Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 806–810, January 1995
Biochemistry

Endonuclease-induced, targeted homologous extrachromosomal recombination in *Xenopus* oocytes

(gene targeting/oocyte injection/single-strand annealing/I-*Sce* I)

DAVID J. SEGAL AND DANA CARROLL

Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT 84132

Communicated by Martin Gellert, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, October 13, 1994

ABSTRACT **Homologous recombination in gene targeting in most organisms occurs by an inefficient mechanism. Inducing a double-strand break in the chromosomal target may increase this efficiency by allowing recombination to proceed by the highly efficient single-strand annealing mechanism. A gene targeting experiment was modeled in *Xenopus* oocytes by using a circular plasmid to mimic the chromosomal target site and a homologous linear molecule (pick-up fragment or PUF) as an analogue of the vector DNA. When those two molecules were simply injected together, no recombination was observed. In contrast, when the circular plasmid was cleaved *in vivo* by injection of the site-specific endonuclease, I-*Sce* I, relatively efficient intermolecular recombination occurred, involving up to 17% of the cleaved molecules. Recombination was dependent on the stability of the PUF; product yield was increased by using longer fragments and by injecting larger amounts of linear DNA, both of which increased the lifetime of the PUF in the oocytes. These results demonstrate that *in vivo* double-strand breaks can induce homologous recombination of reluctant substrates and may be useful in augmenting the efficiency of gene targeting.**

Gene targeting is the process in which an exogenous DNA undergoes homologous recombination with its corresponding chromosomal site. Partial gene duplications, gene replacements, and gene knockouts have been created with this technology, which has the advantage that the modified gene resides at its normal chromosomal locus (1–3). In higher organisms, and in mammalian cells in particular, only very low frequencies of homologous targeting events have been achieved, usually in the range of 10^{-6} per cell. In addition, homologous targeting occurs against a background of nonhomologous events that are 100- to 1000-fold more common (1, 4). Ingenious procedures have been devised to select or screen for the rare successful targeting products (3–5), but the low absolute frequency of favorable events remains a serious limitation.

Available evidence suggests that (inefficient) homologous targeting in mammalian cells proceeds by a conservative recombination mechanism (6). The search for homology does not appear to be limiting in these reactions (7, 8); therefore, the reasons underlying their inefficiency must lie elsewhere. In these same cells, extrachromosomal homologous recombination is accomplished by a more efficient, nonconservative single-strand annealing (SSA) mechanism (9, 10). Briefly, this mechanism requires a strand-specific exonuclease to expose complementary strands of homologous sequences, which then anneal and are repaired to form covalently closed products.

It has occurred to us (11) and to others (12) that the efficiency of gene targeting could be improved if it could be induced to utilize the SSA mechanism. Because the production of complementary strands is mediated by exonuclease, all participants in SSA recombination must have ends. The exogenous vector DNA in a targeting experiment typically fulfills this criterion, but the chromosomal target does not. If a double-strand break (DSB) could be introduced specifically at the desired site, this could potentially activate it for SSA. A DSB generated by a site-specific endonuclease *in vivo* was shown to stimulate nonconservative recombination (presumably via SSA) for chromosomal and extrachromosomal intramolecular events in yeast (13–16) and for extrachromosomal events in plant cells (17) and mammalian cells (18). However, the details and parameters of these reactions have not been analyzed.

Xenopus oocytes support efficient homologous recombination of injected DNA substrates, and a large body of direct evidence indicates that SSA (also referred to as resection annealing) is the responsible mechanism (11, 19, 20). In this study we have extended the observation of endonuclease-induced, intramolecular recombination to the *Xenopus* system. In addition, we have shown that a model targeting experiment works with very good efficiency. We have shown that cleavage of an inert, stable circular DNA *in vivo* activates it for recombination with a homologous linear partner.

MATERIALS AND METHODS

DNA Substrates. pSce2 (5636 bp) was constructed by cloning complementary oligonucleotides (5′-TCGAGACGC-TAGGGATAACAGGGTAATACG-3′, 5′-TCGACGTATT-ACCCTGTTATCCCTAGCGTC-3′) containing the I-*Sce* I target sequence (16) into the *Xho* I site of pRW4, which is essentially pBR322 with a direct 1.25-kb duplication of the tetracycline-resistance gene (21). One *Xho* I site was reconstructed in the process. pSce1 (4412 bp) was constructed by removing one copy of the tetracycline-resistance duplication of pSce2 (by cleavage with *Xho* I and *Cla* I), filling in the ends with Klenow polymerase, and ligating at low DNA concentration.

