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Relationship between RNA Lariat Debranching and Ty1 Element Retrotransposition

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The *Saccharomyces cerevisiae DBR1* gene encodes a 2′-5′ phosphodiesterase that debranches intron RNA lariats following splicing. Yeast *dbr1* mutants accumulate intron lariats and are also defective for mobility of the retrotransposons Ty1 and Ty3. We used a mutagenic PCR method to generate a collection of *dbr1* mutant alleles to explore the relationship between the roles of *DBR1* in transposition and debranching. Eight mutants defective for Ty1 transposition contained single amino acid changes in Dbr1p. Two mutations, G84A and N85D, are in a conserved phosphoesterase motif that is believed to be part of the active site of the enzyme, supporting a connection between enzymatic activity and Ty1 transposition. Two other mutations, Y68F and Y68D, occur at a potential phosphorylation site, and we have shown that Dbr1p is phosphorylated on tyrosine. We have developed an RNase protection assay to quantitate intron RNA accumulation in cells. The assay uses RNA probes that hybridize to *ACT1* intron RNA. Protection patterns confirm that sequences from the 5′ end of the intron to the lariat branch point accumulate in *dbr1* mutants in a branched (lariat) conformation. RNase protection assays indicate that all of the newly generated *dbr1* mutant alleles are also deficient for debranching, further supporting a role for 2′-5′ phosphodiesterase activity in Ty1 transposition. A Ty1 element lacking most of its internal sequences transposes independently of *DBR1*. The existence of Dbr1p-dependent Ty1 sequences raises the possibility that Dbr1p acts on Ty1 RNA.

Saccharomyces cerevisiae Ty1 is a long terminal repeat (LTR) retroelement with a life cycle similar to that of retroviruses (2). Like retroviruses, Ty1 replicates via reverse transcription of an RNA intermediate, subsequently integrating element cDNA into the host genome. Retroviruses and LTR retrotransposons rely on their hosts to provide most of the factors necessary for propagation. We identified *dbr1* mutants in a screen to identify host cell genes required for Ty1 transposition (15). The *dbr1* mutant had been identified previously in a similar screen by others (6). Although *dbr1* cells produce wild-type levels of Ty1 proteins, they accumulate Ty1 cDNA at a slower rate than do wild-type cells, suggesting that Dbr1p plays a role in Ty1 reverse transcription or cDNA stability (15).

In its cellular role, Dbr1p acts at the end of the mRNA splicing process, cleaving the 2′-5′ linkage at the branch point of intron RNA lariats released after exon ligation, converting them to linear RNAs that are rapidly degraded (6, 23). In a *dbr1* mutant, intron RNAs accumulate as lariats (6). The uncleaved lariat branch point blocks the progression of 3′ exonucleases, and there is no 5′ end available on which a 5′ exonuclease can act. *DBR1* is highly conserved, and homologues have been cloned from *Schizosaccharomyces pombe*, *S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* (6, 17, 18, 24). Deletion of *DBR1* has no significant effect on the growth rate of *S. cerevisiae*, but *S. pombe dbr1* null mutants display severe growth defects and abnormal cell morphology (24). The differential effect of the loss of *DBR1* function in these organisms could be related to numbers of introns that are processed by the spliceosome and released as lariats. The *S. pombe* genome contains ~4,730 introns distributed among 43% of the genes (34), while the *S. cerevisiae* genome contains ~255 introns distributed among 5% of the genes (11, 21). However, almost half of the introns in *S. cerevisiae* are in highly expressed genes encoding ribosomal proteins. Therefore, the difference between *S. cerevisiae* and *S. pombe dbr1* mutants in the amount of intron RNA lariat accumulation is less than that indicated by the numbers of introns encoded in their genomes.

The role that Dbr1p plays in Ty1 transposition is not understood, and it is not known if Dbr1p's 2′-5′ phosphodiesterase activity, which catalyzes the debranching of intron RNA lariats, is necessary for Ty1 transposition. In work presented here, a library of *dbr1* missense mutants was created and tested for both Ty1 transposition and debranching activity. The purpose of this screen was to identify amino acid residues in Dbr1p that are necessary for Ty1 transposition and to determine if *dbr1* mutants can be identified that are defective for Ty1 transposition but can still debranch intron RNA lariats. The existence of such mutants could be an indication that 2′-5′ phosphodiesterase activity is not needed for Ty1 transposition. Further experiments would then be warranted to test that possibility.

Although screening is ongoing, all of the *dbr1* missense mutants created in this study are defective for both Ty1 transposition and debranching intron RNA lariats. Two of the mutants have changes in the Dbr1p phosphoesterase signature motif, which is believed to form part of the enzyme active site. The transposition deficiency of these two mutants supports the hypothesis that 2′-5′ phosphodiesterase activity is necessary for Ty1 transposition. Experiments with a Ty1 deletion element

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pCR4-TOPO	TOPO cloning vector	Invitrogen
pYES2.1-TOPO	TOPO cloning shuttle vector; *URA3* 2μm	Invitrogen
pBJC35	p*GAL1*-Ty1::*his3AI*; *URA3* 2μm	9
pDG784	p*GAL1*-Ty1::*his3AI*; *TRP1* 2μm	10
pX3	p*GAL1*-Ty1::*TRP1*; *URA3* 2μm	35
pX100	p*GAL1*-Ty1(Δ620-5562)::*TRP1*; *URA3* 2μm	36
pTM235	*DBR1* ORF in pYES2/GS; *URA3* 2μm	Invitrogen
pTM263	*DBR1*-V5-6xHis in pRS314	This work
pTM400	*ACT1* lariat probe template	This work
pTM411	*ACT1* 3′ intron probe template	This work
pTM426	*ACT1* 5′ intron probe template	This work
pTM428	*dbr1-N8* derivative of pTM235	This work
pTM431	*dbr1-N14* derivative of pTM235	This work
pTM432	*dbr1-N16* derivative of pTM235	This work
pTM434	*dbr1-N20* derivative of pTM235	This work
pTM435	*dbr1-N29* derivative of pTM235	This work
pTM436	*dbr1-C30* derivative of pTM235	This work
pTM442	*dbr1-N52* derivative of pTM235	This work
pTM444	*dbr1-N70* derivative of pTM235	This work
pTM454	*dbr1-C86* derivative of pTM235	This work

TABLE 3. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′-3′)
99	CGGATCGGACTACTAGCAGCT
100	CCATTGAGGCCGCAAATCTCT
101	GCTGAGCCACGATTGGCCCAA
102	GATGCGGCCCTCTAGAAACTC
146	CTCTCCCATAACCTCCTA
147	CTCTCGAGCAATTGGGACCG
219	GGATCCTCAAACCAAGAAGAA AAAGAAA
220	TCGTGGTTATTACAGATCAGTCA
221	GGATCCGTCCCAATTGCTCGAG AGATTT
222	ATAGCAACAAAAAGAATGAAGC
237	GTAAGGGTAGACCAAGACACC AAGGT
238	TAATACGACTCACTATAGGAAG AGACCGGAAGAGTACAAGGACAA

lacking most of the Ty1 internal sequences suggest that Dbr1p may act on Ty1 RNA.

MATERIALS AND METHODS

Yeast and bacterial strains, plasmids, and general procedures. The plasmids and yeast strains used in this study are described in Tables 1 and 2, respectively. For plasmid manipulations, *Escherichia coli* strain TOP10 was used (F-*mcrA* Δ[*mrr-hsdRMS-mcrBC*] ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* D[*ara-leu*]*7697 galU galK rpsL* [Str^r] *endA1 nupG*). When not specifically described, general molecular techniques (1) and standard yeast media and general procedures (14) were used. DNA oligonucleotides used in the procedures described below are listed in Table 3.

Transposition assays. Ty1 transposition was measured with a replica-plating assay, a cell suspension spotting assay, and a quantitative plating assay.

Replica-plating assays have been described previously (15, 29, 35). Briefly, patches of cells containing p*GAL1*-Ty1 are replica plated to medium containing 2% D-(+)-galactose to induce transcription of Ty1. After the induction period, the patches are replica plated to medium that selects for Ty1 transposition events. For p*GAL1*-Ty1::*his3*AI (pBJC35 and pDG784), the induction period is 16 to 20 h at 26°C and Ty1 transposition events are indicated by His$^+$ colonies after 48 h on SD-His selection plates (14). For p*GAL1*-Ty1::*TRP1* (pX3), the induction period is 5 days at 22°C. After the Ty1 expression period is finished for pX3, patches are grown nonselectively for 16 to 24 h to allow passive loss of the plasmid. Ty1 transposition events are then selected by growth on plates that contain 5-fluoroorotic acid (+ FOA) and lack tryptophan (− trp). FOA selects against cells containing pX3 because of its *URA3* marker (3), while the lack of tryptophan selects for the presence of transposed Ty1::*TRP1* elements.

The cell suspension spotting assay is semiquantitative, providing a visual comparison of Ty1 transposition rates between strains. For this assay, cells containing p*GAL1*-Ty1 are grown in patches as described above or in liquid cultures (YNB/CAA medium) (16). For liquid cultures, Ty1 transcription is induced by subculturing into medium containing 2% D-(+)-galactose and growing for 16 to 18 h at 26°C. After the Ty1 expression period in either liquid medium or on plates, equivalent numbers of cells for each strain are spotted in a series of dilutions onto media to select for Ty1 transpositions and separately to show cell titers (complete medium). The starting suspension of each dilution series has an optical density at 600 nm of 0.5. For cells growing on solid medium, patches of cells are scraped off the plates after the Ty1 expression period and suspended in sterile water prior to dilution and spotting.

The quantitative plating assay provides Ty1 transposition frequencies. For this assay, cells are grown in patches or in liquid cultures as described above. After the Ty1 expression period, liquid dilutions of cells are plated for single colonies onto media to select for Ty1 transpositions and separately to show cell titers. Colony counts yield the number of cells containing Ty1 transposition events out of the total number of cells in the population (transposition frequency).

