MOLECULAR AND CELLULAR BIOLOGY, Dec. 2003, p. 8809–8819 Vol. 23, No. 23
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.23.8809–8819.2003

The DIVa Maturase Binding Site in the Yeast Group II Intron aI2 Is Essential for Intron Homing but Not for In Vivo Splicing

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Received 23 June 2003/Returned for modification 18 August 2003/Accepted 27 August 2003

Splicing of the *Saccharomyces cerevisiae* mitochondrial DNA group II intron aI2 depends on the intron-encoded 62-kDa reverse transcriptase-maturase protein (p62). In wild-type strains, p62 remains associated with the excised intron lariat RNA in ribonucleoprotein (RNP) particles that are essential for intron homing. Studies of a bacterial group II intron showed that the DIVa substructure of intron domain IV is a high-affinity binding site for its maturase. Here we first present in vitro evidence extending that conclusion to aI2. Then, experiments with aI2 DIVa mutant strains show that the binding of p62 to DIVa is not essential for aI2 splicing in vivo but is essential for homing. Because aI2 splicing in the DIVa mutant strains remains maturase dependent, splicing must rely on other RNA-protein contacts. The p62 that accumulates in the mutant strains has reverse transcriptase activity, but fractionation experiments at high and low salt concentrations show that it associates more weakly than the wild-type protein with endogenous mitochondrial RNAs, and that phenotype probably explains the homing defect. Replacing the DIVa of aI2 with that of the closely related intron aI1 improves in vivo splicing but not homing, indicating that DIVa contributes to the specificity of the maturase-RNA interaction needed for homing.

Group II introns are found in bacteria and in the organelle genomes of fungi and plants. They have conserved secondary structures comprised of six helical domains (domains I to VI or DI to DVI) separated by short joining sequences (28). Some group II introns self-splice in vitro, most at nonphysiological temperatures and concentrations of salt and Mg^{2+} ions. Splicing occurs by two transesterification reactions via a branched intermediate, similar to the mechanism used by spliceosomal introns (39). The functional core of the intron for the first step of splicing is comprised of the 5′ exon and domains I and V (24, 29). The EBS1 and EBS2 sequences in DI base pair with the IBS1 and IBS2 sequences in the 5′ exon to define the 5′ splice site (21). Domain V is essential for catalysis (37) and is positioned near the 5′ splice junction via a docking site in DI (5).

Some group II introns contain an open reading frame (ORF), most of which is located in the part of domain IV, now known as DIVb, that is looped out from the intron core (45). The group II intron-encoded proteins (IEPs) typically contain four domains called RT, X, D, and En, having reverse transcriptase (RT), maturase (RNA splicing) (X), DNA-binding (D), and DNA endonuclease (En) activities (25). The RT domain is related to the RTs of non-long terminal repeat retroelements, a family that includes human LINE elements (26). Domain X is present in nearly all group II intron ORFs (32, 47). Although the yeast maturases are largely intron-specific splicing factors, genetic studies suggest that there may be some overlap in their specificity among related introns (1, 7). The D domain is thought to make DNA contacts needed for target site recognition and the endonuclease activity of the En domain (15, 42, 46).

These activities of the IEP allow some group II introns to be highly efficient, site-specific retroelements (25). This mobility has been studied in the greatest detail for introns aI1 and aI2 of the *COXI* gene of *Saccharomyces cerevisiae* mitochondrial DNA (mtDNA) and intron Ll.LtrB of *Lactococcus lactis* (reviewed in reference 3). Using aI2 as an example, when a "donor" yeast strain with wild-type aI2 in its mtDNA is mated to a "recipient" strain lacking the intron, about 90% of the recipient alleles acquire the intron by homing (13, 33). The yeast aI2 IEP is a 62-kDa polypeptide (p62) that appears to be processed from a preprotein translated from *COXI* pre-mRNA. After splicing, p62 remains bound tightly to the excised intron RNA lariat in a ribonucleoprotein (RNP) particle. Following mating to a recipient strain, the RNP particle catalyzes the efficient reverse splicing of the intron lariat into a specific target in the sense strand of the recipient mtDNA. Next, the antisense strand of the target DNA is cleaved by the DNA endonuclease activity of p62, and then its RT activity makes cDNA by using the cleaved antisense strand as primer and reverse-spliced intron RNA as template. For wild-type aI2, double-strand break repair completes the intron insertion in most events. We have recently reported several variables that activate a variation of the main aI2 retrohoming pathway in which the reverse-spliced intron RNA serves as the template for full-length cDNA synthesis (13, 14). That pathway resembles the major retrohoming pathway used by the Ll.LtrB intron and does not appear to involve double-strand break repair for completion (11).

