RESEARCH COMMUNICATION

RAD51-independent break-induced replication to repair a broken chromosome depends on a distant enhancer site

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Without the *RAD51* strand exchange protein, *Saccharomyces cerevisiae* cannot repair a double-strand break (DSB) by gene conversion. However, cells can repair DSBs by recombination-dependent, break-induced replication (BIR). *RAD51*-independent BIR is initiated more than 13 kb from the DSB. Repair depends on a 200-bp sequence adjacent to *ARS310*, located ~34 kb centromere-proximal to the DSB, but does not depend on the origin activity of *ARS310*. We conclude that the ability of a recombination-induced replication fork to copy >130 kb to the end of the chromosome depends on a special site that enhances assembly of a processive repair replication fork.

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Break-induced DNA replication (BIR) plays a key role in the repair of double-strand breaks (DSBs) in eukaryotic chromosomes. BIR is likely to be important in restarting DNA replication after the collapse of a replication fork (for reviews, see Haber 1999; Michel 2000), but it also functions to repair DSBs created in other ways. In *Saccharomyces cerevisiae*, when there is homology with another chromosomal template only centromere-proximal to the DSB, repair may occur by recombination-dependent DNA replication, forming a nonreciprocal translocation (Bosco and Haber 1998). Similar types of events have been documented when linearized plasmids are introduced into yeast cells and initiate DNA replication from a template chromosome, travelling as much as sev-

eral hundred kilobases to the end of the chromosome (Dunn et al. 1984; Morrow et al. 1997). BIR also may account for the analogous ALT mechanism of telomere maintenance of human tumor cells in the absence of telomerase, as well as similar types of telomere maintenance in budding yeast (Lundblad and Blackburn 1993; Le et al. 1999; Teng and Zakian 1999; Dunham et al. 2000; Teng et al. 2000).

BIR is a recombination-dependent mechanism to initiate DNA synthesis. By creating a DSB in G₁ cells, we showed that this replicative process can occur before the initiation of normal DNA replication, at least in wild-type cells (Bosco and Haber 1998). BIR may be closely related to the mechanism of gene conversion induced by a DSB, based on gene conversion that appears to involve both leading- and lagging-strand DNA synthesis (Holmes and Haber 1999). Both processes may initiate in the same way, by the invasion of a single-stranded DNA end that is produced by 5' to 3' resection of the DSB, to establish a modified replication fork. In gene conversion, this process would terminate when the second end of the DSB engaged the replication structure, whereas in BIR, the replication process would continue to the end of the chromosome. In some circumstances, BIR and gene conversion appear to be alternative, competing outcomes of DSB-initiated recombination (Esposito 1978; Voelkel-Meiman and Roeder 1990; Malkova et al. 2000).

Nevertheless, there are significant differences between BIR and gene conversion (Malkova et al. 1996). Surprisingly, BIR can occur in the absence of the Rad51p strand exchange protein, whereas gene conversion is completely abolished (Fig. 1). However, BIR still requires the *RAD52* protein (Malkova et al. 1996) and thus is apparently a recombination-dependent process. Similarly, in strains in which Rad54p, Rad55p, or Rad57p is deleted, BIR still occurs, but gene conversion is eliminated (Signon et al. 2001). In the absence of these recombination proteins, BIR is a relatively inefficient process, occurring in ~10–15% of cell divisions. In colonies derived from single cells experiencing a DSB, ~80% of them are sectorized; some cells have lost the broken chromosome entirely whereas others retained it, by BIR (see Fig. 1). The *RAD51*- and *RAD54*-independent pathway of BIR depends on *RAD59*, *TID1*(*RDH54*), *MRE11*, *RAD50*, and *XRS2* (Signon et al. 2001). Double mutant combinations *rad51Δ rad50Δ*, *rad51Δ rad59Δ*, and *rad54Δ tid1Δ* all severely impaired in BIR, although not as strongly as in *rad52Δ* strains (Signon et al. 2001).

