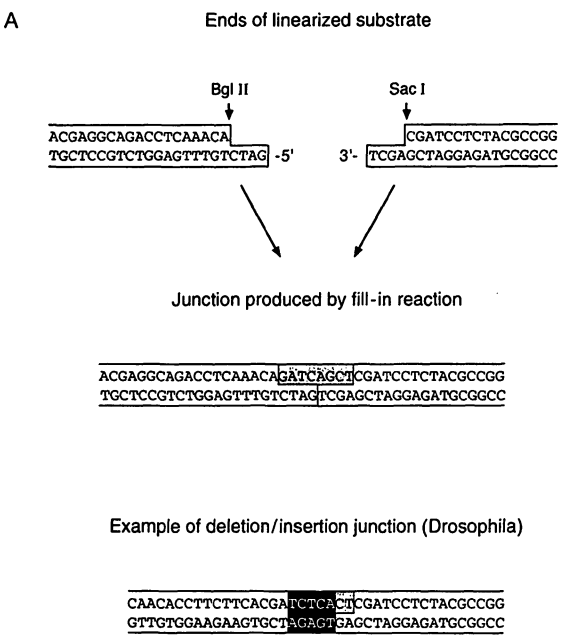


Fig. 3 Preponderance of DNA-End Joining over Homologous Recombination also in *Drosophila* Early Embryos.
Increasing concentrations of linearized pReco-σ substrate (60 ng/μl, 200 ng/μl, and 600 ng/μl for (A), (B), and (C), respectively) were injected into freshly fertilized eggs of *Drosophila*, typically less than 1 h after egg laying, and incubated for the indicated time periods. The amount of end-joined plasmids conferring Kan-resistance (circles) increased with higher amounts of injected DNA. Homologous recombination, as indicated by the number of Tet^r-colonies (triangles), was much lower than DNA-end joining.

genic zebrafish (P. Spaniol and T. Gerster, unpublished). Spermine/spermidine may protect DNA against degradation and/or keep it accessible to repair enzymes. Fertilized eggs of *Drosophila* were tested with increasing amounts of DNA (60, 200, 600 pg of linearized DNA per embryo) (Figure 3). Some of the fertilized eggs/early embryos were lysed immediately after injection with detergent and/or deep freezing, while others were incubated for various time periods as indicated before lysis. DNA was then extracted, competent *Escherichia coli* DH5α cells were transformed by electroporation and plated under different selective conditions (chloramphenicol as an internal control for DNA recovery, ampicillin to determine the total number of colonies, kanamycin to determine DNA-end joining, and tetracycline to determine homologous recombination). In both organisms, there was a rapid increase in the number of Kan^r-colonies indicative of efficient DNA-end joining. This joining activity was approximately proportional to the amount of injected DNA (Figures 2 and 3). With coinjected spermine/spermidine, the number of Kan^r-colonies was still increasing after the one-hour time point, while it leveled off in their absence. Therefore, spermine/spermidine enhance DNA-end joining, and apparently also homologous recombination, in zebrafish (Figure 2A and B vs. Figure 2C).

The observed end joining activity was verified by direct sequencing of a number of colonies from different experiments. The sequences of junctional regions, as shown in Figure 4A for both a completely filled-in and an imperfectly repaired junction, were compared to those recovered from *Xenopus* fertilized eggs. The results show that the junctions were quite heterogeneous, typically with small deletions at the junction point (Figure 4B). However, a closer inspection revealed some differences between species, particularly between vertebrates on one hand, and *Drosophila* on the other. The range of deletions was smallest in zebrafish, i.e., zebrafish embryos are able to perform quite faithful DNA-end joining with a relatively low error load. In fact, completely filled-in junctions were only recovered from zebrafish. While such filled-in junctions were obtained from DNA-end joining reactions in *Xenopus* egg extracts (Thode *et al.*, 1990) they are hardly, if at all, produced upon microinjection into fertilized *Xenopus* eggs (Lehman *et al.*, 1994 and Figure 4B). The greatest junctional variability was produced in *Drosophila*, and in addition there was a correlation between the amount of DNA injected and the extent of the deletion, suggesting

