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Site-Specific Recombination Determined by I-*Sce*I, a Mitochondrial Group I Intron-Encoded Endonuclease Expressed in the Yeast Nucleus

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ABSTRACT

The *Saccharomyces cerevisiae* mitochondrial endonuclease I-*Sce*I creates a double-strand break as the initiating step in the gene conversional transfer of the omega$^+$ intron to omega$^-$ DNA. We have expressed a galactose-inducible synthetic I-*Sce*I gene in the nucleus of yeast that also carries the I-*Sce*I recognition site on a plasmid substrate. We find that the galactose-induced I-*Sce*I protein can be active in the nucleus and efficiently catalyze recombination. With a target plasmid containing direct repeats of the *Escherichia coli lacZ* gene, one copy of which is interrupted by a 24-bp cutting site, galactose induction produces both deletions and gene conversions. Both the kinetics and the proportion of deletions and gene conversions are very similar to analogous events initiated by a galactose-inducible HO endonuclease gene. We also find that, in a *rad52* mutant strain, the repair of double-strand breaks initiated by I-*Sce*I and by *HO* are similarly affected: the formation of deletions is reduced, but not eliminated. Altogether, these results suggest either that the two endonucleases act in the same way after double-strand break formation or that the two endonucleases are not involved in subsequent steps.

ONE of the key issues in understanding genetic recombination is the nature of the initiation step. Studies of homologous recombination in bacteria and fungi have led to the proposal of two types of initiation mechanisms. In the first model, a single-strand nick initiates strand assimilation and branch migration (Meselson and Radding 1975). Alternatively, a double-strand break may occur, followed by a repair mechanism that uses an uncleaved homologous sequence as a template (Resnick and Martin 1976). This latter model has gained support from the fact that integrative transformation in yeast is dramatically increased when the transforming plasmid is linearized in the region of chromosomal homology (Orr-Weaver, Szostak and Rothstein 1981) and from the direct observation of a double-strand break during mating type interconversion of yeast (Strathern *et al.* 1982). Recently, double-strand breaks have also been characterized during normal yeast meiotic recombination (Sun *et al.* 1989; Sun, Treco and Szostak 1991; Alani, Padmore and Kleckner 1990).

Several double-strand endonuclease activities have been characterized in yeast: HO and the intron-encoded endonucleases (see below) are associated with homologous recombination functions, while others still have unknown genetic functions (Endo-*Sce*I, Endo-*Sce*II) (Shibata *et al.* 1984; Morishima *et al.* 1990).The HO site-specific endonuclease initiates mating-type interconversion by making a double-strand break near the YZ junction of *MAT* (Kostriken *et al.* 1983). The break is subsequently repaired using the intact *HML* or *HMR* sequences, resulting in ectopic gene conversion. The HO recognition site is a degenerate 24-bp nonsymmetrical sequence (Nickoloff, Chen and Heffron 1986; Nickoloff, Singer and Heffron 1990). This sequence has been used as a "recombinator" in artificial constructs to promote intra- and intermolecular mitotic and meiotic recombination (Nickoloff, Chen and Heffron 1986; Kolodkin, Klar and Stahl 1986; Ray *et al.* 1988; Rudin and Haber 1988; Rudin, Sugarman and Haber 1989).

The two site-specific endonucleases, I-*Sce*I (Jacquier and Dujon 1985) and I-*Sce*II (Delahodde *et al.* 1989; Wenzlau *et al.* 1989), that are responsible for intron mobility in mitochondria of yeast, initiate a gene conversion that resembles HO-induced conversion [see Dujon (1989) for review]. I-*Sce*I, which is encoded by the optional intron Sc LSU.1 of the 21S rRNA gene, initiates a double-strand break at the intron insertion site (Macreadie *et al.* 1985; Dujon *et al.* 1985; Colleaux *et al.* 1986). The recognition site of I-*Sce*I extends over an 18 bp nonsymmetrical sequence (Colleaux *et al.* 1988). Although the two proteins are not obviously related by their structure (HO is 586 amino acids long while I-*Sce*I is 235 amino acids long), they both generate 4-bp staggered cuts with 3′-OH overhangs within their respective recognition sites.

TABLE 1

Strains

Number	Genotype	Origin
CG379	*MATα ade5 his7-2 leu2-3, 112 can1 ura3-52 trp1-289* [KIL-0]	Yeast Genetic Stock Center
WAP240	*rad 52::TRP1* disruption of CG379	This work
AP308	CG379 cotransformed by pTAR303 and pPEX408	This work
AP317	CG379 cotransformed by pTAR303 and pPEX417	This work
AP208	WAP240 cotransformed by pTAR303 and pPEX408	This work

Thus far all the results on the repair of double-strand breaks initiated *in vivo* have been obtained with *HO*. The issue of the precise role of *HO* seems relevant: Does it act only as an endonuclease at the initiation step or is it also involved in the subsequent steps of the repair process? To answer this question, we decided to test whether the mitochondrial I-*Sce*I endonuclease exerts a recombinogenic activity on nuclear sequences similar to that described for HO. To allow direct comparison with HO, we have used the same system as the one developed by RUDIN, SUGARMAN and HABER (1989). Our results demonstrate, for the first time, that a mitochondrial intron-encoded endonuclease, transcribed in the nucleus and translated in the cytoplasm, generates a double-strand break at a nuclear site. The repair events induced by I-*Sce*I are identical to those initiated by HO.

MATERIALS AND METHODS

Strains and media: *Saccharomyces cerevisiae* strains are listed in Table 1. Strain WAP240 was constructed by gene disruption of the wild type *RAD52* gene (SCHILD *et al.* 1983) in CG379. The disruption was performed by insertion at the *RAD52* locus of a *RAD52 Bam*HI fragment disrupted with a *Bgl*II-*Bam*HI *TRP1* fragment at the *Bgl*II site. The fragment used was derived from the R535 plasmid (a gift from S. ROEDER).