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Domain IV of the Ll.LtrB intron was shown to contain the major high-affinity binding site for that intron's maturase (45). That finding was surprising because DIV is not required for self-splicing (18, 22). By analogy with proteins that assist group I intron splicing, it had been thought that group II intron maturases would bind primarily to intron core elements. Using in vitro transcripts of the *L. lactis* Ll.LtrB intron and recombinant LtrA protein, the binding site was further mapped to DIVa, the 5′-most substructure of DIV, which together with the DIV stem and the joining sequence LIV was found to be necessary and sufficient for in vitro binding (45). LtrA promotes in vitro splicing of Ll.LtrB at near-physiological Mg^{2+} concentrations that do not support self-splicing (40, 45), and deletion of DIVa strongly inhibits but does not eliminate that splicing (45). This residual splicing showed that lower-affinity contacts exist that can support some splicing in vitro. Subsequently, putative interactions were mapped to positions in intron domains I, II, and VI (27). Those authors proposed a model in which the maturase first binds with high affinity to DIVa and then makes other weaker contacts with conserved regions as it folds the intron RNA into its active structure for splicing. In the Ll.LtrB intron, DIVa also contains the ribosome binding site and initiation codon of the LtrA ORF, and the binding of LtrA to DIVa has been shown to downregulate its own translation (43). This situation differs from the yeast aI1 and aI2 introns, where the maturases are synthesized as fusion proteins with polypeptides encoded by their upstream exons, and thus it was possible that DIVa might play different roles in the two types of introns.

Here we investigated the generality of the findings for the Ll.LtrB intron by analyzing the function of DIVa in the yeast mitochondrial system both in vitro and in vivo. We find that DIVa of the yeast aI2 intron is important for both the in vitro and in vivo interaction of the excised intron RNA with its encoded protein, p62. In vivo, however, we find that DIVa is not necessary for aI2 splicing but is essential for homing.

MATERIALS AND METHODS

Yeast strains and constructs. Yeast strains have the nuclear background of strain ID41-6/161 (*MAT***a** *ade1 lys1*) (33), except as noted below. The recipient strain in homing experiments had the nuclear background of strain GRF18 (*MAT*α *leu2 his3*) (33). Strains with aI2 mutations were constructed using methods described previously (6, 33). The nomenclature for mtDNA introns is 1^x2^x, where 1 and 2 denote aI1 and aI2, respectively. A superscript "0" indicates the absence of that intron, and a superscript "+" indicates a wild-type intron. Other designations refer to particular mutant intron alleles. All of these aI2-containing mtDNAs are derived from the mtDNA of strain 1^02^+ (33). The recipient allele, denoted 1^02^0 E2-8G E3 + 2T + 5G, has the indicated nucleotide sequence changes that make it somewhat more efficient for aI2 homing than the originally analyzed recipient allele (1^02^0 E2-8A E3 + 2C + 5A) (13).

Recombinant plasmids. $pBS1^02^{SZD2}$ was made by replacing the *Eco*RI-*Bgl*II fragment of pJVM164 (35) with the *Eco*RI-*Bgl*II fragment of pSZD2 (provided by S. Zimmerly). Positions of nucleotide changes or deletions in constructs used to generate in vitro RNAs for the reverse splicing assays are listed in Table 1 and were made by standard methods. For in vivo constructs, a sequence coding for a triple hemagglutinin (3HA) tag was inserted into pJVM164 at the C terminus of p62 to yield $pBS1^02^{3HA}$, and mutant alleles of that full-length aI2 were derived from that plasmid. The in vitro and in vivo ΔDIVa constructs were deleted for 33 nucleotides (nt) of DIVa (see Fig. 2A) so as not to disrupt the intron reading frame. The aI1-DIVa construct analyzed in vitro replaced 37 nt of aI2 DIVa in pSZD2 with 38 nt of aI1-DIVa (see Fig. 2A) while in the in vivo aI1-DIVa construct the 38 nt of aI1 DIVa replaced the 37 nt of aI2 DIVa plus 1 nt of LIV (nt 561 to 598) to retain the integrity of the intron reading frame. Plasmids were transformed into mitochondria as described elsewhere (7), and each final construction was confirmed by sequencing the relevant amplified segment of mtDNA.

TABLE 1. aI2 alleles and constructs used in the reverse-splicing assay

aI2 allele	Construct name	Mutation[a]
Wild type	pJVM164[b]	None
SZD2	pMYC103[c]	Δ834–2144[e]
ΔDIVa	pMYC112	Δ563–595[e]
		Δ834–2144
DIVap1[d]	pMYC113	Δ576–583[e]
		Δ834–2144
aI1-DIVa	pMYC115	Replaces nt 561–598 with 670–707 from aI1[e]
		Δ834–2144
ΔDIVb	pMYC116	Δ604–2377
DIVbp1	pMYC121	Δ620–2144
DIVbp2	pMYC122	Δ613–2144
		Δ2151–2225
DIVbp3	pMYC123	Δ613–2144
		Δ2151–2300
DIVbp4	pMYC124	Δ613–2144
		Δ2151–2366
DIVbp5	pMYC128	Δ612–2369
DIVbp6	pMYC129	Δ612–2369, Δ609, 2372
DIVbp7	pMYC131	Δ612–2369, Δ608, 609, 2372, 2373
DIVbp8	pMYC132	Δ612–2369, Δ607–609, Δ2372–2374

[a] aI2 from strain ID41-6/161 ρ^+ is 2,483 nt long, and the nucleotides indicated in the table refer to positions starting at the first nucleotide of the intron.
[b] Reference 33.
[c] Based on pSZD2 (S. Zimmerly, unpublished data). See Materials and Methods for details.
[d] The lowercase p denotes the partial deletion of a substructure.
[e] The sequence is shown in Fig. 2A.