A



B

deletion in bp leftward (Bgl II)	rightward (Sac I)	total Δ	insert in bp (sequence)	*Xenopus* Σ 12	zebrafish Σ 24	*Drosophila* Σ 24
0	0	0			3	
0	-1	-1		2	3	
-1	0	-1			1	
0	-2	-2				1
-2	0	-2				1
0	-3	-3			2	1
0	-4	-4			3	
-1	-3	-4			1	
0	-5	-5			3	3
-3	-2	-5		2		
-5	0	-5	+1 (T)			1
0	-6	-6			2	
-3	-3	-6	+1 (G)			1
-2	-5	-7				1
0	-8	-8			1	
-4	-4	-8		2		
0	-9	-9			1	6
0	-10	-10			2	
-3	-7	-10				1
-5	-5	-10		1		
-4	-7	-11		2		
0	-12	-12				1
-4	-8	-12		1	1	
0	-13	-13				1
-2	-11	-13	+1 (C)			1
-1	-13	-14	+3 (GAT)			1
0	-15	-15			1	
-5	-10	-15		1		
-19	-2	-21	+5 (TCTCA)			1
-24	0	-24		1		
-20	-7	-27				1
-23	-36	-59	+4 (TCAG)			1
-30	-47	-77				1
Average deletion at junction:				**9 bp**	**5 bp**	**14 bp**

Fig. 4 Analysis of End-Joined Junctions in *Xenopus*, Zebrafish, and *Drosophila* Early Embryos.
(A) The end structure of the linear pReco-σ substrate (top) and a completely filled-in junction, as recovered from injected zebrafish eggs (middle), are shown. The bottom depicts one example from *Drosophila* with a deletion of 21 bp and concomitant insertion of 5 bp at the junction site; from the left hand side, 19 bp were deleted, i.e., the leftmost ACGA in the top strand of the upper two panels corresponds to the ACGA abutting the insert.
(B) Sequence analysis of the junction in Kan^r-colonies recovered from *Xenopus*, zebrafish, and *Drosophila*. Deletions (in bp) on either end, as well as in total are given. Insertions of a few base pairs were only found in *Drosophila*; the TCTCA insert is shown as an example under (A). The average deletion in these experiments was shortest in zebrafish (5 bp) and almost 3-times longer in *Drosophila* (14 bp).

that the repair system is heavily taxed by high concentrations of free DNA ends (Figure 4B and data not shown). In the process of DNA end repair, microhomologies, i.e. sequence complementarities of 1–6 bases at the two ends, can facilitate joining (Thode *et al.*, 1990; Ramsden and Gellert, 1998). Such microhomologies may also have played a role here: one specific 9 bp deletion found in seven independent isolates (six from *Drosophila*, one from zebrafish, designated 0/-9/-9 in Figure 4B) could have been generated by an overlap of the sequence GATC. Curiously, in *Drosophila* we observed also insertions of extra nucleotides at the junctional breakpoint (Figure 4A and B). These extra nucleotides were not obviously related to other sequences in their vicinity. This phenomenon is reminiscent of the addition of so-called N nucleotides by terminal deoxynucleotidyl transferase (TdT) during V(D)J recombination of vertebrate immune receptor genes (reviewed in Lewis, 1994; Gilfillan *et al.*, 1995; Hagmann, 1997). However, TdT may not be the only enzyme capable of producing 'filler' sequences, since even in non-lymphocyte mammalian cells, DNA-end joining occasionally resulted in oligonucleotide sequences not related to the junction (Roth *et al.*, 1991).