Complete medium (YP) is 1% w/v yeast extract Difco, 1% w/v Bacto-peptone Difco supplemented with glucose (2% w/v, YPglu) or glycerol and lactate (2% w/v each, YPgly+lac) or glycerol, lactate and galactose (2% w/v each, YPgal). Synthetic complete minimal medium (SC) is 6.7 g/liter Yeast Nitrogen Base w/o amino acids from Difco supplemented with either glucose 2% (SCglu) or glycerol 2%, lactate 2% and galactose 2% (SCgal) and all the 20 amino acids plus uracil and adenine. Selective media lack one or several additions [*e.g.*, SCglu-U (uracil), -LWU (leucine, tryptophan, uracil)]. Bacto-Agar (DIFCO) was added (2% w/v) for solid media.

Galactose induction was done in two different ways. First, a glucose grown colony was inoculated into SCgal (see Tables 2 and 4, and Figure 3). For the kinetics experiments cells were first grown in SCglu-UL for 24 hr, then the culture was diluted at 1/500 in SCgly+lac and incubated. At OD = 0.6–0.8 galactose was added to 2% w/v final concentration (see Table 3, and Figures 5 and 6).

β-Galactosidase activity in yeast colonies was scored by an X-Gal assay. Colonies were replica plated on 3 MM Whatman filters, the filters were frozen flat in liquid nitrogen and laid on 0.1 M Na-phosphate buffer, pH 7.0, containing 0.7% agar, 0.001 M $MgSO_4$, 120 μg X-Gal (MILLER 1972). ($MgSO_4$ and X-Gal (Appligene) were added to the melted agar just before pouring the plates). Lac$^+$ colonies turned blue overnight.

***Escherichia coli* and yeast transformation:** *E. coli* was transformed either by the method described by HANAHAN (1985) or by electroporation according to the manufacturer's instructions (Bio-Rad). Yeast transformations were performed by either of the three following methods: spheroplast transformation (HINNEN, HICKS and FINK 1978), lithium acetate (ITO *et al.* 1983) or electroporation (SIMON and MCENTEE 1989), with minor modifications. For electroporation, strains were grown in YPglu until OD_{600} = 1, washed with 25 mM dithiothreitol (DTT) and incubated for 10 min at 30°, then centrifuged and resuspended in 270 mM sucrose, 10 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, at 10^9 cells/ml. Plasmid DNA (20–50 ng) was added to aliquots of 100 μl of the cell suspension. Electroporation conditions were: 2250 V/cm, 250 μF capacitance, and a derivation of 200Ω using the Bio-Rad gene pulser. Cells were plated directly on selective media.

Plasmid constructions: Two plasmids were built: a target plasmid and an expression vector (Figure 1). The target plasmid contained the I-*Sce*I recognition site introduced from a pair of 24-bp synthetic oligonucleotides (G13: 5′-GTATTACCCTGTTATCCCTAGCGT-3′ and G15: 5′-ACGCTAGGGATAACAGGGTAATAC-3′). The expression vector carried a synthetic gene encoding the I-*Sce*I endonuclease (A. THIERRY and B. DUJON, unpublished data) placed under the control of a galactose inducible *GAL1-CYC1* promoter (see Figure 1).

Plasmid pTAR303 (Figure 1A) is similar to pNR18 of RUDIN (RUDIN SUGARMAN and HABER 1989) except for the presence of the I-*Sce*I site instead of the *HO* site and for the deletion of the *URA3* sequence. pTAR303, was built from pJH262 and pJH271 (described, but not named, in MATERIALS AND METHODS of RUDIN, SUGARMAN and HABER 1989) as described below. Reannealed G13-G15 was inserted at the *Bcl*I site of the *lacZ* gene (KALNINS *et al.* 1983) in pJH262. This insertion did not destroy the *Bcl*I site. The resulting plasmid was deleted for the 0.7-kb *Eco*RV-*Sma*I fragment of *URA3*. The 5.9-kb *Hin*dIII fragment including the disrupted *lacZ* gene under the *LEU2-CYC1* promotor was cloned into the *Hin*dIII site of pJH271 in the same orientation as the other *lacZ* copy. The expression plasmid, pPEX408, was built by cloning a 750-bp *Bam*HI synthetic DNA fragment encoding the I-*Sce*I endonuclease under *GAL1-CYC1* control at the *Bam*HI site of the 2 μm, *URA3* plasmid, pLGSD5 (without ATG) (GUARENTE, YOKUM and GIFFORD 1982). This plamid was also deleted for most of the *lacZ* gene (a 2.9-kb *Pvu*II deletion). The construction with the I-*Sce*I gene in the opposite orientation is called pPEX417.

Experiments with HO endonuclease were carried out using plasmid pSE271::GAL10-HO, a gift from F. HEFFRON (NICKOLOFF, CHEN and HEFFRON 1986). The plasmid pJF6, provided by J. FISHMAN-LOBELL, is identical to pNR18 (RUDIN, SUGARMAN and HABER 1989) except that the HO