In vitro reconstitution of reverse splicing. RNP particles were prepared from cultures of strain ΔDV grown on raffinose medium and tested for reconstitution of reverse splicing activity by adding control or in vitro-made DIV mutant lariat RNAs as described previously (15), except that only 75,000 cpm of substrate was used. Strain ΔDV is blocked for splicing due to the deletion of the catalytically essential DV structure, but it expresses wild-type p62 at an elevated level, and RNP particles containing p62 associated with *COXI* pre-mRNA accumulate. RNAs used in those assays were transcribed from *Bst*EII-digested plasmid DNAs (Table 1) with T3 RNA polymerase (Stratagene) and spliced in vitro in 100 mM $MgCl_2$–40 mM Tris-HCl (pH 7.5)–2 M NH_4Cl for 2 h at 42°C to generate RNA samples containing lariat RNA for analysis in the reconstitution experiments. After self-splicing, the RNA was phenol extracted, purified over a G-50 Sepharose spin column, ethanol precipitated, and suspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a concentration of 0.5 mg/ml, as determined by A_{260}. The presence of lariat was assessed by parallel splicing experiments using [α-^{32}P]UTP-labeled RNA and analyzing the splicing products on a denaturing 4% polyacrylamide gel (8, 38).

Northern blotting. Fifty-milliliter yeast extract-peptone (YEP)–2% raffinose cultures were inoculated with cells from a starter culture and grown overnight, and whole-cell RNA was purified as described previously (9), with the modification that the RNA was additionally extracted with phenol-chloroform twice and precipitated before use. Approximately 10 μg of RNA per sample was denatured and run in each lane of a standard 1.5% agarose (SeaKem LE) formaldehyde-MOPS (morpholinepropanesulfonic acid) gel (41). The RNA was then blotted to a nylon membrane (Schleicher & Schuell Nytran) by capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer overnight, UV cross-linked using a Stratalinker (Stratagene), and hybridized to 5′-end-labeled oligonucleotides with RapidHyb buffer (Amersham). Samples were first probed with a *COB* exon 6-specific oligonucleotide (antisense to nt 3170 to 3192 of sequence in reference 36) and quantified with a Molecular Dynamics PhosphorImager. Then, balanced amounts of RNA from each strain were analyzed using probes complementary to *COXI* exon 6 and intron aI2 (antisense to nt 9932 to 9951 and 3472 to 3491, respectively, of sequence in reference 4).

RFLP analysis of homing activity. Homing experiments were performed as described previously (13, 33). In brief, donor and recipient strains were mated

and outgrown in YEP-dextrose medium for 12 to 16 h, diluted 1/100 into yeast nitrogen base–10% glucose medium and incubated for 48 h (twice), and diluted 1/100 into 200 ml of YEP–10% dextrose medium and incubated overnight. mtDNA was then purified on a CsCl gradient containing bisbenzimide (20). *Hpa*II-*Bam*HI digests were probed with a *COXI* exon 1-specific oligonucleotide to detect parental and recombinant *COXI* alleles. The mtDNA input from each parent was determined by measuring the ratio of polymorphic *COB* alleles using a *Hin*cII digest probed with a *COB* intron 4-specific oligonucleotide (33). Blots were quantitated using a PhosphorImager (Molecular Dynamics). Percent homing is the fraction of recipient *COXI* alleles that acquired aI2 by homing and is estimated by measuring the percentage of recipient alleles that were "lost" in the cross (see reference 14). To test whether the control cross using the $1^0 2^{3HA}$ donor strain has retrohoming activity, an alternative restriction fragment length polymorphism (RFLP) analysis was done in which mtDNA from progeny cells was digested with *Dde*I and hybridized with an oligonucleotide containing nt 1905 to 1921 of aI2 (4). *COXI* alleles of donor cells or progeny resulting from retrohoming lack the *Dde*I site in exon 3 and yield a 1.4-kb fragment, while progeny resulting from RT-independent homing (i.e., recombination following cleavage of the target site by reverse splicing and endonuclease cleavage) have acquired the *Dde*I site in exon 3 and so yield a 0.56-kb fragment that is indistinguishable from the donor allele. Unlike the other RFLP digests used in this and previous studies, this digest yields only a semiquantitative measure of retrohoming.