Both zebrafish and *Drosophila* yielded in the pReco-σ assay only few Tet^r-colonies indicative of homologous recombination (Figures 2 and 3). Their numbers were too small for an unambiguous assignment, especially since the recombination substrate always yields a background of spontaneous homologous recombination within the bacterial host (as discussed in Hagmann *et al.*, 1996). To overcome the background problem and to specifically detect homologous recombination, we devised a bimolecular substrate assay that is based on separately cloned partner molecules. One of the substrates was a plasmid containing an Amp^r-gene that had been mutagenized by introducing either a frameshift point mutation or a UAA Stop codon, yielding pMut-F and pMut-S, respectively (Figure 1B; for sequences see Materials and Methods). Suitable restriction sites were also inserted into the plasmid to allow its linearization at the site of mutation. The partner molecule consists of a 478 bp fragment of wild-type sequence covering the mutated region of the β-lactamase gene ('trAmp'; Figure 1B). With this bimolecular system, homologous recombination turned out to be readily detectable even within an overall excess of DNA-end joining.

The truncated Amp^r gene (trAmp) fragment was added at different concentrations to linearized or circular pMut-plasmid, and both were coinjected into stage VI oocytes of *Xenopus*, or fertilized eggs of zebrafish or *Drosophila*. As a recipient, we first tested the so-called pMut-F plasmid which harbours a single nucleotide insertion in the β-lactamase gene resulting in a frameshift (see Materials and Methods). Unexpectedly, this construct gave rise to spontaneous phenotypic reversion to Amp-resistance even in the absence of any wild-type DNA sequence. False positives were much higher in zebrafish than in *Drosophila* (not shown). Apparently, the efficient DNA-end repair in zebrafish as seen in Figure 4 had restored the Amp-resistance. Indeed, sequencing of several Amp^r plasmids recovered from zebrafish injections revealed that in each of them a small number of nucleotides were deleted at the repair junction, thereby restoring the original reading frame (data not shown). The generally larger deletions in *Drosophila* (see Figure 4B) seem to have precluded functional rescue by simple DNA-end joining. For most of the experiments, we thus used the pMut-S plasmid, which is more severely mutated in that it carries a Stop codon in the Amp-resistance gene. In pMut-S, Amp-resistance could not be easily restored in the absence of homologous recombination with a wild-type sequence.

Xenopus oocytes are known to support efficient homologous recombination (Carroll, 1983; Hagmann *et al.*, 1996), which was confirmed by the pMut-trAmp assay (Figure 5A). As expected, the more trAmp fragment was used the more Amp^r-colonies were recovered. However, the net increase was not strictly proportional to the amount of coinjected trAmp. The kinetics of the reaction were also altered: while the recombination reaction was virtually completed after 1 h with low amounts of trAmp, the number of Amp^r-colonies at higher trAmp-concentrations was highest at the 6 h time point and might have further increased upon prolonged incubation (Figure 5A). In addition to the double-stranded trAmp-fragment, we also tested two single-stranded oligonucleotides of wild-type sequence, spanning the mutated region, either unmethylated or methylated at all cytosine residues (for sequences, see Materials and Methods). Since double-stranded DNA with 5-methylcytosine is particularly stable (Butkus *et al.*, 1987), we were interested to see whether methylated DNA might facilitate strand invasion during homologous recombination. Both these oligonucleotides, irrespective of methylation, also yielded some colonies indicative of homologous recombination, but their number was only about 5% of the value observed with the 478 bp trAmp DNA segment (Figure 5A). Recombination with the single-stranded oligonucleotides seemed to be slightly more efficient in *Xenopus* compared to the other two species in that, at least with the methylated oligonucleotide, levels were in the same range as those with the lowest concentration of trAmp (Figure 5A). Nevertheless, on a molar basis the trAmp fragment was about 1000 fold more efficient than the single-stranded oligonucleotides, which were injected at very high concentrations. It remains to be seen whether recombination with oligonucleotides was inefficient due to the short sequence homology, or to rapid degradation of DNA ends in *Xenopus* oocytes which is well documented (see, e.g., Hagmann *et al.*, 1996). There is a report on efficient homologous gene targeting by synthetic oligonucleotides in lymphocytes (Cole-Strauss *et al.*, 1996); however, these findings have been questioned (Stasiak *et al.*, 1997).