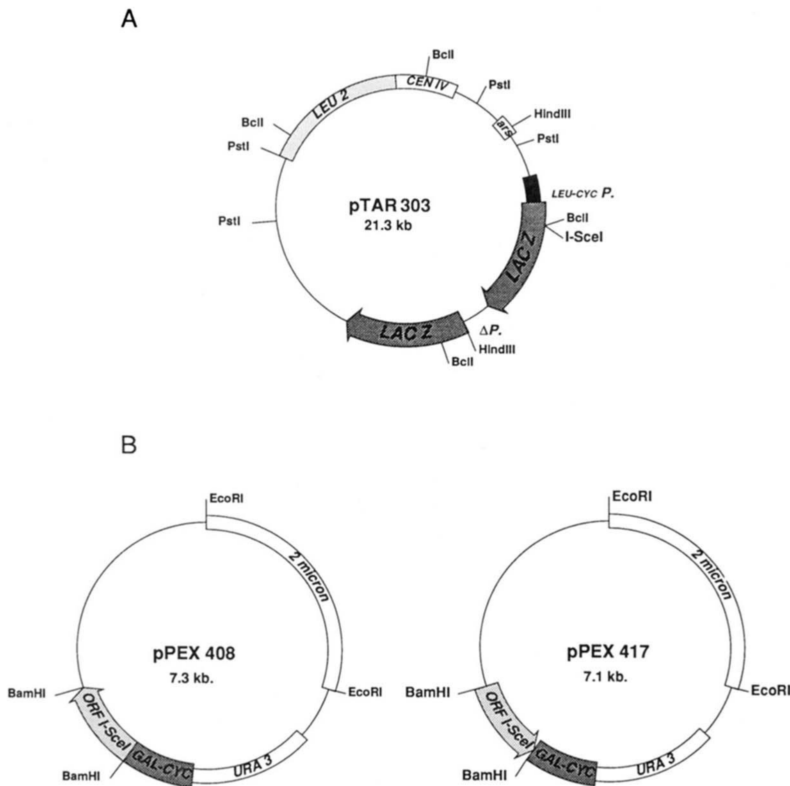

FIGURE 1.—Vectors used in these experiments. A. Target plasmid pTAR303 is a centromeric plasmid carrying the *LEU2* marker and containing a direct duplication of *lacZ* (see MATERIALS AND METHODS). The I-*Sce*I recognition site is cloned, from a synthetic oligonucleotide, at the *Bcl*I site (the insertion restores the *Bcl*I site) of the *lacZ* copy which is under the control of a $CYCI_{UAS}$-*LEU2* hybrid promoter. The other *lacZ* gene is promoterless (ΔP). B. Expression vector pPEX408 is a 2-μm based plasmid derived from pLG-SD5 (GUARENTE, YOCUM and GIFFORD 1982) deleted for its *lacZ* sequence. It contains a synthetic open reading frame coding for the I-*Sce*I endonuclease (A. THIERRY and B. DUJON, unpublished data) placed under a galactose inducible promoter. The control vector pPEX417 has the open reading frame in the opposite orientation.

endonuclease target sequence is present in one copy in pJF6 instead of two repeated copies in pNR18.

Minipreparation of total yeast DNA and Southern analysis: Minipreparations of total yeast DNA were prepared following the protocol in SHERMAN, FINK and LAWRENCE (1979) with minor modifications. Samples were digested and electrophoresed on 0.7% agarose gels, transferred to Hybond-N membrane (Amersham) by vacuum blotting, exposed to UV (0.16 kJ at 254 nm) and hybridized to a [α-^{32}P]ATP-labeled probe (CHURCH and GILBERT 1984). Radioactive probes were prepared by random priming (HODGSON and FISK 1987) from the 2.5-kb *Pvu*II fragment containing *lacZ*.

RESULTS

The mitochondrial I-*Sce*I site-specific endonuclease expressed from a nuclear plasmid can act on a nuclear target site and induce genetic recombination: We have expressed the intronic endonuclease I-*Sce*I in yeast from a nuclear expression plasmid and asked the following questions. (i) Does the endonuclease synthesized in the cytoplasm promote double-strand breaks and site-specific recombination between nuclear target sequences? (ii) If so, is this induced recombination similar to that observed in the same conditions with *HO*? To answer these questions, we have used the experimental system previously developed by RUDIN, SUGARMAN and HABER (1989) except that we have replaced the *HO* gene and its target site by the I-*Sce*I gene and its target site, respectively. Yeast cells were cotransformed with two plasmids: a target plasmid, pTAR303, containing the site of the endonuclease and an expression plasmid, pPEX408, producing the I-*Sce*I endonuclease under the control of a galactose inducible promoter. The target plasmid contains two inactive copies of *lacZ* in tandem: one placed under a yeast promoter but interrupted by the I-*Sce*I recognition site, the second, intact but promoterless.

Double-strand cleavage at the I-*Sce*I site of the target plasmid by the endonuclease may result in two different outcomes (Figure 2). Either the linearized plasmid can be lost, or the break can be repaired by using the uncleaved homologous gene as a template. Repair can yield either a simple gene conversion or a deletion between the two *lacZ* direct repeats. Plasmid loss is easily monitored by the appearance of a Leu$^-$ phenotype. Repair by gene conversion or by deletion both result in a Leu$^+$ Lac$^+$ phenotype but these can be distinguished from each other at the molecular level.

In the first set of experiments, we have compared the frequency of Lac$^+$ cells, with or without induction of the I-*Sce*I endonuclease, in medium selective for both plasmids. We can see in Table 2 that the fraction of blue clones (Lac$^+$) strikingly increases from about 4.7% to about 78% when the endonuclease I-*Sce*I expression is induced. Thus, we conclude that expression of the endonuclease I-*Sce*I from cytoplasmic ribosomes induces homologous recombination between two direct repeats on a nuclear plasmid, indicating that the endonuclease, although of mitochondrial origin, can enter the nucleus.