For experiments with the Ty1 deletion element, the frequency of Ty1 transposition was determined by a method described by Boeke and coworkers (20). Transformants of strain TMY43 and TMY380, carrying either pX3 or pX100, were patched onto SD-Ura (to maintain selection for the plasmids) and grown for 2 days at 30°C. Patches were replica plated to SG-Ura to induce transcription of the plasmid-borne Ty1 elements. SG-Ura plates were incubated at 22°C for 5 days, replica plated to SD-Ura for overnight growth at 30°C, and then replica plated to YPD (14) for overnight growth at 30°C again. Patches grown on YPD were scraped and suspended in water. Dilutions of the suspensions were plated onto YPD at a density of 200 to 400 colonies per plate. When colonies were grown, they were replica plated to SD-Ura and SD-Trp. For a given patch, the number of Ura-Trp$^+$ cells relative to the total number of Ura$^-$ cells is used as the measure of Ty1 transposition. At least three independent transformants were tested for each strain-plasmid combination. The results for each of the transformants for a strain-plasmid combination were combined to generate an overall frequency.

PCR mutagenesis of *DBR1*. The *DBR1* gene was mutagenized by error-prone PCR (4, 5) with the GeneMorph kit from Stratagene (La Jolla, Calif.). The kit contains an error-prone thermophilic DNA polymerase that produces all possible transition and transversion mutations. Mutation frequency is determined by the DNA polymerase error rate and the number of target DNA duplications. In this procedure, the initial target DNA concentration in the PCR dictates the number of target duplications. Few target duplications produces a low mutation frequency (one to three mutations per kilobase of target). The pTM235 plasmid, linearized with *Eco*RI, was used as the PCR template. Mutagenic PCRs were

TABLE 2. Yeast strains used in this study

Strain	Genotype	Reference
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	31
TMY30	*MAT***a** *ura3-52 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	15
TMY43	*MAT***a** *trp1-H3 ura3-52 his3-200 ade2-101^{oc} lys2-1 leu1-12 can1-100 bar1::hisG spt3-Δ202 GAL3$^+$ ΔTy3*	16
TMY60	*MAT***a** *dbr1::neo^r ura3-52 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	29
TMY176	*MAT***a** *rad52::hisG ura3-52 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	This work
TMY344	*MATa dbr1::neo^r rad52::hisG ura3-52 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	This work

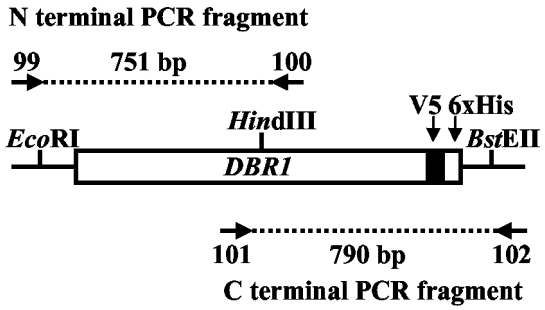

FIG. 1. PCR mutagenesis of *DBR1*. The pTM235 plasmid was linearized and used for PCR with either primers 99 and 100 or primers 101 and 102 for the N- and C-terminal regions of *DBR1*, respectively. Sizes of PCR products are shown. Most point mutations form a fusion with V5 and a six-His tag (6xHis), facilitating Western analysis and protein purification.

carried out separately for the 5′ and 3′ regions of the *DBR1* open reading frame (ORF). Primers 99 and 100 were used for mutagenic PCR of the 5′ region of the *DBR1* ORF (Fig. 1). Primer 99 corresponds to bp 453 to 474 of pYES2/GS (Invitrogen, Carlsbad, Calif.), and primer 100 corresponds to nucleotides (nt) 679 to 658 of the *DBR1* ORF. Primers 101 and 102 were used for mutagenic PCR of the 3′ region of the *DBR1* ORF (Fig. 1). Primer 101 corresponds to bp 528 to 549 of *DBR1*, and primer 102 corresponds to bp 635 to 614 of pYES2/GS. PCR conditions were as follows (amounts specified by the GeneMorph kit): 41.5 μl of H_2O, 5 μl of 10× DNA polymerase buffer, 1 μl of a deoxynucleoside triphosphate mixture, 1 μl of mutazyme DNA polymerase, 1 μl (60 ng) of pTM235 template DNA, and 0.25 μl (250 ng) of each primer. Cycling conditions were as follows: 94°C, 40 s; 52°C, 40 s; 72°C, 2 min (35 cycles).

Purification of Dbr1p-V5-6xHis. Wild-type and mutant versions of Dbr1p were purified under denaturing conditions with the ProBond Purification System (Invitrogen). The ProBond system is designed for purification of proteins tagged with six tandem histidine residues (six-His tag). Whole-cell extracts were prepared from yeast cells (TMY60) containing either pTM235 (*DBR1*) or *dbr1* mutant derivatives (pTM434 and pTM435). Cells were initially grown in YNB/CAA medium lacking uracil and containing 2% D-(+)-raffinose. Expression of Dbr1p-V5-6xHis was induced prior to extract preparation by addition of D-(+)-galactose to 2% and incubation at 26°C for 16 to 20 h. Cells were collected by centrifugation and lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 3 mM $MgCl_2$, 0.2% Triton X-100, 0.1% β-mercaptoethanol, yeast protease inhibitors (Sigma, St. Louis, Mo.), 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and 50 mM sodium fluoride. In some cases, the phosphatase inhibitors (sodium orthovanadate and NaF) were omitted. Dbr1p-V5-6xHis was purified over ProBond columns under conditions recommended by the manufacturer. Multiple fractions containing purified Dbr1p-V5-6xHis were combined and concentrated with Centricon filter devices (Millipore; Billerica, Mass.). The amount of Dbr1p-V5-6xHis recovered from individual ProBond fractions was estimated with Western blot assays done with anti-V5 antibody (1/2,500 dilution). Tyrosine phosphorylation of Dbr1p-V5-6xHis was assessed with an antiphosphotyrosine antibody (PY99; 1/1,000 dilution) from Santa Cruz Biotech (Santa Cruz, Calif.). Blots probed with the PY99 antibody were blocked in TBS-T with 1% bovine serum albumin (1). In vitro phosphatase treatment of Dbr1p-V5-6xHis was performed with λ protein phosphatase (New England Biolabs, Beverly, Mass.). Phosphatase reactions were performed at 30°C for 30 min under conditions recommended by the manufacturer.

RNase protection assays. Plasmid pTM426 was used as a template to create the 5′ *ACT1* intron probe, which spans the 5′ border of the *ACT1* intron. The PCR product generated with oligonucleotides 219 and 220 was cloned into pCR4-TOPO to create pTM426. Plasmid pTM411 was used as a template to create the 3′ *ACT1* intron probe, which includes the lariat branch point of the *ACT1* intron. The PCR product generated with oligonucleotides 221 and 222 was cloned into pYES2.1-TOPO to create pTM411. Yeast genomic DNA was used as the template for both PCRs. Plasmid pTM400 was used as a template to create the *ACT1* lariat probe, which contains sequences on either side of the 2′-5′ lariat branch linkage. The RT-PCR product generated with oligonucleotides 146 and 147 was cloned into pYES2.1-TOPO to create pTM400. The RT-PCR of total yeast RNA was performed with the One-Step kit from Qiagen (Valencia, Calif.). The PCR and RT-PCR products described above were gel purified and cloned into pCR4-TOPO or pYES2.1-TOPO, as appropriate, with TOPO cloning kits from Invitrogen.

The PCR product generated with oligonucleotides 237 and 238 was directly used as a template to create the *ACT1* 3′ exon probe, which hybridizes to sequences in the 3′ exon of *ACT1* RNA. Oligonucleotide 238 includes the sequence of the T7 promoter at its 5′ end.

RNA probes for RNase protection assays were generated from the various templates described above by in vitro transcription reactions with T7 polymerase with the MAXIscript kit from Ambion (Austin, Tex.). Plasmid templates were cleaved immediately downstream of probe sequences prior to transcription to limit transcript length. Plasmids pTM411 and pTM426 were cleaved with *Bam*HI; pTM400 was cleaved with *Sau*3AI. [α-^{32}P] CTP (Amersham Biosciences, Piscataway, N.J.) was used in transcription reactions to radioactively label the probes, which were subsequently gel purified (1).

RNase protection assays were performed with the RPA III kit from Ambion. Hybridizations of probes with RNA samples were performed at 42°C for 16 to 24 h and were followed by digestion with RNaseT1 to remove probe sequences that did not hybridize. Probe amounts in RNase protection assays were ~10^5 cpm. Yeast total RNA amounts ranged from 1 to 10 μg, depending on the experiment. RNase protection assay products were run on 6% polyacrylamide gels. RNA size markers were generated by in vitro transcription of RNA Century Marker templates from Ambion incorporating [α-^{32}P]CTP (Amersham Biosciences). Phosphor screens exposed to the gels were read on a Storm phosphorimager (Amersham Biosciences), and the signal was quantified. Size estimates for the products of RNase protection assays were based on careful determination of mobilities, as well as established knowledge about the properties of the *ACT1* RNA-processing pathway. The products of RNase protection assays were run on large DNA sequencing gels 45 cm in length. Size standards on each gel included 100-, 200-, 300-, 400-, and 500-nt species. Mobilities were carefully plotted on semilog graphs, and size estimates for the undigested probes match expectations within 5 nt. The identities and precise sizes of the protected products were assigned on the basis of the known sizes and structures of *ACT1* RNA splicing products derived from 20 years of intense study by many laboratories.

RESULTS

PCR mutagenesis of *DBR1*. *DBR1* is required for debranching of intron RNA lariats and for full levels of transposition of the yeast LTR retrotransposon Ty1. Comparison of *DBR1* genes from different organisms shows strong conservation of coding sequences. However, the importance of specific amino acid residues for Dbr1p function has not been demonstrated. Furthermore, the relationship between Dbr1p's roles in debranching and Ty1 transposition is unknown. If the 2′-5′ phosphodiesterase activity of Dbr1p is required for both processes, it would suggest that Ty1 transposition involves an RNA with this type of linkage, for which there is no evidence at present. Alternatively, Dbr1p may act differently in Ty1 transposition and debranching. In this case, *dbr1* mutant alleles could be identified that affect one process but not the other.