The pick-up fragments (PUFs) were generated by digestion of the plasmids, pN5, pG20, and pN3, each of which contains a *Xenopus laevis* genomic DNA fragment inserted at the *Hin*dIII site of pBR322 (22, 23). The insert sizes are as follows: pN5, 0.73 kb; pG20, 1.25 kb; pN3, 4.88 kb. After the appropriate restriction enzyme digestions, the PUFs were sometimes purified by 1% agarose gel electrophoresis and recovered with Geneclean (Bio 101). In other experiments, the PUFs were not purified from their vector fragment. pHSS6 was included in all injections as a recovery control (24). All DNAs were extracted with phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), and then ether, followed by ethanol precipitation and resuspension in TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA).

Oocyte Injections. Injections of materials into the nuclei of stage VI *Xenopus* oocytes were conducted by procedures described in detail elsewhere (21). In some cases, blue dextran

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DSB, double-strand break; SSA, single-strand annealing; PUF, pick-up fragment.

(10 mg/ml; Sigma) was coinjected to monitor the success of nuclear injection. In a typical experiment, 1 ng of pSce2 and 1 ng of pHSS6, or 0.5 ng of pSce1 and 1.5 ng of pHSS6, were coinjected into 10–30 oocytes in a 10-nl volume and the oocytes were incubated for at least 3 hr at 18°C. These amounts were chosen to maximize eventual endonuclease cleavage *in vivo*, while ensuring proper chromatin assembly (25). I-*Sce* I (10 units/μl; Boehringer Mannheim) was diluted 1:1 with a 1× buffer supplied by the manufacturer. $MgCl_2$ was added to 2.5 mM, and 50 milliunits were injected in a volume of 10–50 nl, depending on whether PUF was coinjected. For the experiment shown in Fig. 2, the molar ratio of PUF:target was 2:1, corresponding to 0.55, 0.70, and 1.56 ng per oocyte for PUF1, PUF2, and PUF3, respectively. For the experiment shown in Fig. 3, the molar ratio of PUF:target was 14:1, corresponding to 8, 9, and 14.8 ng per oocyte for PUF1, PUF2, and PUF3, respectively. Five nanograms of double-stranded M13mp19 replicative form DNA, cleaved by *Cla* I (fragment sizes, 4.4 kb and 2.9 kb), was coinjected in the latter experiments (see text).

After injection, oocytes were incubated at room temperature overnight (at least 20 hr) or for the time indicated. Nuclei were removed and the DNA was prepared as described (21). All extracted DNAs were digested with analytical restriction enzymes, separated by 1.2% agarose gel electrophoresis in TBE buffer (89 mM Tris·HCl/89 mM boric acid/2 mM EDTA, pH 8), immobilized on Zeta-Probe nylon membranes (Bio-Rad) by overnight capillary transfer in 0.4 M NaOH, and hybridized by the method of Church (26) with random-primed ^{32}P-labeled pBR322 (27). This probe hybridizes to the recombination substrates, pHSS6, the PUFs, and their vector fragments (when present) but not with M13mp19. For the experiment shown in Fig. 3, the probe was constructed from only the large fragment of a *Pst* I and *Sal* I digest of pBR322, which hybridizes with all of the above molecules except the unrecombined PUFs. Quantitation for Fig. 3 was aided by the use of a probe constructed from the small fragment of a *Pst* I digest of pHSS6, which hybridizes with pHSS6 only (data not shown). Autoradiography employed Kodak X-AR film, and quantitation was performed on a Molecular Dynamics PhosphorImager. Nominally identical uninjected samples were found to vary upon quantitation by ±25%. We expect a similar degree of accuracy for other samples.

RESULTS

Intramolecular Recombination. Injected circular DNA molecules are stable in *Xenopus* oocyte nuclei, but simple linear molecules are degraded (21, 28–30). Linear molecules containing appropriately placed homologous sequences become substrates for efficient recombination by a nonconservative SSA mechanism (11, 20, 21, 29). Circular DNAs with homologous repeats, however, are inert in recombination (29, 30). We understand this in terms of the inaccessibility of circular molecules to the 5′ → 3′ exonuclease that mediates SSA recombination (11, 21).