In additional induction experiments (Table 3), we

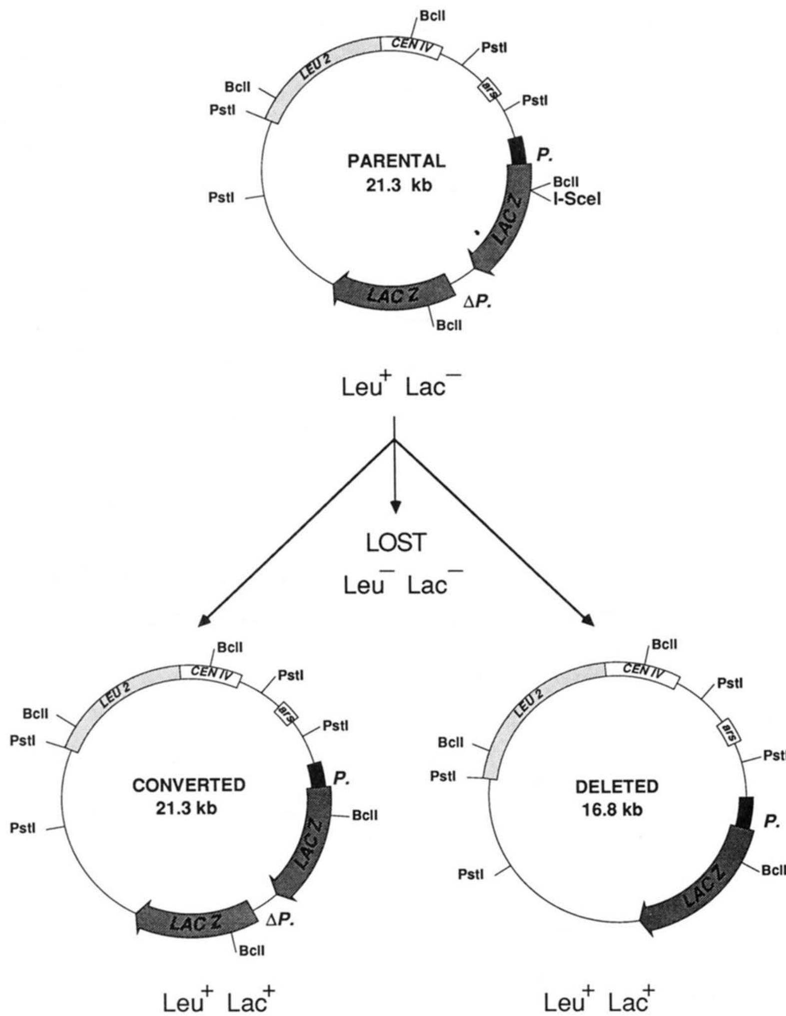

FIGURE 2.—Products expected after double strand break repair of pTAR303 induced by the I-*Sce*I endonuclease. After cleavage by I-*Sce*I, the plasmid pTAR303 can be lost (giving rise to a Leu$^-$ Lac$^-$ cell) or repaired (giving rise to a Leu$^+$ Lac$^+$ cell). If the repair occurs by gene conversion without exchange the resulting plasmid remains identical in size to the parental plasmid (except for the 24-bp I-*Sce*I recognition site). If the repair induces crossing over between the two *lacZ* repeats, the resulting plasmid will be deleted by 4.5 kb. The different products can be easily distinguished by *Pst*I restriction analysis and hybridization using a *lacZ* specific probe: the *Pst*I fragment spanning the *lacZ* sequence is 11.1 kb long in the parental plasmid pTAR303 and in the converted product and is 6.6 kb long in the deleted plasmid.

have estimated the efficiency of repair by measuring both plasmid loss and the formation of Lac$^+$. After 420-min induction (about two cell generations) in nonselective medium, the percentage of loss of the target plasmid (Leu$^-$) increased from 6.6 to 21% while the percentage of Lac$^+$ among Leu$^+$ colonies increased in the same proportion as before (from *ca.* 13 to *ca.* 97%) (Table 3A). Hence repair is more frequent than plasmid loss.

Both plasmid loss and the formation of Lac$^+$ colonies during induction show similar kinetics (Table 3B). The percentages of plasmid loss and of Lac$^+$ clones were again low in the absence of induction. Plasmid loss increased from about 7% to a maximum of 24% in less than 4 hr. During the same time, the percentage of Lac$^+$ clones among the colonies which have kept both plasmids increased from 5.6 to 80%, again in agreement with the previous experiments. Similar proportions of plasmid loss were also observed by RUDIN, SUGARMAN and HABER (1989) using *HO*.

Since the strain used in these experiments is omega$^+$ type 1 [see JACQUIER and DUJON (1985) for definition; data not shown] we examined the possibility that the mitochondrially synthesized endonuclease leaking to the nucleus may account for the relatively high background of Lac$^+$ recombinants observed in the presence of glucose or prior to galactose induction. The frequency of Lac$^+$ recombinants in the same strain (CG379) transformed only with the target plasmid pTAR303 (I-*Sce*I site) is shown in Table 4. The frequency of Lac$^+$ cells (about 0.1%) is much lower than the 5.6–12.9% found in glucose grown cells harboring the galactose-inducible plasmid pPEX408 (Table 3). This suggests that the high level of Lac$^+$ colonies when strain AP308 is grown on glucose results from a low level of expression of I-*Sce*I enconuclease gene carried by the pPEX408 plasmid rather from the mitochondrially ecoded I-*Sce*I enzyme. As a further control, the same experiment was performed with strain CG379 transformed with pJF6 (containing the *HO* site, see MATERIALS AND METHODS). It can be seen that the rates of spontaneous recombination are similar for both plasmids, in glucose as well as in galactose media. Thus the recombination events observed when strain AP308 is induced by the addition of galactose do not result from the participation of the mitochondrial I-*Sce*I or from an effect of growth on galactose medium *per se* but are indeed due to the activity of the I-*Sce*I

TABLE 2

Site-specific recombination between *lacZ* repeats induced by I-*Sce*I endonuclease expression

Growth medium	Experiment No.	No. of colonies tested: Blue	Sectored	White	% Lac^{+a}
SCglu-UL	1	1	0	28	4.8
	2	1	1	31	
	3	6	18	472	
SCgal-UL	1	637	N.D.	189	78
	2	54	0	28	
	3	191	0	27	

Cells of strain AP308 were cultivated for 24 hr in either SCglu-UL or SCgal-UL media (*i.e.*, selective for both plasmids). The cultures were then plated on SCglu-UL to form colonies that were subsequently tested for their Lac$^+$ phenotype using the X-gal color test (see MATERIAL AND METHODS).