In order to study how Dbr1p functions in Ty1 transposition, a library of *dbr1* alleles was generated by mutagenic PCR. The method used (GeneMorph; Stratagene) was chosen because it can produce all possible transition and transversion mutants with minimal bias. Mutations were introduced in the *DBR1* ORF present in pTM235. This plasmid contains the 1,218-nt *DBR1* ORF fused at its 5′ end to the yeast *GAL1* promoter and at its 3′ end to a V5 epitope tag followed by a six-His tag (1) (Invitrogen) (Fig. 1). Mutagenic PCR was performed with two different sets of primers: one set (primers 99 and 100) for the 5′ portion of the *DBR1* ORF (nt 1 to 679) and the other set (primers 101 and 102) for the 3′ portion of the ORF (nt 528 to 1218). PCR conditions were adjusted to limit multiple mutations in the *DBR1* gene in order to generate a collection of single-point mutations (see Materials and Methods). The PCR products were digested either with *Eco*RI and *Hin*dIII (5′

region of *DBR1*) or with *Hind*III and *Bst*EII (3′ region of *DBR1*), and purified fragments were used to replace the corresponding portions of pTM235. Because the mutation rate in the PCR is low, many plasmids in the resulting collection of pTM235 derivatives contain a wild-type *DBR1* allele. Therefore, the collection of plasmids was screened to identify those containing *dbr1* mutant alleles. Yeast strain TMY60 (*dbr1*Δ) was cotransformed with the collection of pTM235 derivatives and pDG784 (p*GAL1*-Ty1::*his3*AI). Individual transformants were screened for the ability to support Ty1 transposition with a replica-plating assay (see Materials and Methods). In this assay, a pTM235 derivative is the only source of Dbr1p in the cell and expression of both Dbr1p and Ty1 is activated by D-(+)-galactose. Positive and negative controls were patched on each plate containing transformants being screened. The positive control (wild-type Ty1 transposition phenotype) was a TMY60 transformant containing pDG784 and the original (unmutagenized) pTM235 plasmid (p*GAL1-DBR1*-V5-6xHis). The negative control (*dbr1* mutant Ty1 transposition phenotype) was a TMY60 transformant containing pDG784 and a vector plasmid (pYES2.1) containing no *DBR1* sequences. Patches of cells unable to support wild-type levels of Ty1 transposition were single colony purified and retested.

The pTM235 derivatives from those TMY60 transformants that retained a mutant phenotype for Ty1 transposition were rescued into *E. coli*. Purified plasmids were subjected to restriction enzyme analysis to eliminate those with apparent deletions from further studies. Full-length plasmids were reintroduced into TMY60 and retested for Ty1 transposition. Only transformants with a Ty1 transposition defect were analyzed further.

Transformants were subjected to Western blot analysis (1) with α-V5 antibody (Invitrogen). For structure-function studies, we are interested in identifying missense mutants that express wild-type levels of full-length Dbr1p. The expression of the V5 epitope on an ~50 kDa protein indicates Dbr1p-V5-6xHis expression because V5 is expressed as a C-terminal tag on the plasmid-encoded Dbr1p proteins. Only those mutants that express Dbr1p-V5-6xHis were analyzed further.

A total of 1,098 pTM235 derivatives created by replacement of the 5′ portion of the *DBR1* ORF with a mutagenic PCR fragment were screened by the process described above. From this collection, 15 plasmids were identified that contain *dbr1* missense alleles resulting in a Ty1 transposition deficiency and of these, 7 plasmids encoded single amino acid substitutions. A total of 955 pTM235 derivatives created by replacement of the 3′ portion of the *DBR1* ORF with a mutagenic PCR fragment were screened. From this collection, seven plasmids were identified that contain *dbr1* missense alleles resulting in a Ty1 transposition deficiency, and of these, one plasmid encoded a single amino acid substitution. The transposition phenotypes of the eight single amino acid substitution alleles are shown in Fig. 2 along with the phenotypes of control strains that include mutant *dbr1-C30*, a nonsense allele that produces a truncated form of Dbr1p.

All of the mutations that encode single amino acid changes in Dbr1p were assessed for dominance or recessiveness. Yeast strain TMY30 (*DBR1*) was cotransformed with plasmids bearing the *dbr1* point mutations and pDG784 (p*GAL1*-Ty1::*his3*AI). Individual transformants were screened for the ability to support Ty1 transposition by the replica-plating assay used in the initial mutant screen. Because the TMY30 strain expresses wild-type Dbr1p, recessive *dbr1* mutations on the plasmid will not alter the wild-type phenotype of the strain, but dominant *DBR1* mutations will produce a mutant phenotype. All of the mutations encoding single amino acid changes in Dbr1p are recessive (data not shown).

Mutations in *DBR1* lie in conserved regions. Amino acid changes in each of the *dbr1* missense mutants are shown in Table 4. Eight of the missense mutations result in single amino acid changes in Dbr1p. The positions of these single amino acid changes are shown in Fig. 3, in which the *S. cerevisiae* Dbr1p sequence is shown aligned with amino acid residues conserved in at least 10 of the 12 Dbr1p sequences available in the GenBank database (Conserved in Fig. 3). Importantly, two mutations (*dbr1-N16* and *dbr1-N30*) map to a GD/GNH phosphoesterase signature motif (19) (Phos in Fig. 3). This motif has been identified in all enzymes that catalyze phosphate bond cleavage. Two independent mutations (*dbr1-N20* and *dbr1-N29*) were found to reside in a potential tyrosine phosphorylation site at Y68 (pattern [RK]-X[2,3]-[DE]-X[2,3]-Y) (8, 13, 26).

Dbr1p is a phosphoprotein. Identification of two mutations that produce changes in the Y68 residue prompted us to investigate the possibility that Dbr1p is phosphorylated. Dbr1p-V5-6xHis was purified from TMY60 cells containing *DBR1* (pTM235), *dbr1-N20* (pTM434), and *dbr1-N29* (pTM435) as outlined in Materials and Methods. The wild-type and mutant forms of Dbr1p were purified with the ProBond system (Invitrogen) in the presence of phosphatase inhibitors. The wild-type and mutant forms of Dbr1p were detected on Western blots with anti-V5 antibody (Fig. 4A, left side) (data for N29 mutant not shown). Western analysis with antiphosphotyrosine antibody indicates that the wild-type and mutant forms of Dbr1p are phosphorylated (Fig. 4A, right side) (data for N29 mutant not shown). When purified Dbr1p proteins are treated with λ protein phosphatase, they no longer react with antiphosphotyrosine antibody (Fig. 4B). λ protein phosphatase removes phosphates from tyrosine, serine, and threonine residues. These data indicate that Dbr1p is phosphorylated on at least one tyrosine other than Y68. Although wild-type Dbr1p may also be phosphorylated on Y68, that cannot be determined from this experiment. As expected, when phosphatase inhibitors were absent during purification, antiphosphotyrosine antibody did not react with either wild-type or mutant Dbr1p (data not shown).

Intron lariat RNA accumulation within cells. We have developed an RNase protection assay, partly based on the lariat protection assay of Vogel and coworkers (33), to measure the accumulation of intron RNA lariats within cells. The assay uses RNA probes that hybridize to *ACT1* RNA. *ACT1* contains an intron that accumulates in *dbr1* strains after it is spliced from *ACT1* pre-mRNA (6). Hybridizations of the probes described below with *ACT1* RNA are shown in Fig. 5. The 5′ intron probe hybridizes to 283 nt in *ACT1* RNA: 145 nt at the 5′ end of the *ACT1* intron and an adjacent 138 nt in the 5′ exon. The 3′ intron probe hybridizes to 244 nt within the *ACT1* intron: 30 nt downstream of the lariat branch point and 214 nt upstream of and including the lariat branch point. The lariat probe hybridizes to 206 nt within the *ACT1* intron: 69 nt at the 5′ end