Western blotting. Mitochondrial fractions were prepared from 50-ml cultures (YEP medium with 2% raffinose) as described previously (23). The mitochondrial pellet was resuspended in sample buffer (2% sodium dodecyl sulfate [SDS], 25 mM Tris-HCl [pH 6.8], 6% glycerol, 1% β-mercaptoethanol, 0.005% Serva blue G). Aliquots (~30 μg of protein) were separated in an SDS–7.5% polyacrylamide gel and transferred to nitrocellulose using a semidry blotting apparatus. 16B12 anti-HA antibody (BAbCo) was used with a 1:1,000 dilution in 5% milk–phosphate-buffered saline (PBS-T) (1× PBS, 0.05% Tween 20). After incubation with an horseradish peroxidase-coupled secondary antibody (Bio-Rad) for 30 to 60 min at room temperature, an ECL kit (Amersham Biosciences) was used for chemiluminescent detection of p62-3HA. A monoclonal antibody (Molecular Probes) was used to detect porin. Blots were washed three times for 5 min in PBS-T at room temperature after primary and secondary antibody incubations.

Fractionation of mitochondrial lysates. Flotation gradient-purified mitochondria were isolated as described previously (23) and suspended at ~1 mg of mitochondrial protein/ml in lysis buffer (25 mM Tris-HCl [pH 7.5], 25 mM $CaCl_2$, and 5 mM dithiothreitol [DTT]) containing 150 or 500 mM KCl, as indicated. NP-40 was then added to a final concentration of 1%. Lysates were centrifuged at 20,000 × *g* for 1 h, and the resulting supernatant was centrifuged at 100,000 × *g* for 1 h. Each pellet was suspended in SDS-polyacrylamide gel electrophoresis sample buffer, and balanced aliquots were analyzed on SDS–7.5% polyacrylamide gels followed by Western blotting. Mitochondrial preparations were balanced using an antibody to porin, and relative amounts of p62 in various strains were determined by analyzing Western blots containing dilutions of the wild-type or ΔD5 fractions.

RT assays. RT activity in RNP particle preparations was assayed by using poly(rA)-oligo$(dT)_{18}$ substrate and measuring polymerization of [α-^{32}P]dTTP into high-molecular-weight material retained on DE81 paper (33, 48). RNP particles were isolated from flotation-gradient-purified mitochondria from wild-type and mutant cells grown in raffinose medium as described previously (33) with several modifications. Mitochondria were lysed with NP-40 in the standard buffer containing 150 mM KCl instead of 500 mM KCl. In previous experiments, aliquots containing 0.025 A_{260} units were assayed from sets of strains, while here aliquots were balanced for their content of p62 by Western blotting. Complete reactions were carried out in triplicate as described elsewhere (33, 48) in reaction medium containing a standard salt solution (50 mM Tris-HCl [pH 8.5], 100 mM KCl, 2 mM $MgCl_2$, 5 mM DTT), poly(rA)-oligo$(dT)_{18}$ substrate, RNase A, and 10 μCi of [α-^{32}P]dTTP. Reactions were initiated by addition of RNP particles, and reaction mixtures were incubated for 10 min at 37°C. The labeled cDNAs were then collected on DE81 paper disks and quantified by Cerenkov counting.

RESULTS

In vitro mapping of RNA domains required for interaction with p62. Because we lack a source of active recombinant p62 for in vitro binding or splicing assays, we used the in vitro reverse splicing RNA complementation assay diagrammed in Fig. 1 (15) to map aI2 intron sequences that are important for p62 binding and function. Endogenous aI2 lariat RNA in RNP particles purified from wild-type strains containing the intron (e.g., strain $1^0 2^+$) can reverse splice into DNA target sites, and this reaction is assayed readily by the reduced electrophoretic mobility of a short labeled double-stranded DNA substrate (Fig. 1 and 2B, lane 1). Deletion of DV from aI2 (strain $1^0 2^{\Delta DV}$) completely blocks splicing but permits synthesis of wild-type p62 that accumulates in RNP particles with *COXI* pre-mRNA instead of lariat RNA. The ΔDV RNP particles have RT activity in vitro (33, 35) but no reverse-splicing activity (46). Adding self-spliced wild-type lariat RNA to $1^0 2^{\Delta DV}$ RNP particles reconstitutes reverse-splicing activity (16) (Fig. 1 and 2B, lane 3). This assay depends on the transfer of p62 from ΔDV pre-mRNA to the in vitro-synthesized lariat RNA and measures functionally effective binding of p62 to the lariat RNA.

To map sequences needed for this interaction, plasmids with various deletions of DIV were constructed, and in vitro self-spliced lariat RNAs from them were tested for their ability to bind p62 and reconstitute reverse-splicing activity (Table 1; Fig. 2A and C). Consistent with the previous finding that DIV is not essential for self-splicing of other group II introns (18, 22, 24), all of the deletions analyzed here self-spliced well, with branching (8). Because lariat RNA from plasmid pSZD2, which has 1,310 bp of the intron ORF deleted (positions 834 to 2144, Fig. 2A and Table 1), was active in this assay (Fig. 2B, compare lanes 3 and 4), all further constructs were derived from pSZD2. Deletion of DIVa (ΔDIVa; Fig. 2A) blocked the reverse-splicing activity (Fig. 2B, lane 5), as did a partial deletion that removed just the terminal loop region (DIVap1; Fig. 2A and B, lane 6). These findings show that aI2 DIVa is required for the functional interaction of aI2 RNA with p62.