[a] The Lac$^+$ frequency is an overestimate, because sectored colonies are counted as entirely Lac$^+$. Although some of these sectored colonies may have arisen by the segregation of a Lac$^+$ plasmid to only one of the two mitotic cells following the first cell division after plating, it is equally likely that they represent two independent cells (or a mother and daughter chromosome in G2, prior to mitosis) plated as a single colony forming unit. In this case, the total number of cells plated, including colonies that were entirely Lac$^-$, is underestimated and the frequency of Lac$^+$ events is overestimated.

TABLE 3

Plasmid loss and site-specific recombination induced by I-*Sce*I endonuclease expression

Induction time (min)	No. of colonies tested	Percent pTAR303 loss	% Lac$^+$
A. −24	150	6.6	12.9
420	200	21	97.4
B. −15	96	7.3	5.6
45	48	12.5	33.3
95	93	16.1	60.2
145	87	18.4	84.5
225	95	23.1	68.4
600	78	24.3	69.5
1440	174	24.1	80.3

AP308 cells were induced in YPgal media for the time shown. Uninduced cells were taken prior to induction (−15 or −24 min) as control. The culture was diluted and plated on YPglu (A) or SCglu-U (B) to obtain individual colonies which were picked at random and tested on SCglu-U, SCglu-L and X-Gal. pTAR303 loss is expressed as the percentage of Ura$^+$ Leu$^-$ colonies over the total number of colonies tested. Frequency of Lac$^+$ is expressed as the percentage of blue clones over the total number of Ura$^+$ Leu$^+$ colonies.

endonuclease expressed from a nuclear plasmid.

The I-*Sce*I endonuclease induces gene conversion as well as deletions: The results reported above show that the action of the site-specific endonuclease I-*Sce*I promotes homologous recombination in the yeast nucleus. To distinguish between gene conversion and crossing over, we have examined the molecular structure of the Lac$^+$ plasmids by Southern blot. Gene conversion alone would lead to a "parental size" product while crossing over would produce a plasmid deleted of 4.5 kb between the *lacZ* repeats (see Figure 2).

TABLE 4

Spontaneous recombination between *lacZ* repeats containing the I-*Sce*I or HO target site in the absence of any endonuclease expression vectors

Target site	Growth medium	No. of colonies tested: Blue	Sectored	White	% Lac^{+a}
I-*Sce*I	SCglu-UL	0	1	922	0.1
	SCgal-UL	0	0	921	<0.1
HO	SCglu-UL	0	3	705	0.4
	SCgal-UL	0	1	646	0.15

Cells of strain CG379 transformed by either pTAR303 or pJF6 were cultivated either in SCglu-UL or in SCgal-UL for 9–11 generations. The cultures were then plated on SCglu-L to form colonies that were tested for their Lac$^+$ phenotype as in Table 2. The results are pooled from five experiments.

[a] As noted in Table 2, the frequency of Lac$^+$ cells is overestimated.

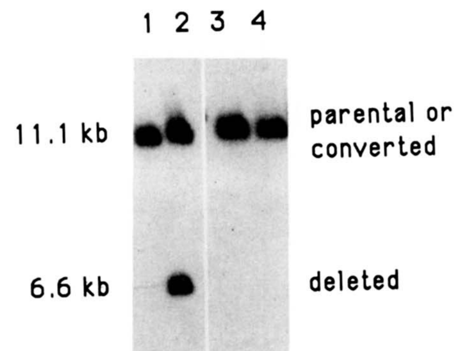

FIGURE 3.—Southern blot analysis of I-*Sce*I induced double strand break repair products. Total DNAs were extracted from strain AP308 (lanes 1 and 2) or from AP317 (lanes 3 and 4). Cells were grown either on SCglu-UL (lanes 1, 3) or on SCgal-UL (lanes 2 and 4) as described in Table 2. DNAs were digested by *Pst*I, run on 0.7% agarose gel and probed with the 2.5-kb *Pvu*II *lacZ* fragment.

It can be seen in Figure 3 that when the cells were grown on glucose medium (lanes 1 and 3), only the 11.1-kb band of parental size is visible. On the contrary, an additional band is clearly visible in the strain AP308 grown on galactose to induce I-*Sce*I expression (lane 2). This band corresponds to a size of 6.6 kb as expected of a deletion between the two *lacZ* tandem repeats. This band is not visible in the control strain AP317 grown in the same galactose medium (lane 4) (confirming that only the endonuclease produced from the nuclear vector is active). We conclude therefore that expression of the I-*Sce*I endonuclease induces crossing over between the repeated *lacZ* sequences.

To estimate the relative frequency of crossing over (deletion) *vs.* gene conversion we analyzed individual clones obtained after I-*Sce*I induction. In a first experiment, 12 clones (3 white and 9 blue) from the induction experiment of Table 2 were analyzed (Figure 4A): the 3 white clones show, as expected, only a parental size band (lanes 8, 11 and 12), while 8 of the 9 blue clones show only the 6.6-kb band (lanes 1, 2, 4