To test whether delivery of a DSB to a circular DNA *in vivo* would activate it for recombination, we designed a substrate molecule, pSce2, with a cleavage site for the site-specific endonuclease, I-*Sce* I, between two direct 1.25-kb repeats (see Fig. 1*A*). I-*Sce* I was chosen because it has been previously demonstrated to cleave chromatin *in vivo* (16). In addition, its 18-bp recognition sequence is not expected to occur in the *Xenopus* genome (31).

pSce2 was injected into oocyte nuclei and incubated for 3 hr to allow chromatin formation (25, 32). I-*Sce* I was then injected into the nuclei of these same oocytes, and incubation was continued for various times before the nuclei were removed and the DNA was extracted. DNA recoveries were normalized to that of coinjected circular pHSS6, indicated as fragment R in Fig. 1*B*.

As shown in Fig. 1*B*, *in vivo* cleavage resulted in the formation of recombination products. Cleavage by I-*Sce* I was evidenced in

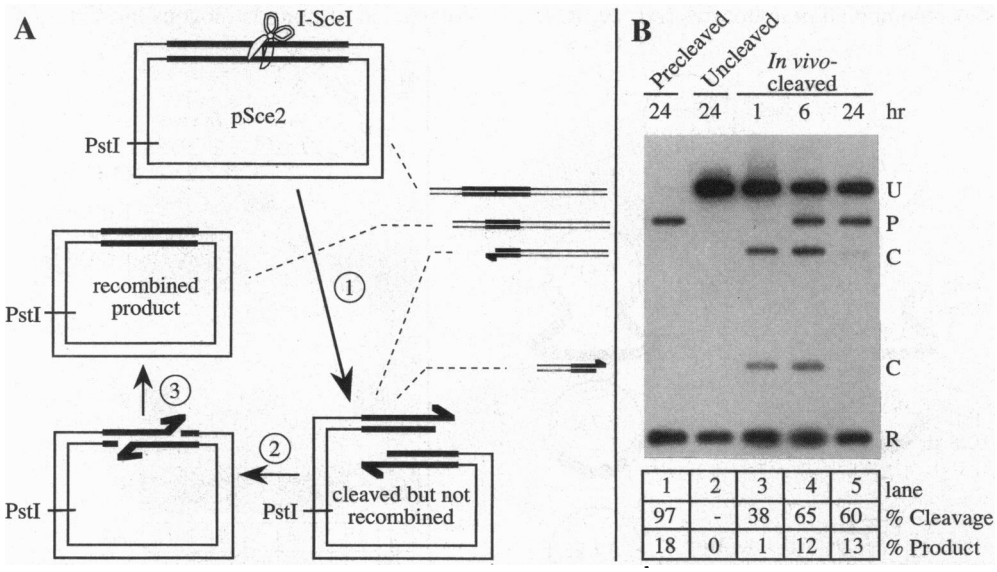


FIG. 1. I-*Sce* I-induced, intramolecular recombination. (*A*) Diagram of the pSce2 substrates and recombination intermediates and products generated by the SSA mechanism. Dashed lines are drawn from each of these species to fragments they yield upon analytical digestion with *Pst* I; the fragments are shown next to their positions in the autoradiogram in *B*. The dark lines on pSce2 represent the directly oriented 1.25-kb repeats. Half arrowheads indicate the 3′ end of each strand. Cleavage by I-*Sce* I (scissors) between the two repeats produces molecular ends (1), which are degraded by an endogenous 5′ → 3′ exonuclease to expose complementary strands. The strands anneal (2) and are repaired and ligated to form recombined product (3). Intermolecular events are also possible with this substrate. (*B*) Autoradiogram of a typical time course experiment. Labels at the right indicate the position of molecules that are uncleaved by I-*Sce* I (U), recombination products (P), molecules cleaved by I-*Sce* I but not recombined (C), and the recovery control plasmid (R). Quantitation of the percent of pSce2 cleaved by I-*Sce* I (% Cleavage) and percent of pSce2 converted to product (% Product) appear below the figure. The original amount of pSce2 was determined from uninjected samples (not shown), and all values were normalized by comparison to the recovery control plasmid. Lanes: 1, pSce2 cleaved by I-*Sce* I prior to injection; 2, pSce2 injected in the absence of I-*Sce* I; 3–5, pSce2 cleaved by I-*Sce* I *in vivo*. pSce2 was injected 3 hr prior to I-*Sce* I, after which incubation continued for the times indicated.

this analysis by the appearance of two linear fragments, labeled C in Fig. 1*B* (lanes 3 and 4). Cleavage reached a maximum level of about 60% of the substrate DNA several hours after enzyme injection. Products of homologous recombination, as evidenced by the unique band labeled P, are very faintly visible within 1 hr and level off at 12–13% of total injected pSce2 by about 6 hr after I-*Sce* I injection (Fig. 1*B*, lanes 3–5). Substrate molecules that were cleaved but did not recombine were degraded during prolonged incubation (lane 5).