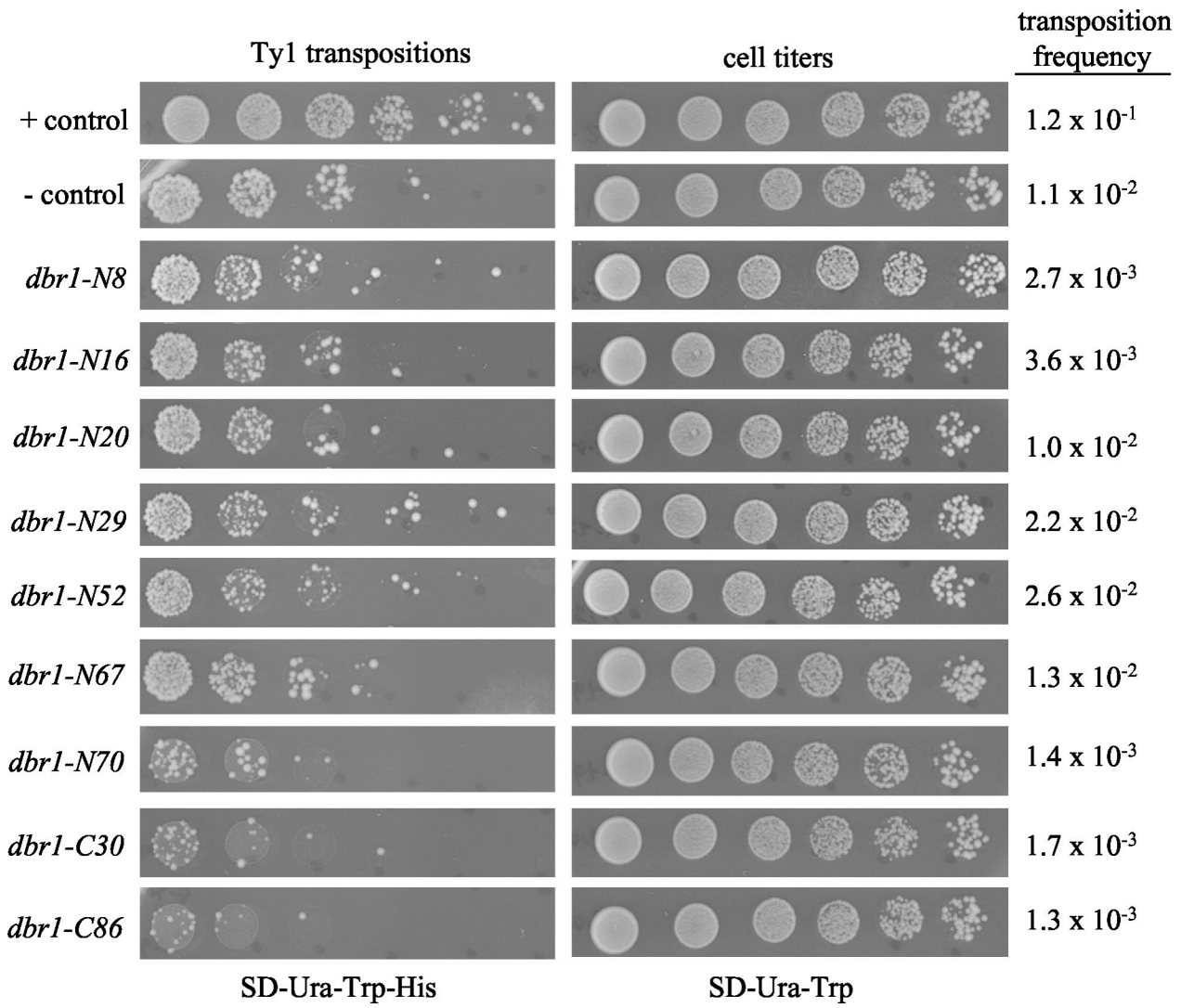

FIG. 2. Ty1 transposition phenotypes of *dbr1* mutants. Transposition of Ty1 was measured with the *pGAL1*-Ty1::*his3*AI element as described in Materials and Methods. Individual transformants containing pDG784 and a derivative of pTM235 (with mutagenized *dbr1* DNA) were grown in SD-Ura-Trp before induction of transcription of Ty1 and *DBR1* with galactose for 16 to 20 h. Serial dilutions were spotted on either SD-Ura-Trp-His (to select for transpositions) or SD-Ura-Trp (for cell titers). The calculated transposition frequency of each mutant is the average of three independent experiments. The positive (+) control is pTM235 (*DBR1*) cotransformed with pDG784. The negative (−) control is pYES2.1 (vector) cotransformed with pDG784.

of the intron, as well as 137 nt starting at the lariat branch point and extending upstream. As a control for the amount of protection observed by intron RNA sequences, a 3′ exon probe was used in some experiments. This probe hybridizes to 333 nt of the *ACT1* 3′ exon.

With RNase protection assays, we have measured the accumulation of *ACT1* RNA species in both a *dbr1*Δ mutant (TMY60) and the wild type (TMY30) (Fig. 5D). Cells of each strain were grown in rich medium (YPD) to mid-log phase. Total RNA was isolated from the cells and used for RNase protection assays. Extensive accumulation of particular *ACT1* RNA species occurs in the *dbr1* mutant compared to the wild type, evidenced by the extensive probe protection by *dbr1* RNA seen in Fig. 5D, lanes 2, 4, and 6, compared to the minimal protection by wild-type RNA seen in lanes 1, 3, and 5. The sizes of the major protected fragments in lanes 2, 4, and 6 correspond to expectations for protection by *ACT1* intron lariat RNA (positions of black arrows). The relative amounts of protected fragments indicate that introns accumulate in the *dbr1* mutant at levels 50 to 100 times the levels in the wild type (data not shown).

For the lariat probe, the observed protection of ~211 nt (Fig. 5D, lane 6) is expected if the 2′-5′ phosphodiester bond between the 5′ end of the intron and the lariat branch point is intact and the probe hybridization spans the lariat branch point. Also in line with expectations, enhanced protection of 138- and 74-nt fragments, predicted if intron lariats are debranched, is not observed for the *dbr1* mutant. Debranched introns do not accumulate in the wild-type strain either, consistent with their rapid degradation.

As mentioned above, the *ACT1* intron lariat protects 206 nt of the lariat probe. The extra nucleotides that bring the pro-

TABLE 4. *dbr1* missense mutants used in this study

Mutant name	Mutated residue(s) in Dbr1p
dbr1-N1	I26N, I37N, K135M, W148R
dbr1-N8	H97Y
dbr1-N10	I44T, G126W
dbr1-N14	D180Y
dbr1-N16	G84A
dbr1-N20	Y68F
dbr1-N29	Y68D
dbr1-N30	N85D
dbr1-N31	I66L, H92L
dbr1-N33	G38V, A75V
dbr1-N37	I26N, I37N, K135M
dbr1-N39	S52L, G99D, H171Y
dbr1-N51	I32M, L124S
dbr1-N52	S212C
dbr1-N70	I37T, S43C, N182Y
dbr1-C5	I240S, D273N, G280N, K290E, P304S, E335G, E361K
dbr1-C7	F236I, A298V, N353S
dbr1-C8	M330I, E335G, E361K
dbr1-C9	E284D, K290E
dbr1-C33	I240S, D273N, G277D, P304S
dbr1-C68	L218Q, L268S, M330I
dbr1-C86	E284D

tected fragment size to 211 nt are due to the positions of the first RNase T1 cut sites on either site of the hybridizing region. Differences for other probes between the extents of probe hybridization with *ACT1* RNAs and the size of protected fragments in the RNase protection assays are also due to the positions of the RNase T1 cut sites.

RNase protection assays with the 5′ intron probe indicate that a fragment of ~156 nt is extensively protected by RNA from the *dbr1* strain (Fig. 5D, lane 2). This result is expected if the 5′ portion of the probe, the part that hybridizes to the 5′ portion of the *ACT1* intron, is protected because of accumulation of *ACT1* intron RNA (Fig. 5A). Mature *ACT1* mRNA will protect only the 3′ part of the probe, a fragment of 143 nt. Evidence of such protection by the mature *ACT1* mRNA is indicated in lanes 1 and 2 of Fig. 5D by the stippled arrow. Note that this fragment is present in roughly equal amounts in the *dbr1* mutant and the wild type. This result indicates that production of mature *ACT1* mRNA is not grossly affected in the *dbr1* mutant.

RNase protection assays with the 3′ intron probe indicate that a fragment of ~215 nt is extensively protected by RNA from the *dbr1* strain (Fig. 5D, lane 4). This result is expected if ~30 nt at the 5′ end of the probe are not protected. The hybridizations diagrammed in Fig. 5B show both the entire intron lariat and a trimmed intron lariat in which the 3′ end is 1 base downstream of the lariat branch point. Our experimental results indicate that the 3′ tail of the intron lariat is degraded to the branch point or just downstream of it.

Accumulation of *ACT1* intron lariat RNA in *dbr1* mutants. We have performed RNase protection assays to measure the accumulation of *ACT1* intron lariat RNA in the newly generated *dbr1* mutants. These RNase protection assays are a means by which to assess the debranching capability encoded by the various *dbr1* mutant alleles. The newly generated alleles are carried on plasmids in a *dbr1*Δ strain (TMY60) and are under the control of the *GAL1* promoter. TMY60 cells containing the various plasmids were pregrown on selective raffinose medium to early log phase. The precultures were split into two portions. D-(+)-Galactose was added to one portion to a final concentration of 2% to induce expression of the plasmid-borne *dbr1* alleles. D-(+)-Glucose was added to the other portion to a final concentration of 2% to repress expression of *dbr1* (negative control).

All of the *dbr1* point mutant alleles identified in this study on the basis of their Ty1 transposition deficiencies are also deficient for debranching of intron lariats (Fig. 6). The extent of the debranching deficiency of each mutant is consistent with a severe loss of function relative to the wild-type allele. Figure 6 depicts RNase protection assays with both the 5′ intron probe and the 3′ intron probe. The black arrows in each panel indicate the fragments that represent accumulation of intron RNAs.

The sensitivity of the RNase protection assay uncovers the fact that the Dbr1p-V5-6xHis fusion protein is not as efficient in debranching intron RNA lariats as native Dbr1p. Two different alleles encoding this fusion protein were tested: one allele has the *GAL1* promoter driving expression of the fusion protein (pTM235), and the other allele has the native *DBR1* promoter (pTM263). As shown in lanes 12 and 13 of Fig. 6A, the 156-nt fragment of the 5′ intron probe is readily detected in RNA samples from strains containing the two different *DBR1*-V5-6xHis fusion alleles, indicating protection by *ACT1* intron sequences. The p*GAL1*-driven fusion needs galactose for full expression, and the result in lane 12 of Fig. 6A is for an RNA sample from cells grown for 18 h in 2% galactose. This debranching inefficiency can be contrasted with the absence of the 156-nt fragment in lane 1 of Fig. 5D, for which RNA from a strain bearing native, wild-type *DBR1* was used. Interestingly, in Ty1 transposition assays, the wild-type Dbr1p-V5-6xHis fusion protein supports levels of transposition that are not readily distinguishable from levels supported by native, wild-type Dbr1p (data not shown).

Despite the inefficiency of the wild-type Dbr1p-V5-6xHis fusion protein in debranching intron RNA lariats, all of the mutants are clearly much less active. In terms of absolute levels of RNA lariat accumulation, the results for the RNase protection assays fluctuate slightly from one experiment to the next. This is most likely due to slight differences in growth state or other factors that vary from experiment to experiment. The sensitivity of the RNase protection assay then displays this variation. However, relative comparisons of the mutants to the wild type clearly show the correlation between Ty1 transposition and debranching activity.