To test whether DIVa influences the specificity of p62 binding, we replaced the DIVa of aI2 with that of the closely related group II intron aI1. aI1 DIVa is 1 nt longer than aI2 DIVa, and it is 58% identical in sequence (Fig. 2A). This hybrid intron (aI1-DIVa) self-splices well but only weakly reconstitutes reverse-splicing activity (Fig. 2A, lane 12), suggesting that DIVa influences the specificity of maturase interactions with group II intron RNAs.

For the *L. lactis* Ll.LtrB intron, deletion of DIVb has little effect on RNA binding and decreased the rate of protein-dependent splicing only two- to threefold (45). Thus, it was striking that removal of the remnant of aI2 DIVb from SZD2 RNA (ΔDIVb; Fig. 2B, lane 7) blocked reconstitution of reverse-splicing activity. We analyzed a set of partial deletions of the DIVb sequence (Fig. 2B, lanes 8 to 11 and 13 to 16, and 2C), and the smallest mutant construct that supports reverse splicing is DIVbp6, in which DIVb consists of a 6-bp stem with a 4-nt loop (Fig. 2B, lane 14, and 2C). Further shortening of the DIVb stem to 5 bp (DIVbp7) or 4 bp (DIVbp8) eliminated the activity (Fig. 2B, lanes 15 and 16, and 2C). These effects of DIVb length may reflect a direct role for DIVb in maturase binding or an indirect effect on the configuration of an adjacent element. Taking the results together, we conclude that the bacterial and yeast introns both bind to their respective maturases via DIVa and that there may be a differential role for DIVb for these two introns.