Essentially all DSB-induced mitotic recombination requires the Rad52 protein (reviewed in Pâques and Haber 1999), which has strand-annealing activity in vitro (Mortensen et al. 1996). Rad52p physically and genetically interacts with Rad51p and with the single-strand DNA-binding protein complex, RPA. However, several types of DSB-induced recombination can occur in the absence of Rad51p, Rad54p, Rad55p, and Rad57p. These events include single-strand annealing (SSA) of homologous sequences flanking a DSB (Ivanov et al. 1996) and maintenance of chromosome ends in the absence of telomerase (Le et al. 1999). On centromeric plasmids, but not on chromosomes, even gene conversion between inverted repeats can occur without Rad51p (Ivanov et al. 1996). It has been suggested that such recombination could occur by two Rad51p-independent processes, BIR

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coupled to SSA (Kang and Symington 2000). In recombining plasmids, the requirement for Rad51p depends on a favorable chromatin structure of the recombining sequences (Sugawara et al. 1995). When donor sequences are heterochromatic, Rad51p is required, whereas when the sequences are in a less constrained chromatin state, Rad51p is not needed. Rad52p is needed in every situation. These experiments suggest that successful BIR might only be able to be initiated at special locations along the chromosome, at sites that are sufficiently accessible to allow strand invasion in the absence of Rad51p.

We asked if BIR initiated only at a limited number of locations. Surprisingly, repair did not initiate anywhere in the first 13 kb proximal to the DSB. In the absence of Rad51p, most of the BIR events on chromosome III depend on sequences adjacent to an origin of DNA replication, but the sequences needed for origin function are not required. Moreover, we show that this sequence does not provide an especially open site for strand invasion of the template, because it is only needed on the broken chromosome. This *cis*-acting site may enable the BIR process to perform the extensive replication that occurs from this point to the end of the chromosome, >130 kb away.

Results

RAD51-independent BIR initiates nonrandomly along the right arm of chromosome III

To determine where BIR occurs, we constructed a set of diploids homozygous for *rad51Δ* in which a *URA3* marker was inserted centromere-proximal to the *MATa* locus, the site of the DSB induced by HO endonuclease (Fig. 1). Cleavage takes place ~202 kb from the left end of chromosome III (Kostriken and Heffron 1984). The *MATα-inc* locus on the homologous chromosome is not cleaved by HO, which is expressed from a galactose-inducible promoter. Loss of the broken chromosome yields *Ade⁻ Thr⁻* cells, but BIR produces *Ade⁺ Thr⁻* cells (Malkova et al. 1996). Many colonies are, in fact, multiply sectored, having sectors that have lost the broken chromosome and one or more sectors that have retained the left arm of the broken chromosome, by BIR. A few *Ade⁺ Thr⁺* cells appear to be the result of nonhomologous end-joining of the HO-cleaved *MATa* locus rather than authentic gene conversion events (data not shown). A few *Ade⁺ Thr⁻* cells also could occur because of a combination of two different Rad51p-independent processes, BIR and SSA (Kang and Symington 2000), but if this occurs, it is quite rare.

When the *URA3* gene was inserted into the top chromosome, either 3 or 13 kb centromere-proximal to the DSB, none of the BIR events retained the *URA3* marker (Fig. 2). For example, when *URA3* was 13 kb proximal to the DSB at *MAT*, there were no *Ura⁺* colonies or sectors among 650 colonies that had undergone BIR. This argues that repair of the DSB had occurred more centromere-proximally, far from the site of the DSB. However, as seen in Figure 2, when *URA3* was inserted 162 kb from the left end of the chromosome (40 kb proximal to the cleavage site, respectively), ~48% of the colonies yielded BIR events that retained the *URA3* marker (*Ade⁺ Ura⁺ Thr⁻*), either as an entire colony or as one or more sectors against a background of cells that had lost the broken