With the pMut-S construct the background in zebrafish was very low, and homologous recombination could be easily detected (Figure 5B). Similar to the situation with *Xenopus*, recombination was most efficient and dose-de-

pendent with the double-stranded trAmp-fragment, while both single-stranded oligonucleotides were hardly active. Also in *Drosophila*, homologous recombination with the double-stranded trAmp fragment was quite impressive (Figure 5C). Even though homologous oligonucleotides have been used for gap repair in *Drosophila* (Banga and Boyd, 1992), in our hands the oligonucleotides were only marginally active (Figure 5C).

In all cases homologous recombination was strictly dependent upon linearization of the plasmid substrate. In other words, if one of the partner molecules was circular, i.e., uncut plasmid, homologous recombination occurred only at background levels, despite the second recombination partner being linear. Furthermore, coinjection into *Xenopus* eggs of bacterial proteins involved in recombination processes, namely purified RecA protein and/or single strand binding protein (SSB), also did not yield any significant homologous recombination with uncut plasmid DNA. In the case of a linearized substrate, RecA led to a moderate increase in the recombination efficiency (at most 2-fold; data not shown). The requirement for two linear substrates to achieve homologous recombination is not unexpected: in both murine somatic cells and embryonic stem cells, homologous recombination can be boosted 50- to 100-fold if the chromosomal gene is linearized by a rare-cutting restriction enzyme (Smih *et al.*, 1995 and references therein).

Our analysis of the fate of microinjected DNA shows that there is a significant degree of homologous recombination in all three systems of rapidly developing zygotes/early embryos, namely in *Drosophila*, zebrafish and *Xenopus*. Nevertheless, there is an overwhelming preponderance of DNA-end joining in the fertilized eggs of these organisms. This DNA-end joining activity is most faithful in zebrafish under the conditions tested since deletions are shorter when compared to the other two species.

Since both *Drosophila* and zebrafish have gained wide recognition as genetic model organisms, our studies might be viewed as preliminary experiments towards experimental induction of homologous recombination, e.g., to achieve targeted gene disruption in these animals. Recently, a phenomenon designated 'parahomologous' recombination has been described in *Drosophila*, which leads to the chromosomal integration of introduced DNA in the vicinity of homologous sequences (Cherbas and Cherbas, 1997; see also Hama *et al.*, 1990; Whiteley and Kassis, 1997). Despite generally targeting a particular chromosomal locus, this type of recombination does not allow precise predetermined alterations of the genome. To achieve and optimize true gene targeting by homologous recombination, model experiments might have to include chromosomally integrated target DNA, preferably with the possibility of linearizing it to facilitate recombination, as discussed above (Smih *et al.*, 1995).

In *Drosophila*, where true gene targeting of any chosen gene has not been achieved so far, the DNA break generated by the mobilization of a P element transposon is indeed amenable to repair by homologous sequences (Ban-