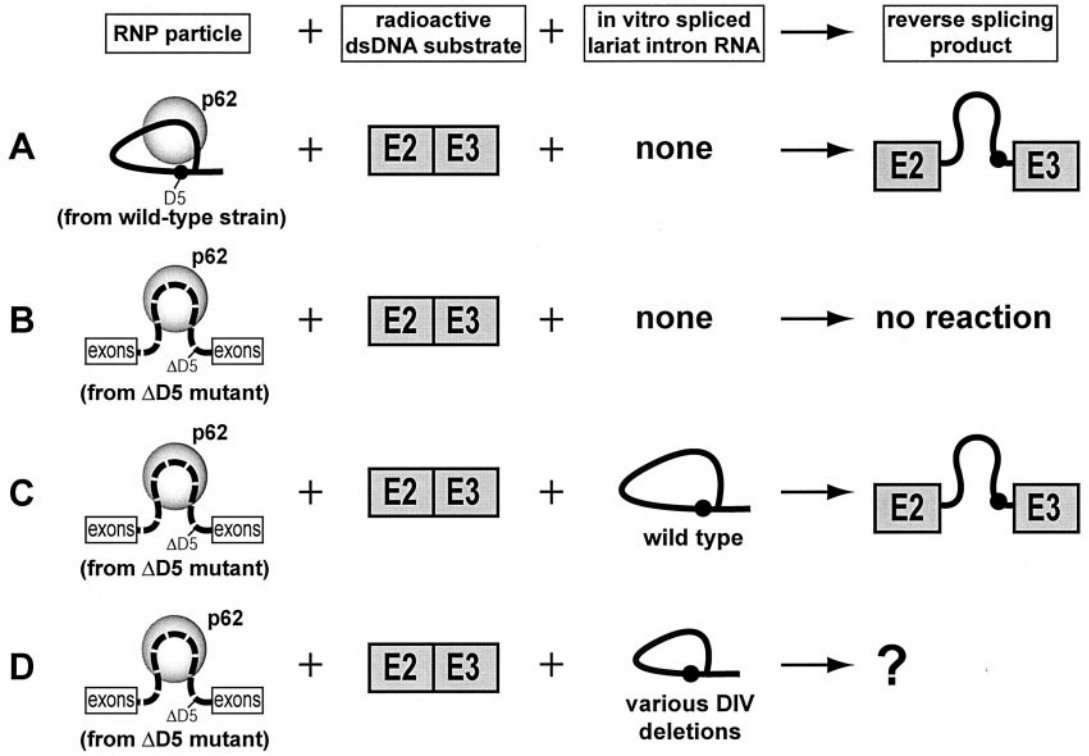

FIG. 1. Diagram of in vitro assay for complementation of reverse-splicing activity. Line A illustrates the basic features of the in vitro assay of reverse-splicing activity of RNP particle fractions isolated from yeast mitochondria (46). Each sample has an aliquot of RNP particles in which the IEP, p62, is bound to aI2 lariat RNA. The substrate is a radiolabeled double-stranded DNA (dsDNA) that contains the active aI2 homing site, i.e., the ligated exon 2-exon 3 junction of the *COXI* gene. In the experiments of Fig. 2 the DNA substrate contains 205 bp of the upstream exons and 35 bp of the downstream exon. The reverse-splicing reaction covalently joins the intron RNA to the sense strand of the DNA target, and the reaction is detected as a shift of fast-migrating labeled substrate to a much slower migrating position on an agarose gel. The product of full reverse splicing is shown, and partial reverse splicing yields slightly shorter products in which only exon 3 is joined to the 3′ end of the intron lariat. Line B shows that RNP particles from strain ΔDV, having the catalytic DV domain deleted, contain p62 bound to unspliced pre-mRNA and that they lack reverse-splicing activity. Line C shows that adding in vitro-made wild-type intron lariat RNA to the ΔDV RNP particles reconstitutes the reverse-splicing activity (15). Line D shows that lariat RNAs having various segments of the intron deleted can be tested for reverse-splicing activity in this complementation assay (Fig. 2). The assay is ideally suited for analysis of deletions of DIV because that large substructure is not essential for self-splicing of two yeast group II introns (18, 22).

DIVa is not required for splicing in vivo. We next analyzed the role of DIVa in aI2 splicing in vivo. In order to detect p62 protein in these experiments, we added a 3HA tag to the C terminus of wild-type p62 (p62-3HA). Control experiments showed that this tag has little effect on the in vivo splicing of the intron, as judged by the level of spliced *COXI* mRNA and the absence of an elevated amount of unspliced pre-mRNA (Fig. 3B, compare lanes 1 and 2), while permitting ready detection of the protein in cell extracts (see below). Although the 3HA tag lowers the level of excised intron RNA about 50% (Fig. 3C, lane 2), RNP particles from that strain have reverse splicing (8) and RT activities (Table 2). Based on those results, all of the strains analyzed below are derivatives of the 3HA-tagged wild-type strain, and results obtained with those mutant strains are compared with the data obtained with the 3HA control strain. Each mutant allele was constructed in the full-length intron, transformed into mitochondria, and placed in wild-type mtDNA by recombination.

Strain $1^0 2^{3HA\Delta DIVa}$ has 33 of the 37 nt of DIVa deleted (nt 563 to 595, Fig. 2A) so as to preserve the intron reading frame. The mutation does not interfere with growth on glycerol medium, and it inhibits splicing of aI2 in raffinose-grown cells only modestly (Fig. 3B, lane 4). An RT-PCR assay showed that the ΔDIVa strain splices aI2 accurately (8). By using the level of the mRNA of the mitochondrial *COB* gene to balance the RNA inputs (Fig. 3D), the amount of spliced mRNA in strain ΔDIVa is ~70% of that in the control strain (lane 2), indicating ~30% inhibition of splicing. Alternatively, the extent of splicing can be estimated from each blot as the fraction of total *COXI* transcript signal that is spliced mRNA. By that measure, 85% of the signal in Fig. 3B, lane 4, is spliced mRNA, suggesting only about 15% inhibition. As a control, lane 3 shows that a strain in which aI2 splicing is blocked by deletion of DV ($1^0 2^{3HA\Delta DV}$) accumulates only *COXI* pre-mRNA. Excised group II intron RNAs are known to accumulate in yeast mitochondria (2, 16, 19), and strain ΔDIVa accumulates about 67% of the expected amount of intron RNA based on the level of spliced mRNA (Fig. 3C, lane 4 versus lane 2).

To determine whether the aI2 splicing in the ΔDIVa strain depends on p62, we mutated three codons near the beginning of the RT domain to stop codons (strain $1^0 2^{\Delta DIVa\text{-}stop}$), thereby