Figure 1. Repair of a DSB in a *MATa/MATα-inc* diploid. In the absence of Rad51p, gene conversion (A) is virtually eliminated. Repair occurs by break-induced replication, producing *Ade⁺ Thr⁻* cells that may either retain a *URA3* marker (B) or else occur such that *URA3* is lost (C). If no repair occurs, the entire broken chromosome is lost, and cells become *Ade⁻ Thr⁻* (D). Many colonies repair the DSB after one or more cell divisions, so that the colonies often are sectored *Ade⁺ Thr⁻/Ade⁻ Thr⁻*.

chromosome. When the *URA3* marker was placed more centromere-proximal, at kilobase 133, 78% of the colonies showing evidence of BIR events retained *URA3*. In some cases, the colonies were sectored *Ura3⁺/Ura3⁻*. These data indicate that *RAD51*-independent recombination is successful only when it is initiated at nonrandom sites along the chromosome, >13 kb proximal to the site of the DSB.

Most BIR events depend on a sequence near ARS310

Inspection of the DNA sequences between 162 and 189 kb revealed one possibly interesting *cis*-acting element that might account for the success of BIR in that region: the origin of DNA replication, *ARS310*, at kilobase 166. Although there are two other sequences closer to *MAT* that function as origins of DNA replication on plasmids, *ARS310* is the nearest centromere-proximal origin that is functional on an intact chromosome III (Newlon et al. 1993). To determine if the region containing *ARS310* was indeed important for the initiation of BIR, we constructed a diploid homozygous for a 845-bp deletion that includes *ARS310* (*ars310-Δ1*, Fig. 3A). The frequency of colonies that lacked any BIR sectors increased from 19% to 50% of the colonies, and equally strikingly, it dramatically reduced the proportion of BIR events that retained *URA3* at kilobase 133 from 78% to 36% (Fig. 4A). Thus, in *ars310-Δ1*, BIR occurs less often, and, when it occurs, it usually is initiated even further from the site of the DSB. However, as shown below, the sequences that facilitate BIR are actually adjacent to *ARS310* and not dependent on its activity.

The enhancer sequences are only required on the broken chromosome

If the function of the region including *ARS310* were to provide a site that was particularly accessible for strand invasion or strand annealing without *RAD51*, it might only be required on the recipient (bottom) chromosome. We created additional *rad51Δ* diploids in which the 845-

Figure 2. Position of BIR as determined by the retention of a *URA3* marker placed centromere-proximal to the DSB at *MAT*. The retention of *URA3* markers inserted 3, 10, 40, and 69 kb proximal to the DSB was determined among colonies in which BIR had occurred. (BIR) break-induced replication; (DSB) double-strand break.

Figure 3. Deletion analysis of the *ARS310* region. (A) Map of the region containing *ARS310* and the locations of three deletion/modifications created in this region. (B) Two hundred base pair sequence of FBI, the facilitator of *rad51*-independent BIR. (FBI) facilitator of BIR; (BIR) break-induced replication.

bp region was deleted only on the top or bottom chromosome (Fig. 4A). The results (Fig. 4A) indicate that the region removed in *ars310-Δ1* is important only on the broken chromosome. Deletion of this region from the recipient chromosome gave results indistinguishable from the wild-type diploid, whereas deleting it from the top chromosome gave results indistinguishable from the homozygous deletion.

Most BIR events initiate distal to the enhancer region

To determine more precisely where BIR events initiated, we used the diploid that retained *ARS310* on the top chromosome but carried the *ars310-Δ1* deletion on the unbroken chromosome (Fig. 4A). We then examined cells that had retained *URA3* at kilobase 162 (which is 4 kb to the left of *ARS310*) to determine if *ARS310* on the top chromosome had been retained or if the region had become homozygous for *ars310-Δ1* (data not shown). In 14 of 15 cases, the region remained heterozygous for the presence of *ARS310*, indicating that BIR must have initiated to the right of *ARS310*.