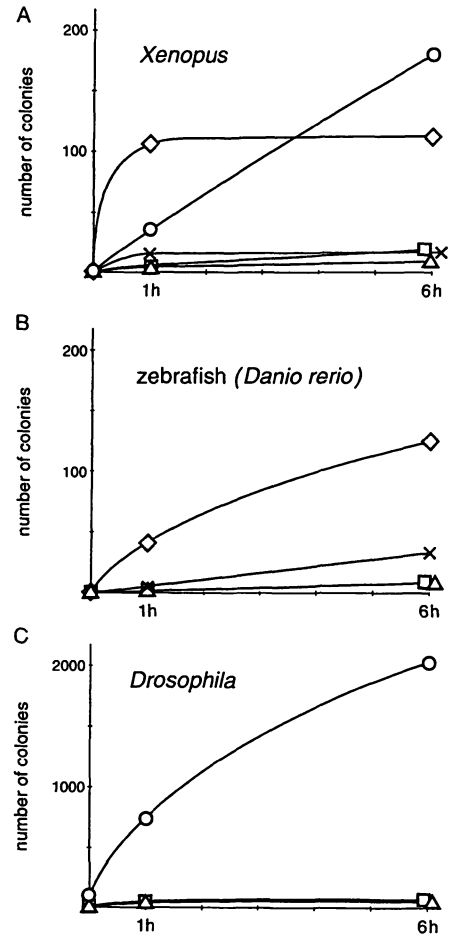


Fig. 5 Double Strand Gap Repair by Homologous Recombination in *Xenopus*, Zebrafish, and *Drosophila*.
(A) *Xenopus* stage VI oocytes as a positive control for homologous recombination (see also Carroll, 1983; Hagmann *et al.*, 1996). A schematic overview of the bimolecular reaction is given in Figure 1 B. At a given concentration of 100 ng/μl pMut-S recipient plasmid, increasing concentrations of trAmp fragment were coinjected (10 ng/μl, 100 ng/μl or 1 mg/μl, corresponding to molar ratios trAmp/pMut-S of 0.8, 8, or 80, respectively). The efficiency of homologous recombination was dose-dependent: the more double-stranded trAmp fragment was injected [cross (10 ng/μl), diamond (100 ng/μl), and circle (1 μg/μl)], the more Amp^r-colonies were recovered. With either methylated (square) or unmethylated (triangle) single-stranded oligonucleotides (1 μg/μl), the frequency of homologous recombination was about 5–10% of the value seen with the 478 bp trAmp fragment.
(B) In zebrafish, homologous recombination between trAmp and pMut-S recipient DNA was also more efficient with high concentrations of trAmp (10 ng/μl; cross vs. 100 ng/μl; diamonds, corresponding to molar ratios trAmp/pMut-S of 0.8 and 8, respectively). Results with methylated and unmethylated single-stranded oligonucleotides are indicated by squares and triangles, respectively. As in Figure 2A and B, the injection buffer contained spermine/spermidine.
(C) In *Drosophila*, coinjection of linearized pMut-S plasmid (100 ng/μl) with the double-stranded trAmp fragment (500 ng/μl; circles) revealed efficient homologous recombination. Methylated (square) or unmethylated (triangle) single-stranded oligonucleotides were again inefficient recombination substrates. The absolute number of colonies may not be directly comparable between species because of differences in amount and concentration of DNA and/or protocol of injection.

ga and Boyd, 1992; Lankenau, 1995; Keeler *et al.*, 1996).

In any case, our studies show that in order to obtain homologous recombination at appreciable frequency in zebrafish and *Drosophila*, one would have to inhibit or bypass the predominant DNA-end joining activity in these species. One possibility might be to use a different developmental stage of the germ cells: in our previous report we found that *Xenopus* oocytes, in marked contrast to mature eggs and early embryos, lack any appreciable DNA-end joining activity. Thus oocytes preferentially repair DNA by homologous recombination, and we have proposed to consider oocytes and/or other germ cells for gene targeting studies (Hagmann *et al.*, 1996).

Alternatively, one would have to specifically inhibit the DNA-end joining apparatus in zygotes/early embryos, e.g., by substrate inhibition (see also Hagmann *et al.*, 1996), or by specific targeting of the proteins involved. For example, Ku antigen, a heterodimeric protein, has been shown to be involved in DNA double strand break repair. Ku is required for proper alignment of DNA ends in V(D)J rearrangement, and cells lacking Ku antigen have to resort to microhomologies for DNA end joining (Ramsden and Gellert, 1998). Ku, which is part of the multisubunit DNA-dependent protein kinase (DNA-PK) (Jackson and Jeggo, 1995; Weaver, 1995) might thus be a possible target for manipulation of cellular recombination systems, as is poly-ADP-ribose polymerase (PARP) (Morrison *et al.*, 1997). Elimination of the PARP gene by targeted gene disruption results in a greatly increased frequency of homologous recombination, at least between sister chromatids (Wang *et al.*, 1997; Ménissier de Murcia *et al.*, 1997). Interestingly, PARP activity in *Xenopus* appears at the oocyte-to-egg transition stage (Aoufouchi and Shall, 1997), which parallels the transition to efficient DNA-end joining (Goedecke *et al.*, 1992; Hagmann *et al.*, 1996). Finally, gene targeting may be facilitated by selective overexpression of proteins promoting homologous recombination (Bezzubova *et al.*, 1997).