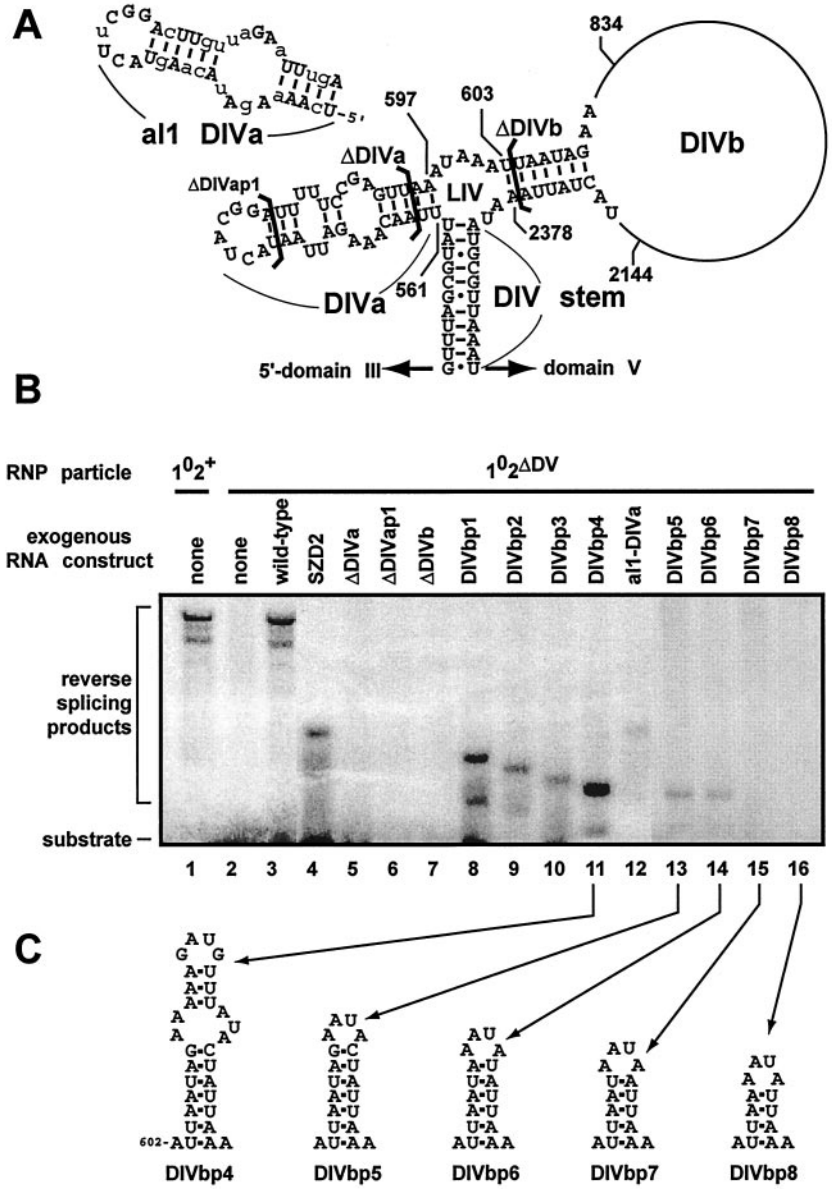

FIG. 2. In vitro mapping of the DIV sequences needed for functional p62 binding. (A) Structure of DIV of aI2. DIV extends from the central wheel of the group II intron as the DIV stem, DIVa stem-loop, LIV, and the long DIVb, as indicated. The nucleotides of DIV present in the minimal active substrate DIVbp6 are shown as uppercase letters. The brackets indicate the breakpoints of the ΔDIVa and ΔDIVb constructs. Numbers indicate nucleotide positions counting from the first nucleotide of aI2 from strain ID41-6/161. Also shown are the sequence and predicted secondary structure of the DIVa substructure of the related group II intron aI1 from the same strain. (B) Reverse-splicing assays. Various deletion or mutant RNAs were used to map sequences important for p62 binding, by the in vitro reverse-splicing reconstitution assay (15) (see Materials and Methods for details). Lanes 4 to 12 are overexposed relative to the other lanes to accentuate the low level of activity of aI1-DIVa RNA (lane 12). DIVbp6 (lane 14) is the smallest active substrate in this assay. In most active samples there are three product bands; the slowest-migrating band results from full reverse splicing while the faster-migrating doublet results from partial reverse-splicing reactions (13, 15). (C) Secondary structures illustrating some of the DIVb partial deletions analyzed.

truncating the p62 ORF (Fig. 3A). In this mutant strain, no spliced mRNA or excised intron RNA is detected on Northern blots (Fig. 3B and C, lane 5), showing that the substantial splicing activity in the ΔDIVa mutant depends on synthesis of the maturase protein.

Next we found that the DIVa from aI1 can rescue the partial splicing defect of ΔDIVa. As shown in lane 6 of Fig. 3B and C, strain aI1-DIVa has more spliced mRNA (~96% of the control level in this gel) and more excised intron RNA (~100% of control) than does strain ΔDIVa. These observations are consistent with the above in vitro finding that aI1-DIVa can partially substitute for aI2-DIVa in the reverse-splicing assay (Fig. 2B, lane 12). We also found that adding stop codons to the aI1-DIVa allele inhibits splicing (Fig. 3B, lane 7).

Based on the in vivo splicing phenotypes of these mutant strains, we conclude that DIVa is not required for aI2 splicing