ARS310 is not required to stimulate *RAD51*-independent BIR

To establish whether *ARS310* and its surrounding sequences were unique, we created two different alterations of the region. For these experiments, we used diploids in which only the top (broken) chromosome was modified. First, we inactivated *ARS310* by introducing pairs of base pair substitutions in each of the three ACS sequences that contribute to the activity of *ARS310* (Theis and Newlon 2001). These mutations eliminated origin firing as determined by two-dimensional gel analysis (data not shown). In two independent strains, BIR occurred to the same extent as in a wild-type strain (Fig. 4D). Thus, origin activity per se is not required.

We then created a second, smaller deletion of *ARS310* (*ars310-Δ2*), a *HpaI-SpeI* deletion that removes 500 bp of *ARS310* but leaves the adjacent 200 bp intact (Fig. 4C). The results show that these *ARS*-adjacent sequences have most or all of the activity needed to promote efficient BIR. Compared with a deletion of *ars310-Δ1*, in which 50% of the cells lost the broken chromosome and only 36% of the BIR events retained the *URA3* marker located at kilobase 133, the presence of the 200-bp segment reduced chromosome loss to 23% and increased *URA3* retention to 74%. Thus, neither *ARS* activity nor the 500-bp fragment containing *ARS310* is needed to stimulate BIR.

We also replaced 649 bp of *ARS310* with *ARS309* (see Fig. 3A), by using a construct that retains the same 200-bp region as in the *ars310-Δ2* deletion. We confirmed that *ARS309* was a functional origin of DNA replication in this new location (data not shown). The *ars310Δ::ARS309* replacement restored BIR events retaining the *URA3* marker at kilobase 133 to nearly the same level as with the intact *ARS310* and its surrounding sequences (Fig. 4B). From these experiments, we concluded that the ability to promote BIR resided in the 200 bp distal to *ARS310* (and retained in the *ars310Δ::ARS309* construct).

Discussion

The key question that we have attempted to address in this study is how recombination-mediated DNA replication can occur in the absence of Rad51p. Rad51p is the only protein expressed in mitotic cells that is homologous with the RecA strand exchange protein of bacteria, the only known family of strand exchange proteins (Shinohara et al. 1992). Accordingly, gene conversion between an HO-cleaved *MAT* locus and an intact template on a homologous chromosome is abolished in *rad51Δ* diploids. Nevertheless, these cells are able to repair a DSB by a recombination-dependent (i.e., *RAD52* dependent) process that we have termed break-induced replication, analogous to recombination-dependent processes that function in bacteriophage T4 replication initiation and that can accomplish recombination in phage λ (for reviews, see Kowalczykowski 2000; Kreuzer 2000). BIR also has been invoked to explain how cells with a broken replication fork in both prokaryotes and eukaryotes can

Figure 4. Role of the ARS310 region in facilitating *rad51Δ*-independent BIR. (A) Effect of homozygous and heterozygous *ars310-Δ1* deletions. (B) Effect of the *ars310Δ::ARS309* replacement of part of the ARS310 region. (C) Effect of the *ars310-Δ2* deletion, which leaves the FBI region (indicated by rectangle) intact. (D) Effect of 3-bp substitutions in each of the three ACS sequences within ARS310. (BIR) break-induced replication.

reestablish replication (Kogoma 1996; Haber 1999; Michel 2000) and also how the stability of chromosome ends can be maintained in the absence of telomerase (Walmsley et al. 1984; Lundblad and Blackburn 1993; Bosco and Haber 1998; Le et al. 1999; Teng and Zakian 1999; Dunham et al. 2000; Teng et al. 2000). As with the BIR system described here, maintenance of *S. cerevisiae* cells in the absence of telomerase also requires RAD52 but is independent of RAD51. Both the single BIR event that we have studied and telomere maintenance without telomerase occur by two RAD52-dependent pathways, one of which requires RAD51, RAD54, RAD55, and RAD57 and the other of which involves RAD50, MRE11, XRS2, RAD59, and TID1 (Lundblad and Blackburn 1993; Le et al. 1999; Teng and Zakian 1999; Teng et al. 2000; Signon et al. 2001).