Integration versus recombination of Ty1 cDNA in *dbr1* mutant strains. Ty1 cDNA produced by reverse transcription of Ty1 RNA can become incorporated into yeast chromosomal DNA by two independent processes. One process, integration, requires the Ty1-encoded integrase protein IN. IN binds the ends of the Ty1 cDNA, cleaves the chromosomal target sequence, and joins the Ty1 cDNA to the target DNA. Cellular repair enzymes complete the integration process (fill in gaps and ligate nicks). Interactions between homologous DNA sequences in the Ty1 cDNA and the chromosomal target are not part of the integration process. Integration sites seem to be chosen by protein-protein interactions, possibly between IN and a protein bound to target DNA. Homologous recombina-

```
Dbr1p   1 MTKLRIAVQG CCHGQLNQIY KEVSRIHAKT PIDLLIILGD FQSIRDGQDF
Phos           I   G D H                      D  L  GD   D
Conserved    MRIAV G C HG LD IY   I         VDLLICCGD FQ VRN  D

                              *                   **           *
Dbr1p  51 KSIAIPPKYQ RLGDFISYYN NEIEAPVPTI FIGGNHESMR HLMLLPHGGY
Phos                                           GNHE
Conserved   M VP KY   M  F  YYS G   APVLTI FIGGNHEA    L EL YGGW

Dbr1p  101 VAKNIFYMGY SNVIWFKGIR IGSLSGIWKE WDFNKQRPDW NDLENNNWKA
Phos
Conserved  VAPNIYYMG    VV    VR IGGISGIY     F K   E   PY

                                    *
Dbr1p  151 NIRNLYHVRI SDIAPLFMIK HRIDIMLSHD WPNGVVYHGD TKHLLKLKPF
Phos                                  I   H
Conserved    RS YHVR   DV KL  L    VDI LSHD WP  I   G     LLR K

               *
Dbr1p  201 FEQDIKEGKL GSPVTWQLLR DLRPQWWLSA HLHVRFMASI KHNKRSHEPP
Phos                               D I  G H H
Conserved  F  DI    L G      LL   L P YW  A HLH KF A V  H

                                                *
Dbr1p  251 NKSTSKTKKN NNEIDLDLSS DEDERSGIMN CQEENEYDSK YGETRFLALD
Conserved                                                TKFLALD

Dbr1p  301 KCLPRRRWLE ILEIEPDTSH ASWKDENHRM FWDPEFINNL VICQKNKNLL
Conserved  KCLP R FLQ ILEIE               L  YD EWL I

Dbr1p  351 SNKPFNSVNW IELSQSNREE GRDIDWENYA IPAYTLDIQK DEVRQTKAFI

Dbr1p  401 SKFMT
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FIG. 3. Single-point mutations in *dbr1* lie mostly in conserved residues of Dbr1p. The amino acid sequence of *S. cerevisiae* Dbr1p is shown on the top line of each sequence block. Positions of amino acid changes in the point mutations are indicated by asterisks above the Dbr1p sequence. The phosphoesterase domain is indicated by underlined residues in Dbr1p (amino acids 4 to 238). Residues conserved in phosphoesterases are indicated on the "Phos" line. The GD/GNH phosphoesterase signature domain is part of this conserved sequence. Residues conserved in debranching enzymes are indicated on the "Conserved" line. The 12 organisms for which Dbr1p sequences are available are *S. cerevisiae*, *S. pombe*, *Neurospora crassa*, *Plasmodium falciparum*, *Arabidopsis thaliana*, *D. melanogaster*, *Anopheles gambiae*, *C. elegans*, *Xenopus laevis*, *Rattus norvegicus*, *M. musculus*, and *H. sapiens*. Residues identical in all 12 proteins are underlined in the "Conserved" row, while residues conserved in at least 10 of the 12 proteins are shown without underlining.

tion is the second process for incorporation of Ty1 cDNA into host chromosomes. This process can only occur at sites where Ty1 sequences are already present. Rad52p is an essential component of the homologous recombination machinery, so *rad52* mutants exhibit severe recombination defects (32), including Ty1 cDNA recombination. Previous work has shown that incorporation of Ty1 cDNA into the host chromosomes can occur in a *rad52* mutant by the integration process described above (30). Likewise, incorporation of Ty1 cDNA for an IN mutant element can occur very efficiently by homologous recombination (30). However, incorporation of Ty1 cDNA for an IN mutant element does not occur in a *rad52* mutant yeast strain because both integration and recombination are defective (30; data not shown). In addition, Ty1 mutants that produce alterations of the sequences at the Ty1 cDNA ends transpose only by homologous recombination. IN can no longer bind the cDNA ends in a way that promotes integration. Therefore, Ty1 mutants that produce altered cDNA ends do not transpose in a *rad52* mutant.

We have compared the transposition rates of *dbr1* and *dbr1 rad52* strains to infer structural aspects of the Ty1 cDNA produced in a *dbr1* mutant background. Strains YPH499 (wild type), TMY60 (*dbr1*), TMY176 (*rad52*), and TMY344 (*dbr1 rad52*) were transformed with the p*GAL1*-Ty1::*his3*AI plasmid (pBJC35) and, separately, the p*GAL1*-Ty1::*TRP1* plasmid (pX3). Three transformants for each strain-plasmid combination were tested for Ty1 transposition. The results for transformants containing pX3 are depicted in Fig. 7. We observed the expected reduction in Ty1 transposition in the *dbr1* mutant strain compared to the wild type. However, the frequency of

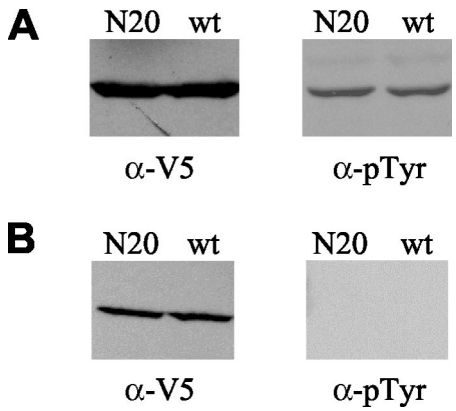

FIG. 4. Dbr1p is a phosphoprotein. (A) Western blots of Dbr1p-V5-6xHis following purification of the protein in the presence of phosphatase inhibitors. The left half shows purified wild-type (wt) and N20 mutant Dbr1p probed with anti-V5 antibody (α-V5). The right half shows the same samples probed with antiphosphotyrosine antibody (α-pTyr). (B) Western blots of purified Dbr1p-V5-6xHis after treatment with λ protein phosphatase.

the residual transposition events occurring in the *dbr1* mutant strain is not significantly reduced any further in a *dbr1 rad52* strain. If integration of the Ty1 cDNA was defective in a *dbr1* mutant, transposition would be reduced at least 25-fold in the *dbr1 rad52* strain compared to the *dbr1* strain. This conclusion is based on the fact that Ty1 elements defective for integration transpose at wild-type levels in *RAD52* yeast strains but at least 100-fold less efficiently in *rad52* strains (30; data not shown). Therefore, we conclude that chromosomal incorporation of Ty1 cDNA produced in *dbr1* strains in the absence of the major homologous recombination machinery must be catalyzed by the Ty1 IN protein. Because IN requires a precisely formed cDNA, these results suggest that the Ty1 cDNA produced in *dbr1* strains is full length and contains the normal ends that interact with the Ty1 IN protein.

Loss of *DBR1* dependence by Ty1 deletion elements. We have compared the transposition frequency of a Ty1 element containing an internal deletion (pX100) (36) to a full-length Ty1 element (pX3) (35) to determine if Dbr1p acts on Ty1 RNA (Table 5). Ty1 bases 620 to 5562 are deleted in pX100, and Ty1 proteins are supplied in *trans* by an unmarked, transposition-defective Ty1 element carried on the same plasmid (36). Transposition was measured in wild-type and *dbr1* deletion strains. As expected, the full-length element transposed at a much lower frequency in a *dbr1* strain than it did in the wild-type strain (29% of the wild-type strain frequency in this experiment). However, the deletion element transposed at much more similar frequencies in wild-type and *dbr1* strains (the *dbr1* strain frequency was 72% of the wild-type strain frequency; Table 5). These data are consistent with the loss of *DBR1*-dependent sequences in the deletion element. It is notable, however, that the Ty1 deletion element transposed at an overall lower frequency in both wild-type and *dbr1* mutant strains.

DISCUSSION

The roles of Dbr1p in Ty1 transposition and intron lariat debranching are related. Our goal is to determine the role that Dbr1p plays in Ty1 transposition. On the one hand, Dbr1p's role in Ty1 transposition may involve its 2′-5′ phosphodiesterase activity, which would suggest that an RNA with a 2′-5′ linkage is involved in transposition. Two RNAs are already known to play central roles in Ty1 transposition: Ty1 RNA acts as both an mRNA and the genome of the Ty1 virus-like particle (2), while $tRNA^{imet}$ acts as the primer for the initiation of Ty1 reverse transcription (7). Neither of these RNAs is known to contain a 2′-5′ linkage. On the other hand, Dbr1p's role in Ty1 transposition may not involve its 2′-5′ phosphodiesterase activity. Among the possibilities is one in which only the RNA binding capacity of Dbr1p is used in some way during transposition.