Two control samples confirm previous observations. First, when pSce2 was cleaved prior to injection, essentially only recombination products were recovered (Fig. 1*B*, lane 1). Second, virtually no recombination products were observed when I-*Sce* I was not injected (lane 2). The faint (<1%) "product" band visible in lane 2 was present in uninjected samples and is believed to have arisen from recombination in bacteria during plasmid preparation. This trace amount of recombination is routinely seen with this substrate and is always much less than that obtained when a DSB is present. The yield of products following *in vivo* cleavage was 22% of the cleaved molecules (lane 5), which is quite comparable to the 18% yield obtained with the precleaved sample (lane 1).

Intermolecular Recombination. We next investigated an intermolecular recombination event that is conceptually analogous to a typical gene targeting experiment. The model for the chromosomal target was a circular plasmid DNA, pSce1, which is essentially pBR322 with a recognition sequence for I-*Sce* I inserted at the *Cla* I site. This plasmid carries no repeated sequences, so it is incapable of recombination with itself following I-*Sce* I cleavage. Three model vector DNAs were used, which were linear fragments from pBR322 clones with inserts of different sizes at the *Hin*dIII site (which is only 6 bp away from the *Cla* I site) (Fig. 2*A*). We call these PUFs because they can potentially recombine with and thereby rescue pSce1 after it is cut by I-*Sce* I. Each PUF has 0.66–0.78 kb of perfect homology with pSce1 on each side of the insertion/cleavage site (Fig. 2*A*); this should be sufficient to support efficient SSA recombination in oocytes (ref. 29; R. J. Dawson and D.C., unpublished data). The expected products are analogous to those hoped for in a targeting experiment: the inserts from the linear PUFs are incorporated at the homologous site of pSce1 (Fig. 2*A*).

As before, pSce1 was injected into oocyte nuclei and allowed 3 hr to assemble into chromatin prior to injection of a PUF and/or I-*Sce* I. In the absence of a PUF, I-*Sce* I cleaved about half of the circular molecules *in vivo*, but the cleaved DNA was simply degraded (Fig. 2*B*, lane 1). When PUF3 was injected but no enzyme was provided, the circular pSce1 was stable, the PUF was degraded, and no recombination products were formed (lane 2). When the pSce1 was cleaved prior to injection, it recombined intermolecularly with each of the PUFs (lanes 3–5). At the inputs used in this experiment (0.5 ng of pSce1 and a 2-fold molar excess of PUF in each oocyte), the yield of products increased significantly with the length of the PUF.

The results shown in lanes 6–8 of Fig. 2*B* demonstrate that *in vivo* cleavage of pSce1 activates it for recombination with PUF2 and PUF3. The yields were considerably lower than that seen with the precleaved plasmid, and that for PUF1 was below the limit of detection in this simple Southern blot analysis. Nonetheless, this demonstrates stimulation of homologous intermolecular recombination by site-specific cleavage of the target DNA.

Improving Product Yield. One possible explanation for the low product yields in the preceding experiment is that the PUFs did not persist in the oocytes long enough to recombine with the pSce1 after it was cleaved. We know that injected linear DNAs are degraded rapidly in oocytes (21), whereas I-*Sce* I cleavage of pSce1 may take place over several hours (see Fig. 1*B*). We also have some evidence that DNA cleaved *in vivo* is degraded more slowly than injected linear DNA, perhaps due to the prior assembly of chromatin proteins onto the circular molecules that were injected (data not shown).