Here, we show that Rad51p-independent repair does not occur at random sites proximal to the DSB; rather, most of the repair events initiate between 13 and 34 kb proximal to the DSB. We identified a 200-bp segment adjacent to ARS310 that is necessary for this activity. Whether the activity is entirely contained in these 200 bp or extends distally has not been determined. We have named this Facilitator of BIR as FBI. There is probably at least one additional sequence on chromosome III with similar activity, because approximately one-third of the original BIR events occur in a strain deleted for FBI. This

sequence is not near ARS309, because a deletion of this ARS does not affect BIR in the absence of FBI; moreover, ARS309 inserted 3 kb proximal to MAT does not change the location of BIR events (data not shown).

How FBI, situated 34 kb proximal to the DSB, allows BIR to occur without Rad51p is not clear. Initially, we speculated that the ARS310 region, partly denatured by the persistent binding of origin recognition complex (ORC) proteins (Lee et al. 2000), might provide an especially accessible site for strand invasion in the absence of Rad51p. However, this idea is inconsistent with the finding that FBI is needed only on the broken chromosome. Hence, the template chromosome does not have to be particularly open.

One possibility is that FBI helps to assemble a processive strand-invasion and replication complex that allows BIR to proceed to the chromosome end. We know that DNA synthesis during repair is less processive than in normal DNA replication (Pâques et al. 1998). Moreover, colonies produced by single cells of *rad51Δ* diploids that suffered a DSB are often small and nibbled, as if some cells died and failed to give rise to offspring that would populate that part of the growing colony (Malkova et al. 1996). Because simple chromosome loss is not a lethal event and many cells in the colony are 2n-1, these lethal sectors imply that many cells tried to perform BIR, but these attempts ended in a lethal failure. Perhaps the template chromosome and the partially replicated chromosome became trapped in mitosis, causing cell death.

FBI may provide a way for Mcm proteins or some alternative helicase to associate with the BIR replication fork (Fig. 5C) or to recruit the proteins that act in the absence of Rad51p. Most likely, the repair fork lacks the Mcm proteins that are suggested to act as the helicase at normal replication forks (Aparicio et al. 1997; Labib et al. 2000). Mcm proteins are loaded at origins by interactions with origin-binding proteins (Tanaka et al. 1997); they may not assemble at a replication fork established by recombination.

Alternatively, FBI could restrain degradation of the broken chromosome (Fig. 5A) to allow the assembly of the replication-strand-invasion complex. Another step that must occur is to remove a long 3'-ended single-stranded DNA tail created by 5' to 3' exonuclease degradation (Fig. 5B). This step is needed to provide a 3' end that can act as a primer to initiate new DNA synthesis. Although in some circumstances such a tail can be removed by Rad1p-Rad10p endonuclease (Pâques and Haber 1997), Rad51p-independent BIR is Rad1p independent (Signon et al. 2001). In these respects, FBI has some similarities to the properties of the Chi sequence that modifies the activities of RecBCD nuclease in *Escherichia coli* (for review, see Kuzminov 1999). One difference is that Chi-dependent recombination is promoted further away from the DSB, whereas 14 of 15 repair events occur upstream of *ars310-Δ1*, showing that FBI promotes recombination upstream of this site.

Figure 5. Possible roles of the FBI sequence in promoting Rad51p-independent BIR. (A) DNA is resected by 5' to 3' exonucleases, leaving long 3' single-stranded DNA (ssDNA) tails. FBI may stall the exonuclease, allowing other processing of the ssDNA. (B) The 3'-ended tails must be cleaved off to provide a 3' end that can act as a primer to initiate new DNA synthesis. FBI may participate in this Rad1p-Rad10p independent process. (C) FBI may help recruit processivity or other factors (shown as a black triangle) needed for BIR to the end of the chromosome, >130 kb away. (DSB) double-strand break; (FBI) facilitator of BIR.