Dbr1p can be divided into different regions based on sequence conservation (Fig. 3). On the one hand, the N-terminal 242 amino acids show the strongest conservation with the Dbr1p sequences from 11 other species. The N-terminal region contains the phosphoesterase domain, comprising 20 conserved amino acids. However, the debranching enzymes exhibit additional extensive conservation. The 12 Dbr1p sequences in the GenBank database have 54 identical amino acids in their phosphoesterase domains, with an additional 84 amino acids conserved in at least 10 of the enzymes. A second region of conservation among the debranching enzymes lies outside of the phosphoesterase domain. For *S. cerevisiae* Dbr1p, this region encompasses approximately amino acids 294 to 340. In this region, the 12 Dbr1p sequences in the GenBank database have 6 identical amino acids, with an additional 21 amino acids conserved in at least 10 of the enzymes. The C-terminal regions of the different Dbr1p sequences vary greatly in length and do not contain significant similarities, although potential nuclear localization sequences are present in all of the enzymes.

Of the eight *dbr1* mutations encoding a single amino acid change, seven lie within the phosphoesterase domain. Two *dbr1* alleles (*dbr1-N16* and *dbr1-N30*) have changes that alter the GD/GNH phosphoesterase signature motif, which is thought to form part of the active site of the enzyme (19). If this is the case and other functions of Dbr1p are intact in these mutants (e.g., RNA binding), the decrease in Ty1 transposition for these mutants would be a strong indication of the importance of phosphodiesterase activity for transposition.

Two other *dbr1* alleles (*dbr1-N20* and *dbr1-N29*) have changes that alter a potential tyrosine phosphorylation site at Y68. We have shown that Dbr1p is phosphorylated, although the specific phosphorylated residues remain to be identified. The Y68 mutations encoded by *dbr1-N20* and *dbr1-N29* remain phosphorylated on tyrosine, indicating that Dbr1p may have multiple phosphorylation sites. Alignment of Dbr1p sequences in Fig. 3 shows that the tyrosine represented by Y68 in *S. cerevisiae* is conserved among all of the Dbr1p proteins identified. A potential kinase phosphorylation site associated with this tyrosine is identified only for the *S. cerevisiae*, *S. pombe*, *N. crassa*, and *P. falciparum* proteins with Prosite software (12). Analysis of Dbr1p phosphorylation sites and identification of a Dbr1p kinase(s) will provide more information about the post-translational regulation of Dbr1p.

Among the *dbr1* mutants isolated in this study is one (*dbr1-C86*) that affects a region of Dbr1p that is not conserved and changes a glutamate to an aspartate. This region probably does not participate in the catalytic function of Dbr1p but may be

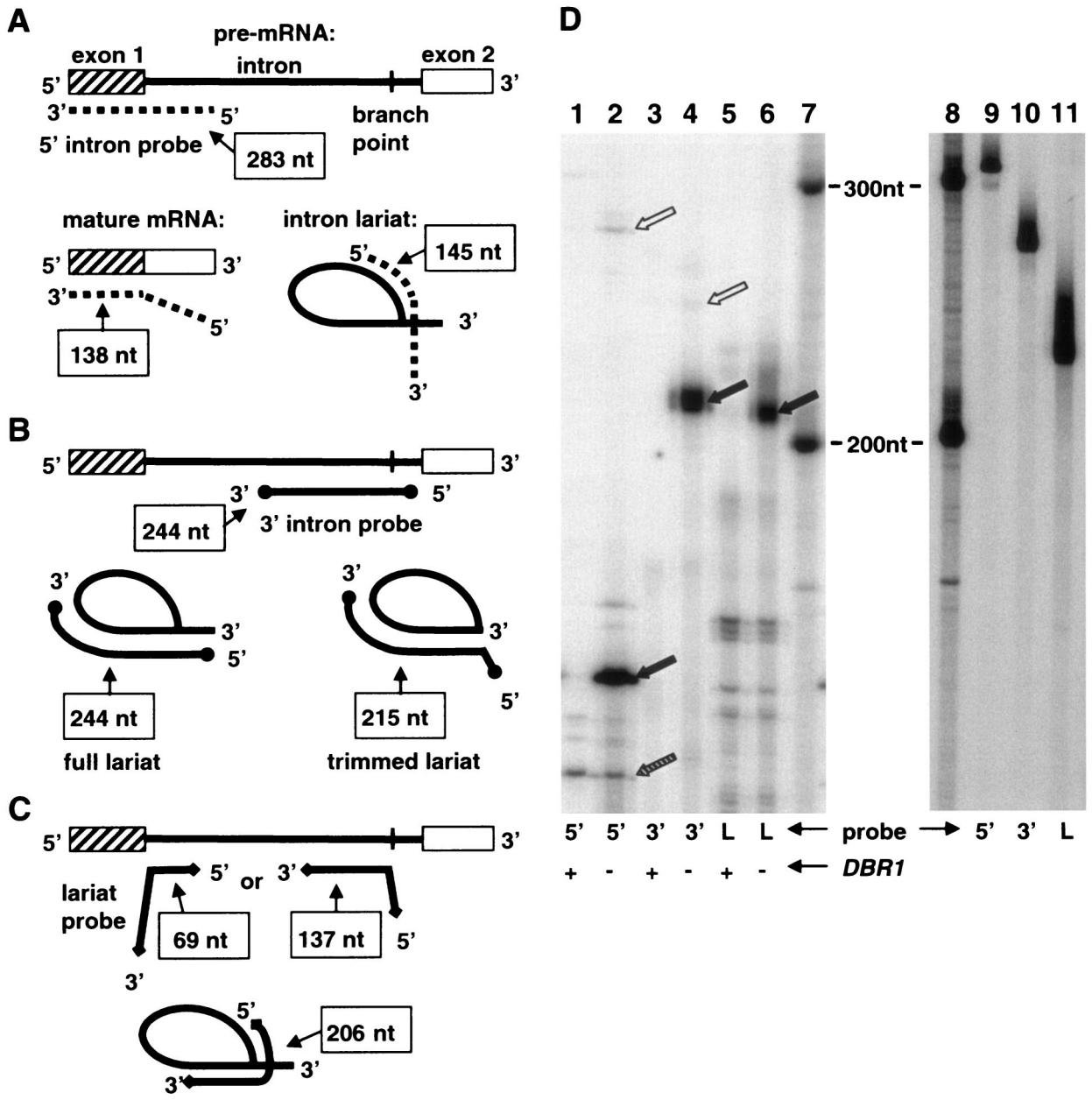

FIG. 5. RNase protection assay. (A) Diagram of the 5′ intron probe annealing to different *ACT1* RNA species. The three different *ACT1* RNA species are pre-mRNA, mature mRNA, and the intron lariat. The different RNA species are not drawn to scale. The extents of probe annealing are indicated in boxes and reflect the sizes of the probe fragments protected in RNase protection assays. The relative amounts of the different RNA species are reflected by the amounts of protected probe fragments in the RNase protection assay. (B) Diagram of the 3′ intron probe annealing to different *ACT1* RNA species. The *ACT1* RNA species are the pre-mRNA and the intron lariat. The intron lariat is also depicted in a form in which all of the nucleotides of the 3′ tail but one have been removed by nucleases (trimmed lariat). (C) Diagram of the lariat probe annealing to different *ACT1* RNA species. The *ACT1* RNA species are the pre-mRNA and the intron lariat. (D) Results of the RNase protection assay for *ACT1* RNA species. Lanes: 1 and 2, protection of 5′ intron probe (indicated by 5′ at bottom of gel); 3 and 4, protection of 3′ intron probe (indicated by 3′ at bottom of gel); 5 and 6, protection of lariat probe (indicated by L at bottom of gel); 7, RNA size markers. Reactions run in lanes 1, 3, and 5 used 2 μg of total RNA from *DBR1* cells (wild type) (indicated by plus sign at bottom of gel). Reactions run in lanes 2, 4, and 6 used 2 μg of total RNA from *dbr1* mutant cells (indicated by minus sign at bottom of gel). Solid black arrows in lanes 2 (156 nt), 4 (215 nt), and 6 (211 nt) indicate fragments protected by *ACT1* intron lariat. Stippled arrow in lane 2 indicates fragment of 5′ intron probe protected by *ACT1* mature mRNA (143 nt). White arrow in lane 2 indicates fragment of 5′ intron probe protected by *ACT1* pre-mRNA (283 nt). White arrow in lane 4 indicates fragment of 3′ intron probe protected by *ACT1* intron RNA containing its full 3′ tail (250 nt). Lanes 9, 10, and 11 are undigested probes: 5′ intron probe, 3′ intron probe, and lariat probe, respectively. Lane 8 contains RNA size markers.

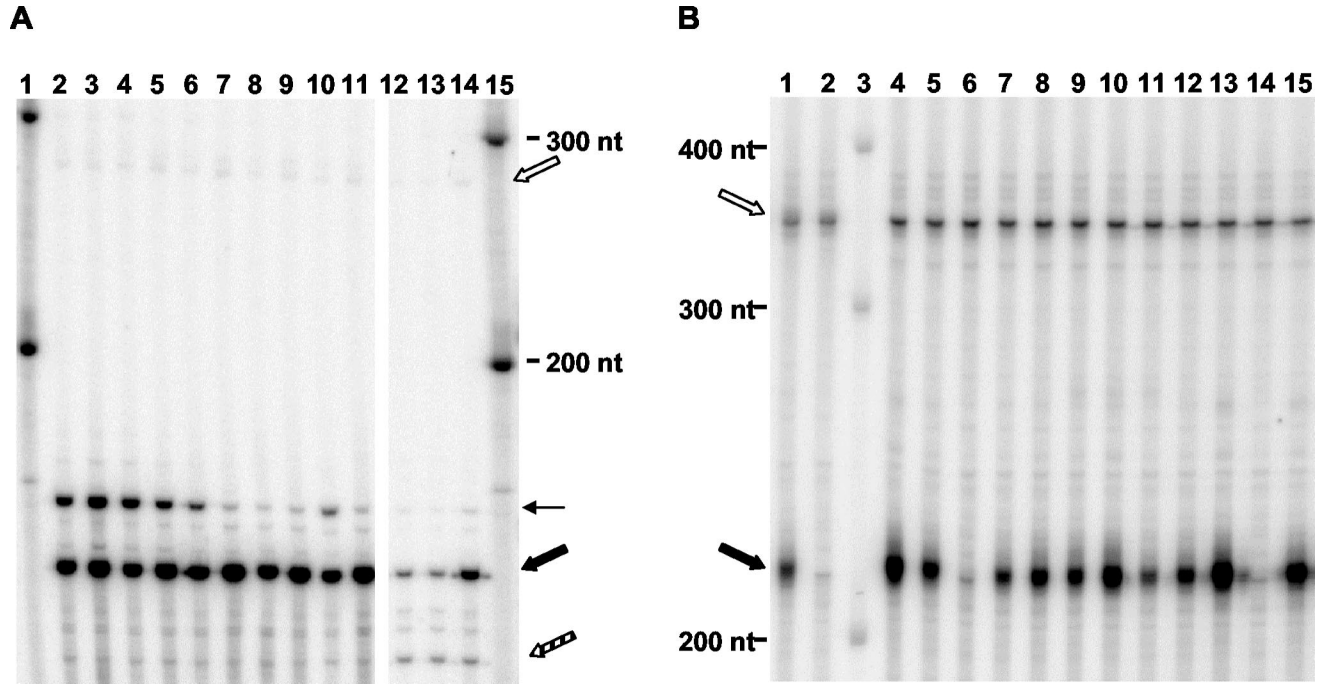

FIG. 6. Intron lariat accumulation in *dbr1* mutant strains. (A) RNase protection assay with the 5′ intron probe and 1.5 μg of total RNA from transformants of *dbr1* strain TMY60 carrying different plasmids. Lanes: 2 and 3, pTM431 (N14 mutant); 4 and 5, pTM432 (N16 mutant); 6 and 7, pTM434 (N20 mutant); 8 and 9, pTM435 (N29 mutant); 10 and 11, pTM436 (C30 mutant); 12, pTM235 (p*GAL1-DBR1*-V5-6xHis); 13, pTM263 (*DBR1*-V5-6xHis); 14, pYES2.1 (vector); 1 and 15, RNA size markers. Reaction mixtures loaded into lanes 2, 4, 6, 8, 10, 12, 13, and 14 contained total RNA from cells grown in galactose for 18 h. Reaction mixtures loaded into lanes 3, 5, 7, 9, and 11 contained total RNA from cells grown in glucose. The solid black arrow indicates fragments protected by the *ACT1* intron lariat (156 nt). The stippled arrow indicates the fragment of the 5′ intron probe protected by *ACT1* mature mRNA (143 nt). The white arrow indicates the fragment of the 5′ intron probe protected by *ACT1* pre-mRNA (283 nt). The band of variable intensity indicated by the thin horizontal arrows is most likely an incompletely digested product of protection by the *ACT1* intron. (B) RNase protection assay with 3′ intron probe and 1 μg of total RNA from control strains and transformants of *dbr1* mutant strain TMY60 carrying different plasmids. Lanes: 1, TMY60 (*dbr1*); 2, TMY30 (*DBR1*), 3, RNA size markers; 4, TMY60 plus pTM442 (N52 mutant); 5, TMY60 plus pTM428 (N8 mutant); 6, TMY60 plus pTM235 (p*GAL1-DBR1*-V5-6xHis); 7, TMY60 plus pTM444 (N70 mutant); 8, TMY60 plus pTM454 (C86 mutant); 9, TMY60 plus pTM436 (C30 mutant); 10, TMY60 plus pTM435 (N29 mutant); 11, TMY60 plus pTM434 (N20 mutant); 12, TMY60 plus pTM432 (N16 mutant); 13, TMY60 plus pTM431 (N14 mutant); 14, TMY60 plus pTM235 (p*GAL1-DBR1*-V5-6xHis); 15, TMY60 plus pYES2.1 (vector). All cultures were grown in galactose for 18 h prior to RNA isolation. The solid black arrow indicates the part of the *ACT1* 3′ intron probe protected by the *ACT1* intron lariat (215 nt). The white arrow indicates the control reaction product, showing protection of the *ACT1* 3′ exon probe by *ACT1* mature mRNA (333 nt). The C30 mutant has a nonsense mutation and does not make full-length Dbr1p.

involved in interactions that Dbr1p has with other proteins (e.g., spliceosomal proteins).

All of the *dbr1* mutants defective for Ty1 transposition identified so far are also defective for RNA lariat debranching. Although we are continuing to identify additional *dbr1* alleles, our results are consistent with a role for 2′-5′ phosphodiesterase activity in Ty1 transposition.

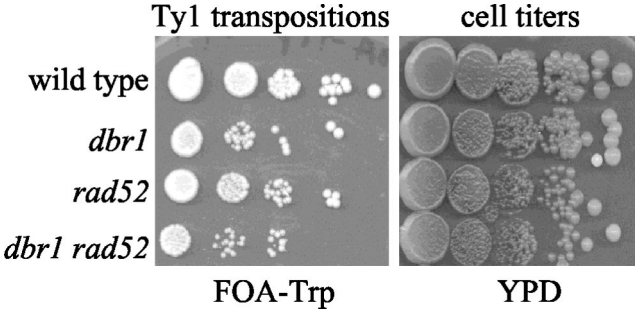