We have shown previously that the rate of DNA degradation is slowed by injection of larger amounts of DNA and by coinjection of nonhomologous linear fragments (33, 34). We

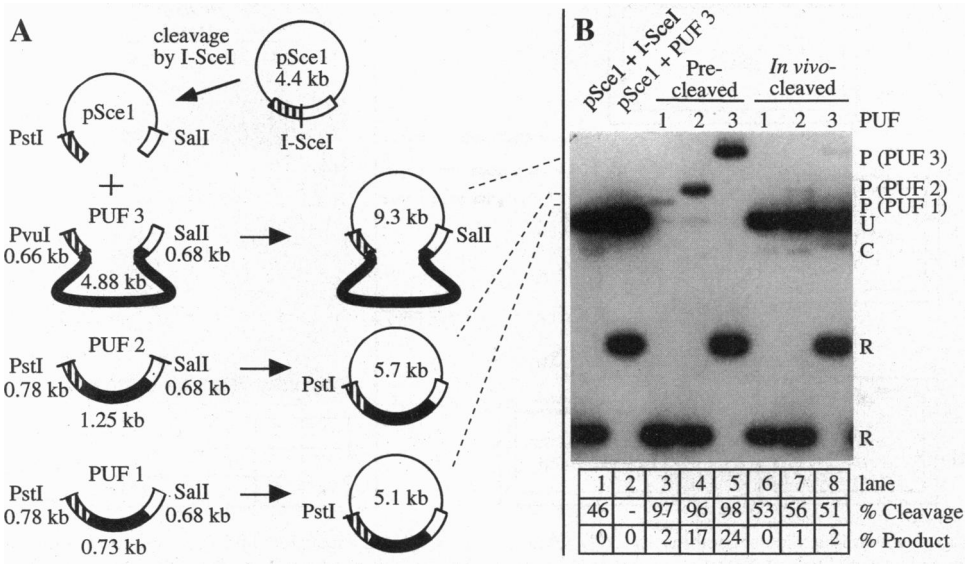
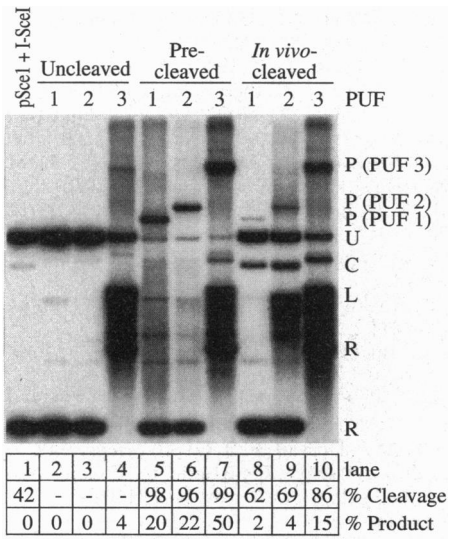


FIG. 2. Intermolecular recombination of pSce1 with the three PUFs. (*A*) Reaction substrates and products. Hatched and open boxes represent regions of homology between pSce1 and the PUFs. The solid black regions on the PUFs represent *Xenopus* genomic inserts of the indicated sizes. The expected positions of the recombination products, after cleavage with *Pst* I (PUF1, PUF2) or *Sal* I (PUF3), are indicated with reference to the autoradiogram in *B*. (*B*) Autoradiogram of an experiment showing induction of intermolecular recombination between pSce1 and PUFs by *in vivo* I-*Sce* I cleavage. Labels are as in Fig. 1*B*. Most samples were analytically digested with *Pst* I before electrophoresis; however, because the insert in PUF3 contains a site for *Pst* I, samples containing this molecule were analytically digested with *Sal* I and *Not* I. This enzyme set altered the position of the recovery control plasmid band (R). If the PUF fragments were not degraded, PUF3 would run just behind the band P (PUF2), PUF2 would run approximately midway between the bands labeled C and R, and PUF1 would run just ahead of the larger R band. Lanes: 1, pSce1 and I-*Sce* I in the absence of any PUF; 2, pSce1 and PUF3 in the absence of I-*Sce* I; 3–5, pSce1 cleaved by I-*Sce* I prior to injection, coinjected with each PUF; 6–8, pSce1 cleaved by I-*Sce* I *in vivo*.

combined these factors by increasing the molar ratio of PUF:target from 2:1 to 14:1 and by including nonhomologous DNA fragments. The latter were of two sorts: (*i*) the PUFs were not purified away from their vector fragments, which effectively doubles the concentration of molecular ends; (*ii*) 5 ng of M13 replicative form DNA cut with *Cla* I was coinjected, bringing the total number of ends in each sample to 8×10^{10}. This is 40 times the end concentration of the experiment shown in Fig. 2.