Materials and Methods

Plasmid Construction and Oligonucleotides

The recombination vector pReco-σ and the internal reference plasmid pCm184 have been described previously (Hagmann *et al.*, 1996). The primers used for the PCR-based detection of recombination products (Tet^r or Kan^r, see Figure 1A) are as follows:

Tet up (27 nt; 5′-end at position 149 in the Tet-resistance gene)

5′-CGCTATATGCGTTGATGCAATTTCTAT-3′

Tet low (19 nt; 5′-end at position 786 in the Tet-resistance gene)

5′-AGCGAGGGCGTGCAAGATT-3′

Kan (20 nt; 5′-end at position 148 in the Kan-resistance gene)

5′-GCGCATCGGGCTTCCCATAC-3′

The recombination vectors pMut-F/S were constructed by replacing the *Pst*I-*Fsp*I fragment within the Amp-resistance gene from pACYC 177 with different double stranded oligonucleotides harboring mutations.

Original sequence:

5′-GCAATGGCAACAACGTTGC-3′ wild type
3′-ACGTCGTTACCGTTGTTGCAACG-5′

pMut-S
5′-GCAATGGCAACA**TAA**TTGC-3′ Stop codon
3′-ACGTCGTTACCGTTGTATTAACG-5′ (ochre)

pMut-F
5′-GCAATGGCAACAAC**C**GTTGC-3′ +1 frame-
3′-ACGTCGTTACCGTTGTTGGCAACG-5′ shift

For the vector containing the 478 bp trAmp ('truncated Ampicillin') fragment, the ampicillin-resistance gene from pBluescript (pBSSK$^-$) was replaced by the *Hin*dIII fragment from pACYC 184 containing the chloramphenicol-resistance gene. Thereafter, a 593 bp *Ban*I-*Hin*cII fragment from pACYC 177 containing the 478 bp *Ahd*I-*Sca*I trAmp fragment was blunted and cloned into the *Eco*RV site of the modified pBluescript vector.

The single-stranded oligonucleotides spanning the mutated region within the Amp-resistance gene are the following:

Unmethylated oligonucleotide (80nt):

5′-AACGACGAGCGTGACACCACGATGCCTGCAGCA ATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACT TACTCT-3′

Methylated oligonucleotide (80nt; 23 cytosine residues are C^5-methylated):

5′-AA^{m}CGA^{m}CGAG^{m}CGTGA^{m}CA^{m}C^{m}CA^{m}CGATG^{m}C^{m}CTG^m CAG^{m}CAATGG^{m}CAA^{m}CAA^{m}CGTTG^{m}CG^{m}CAAA^{m}CTATTAA^m CTGG^{m}CGAA^{m}CTA^{m}CTTA^{m}CT^{m}CT-3′

Zebrafish Egg Injections

The injection buffer and the experimental setup for injections were as previously described (Hauptmann and Gerster, 1996). For most experiments, both spermine (30 μM) and spermidine (70 μM) were included. An amount of about 1 nl of solution was injected at the indicated DNA concentrations into freshly fertilized zygotes. (Although it is difficult to measure the injected volume in absolute terms, care was taken to ensure a constant injection volume into all zygotes of an injection series. In addition, the relative recovery of chloramphenicol^r control plasmid allowed an independent volume estimation). Oligonucleotides were used at a concentration of 1 μg/ml injection buffer in all three species. After the times indicated the zygotes were harvested in lysis buffer and quickly frozen (see below).

Xenopus laevis Oocyte/Egg Injections

Approximately 10 nl DNA solution were injected into each *Xenopus laevis* oocyte/fertilized egg as previously described (Hagmann *et al.*, 1996). The zygotes/early embryos were harvested after the times indicated by resuspension in lysis buffer (see below).