FIG. 7. Ty1 cDNA from a *dbr1* mutant can interact with IN protein. Transposition rates were measured by a dilution-spotting method. Strains YPH499 (wild type), TMY60 (*dbr1*), TMY176 (*rad52*), and TMY344 (*dbr1 rad52*) were spotted onto FOA-Trp (to select for transpositions) and YPD (to indicate cell titers). The dilution factor between adjacent spots is fivefold.

Intron RNA accumulation in *dbr1* mutants. Previous work by others, based on RNA levels and mobility patterns, suggested that intron RNAs accumulate in *dbr1* mutants and maintain their lariat structure (6). RNase protection assays in our work also demonstrate the high levels to which intron RNAs accumulate in *dbr1* mutants and confirm the maintenance of the lariat branch.

Another suggestion from previous work is that intron se-

TABLE 5. Transposition of a Ty1 deletion element

Strain/plasmid	Transposition frequency (Ura^- Trp^+/Ura^- colonies)	Relative frequency (%)
Wild type/pX3	2.88×10^{-1} (312/1,082)	100.0
dbr1/pX3	8.49×10^{-2} (79/931)	29.4
Wild type/pX100	2.98×10^{-2} (63/2,117)	10.3
dbr1/pX100	2.12×10^{-2} (50/2,353)	7.4

quences accumulating in a *dbr1* mutant lack most of their 3′ tails (6), presumably because of the action of exonucleases. The 3′ tail is the RNA sequence from the lariat branch point to the 3′ end of the excised intron. The protection of ~215 nt of the 3′ intron probe in our RNase protection assay experiments demonstrates this directly. Our data do not distinguish whether the 3′ tail of the intron is degraded all the way to the branch point or to a position 1 nt or a few nucleotides downstream of the branch point. However, in RNA lariats, the 2′-5′ phosphodiester bond renders the adjacent 3′-5′ bond resistant to RNase and nuclease P1 (27). In addition, Dbr1p needs the adjacent 3′-5′ phosphodiester bond at the branch point in order to cleave the 2′-5′ phosphodiester bond (25, 28). Therefore, it is likely that the 3′ tail remaining on intron RNA lariats that accumulate in *dbr1* mutant strains extends only 1 nt past the branch point.

If the entire intron lariat, including the 3′ tail, were accumulating in *dbr1* strains, protection of 250 nt of the 3′ intron probe would occur. A fragment of ~250 nt is observed accumulating in the *dbr1* mutant strain (position of white arrow in Fig. 5D, lane 4), although it is present at a much lower level than the 215-nt fragment. This fragment is best observed on long exposures of RNase protection assay gels (data not shown). Enhanced accumulation of the entire intron lariat in the *dbr1* mutant may indicate that other steps in the splicing reaction are slowed when the debranching reaction does not occur and intron lariats accumulate to a high level.

On the basis of what is known about mRNA splicing, debranching of intron lariats occurs after splicing is complete and is part of the process of degrading excised introns. The production of mature mRNA in the *dbr1* mutant strain is expected to occur similarly to that in the wild type. Direct molecular evidence that this is the case is provided by the RNase protection assays with the 5′ intron probe described in Results. Protection of the probe by the mature *ACT1* mRNA produced the 143-nt fragment indicated in lanes 1 and 2 of Fig. 5D by the stippled arrow. The levels of this fragment are very similar for *dbr1* mutant and wild-type samples, especially compared to levels of the probe fragments protected by intron sequences (black arrow). However, on the basis of the accumulation of the 143-nt fragment, there is ~30% more mature *ACT1* mRNA in the wild-type strain than in the *dbr1* mutant. This observation is consistent with slowing of other steps in the splicing reaction for the *dbr1* mutant, such as trimming of the 3′ tail of the *ACT1* intron lariat noted above.

Further support for slowing of other steps in the splicing reaction in the *dbr1* mutant can be seen in the RNase protection assay that uses the 5′ intron probe. *ACT1* pre-mRNA will protect a 283-nt fragment of the 5′ intron probe. In lane 2 of Fig. 5D, enhanced protection of a ~283-nt fragment is observed for the *dbr1* mutant compared to the wild type (position of white arrow). Slowing of the first transesterification step of the splicing reaction would account for the enhanced protection.

A side issue regarding the creation of the *ACT1* lariat probe template (pTM400) is worth mentioning. Construction of pTM400 required the creation of an RT-PCR product that spans the *ACT1* lariat branch point. One way to accomplish this is to reverse transcribe across the 2′-5′ linkage. Szostak and coworkers demonstrated that RTs can read across a lone 2′-5′ linkage in a template in which the remaining bonds are 3′-5′ linkages (22). We performed a similar type of RT reaction in our RT-PCRs. The major difference is that the intron branch point nucleotide with the 2′ linkage also has an adjacent 3′ linkage. Interestingly, in our reactions with the *ACT1* branch, we found that deoxyadenosine was incorporated into the cDNA at the first position RT reads after crossing the branch point in 16 out of 17 RT-PCR clones (data not shown). Thymidine, the deoxynucleoside dictated by Watson-Crick base pairing, was present in the remaining clone. Another group found the same incorporation pattern during RT of a plant intron RNA (33). Szostak's group reported that RT stalls when RT incorporates the first nucleotide after crossing the 2′-5′ bond (22). If the Szostak template and an intron RNA lariat act similarly, the 2′-5′ linkage on the template seems to promote non-Watson-Crick base recognition during incorporation that results in altered nucleotide specificity. However, this type of base recognition appears to have only a minor effect on the rate of incorporation, as evidenced by minor stalling in cDNA synthesis at this position (22). However, once the nucleotide is incorporated, the base pairing interaction may position this nucleotide in a way that does not promote efficient incorporation of the subsequent nucleotide into the cDNA, resulting in very significant stalling in cDNA synthesis. It is not known if stalling occurs in the rare instances in which the correct nucleotide is incorporated (i.e., the nucleotide predicted by Watson-Crick pairing).

Ty1 cDNA produced in a *dbr1* mutant can interact with the Ty1 IN protein. Our previous work showed that Ty1 cDNA accumulates at a slower rate in *dbr1* strains, suggesting that Dbr1p might be involved in Ty1 reverse transcription or cDNA stability. One possibility is that Ty1 RNA is a substrate for Dbr1p. However, because Ty1 reverse transcription is primed by the cellular $tRNA^{imet}$, it is also possible that a 2′-5′ RNA-DNA branch remains on one end of the Ty1 cDNA. This would occur if the tRNA primes from a 2′-OH of instead of a 3′-OH, a possibility suggested previously (6). In work presented here, the Ty1 cDNA accumulating in *dbr1* strains is capable of integration into the host chromosomal DNA in the absence of recombination machinery (*dbr1 rad52* strain). This result indicates that the Ty1 cDNA has ends capable of interacting with the Ty1 IN protein, which rules out a 2′-5′ linkage remaining on one of the two ends. If Dbr1p plays a direct role in Ty1 transposition, these results point to Ty1 RNA as a possible substrate for the enzyme.

Identification of *DBR1*-dependent sequences in Ty1 RNA. A Ty1 deletion element (pX100) missing 4,942 out of the 5,918 bases of Ty1 transposed at approximately the same frequency in wild-type and *dbr1* strains. This result is consistent with the notion that Dbr1p acts on some feature that is part of the missing bases. One possibility is that an RNA branch forms in those bases or that the bases are important for the formation of a branch in the remaining Ty1 RNA sequences. Because RNA branching would not occur when the internal Ty1 sequences are missing, debranching would not be necessary and transposition would be similar in wild-type and *dbr1* strains. An additional, striking feature of the Ty1 deletion element is that it transposes at an overall lower frequency than the full Ty1 element (Table 5) (36). This result indicates that the deleted sequences are important for Ty1 transposition. One possibility