As seen in Fig. 3, this increase in the amount of injected linear DNA substantially increased the yield of intermolecular recombination products. In the samples for which I-*Sce* I was injected to induce cleavage *in vivo*, cutting was slightly more effective than seen previously, and recombination products were easily observed in each case (lanes 8–10). In fact, the product yields for the *in vivo* cleaved DNAs approached to within a few-fold those observed for the precleaved samples (lanes 5–7). The percent cleavage appeared to increase with the size of the PUF, perhaps indicating saturation of the chromatin protein pool and consequent increased availability of the I-*Sce* I cleavage site as the mass of injected DNA increased. It is conceivable that recombination products would have continued to accumulate if the incubations had been extended beyond 24 hr, since there was still some PUF and some cleaved pSce1 (bands labeled C) remaining in these samples. The persistence of the injected linear DNA (bands labeled L and associated smears) was evident, particularly in the case of PUF3, where the total mass of injected DNA was largest.

The control injections again showed that no recombination occurred in the absence of injected PUF (Fig. 3, lane 1) or without cleavage of the target pSce1 (lanes 2–4). The one apparent exception was the uncleaved PUF3 sample (lane 4); however, the apparent product in this case was the result of incomplete cleavage of pN3 by *Pvu* I and *Sal* I prior to injection and was evident also in uninjected samples (not shown). The apparent product yield in this control—4% of pSce1, equivalent to 0.3% of PUF3—was subtracted from the values obtained for the precleaved and *in vivo* cleaved samples.



1	2	3	4	5	6	7	8	9	10	lane
42	-	-	-	98	96	99	62	69	86	% Cleavage
0	0	0	4	20	22	50	2	4	15	% Product

FIG. 3. Improving product yield. Autoradiogram of an experiment similar to that in Fig. 2, except the molar ratio of PUF:pSce1 was increased from 2:1 to 14:1, and nonhomologous linear DNA was added (L). Other labels are as in Fig. 1*B*. Lanes: 1, pSce1 and I-*Sce* I injected in the absence of any PUF; 2–4, pSce1 and PUFs injected in the absence of I-*Sce* I. The apparent product yield from the control in lane 4 was subtracted from the values obtained for the precleaved and *in vivo* cleaved samples (see text); 5–7, pSce1 cleaved by I-*Sce* I prior to injection; 8–10, pSce1 cleaved *in vivo*.

Separate experiments were performed in which only the PUF or only the M13 replicative form DNA concentration was increased to 10 ng of DNA per oocyte ($\approx 7 \times 10^{10}$ total number of ends per sample). A pronounced increase in product formation was observed in each case (data not shown), but not as substantial as that seen when both components were increased together as above.

DISCUSSION

We have shown that I-*Sce* I cleavage *in vivo* can induce intramolecular and intermolecular homologous recombination in *Xenopus* oocytes. Our intermolecular experiments were designed to mimic a gene targeting experiment by using a circular plasmid as a model for the relatively inert chromosomal target and a linear DNA molecule as a model for the targeting vector. We hypothesized that an *in vivo* targeted DSB would provide an opportunity for the inert circular molecule to participate in homologous recombination by the highly efficient SSA mechanism, resulting in a targeting efficiency near unity. In practice, the best efficiency we achieved was 15% of all target molecules and 17% of those that had been cleaved by I-*Sce* I. While this is still shy of unity, it is one-third of the best efficiency we saw with precleaved DNAs, and it is far greater than the 10^{-6} events per cell reported in typical gene targeting experiments (7). Even with direct nuclear injection, efficiencies of only 10^{-2} have been achieved with mammalian cells (35).

Our results corroborate other studies in which the induction of a specific DSB has been shown to stimulate homologous recombination at the cleavage site. This has been accomplished in yeast, plant, and mammalian cells in culture by the use of site-specific endonucleases (13–18) and in flies and nematodes by transposon excision (36, 37) as the DSB-inducing agents. Among these experiments, ours models most closely the molecular format of a gene targeting protocol.

In our experiments we used the site-specific endonuclease I-*Sce* I to deliver the stimulatory DSB and routinely saw cleavage of ≈60% of the I-*Sce* I sites. Incomplete cleavage by I-*Sce* I *in vivo* has been reported by others (16–18) and may be due to suboptimal conditions in the oocyte nucleus, protection of the I-*Sce* I site by nucleosomes, and loss of enzyme activity by degradation, denaturation, or diffusion out of the nucleus. We were able to raise the level of cleavage by injecting very large amounts of linear DNA, which may be due to disruption of chromatin structure. However, raising the cleavage efficiency to 100% would be expected to result in only a 2-fold increase in product yield.