An inspection of the 200-bp FBI sequence shows several putative protein-binding sites, none of which is obviously implicated in DNA replication functions. However, the small size of this region should make it possible to learn what its key features are.

Materials and methods

Strains

Diploids are isogenic to those described by Signon et al. (2001) and are the result of crossing haploid strains EI515 and YLS23. Modifications of the ARS310 region and the insertion of a *URA3* marker (described below) were performed, and then deletions of the *RAD51* gene were introduced into derivatives of strains EI515 and YLS23 (Malkova et al. 1996) using the a linearized restriction fragment of pJH683 (*rad51Δ::URA3*) or pJH1079 (*rad51Δ::LEU2*).

A 1.1-kb *HindIII* *URA3* fragment was introduced 3 and 13 kb centromere-proximal to *MAT* by using linearized fragments of plasmids pAF228 (a gift from B. Dujon, Institut Pasteur) and pJH106, respectively. The same *URA3* marker was introduced at positions 133 and 162 kb from the left end of chromosome III, ~69 and 40 kb centromere-proximal to the HO cleavage site at *MAT* using plasmids pLS96 and pLS98, respectively. Details are available on request.

Construction of ARS deletions and replacements

Plasmids used for deleting or moving ARS elements were constructed in the *URA3* vector, pRS306, and were used for two-step gene replacements (Boeke et al. 1987). Plasmids were digested with an appropriate restriction enzyme to direct integration and were used to transform yeast strains YLS23 and EI515 (Gietz et al. 1992). Transformants were streaked on plates containing 5-fluoro-orotic acid to select strains that had lost the *URA3* marker (Boeke et al. 1984) and in which the wild-type chromosome III segment was replaced by the desired construct.

The *ars310-Δ1* deletion plasmid, pYND54, carried a 5.7-kb *SacII-HindIII* fragment of chromosome III from which the 849-bp *EcoRV* fragment (chromosome III base pairs 166495–167340) containing *ARS310* had been deleted [A. Dershowitz and C.S. Newlon, in prep.]. The *ars310-Δ2* construct was created by deleting the 500-bp *SpeI-HpaI* fragment (chromosome III base pairs 166590–167090) containing *ARS310* from plasmid ARS310BX (Theis and Newlon 2001). To insert *ARS309* in place of *ARS310*, we amplified a 421-bp fragment containing *ARS309* (chromosome III base pairs 131948–132368) and fused it to the appropriate left- and right-flanking fragments, including base pairs 165177–166490 and 167139–169198, respectively. For *ARS309* inserted 3 kb proximal to

MAT, the 421-bp *ARS309* region was between base pairs 198042 and 198110. Plasmid *ΔARS309* (Dershowitz and Newlon 1993) was used to make the *ARS309* deletion.

Media and growth conditions

Rich medium (YEPD) and synthetic complete medium with bases and amino acids omitted as specified were as described (Kaiser et al. 1994). YEP-glycerol and YEP-galactose (YEP-Gal) consisted of 1% yeast extract/2% Bacto peptone media supplemented with 3% (v/v) glycerol or 2% (w/v) galactose, respectively. YEPD medium containing 0.015% (v/v) methyl methane sulfonate was used to assess Rad[−] phenotypes. Cultures were incubated at 30°C.

Analysis of DNA repair

Logarithmically growing cells grown in YEP-glycerol were plated on YEP-Gal and grown into colonies, as described previously (Malkova et al. 1996). Cells were grown overnight in 50 mL of YEP-glycerol to a cell density of $1\text{--}5 \times 10^7$ cells per milliliter. Appropriate dilutions of cells were plated on YEP-Gal, grown to colonies, and analyzed. The colonies then were replica-plated onto nutritional drop-out media to determine the fate of the *ADE1*, *THR4*, and *URA3* markers. Colonies that either were fully Ade⁺ Thr⁺ or contained Ade⁺ Thr⁺ sectors against an Ade[−] Thr[−] background were counted and scored for the presence of the *URA3* marker.

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