is that supplying Ty1 proteins in *trans*, as is the case for the deletion element, is inferior to supplying them in *cis*. Alternatively, just as removal of a branch in Ty1 RNA may be important for transposition, the formation of that branch may also be important. Accordingly, the Ty1 deletion element would not form the RNA branch and would transpose poorly as a result. However, as noted above, the transposition frequency would no longer vary between wild-type and *dbr1* mutant strains.

The results presented here point to the possibility that a branch forms in Ty1 RNA during transposition. In experiments to be reported elsewhere (Z. Cheng and T. M. Menees, unpublished data), we have identified a branch in Ty1 RNA and are working to determine the functions of branch formation and removal in transposition. The relatedness of LTR retrotransposons and retroviruses raises the possibility that a debranching deficiency could similarly decrease the mobility of retroviruses. If such turns out to be the case, it could open new approaches for antiretroviral therapy.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 2003. Current protocols in molecular biology on CD-ROM. Current Protocols Inc., Brooklyn, N.Y.
2. **Boeke, J., and J. Stoye.** 1997. Retrotransposons, endogenous retroviruses and the evolution of retroelements, p. 343–435. *In* J. Coffin, S. Hughes, and H. Varmus (ed.), Retroviruses. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
3. **Boeke, J. D., F. Lacroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:**345–346.
4. **Cadwell, R. C., and G. F. Joyce.** 1994. Mutagenic PCR. PCR Methods Appl. **3:**S136–S140.
5. **Cadwell, R. C., and G. F. Joyce.** 1992. Randomization of genes by PCR mutagenesis. PCR Methods Appl. **2:**28–33.
6. **Chapman, K. B., and J. D. Boeke.** 1991. Isolation and characterization of the gene encoding yeast debranching enzyme. Cell **65:**483–492.
7. **Chapman, K. B., A. S. Bystrom, and J. D. Boeke.** 1992. Initiator methionine tRNA is essential for Ty1 transposition in yeast. Proc. Natl. Acad. Sci. USA **89:**3236–3240.
8. **Cooper, J. A., F. S. Esch, S. S. Taylor, and T. Hunter.** 1984. Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases *in vivo* and *in vitro*. J. Biol. Chem. **259:**7835–7841.
9. **Curcio, M. J., and D. J. Garfinkel.** 1991. Single-step selection for Ty1 element retrotransposition. Proc. Natl. Acad. Sci. USA **88:**936–940.
10. **Garfinkel, D. J., A. M. Hedge, S. D. Youngren, and T. D. Copeland.** 1991. Proteolytic processing of pol-TYB proteins from the yeast retrotransposon Ty1. J. Virol. **65:**4573–4581.
11. **Grate, L., and M. Ares, Jr.** 2002. Searching yeast intron data at Ares lab web site. Methods Enzymol. **350:**380–392.
12. **Hofmann, K., P. Bucher, L. Falquet, and A. Bairoch.** 1999. The PROSITE database, its status in 1999. Nucleic Acids Res. **27:**215–219.
13. **Hunter, T.** 1982. Synthetic peptide substrates for a tyrosine protein kinase. J. Biol. Chem. **257:**4843–4848.
14. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Karst, S. M., M.-L. Rutz, and T. M. Menees.** 2000. The yeast retrotransposons Ty1 and Ty3 required the RNA lariat debranching enzyme, Dbr1p, for efficient accumulation of reverse transcripts. Biochem. Biophys. Res. Commun. **268:**112–117.
16. **Karst, S. M., N. Sadeghi, and T. M. Menees.** 1999. Cell cycle control of reverse transcriptase activity for the yeast retrotransposon Ty3. Biochem. Biophys. Res. Commun. **254:**679–684.
17. **Kim, H. C., G. M. Kim, J. M. Yang, and J. W. Ki.** 2001. Cloning, expression, and complementation test of the RNA lariat debranching enzyme cDNA from mouse. Mol. Cell **11:**198–203.
18. **Kim, J. W., H. C. Kim, G. M. Kim, J. M. Yang, J. D. Boeke, and K. Nam.** 2000. Human RNA lariat debranching enzyme cDNA complements the phenotypes of *Saccharomyces cerevisiae dbr1* and *Schizosaccharomyces pombe dbr1* mutants. Nucleic Acids Res. **28:**3666–3673.
19. **Koonin, E. V.** 1994. Conserved sequence pattern in a wide variety of phosphoesterases. Protein Sci. **3:**356–358.
20. **Lauermann, V., M. Hermankova, and J. D. Boeke.** 1997. Increased length of long terminal repeats inhibits Ty1 transposition and leads to the formation of tandem multimers. Genetics **145:**911–922.
21. **Lopez, P. J., and B. Seraphin.** 2000. YIDB: the yeast intron database. Nucleic Acids Res. **28:**85–86.
22. **Lorsch, J. R., D. P. Bartel, and J. W. Szostak.** 1995. Reverse transcriptase reads through a 2′-5′ linkage and a 2′-thiophosphate in a template. Nucleic Acids Res. **23:**2811–2814.
23. **Nam, K., R. H. Hudson, K. B. Chapman, K. Ganeshan, M. J. Damha, and J. D. Boeke.** 1994. Yeast lariat debranching enzyme. Substrate and sequence specificity. J. Biol. Chem. **269:**20613–20621.
24. **Nam, K., G. Lee, J. Trambley, S. E. Devine, and J. D. Boeke.** 1997. Severe growth defect in a *Schizosaccharomyces pombe* mutant defective in intron lariat degradation. Mol. Cell. Biol. **17:**809–818.
25. **Ooi, S. L., C. Dann III, K. Nam, D. J. Leahy, M. J. Damha, and J. D. Boeke.** 2001. RNA lariat debranching enzyme. Methods Enzymol. **342:**233–248.
26. **Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton.** 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA **79:**973–977.
27. **Ruskin, B., and M. R. Green.** 1990. RNA lariat debranching enzyme as tool for analyzing RNA structure. Methods Enzymol. **181:**180–188.
28. **Ruskin, B., and M. R. Green.** 1985. An RNA processing activity that debranches RNA lariats. Science **229:**135–140.
29. **Sadeghi, N., M. L. Rutz, and T. M. Menees.** 2001. Thermal blockage of viruslike particle formation for the yeast retrotransposon Ty3 reveals differences in the cellular stress response. Arch. Virol. **146:**1919–1934.
30. **Sharon, G., T. J. Burkett, and D. J. Garfinkel.** 1994. Efficient homologous recombination of Ty1 element cDNA when integration is blocked. Mol. Cell. Biol. **14:**6540–6551.
31. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
32. **Symington, L. S.** 2002. Role of *RAD52* epistasis group genes in homologous recombination and double-strand break repair. Microbiol. Mol. Biol. Rev. **66:**630–670.
33. **Vogel, J., W. R. Hess, and T. Borner.** 1997. Precise branch point mapping and quantification of splicing intermediates. Nucleic Acids Res. **25:**2030–2031.
34. **Wood, V., R. Gwilliam, M. A. Rajandream, M. Lyne, R. Lyne, A. Stewart, J. Sgouros, N. Peat, J. Hayles, S. Baker, D. Basham, S. Bowman, K. Brooks, D. Brown, S. Brown, T. Chillingworth, C. Churcher, M. Collins, R. Connor, A. Cronin, P. Davis, T. Feltwell, A. Fraser, S. Gentles, A. Goble, N. Hamlin, D. Harris, J. Hidalgo, G. Hodgson, S. Holroyd, T. Hornsby, S. Howarth, E. J. Huckle, S. Hunt, K. Jagels, K. James, L. Jones, M. Jones, S. Leather, S. McDonald, J. McLean, P. Mooney, S. Moule, K. Mungall, L. Murphy, D. Niblett, C. Odell, K. Oliver, S. O'Neil, D. Pearson, M. A. Quail, E. Rabbinowitsch, K. Rutherford, S. Rutter, D. Saunders, K. Seeger, S. Sharp, J. Skelton, M. Simmonds, R. Squares, S. Squares, K. Stevens, K. Taylor, R. G. Taylor, A. Tivey, S. Walsh, T. Warren, S. Whitehead, J. Woodward, G. Volckaert, R. Aert, J. Robben, B. Grymonprez, I. Weltjens, E. Vanstreels, M. Rieger, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, A. Dusterhoft, C. Fritzc, E. Holzer, D. Moestl, H. Hilbert, K. Borzym, I. Langer, A. Beck, H. Lehrach, R. Reinhardt, T. M. Pohl, P. Eger, W. Zimmermann, H. Wedler, R. Wambutt, B. Purnelle, A. Goffeau, E. Cadieu, S. Dreano, S. Gloux, et al.** 2002. The genome sequence of *Schizosaccharomyces pombe*. Nature **415:**871–880.
35. **Xu, H., and J. D. Boeke.** 1987. High-frequency deletion between homologous sequences during retrotransposition of Ty elements in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **84:**8553–8557.
36. **Xu, H., and J. D. Boeke.** 1990. Localization of sequences required in *cis* for yeast Ty1 element transposition near the long terminal repeats: analysis of mini-Ty1 elements. Mol. Cell. Biol. **10:**2695–2702.