Drosophila melanogaster Egg Injections and DNA Extraction

Approximately 1 nl of DNA solution was injected into *Drosophila rosy*^{506} embryos prior to pole cell formation, which was about 1 hour AEL (After Egg Laying), according to the standard P-element mediated transformation protocol (Rubin and Spradling, 1982). Twenty embryos were injected per time point and incubated at 18 °C for various time periods as indicated. The embryos were frozen quickly in liquid nitrogen, lysed in 50 μl lysis buffer (0.1 M Tris pH 9.0, 0.1 M EDTA, 1% SDS and 0.5 μl diethyl pyrocarbonate), ground for 1 minute and incubated at 68–70 °C for 30 minutes. After RNase A digestion, DNA was extracted serially

with phenol, phenol/dichloromethane and dichloromethane and subsequently precipitated. Finally, DNA was resuspended in 0.1 × TE (TE is 10 mM Tris, 0.1 mM EDTA, pH 8).

DNA Extraction and *E. coli* Electroporation

After the indicated incubation times, pools of 10 oocytes or eggs from *Xenopus laevis* or pools of 20 eggs from *Danio rerio* (zebrafish) were taken up in 400 μl lysis buffer (50 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% SDS, 1 mg/ml Proteinase K), frozen quickly, incubated for 1 h at 37 °C, and frozen at −80 °C. DNA was extracted as described above. DNA and the coextracted RNA were resuspended in TE, after which appropriate amounts were electroporated into competent *E. coli* DH5α cells, and the bacteria were plated on antibiotic-containing LB agar plates (Hagmann *et al.*, 1996). Most data points in Figures 2, 3 and 5 represent the average of three independent electroporations.

Acknowledgements

We are indebted to Drs. Petra Pfeiffer (Essen) and to Markus Noll, Joy Alcedo and Felix Althaus (Zürich) for valuable discussions. This work was supported by the Schweizerischer Nationalfonds and the Kanton Zürich.

References

Aoufouchi, S., and Shall, S. (1997). Regulation by phosphorylation of *Xenopus laevis* poly(ADP-ribose) polymerase enzyme activity during oocyte maturation. Biochem. J. *325*, 543–551.

Banga, S.S., and Boyd, J.B. (1992). Oligonucleotide-directed site-specific mutagenesis in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *89*, 1735–1739.

Bezzubova, O., Silbergleit, A., Yamaguchi-Iwai, Y., Takeda, S., and Buerstedde, J.M. (1997). Reduced X-ray resistance and homologous recombination frequencies in a RAD54$^{(-/-)}$ mutant of the chicken DT40 cell line. Cell *89*, 185–193.

Butkus, V., Klimasauskas, S., Petrauskiene, L., Maneliene, Z., Janulaitis, A., Minchenkova, L.E., and Schyolkina, A.K. (1987). Synthesis and physical characterization of DNA fragments containing N4-methylcytosine and 5-methylcytosine. Nucl. Acids Res. *15*, 8467–8478.

Carroll, D. (1983). Genetic recombination of bacteriophage λ DNAs in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA *80*, 6902–6906.

Cherbas, L., and Cherbas, P. (1997). Parahomologous gene targeting in *Drosophila* cells – an efficient, homology-dependent pathway of illegitimate recombination near a target site. Genetics *145*, 349–358.

Cole-Strauss, A., Yoon, K., Xiang, Y., Byrne, B.C., Rice, M.C., Gryn, J., Holloman, W.K. and Kmiec, E.B. (1996). Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. Science *273*, 1386–1389.

Gaiano, N., Allende, M., Amsterdam, A., Kawakami, K., and Hopkins, N. (1996). Highly efficient germ-line transmission of proviral insertions in zebrafish. Proc. Natl. Acad. Sci. USA *93*, 7777–7782.

Gilfillan, S., Benoist, C., and Mathis, D. (1995). Mice lacking terminal deoxynucleotidyl transferase: adult mice with a fetal antigen receptor repertoire. Immunol. Rev. *148*, 201–219.

Goedecke, W., Vielmetter, W., and Pfeiffer, P. (1992). Activation of a system for the joining of nonhomologous DNA ends during *Xenopus* egg maturation. Mol. Cell. Biol. *12*, 811–816.