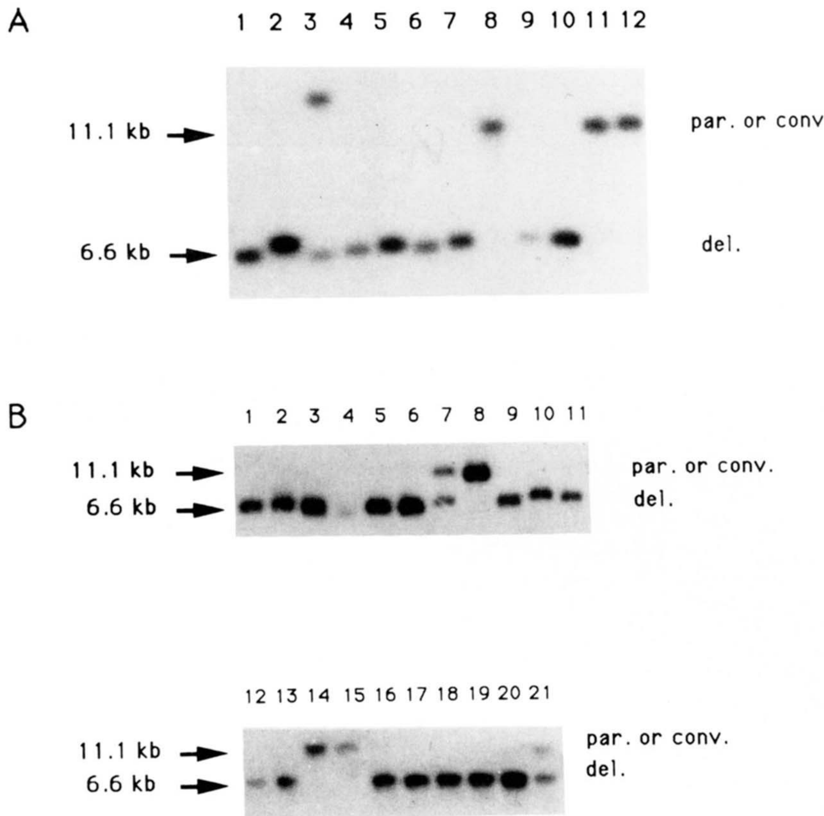

FIGURE 4.—Southern blot analysis of individual clones isolated after I-*Sce*I expression. Total DNAs were extracted from blue or white individual subclones derived from individual colonies in the induction experiment described on Table 2 (4A) or from G1 micromanipulated cells in the induction experiment described in Table 3A (4B). DNAs were digested and analyzed as in Figure 3. Part A: lanes 8, 11 and 12: white clones (Lac$^-$); lanes 1–7, 9 and 10: blue clones (Lac$^+$). Lane 3 may reflect partial digestion by *Pst*I of a deletion event. Part B: all clones analyzed are blue. Par. is the parental configuration, conv. is the gene conversion without crossing over configuration and del. is the deletion product configuration.

to 7, and 9, 10). The last one (lane 3) shows the 6.6-kb band and an additional band representing a partial digest.

In addition, 21 blue clones from the induction experiment in Table 3A were analyzed (Figure 4B). Sixteen of these colonies have plasmids resulting from crossing over (6.6-kb band), 3 have plasmids resulting from gene conversion alone (11.1-kb band) and 2 show a pattern indicating the simultaneous presence of the deleted and parental size plasmids. However these latter two cannot represent mixed colonies issued from budding cells since in this experiment G1 cells were isolated by micromanipulation; they probably represent instances in which the strain initially carried two copies of pTAR303. In summary, 27 out of 30 blue clones tested (90%) contain the crossover product. In similar experiments using HO endonuclease, RUDIN, SUGARMAN and HABER (1989) found that a similar proportion (83%) of Lac$^+$ plasmids contained deletions.

Physical analysis of the double-strand break formation and its repair: The recombination events induced by I-*Sce*I expression can be accounted for by the action of this site-specific endonuclease on the target site inserted into one of the *lacZ* copies. We have looked for the appearance and processing of the expected double-strand break intermediate during induction and compared it to the results obtained with induction of HO. Figure 5A shows that, prior to the start of induction, most of the plasmids pTAR303 are in the parental configuration (as indicated by the 11.1-kb *Pst*I restriction fragment). A faint band of 6.6 kb probably resulted from a low background of recombination in the noninduced cells (see Table 4). Within 30 min after galactose addition, two extra bands, of 8.8 and 2.3 kb, appear; these are the sizes expected for the plasmid linearized at the inserted I-*Sce*I site by a double-strand break. After 60–90 min of induction one observes the appearance of the 6.6-kb band indicating the formation of a deleted product (the product of gene conversion cannot be distinguished here from the parental plasmid). After 180-min induction, the bands corresponding to the cut plasmid start to disappear while the 6.6-kb band remains constant. This confirms that the 8.8-kb and the 2.3-kb band are, as expected, intermediates of the recombination reaction.

We have compared the effects of the endonuclease I-*Sce*I to that of HO by repeating the experiment of RUDIN, SUGARMAN and HABER (1989) in our strain CG379 (carrying pSE271::GAL10-HO and pJF6) (Figure 5B). Comparison of Figure 5, A and B, shows similar kinetics, both for the appearance and disappearance of the intermediates and for the appearance of the final recombined product. We conclude, therefore, that I-*Sce*I and HO induced site-specific double-strand breaks are similarly processed.

I-*Sce*I as well as *HO* double-strand breaks are repaired poorly in *rad52* mutants: The *RAD52* gene which was first shown to be necessary for repair of X-