The most influential factor in determining the yield of recombination products was the stability of the linear vector DNA (PUF). Stability was improved by increasing the overall length of the PUF and the amount of injected linear DNA. It is known that linear DNA in oocyte nuclei is subject to the action of a potent 5′ → 3′ exonuclease (21), and increasing the total mass of linear DNA and the number of molecular ends has been shown to decrease substantially the rate of degradation (33, 34). While we have not surveyed conditions extensively, we obtained good results with high concentrations of PUF, plus inclusion of nonhomologous linear DNA. Other factors, such as a possible differential compartmentalization of the PUF and previously injected pSce1, may contribute but were not examined.

Earlier gene targeting studies have found that targeting efficiency increased exponentially as the length of homology was increased (38, 39). We did not vary the length of homology in our experiments. We predict, however, that this would have relatively little effect on product yield, apart from the noted influence of overall PUF length, because the mechanism of recombination is fundamentally different from that encountered in a traditional gene targeting experiment. The minimum

homology necessary to support efficient SSA recombination in oocytes is <250 bp (ref. 29; R. J. Dawson and D.C., unpublished data), and comparable numbers have been obtained for SSA events in other systems (10). As long as sufficient homology is present, all events should have approximately the same efficiency.

We believe that recombination in these experiments proceeds by the SSA mechanism. Earlier work with precleaved substrates showed that SSA is the only detectable recombination mechanism operating in stage VI *Xenopus* oocytes (11, 19, 20). Direct tests of the alternative double-strand break repair mechanism failed to detect recombination products (19, 20). Although not rigorously proven here, the kinetics and observed intermediates are all consistent with and predicted by our earlier work with precleaved substrates. One difference was that the rate of degradation of *in vivo* cleaved DNA appeared to be slower than that of injected linear DNAs, and we speculate that this may be due to the retention of chromatin proteins that had assembled onto the injected circular DNA (25) and/or I-*Sce* I remaining bound for some period after cleavage (40).

Because this reaction proceeds via the SSA pathway and because SSA recombination has been demonstrated in a wide variety of cell types, we suspect this strategy will be applicable to many other organisms. However, other cell types may have additional pathways of recombination available to them, such as nonhomologous end joining. Indeed, nonhomologous events outnumber homologous targeting events in traditional gene targeting experiments (1, 4). Increasing PUF concentration could be a problem in such cells because illegitimate recombination would be increased. The oocyte system does not allow us to study competition by these other recombination pathways, but such studies are possible in activated *Xenopus* eggs, which have been shown to support homologous and nonhomologous recombination (41).

Endonuclease-induced, targeted homologous recombination could be a useful technique only if the appropriate cleavage site already existed in the target molecule. It would be more desirable to have a generalized reagent that could induce a DSB at any chosen site. Efforts are necessary to develop *in vivo* targeted cleavage reagents based on successful *in vitro* strategies (42–44).

We thank Maria Jasin for communicating results prior to publication and Jim Haber and Bernard Dijon for their discussion of the feasibility of I-*Sce* I cleavage in oocytes. We thank Janet Lindsley, Maria Jasin, Kent Golic, and Peter Glazer for their discussion of this manuscript. We also thank all members of our laboratory for their ideas and contributions, especially Jon Trautman for expert technical assistance with the oocytes and Renée Dawson, Geneviève Pont-Kingdon, Shawn Christensen, and Chris Lehman for their comments on this manuscript. Special thanks go to Renée Dawson for her recommendation to vary overall PUF length while maintaining constant homology length. This work was supported by a grant from the National Science Foundation (MCB-9010130).

1. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51,** 503–512.
2. Capecchi, M. (1989) *Science* **244,** 1288–1292.
3. Koller, B. H. & Smithies, O. (1992) *Annu. Rev. Immunol.* **10,** 705–730.
4. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336,** 348–352.
5. Jasin, M. & Berg, P. (1988) *Genes Dev.* **2,** 1353–1363.
6. Pennington, S. L. & Wilson, J. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9498–9502.
7. Thomas, K. R., Folger, K. R. & Capecchi, M. R. (1986) *Cell* **44,** 419–428.
8. Zheng, H. & Wilson, J. H. (1990) *Nature (London)* **344,** 170–173.
9. Lin, F.-L., Sperle, K. & Sternberg, N. (1984) *Mol. Cell. Biol.* **4,** 1020–1034.
10. Subramani, S. & Seaton, B. L. (1988) in *Homologous Recombination in Mitotically Dividing Mammalian Cells*, eds. Kucherlapati, R. & Smith, G. R. (Am. Soc. Microbiol., Washington, DC), pp. 549–574.
11. Maryon, E. & Carroll, D. (1991) *Mol. Cell. Biol.* **11,** 3278–3287.
12. Lin, M., Sperle, K. & Sternberg, N. (1992) in *Studies on Extrachromosomal Homologous Recombination in Mammalian Cells: Implications for Chromosomal Recombination and Gene Targeting*, eds. Gottesman, M. E. & Vogel, H. J. (Academic, San Diego), pp. 15–28.
13. Rudin, N. & Haber, J. E. (1988) *Mol. Cell. Biol.* **8,** 3918–3928.
14. Rudin, N., Sugarman, E. & Haber, J. E. (1989) *Genetics* **122,** 519–534.
15. Ozenberger, B. & Roeder, G. S. (1991) *Mol. Cell. Biol.* **11,** 1222–1231.
16. Plessis, A., Perrin, A., Haber, J. E. & Dujon, B. (1992) *Genetics* **130,** 451–460.
17. Puchta, H., Dujon, B. & Hohn, B. (1993) *Nucleic Acids Res.* **21,** 5034–5040.
18. Rouet, P., Smih, F. & Jasin, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6064–6068.
19. Jeong-Yu, S. & Carroll, D. (1992) *Mol. Cell. Biol.* **12,** 112–119.
20. Pont-Kingdon, G., Dawson, R. J. & Carroll, D. (1993) *EMBO J.* **12,** 23–34.
21. Maryon, E. & Carroll, D. (1989) *Mol. Cell. Biol.* **9,** 4862–4871.
22. Carroll, D., Garrett, J. E. & Lam, B. S. (1984) *Mol. Cell. Biol.* **4,** 254–259.
23. Garrett, J. E. & Carroll, D. (1986) *Mol. Cell. Biol.* **6,** 933–941.
24. Jeong-Yu, S. & Carroll, D. (1992) *Mol. Cell. Biol.* **12,** 5426–5437.
25. Gargiulo, G. & Worcel, A. (1983) *J. Mol. Biol.* **170,** 699–722.
26. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
27. Feinberg, P. A. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
28. Wyllie, A. H., Laskey, R. A., Finch, J. & Gurdon, J. (1978) *Dev. Biol.* **64,** 178–188.
29. Carroll, D., Wright, S. H., Wolff, R. K., Grzesiuk, E. & Maryon, E. B. (1986) *Mol. Cell. Biol.* **6,** 2053–2061.
30. Sweigert, S. E. & Carroll, D. (1990) *Mol. Cell. Biol.* **10,** 5849–5856.
31. Colleaux, L., D'Auriol, L., Galibert, F. & Dujon, B. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6022–6026.
32. Miller, T. J. & Mertz, J. E. (1982) *Mol. Cell. Biol.* **2,** 1595–1607.
33. Maryon, E. (1990) Ph. D. thesis (Univ. of Utah, Salt Lake City).
34. Maryon, E. & Carroll, D. (1991) *Mol. Cell. Biol.* **11,** 3268–3277.
35. Capecchi, M. (1990) *Nature (London)* **348,** 109.
36. Gloor, G. B., Nassif, N. A., Johnson-Schlitz, D. M., Preston, C. R. & Engels, W. R. (1991) *Science* **253,** 1110–1117.
37. Plasterk, R. H. A. & Groenen, J. T. M. (1992) *EMBO J.* **11,** 287–290.
38. Deng, C. & Capecchi, M. R. (1992) *Mol. Cell. Biol.* **12,** 3365–3371.
39. Hasty, P., Rivera-Perez, J. & Bradley, A. (1992) *Mol. Cell. Biol.* **12,** 2464–2474.
40. Perrin, A., Buckle, M. & Dujon, B. (1993) *EMBO J.* **12,** 2939–2947.
41. Lehman, C. W., Clemens, M., Worthylake, D., Trautman, J. K. & Carroll, D. (1993) *Mol. Cell. Biol.* **13,** 6897–6906.
42. Strobel, S. A. & Dervan, P. B. (1990) *Science* **249,** 73–75.
43. Perrouault, L., Asseline, U., Rivalle, C., Thuong, N. T., Bisagni, E., Giovannangeli, C., Le Doan, T. & Hélène, C. (1990) *Nature (London)* **344,** 358–360.
44. Pei, D., Corey, D. R. & Schultz, P. G. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9858–9862.