Hagmann, M., Adlkofer, K., Pfeiffer, P., Bruggmann, R., Georgiev, O., Rungger, D. and Schaffner, W. (1996). Dramatic changes in the ratio of homologous recombination to nonhomologous DNA-end joining in oocytes and early embryos of *Xenopus laevis*. Biol. Chem. Hoppe-Seyler *377*, 239–250.

Hagmann, M. (1997). RAGged Repair: What's New in V(D)J Recombination. Biol. Chem. *378*, 815–819.

Hama, C., Ali, Z., and Kornberg, T.B. (1990). Region-specific recombination and expression are directed by portions of the *Drosophila* engrailed promoter. Genes Dev. *4*, 1079–1093.

Hauptmann, G., and Gerster, T. (1996). Complex expression of the zp-50 POU gene in the embryonic zebrafish brain is altered by overexpression of sonic hedgehog. Development *122*, 1769–1780.

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y., and Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Devel. Biol. *192*, 289–299.

Jackson, S.P., and Jeggo, P.A. (1995). DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. Trends Biochem. Sci. *20*, 412–415.

Keeler, K.J., Dray, T., Penney, J.E., and Gloor, G. (1996). Gene targeting of a plasmid-borne sequence to a double-strand break in *Drosophila melanogaster*. Mol. Cell. Biol. *16*, 522–528.

Lankenau, D.-H. (1995). Genetics of genetics in *Drosophila*: P elements serving the study of homologous recombination, gene conversion and targeting. Chromosoma *103*, 659–668.

Lehman, C.W., Trautman, J.K., and Carroll, D. (1994) Illegitimate recombination in *Xenopus*: characterization of end-joined junctions. Nucleic acids Res. *22*, 434–442.

Lewis, S.M. (1994). The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv. Immunol. *56*, 27–150.

Long, Q.M., Meng, A.M., Wang, H., Jessen, J.R., Farrell, M.J., and Lin, S. (1997). GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development *124*, 4105–4111.

Ménissier de Murcia, J., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Mark, M., Oliver, F.J., Masson, M., Dierich, A., LeMeur, M., Waltzinger, C., Chambon, P., and de Murcia, G. (1997). Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc. Natl. Acad. Sci. USA *94*, 7303–7307.

Morrison, C., Smith, G.C., Stingl, L., Jackson, S.P., Wagner, E.F., and Wang, Z.Q. (1997). Genetic interaction between PARP and DNA-PK in V(D)J recombination and tumorigenesis. Nature Genetics *17*, 479–482.

Ramsden, D.A., and Gellert, M. (1998). Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double strand breaks. EMBO J. *17*, 609–614.

Roth, D.B., Proctor, G.N., Stewart, L.K., and Wilson, J.H. (1991). Oligonucleotide capture during end joining in mammalian cells. Nucleic Acids Res. *19*, 7201–7205.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science *218*, 348–353.

Smih, F., Rouet, P., Romanienko, P.J., and Jasin, M. (1995). Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. Nucl. Acids Res. *23*, 5012–5019.

Stasiak, A., West, S.C., and Engelman, E.H. (1997). Sickle cell anemia research and a recombinant DNA technique (Letter to the Editor). Science *277*, 460–461.

Thode, S., Schäfer, A., Pfeiffer, P., and Vielmetter, W. (1990). A novel pathway of DNA end-to-end joining. Cell *60*, 921–928.

Wang, Z.-Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K., and Wagner, E. (1997). PARP is important for genomic stability but dispensable for apoptosis. Genes Dev. *11*, 2347–2358.

Weaver, D.T. (1995). What to do at an end: DNA double-strand-break repair. Trends Genet. *11*, 388–392.

Whiteley, M., and Kassis, J.A. (1997). Rescue of *Drosophila* engrailed mutants with a highly divergent mosquito engrailed cDNA using a homing, enhancer-trapping transposon. Development *124*, 1531–1541.

Received March 27, 1998; accepted April 16, 1998