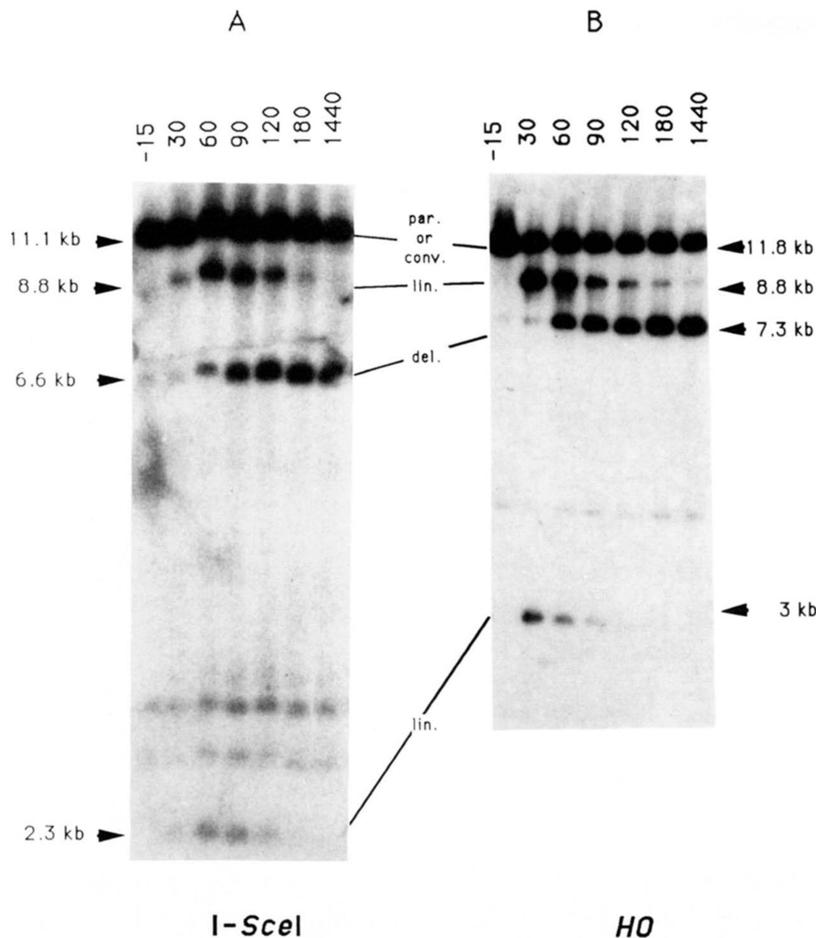

FIGURE 5.—Comparison of I-*Sce*I- and *HO*-induced events after galactose induction. Total DNA was extracted from AP308 cells (A) or from CG379 cells transformed with pJF6 and pSE271::GAL10-HO (B) prior to and during induction in YPgal media. DNAs were digested and analyzed as indicated in figure 3. Notice that the fragments from the pJF6 plasmid are 0.7 kb larger than those from the pTAR303 plasmid since the *URA3* gene of pTAR303 is partially deleted.

ray-damaged DNA (RESNICK 1969; GAME and MORTIMER 1974) is also necessary for mitotic and meiotic homologous recombination (GAME *et al.* 1980), homologous integration of linear plasmids (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981), mating-type switching (MALONE and ESPOSITO 1980; WEIFFENBACH and HABER 1981) and other recombination events initiated by *HO* (RUDIN and HABER 1988). More recently, OZENBERGER and ROEDER (1991) have shown that HO-induced recombination in direct repeats of *rDNA* or *CUP1* genes is reduced but not eliminated in *rad52* strains.

We have tested the effect of a *rad52* disrupted allele on the I-*Sce*I induced recombination. Figure 6 shows the results of an induction experiment in the two isogenic strains AP308 and AP208 (respectively *RAD52* and *rad52::TRP1*). In both strains, double-strand breaks are formed. The wild-type strain shows the same kinetics of appearance of the 6.6-kb *Pst*I deletion fragment as in Figure 5A. In contrast, recombination is very inefficient in the *rad52::TRP1* strain, as indicated by the low intensity of the deleted product. In addition, we have observed a very high rate of pTAR303 plasmid loss when I-*Sce*I is induced in the *rad52::TRP1* strain: 67% of 43 Ura^+ plated colonies became Leu^- (compared to about 20% for the *RAD52* strain). Nevertheless, we have recovered Leu^+ *rad52* colonies that became Lac^+. Out of 14 Leu^+ colonies, 5 were Lac^+. Thus, about 13% of the galactose-induced *rad52::TRP1* cells that were still Ura^+ became Lac^+; this proportion is consistent with the intensity of the 6.6-kb band on the Southern blot. We conclude that the product of the *RAD52* gene is important, but not completely necessary for the repair of double-strand breaks initiated by I-*Sce*I, as with *HO*-mediated events (see DISCUSSION).

DISCUSSION

The results presented here demonstrate that the mitochondrial intron-encoded I-*Sce*I endonuclease, when expressed in the cytoplasm from a nuclear expression vector, is active on a nuclear target. Its action promotes high levels of intramolecular homologous recombination. From those results, we can conclude the following. First, although I-*Sce*I has no known specific nuclear targeting sequence its small size (325 amino acids) and its basic nature are likely sufficient for nuclear import by diffusion. Second, the 24-bp sequence introduced in the target gene in sufficient for recognition and cutting by I-*Sce*I in the yeast nucleus. This was also the case *in vivo* in *E. coli* and in *in vitro* assays (COLLEAUX *et al.* 1986; MONTEILHET *et al.* 1990). Finally, no toxic effect of I-*Sce*I was detected, in viability or growth rate (data not

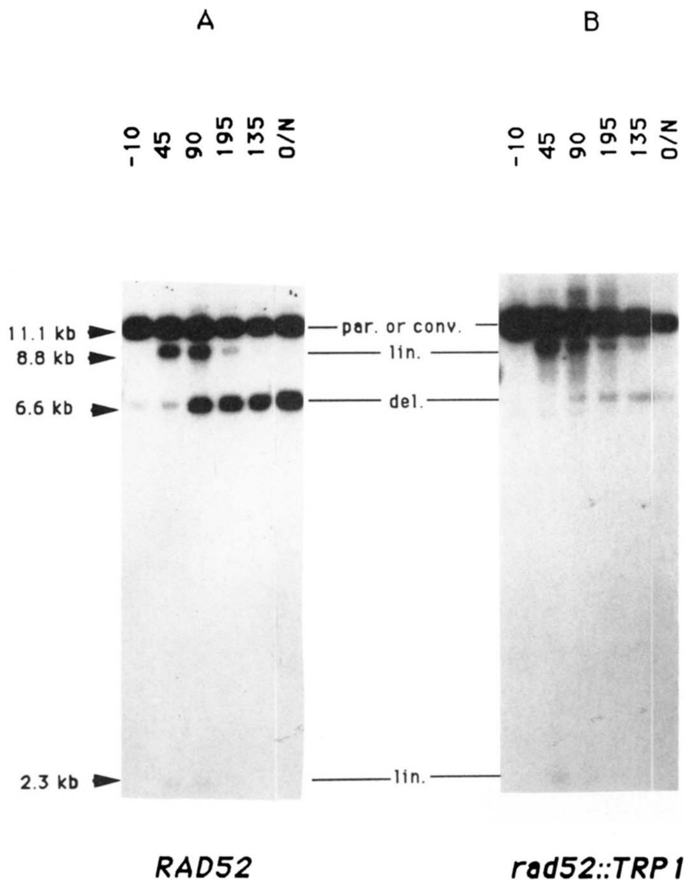

FIGURE 6.—Southern blot analysis of total DNA from wild-type and *rad52* double transformants during an induction. Total DNAs were extracted from strain AP308 (A) or AP208 (B) during an I-*Sce*I induction experiment. DNA was digested and analyzed as indicated in Figure 3.

shown). This is consistent with the recent observation of the absence of natural I-*Sce*I site in the yeast genome (THIERRY *et al.* 1991).

We demonstrated the recombinogenic effect of I-*Sce*I on intramolecular recombination between two direct *lacZ* sequences. After induction, the level of Lac$^+$ clones rose from *ca.* 5–10% to *ca.* 80–95%. In the *RAD52* background, the repair is very efficient compared to the 20–24% of cells that lose the *lacZ* cleaved plasmid. Moreover, the repair is precise, since a functional *lacZ* gene is restored. These results are very similar to those obtained by RUDIN, SUGARMAN and HABER (1989) for HO-induced events using nearly identical substrates.

After I-*Sce*I induction, 60–80% of the cells are repaired within about half a generation time, arguing against the hypothesis that repair of I-*Sce*I induced double-strand breaks would take place only during a short period of the cell cycle. This is in agreement with the results of CONNOLY, WHITE and HABER (1988) and RUDIN, SUGARMAN and HABER (1989) who have shown that double-strand breaks induced by HO under galactose control can efficiently be repaired throughout the cell cycle, and with similar kinetics to those reported here.

The double-strand break repair model postulates that crossing over occurs associated with gene conversion (ORR-WEAVER and SZOSTAK 1983). We have observed here simple gene conversion and a deletion between direct repeats. Although the deletion corresponds to one of the products expected from a reciprocal crossing over, the other expected product–a 4.5-kb circular molecule–has not been detected in our experiments. This reciprocal product is also not observed with *HO*. It now appears likely that the deletions do not result not from "gap-filling" and crossing-over, but from reannealing of single-stranded ends after extensive single-strand degradation (FISHMAN-LOBELL, RUDIN and HABER 1992; SUGAWARE and HABER, 1992). Analogous single-stranded degradation and annealing events (first proposed by LIN, SPERLE and STERNBERG 1984, 1990) have also been recently observed in *Xenopus laevis* oocytes (MARYON and CARROLL 1989, 1991a,b). We believe that there are two different pathways in double-strand break repair between direct repeats: one leading to a gene conversion by repair of the cut and the other leading to a "nonreciprocal crossing over," via extensive single-strand degradation. Both pathways appear to be initiated by I-*Sce*I as well as HO.

WHITE and HABER (1990) have previously shown that a *rad52* mutation prevents the gene conversional repair of a double-strand break during *MAT* switching, but greatly accelerates the 5′- to 3′-exonucleolytic digestion of HO-cut DNA. Our present results indicate that at least some deletion product can still be

formed in a *rad52* strain. Some or all of the deletion visible in Figure 6 must represent completely repaired and ligated product, as *ca.* 10% of the galactose-induced *rad52* colonies contained a Lac$^+$ plasmid.

Recently, OZENBERGER and ROEDER (1991) have also found that HO-induced events can be repaired by deletions in *rad52* strains, when the cutting site is in the midst of multiple tandemly repeated rDNA or *CUP1* chromosomal genes. We have also observed RAD52-independent double-strand break repair by studying HO-induced recombination in pJF6, the plasmid analogous to the I-*Sce*I substrate, pTAR303. We have found essentially identical results with pJF6 as those for pTAR303 reported here (FISHMAN-LOBELL, RUDIN and HABER 1992). We note that when a similar experiment was performed using a direct repeat of *URA3* genes integrated into a chromosome, RUDIN and HABER (1988) did not find such a high proportion of induced *rad52* cells that yielded viable recombinants. We do not know if this reflects an inherent difference between plasmid and chromosomal events or a difference in the size of the homologous regions that were available to undergo recombination by single-strand annealing or some other mode of recombination.

The only differences between I-*Sce*I- and *HO*-induced events appear to be quantitative ones: (i) a slightly lower frequency of recombinant clones is observed after induction by I-*Sce*I, and (ii) the background frequency of recombinants is higher with I-*Sce*I. The first difference can be linked to the slightly lower abundance of the plasmid linearized by I-*Sce*I than by HO. This could result from: (i) a smaller proportion of cells containing the I-*Sce*I expression plasmid (caused, for example, by the lower stability of the multicopy I-*Sce*I vector *vs.* the centromere-containing single-copy *HO* plasmid in nonselective medium), (ii) a lower level of expression of I-*Sce*I, (iii) a lower protein stability, (iv) a lower nuclear targeting efficiency, or (v) a lower cutting efficiency. Since I-*Sce*I is normally a mitochondrial protein, the last three points are not unexpected. The high background frequency of Lac$^+$ clones could again be due to differences between the HO and I-*Sce*I expression vectors. We eliminated the possibility of a leakage of the genuine mitochondrial I-*Sce*I protein to the nucleus.

In conclusion, the striking resemblance between *HO*- and I-*Sce*I-induced events suggests either that the two endonucleases act in the same way after the double-strand break formation or that the two endonucleases are not involved in the subsequent steps. Since the two proteins are not obviously related, we favor the second possibility.

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