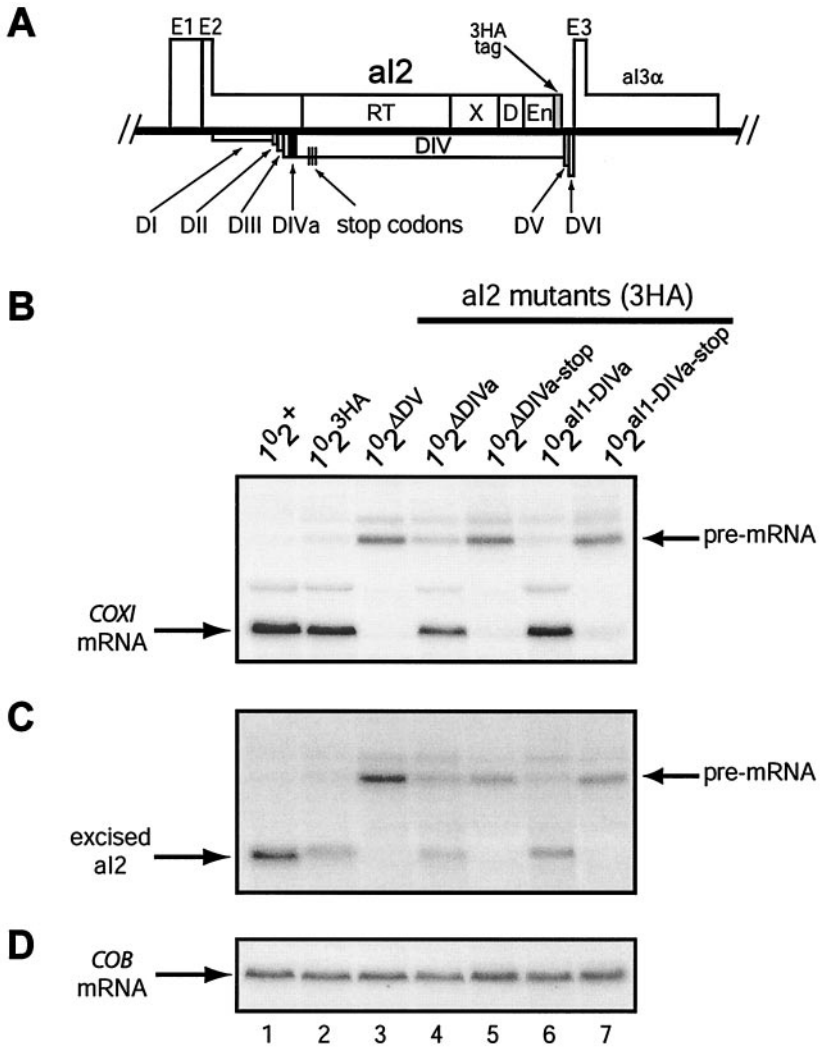

FIG. 3. DIVa mutations partially block splicing in vivo. (A) The organization of aI2 and flanking sequences of the *COXI* gene. The tall rectangles above the line represent *COXI* exons, and the short rectangles represent intron reading frames. RT, X, D, and En denote domains associated with RT, maturase, DNA binding, and DNA endonuclease functions of the IEP (p62), respectively. The rectangles below the line indicate the positions of intron secondary structure domains; DIVa is the shaded box within DIV. Three consecutive stop codons placed in aI2 in strains $1^02^{\Delta DIVa\text{-}stop}$ and $1^02^{aI1DIVa\text{-}stop}$ are at nt 840 to 848 of aI2, as indicated. (B to D) RNA blots. Samples of whole-cell RNA from the strains shown were balanced for their level of *COB* mRNA, separated on a formaldehyde–MOPS–1.5% agarose gel, and transferred to a nylon membrane, which was sequentially probed, stripped, and reprobed with oligonucleotides complementary to *COXI* exon 6 (B), intron aI2 (C), and *COB* exon 6 (D). Strains 1^02^+ (lane 1) and $1^02^{\Delta DV}$ (lane 3) define the positions of *COXI* mRNA and aI2-containing pre-mRNA, respectively. The faint band migrating slower than *COXI* mRNA in lanes 1, 2, 4, and 6 of panel B is the pre-mRNA containing unspliced aI4α; the faint band migrating slower than the aI2-containing pre-mRNA in lanes 3 to 5 and 7 contains unspliced aI4α in addition to aI2.

in vivo, although splicing is somewhat more efficient when wild-type or aI1-DIVa is present. Because maturase-dependent splicing is so efficient in the absence of DIVa, we conclude that DIVa is but one of a number of contacts made between p62 and aI2 pre-mRNA that are important for splicing. These data show that prior binding to DIVa is not essential for establishing those other contacts or for folding aI2 RNA into a configuration that is active for splicing in vivo.

Mutations of DIVa block homing. Next we tested the effects of the same DIVa mutations on homing activity in crosses by using a recipient strain with an efficient target site for aI2 homing (13). In this assay intron donor and recipient strains are mated and grown for about 20 generations to permit segregation of parental and recombinant mtDNAs. mtDNA is then purified from the mixed progeny of the cross and subjected to RFLP analysis to detect parental and recombinant alleles (33). As shown in Fig. 4A, the parental and major recombinant *COXI* alleles in progeny of this cross are readily distinguished in a *Hpa*II-*Bam*HI digest. In the control cross ($1^02^{3HA} \times 1^02^0$), there is about 60% homing (Fig. 4B, lane 4). This level of homing is somewhat lower than the ~90% homing reported for this cross with a donor strain lacking the 3HA tag (13) (e.g., lane 3) but is more than sufficient for our purpose. We showed previously that the homing of the aI2 intron can occur both by RT-dependent retrohoming and by an RT-independent mechanism in which the intron RNPs cleave the

TABLE 2. RT activity in RNP fractions

Expt no.	Fraction	Full system[a]	No RNase[b] (% of full reaction)	Pre-RNase[c] (% of full reaction)
1a	Wild type	35,001 ± 3,412 (100)	9.6	5.1
	ΔDIVa	12,909 ± 391 (37)	32	30
	aI1-DIVa	17,157 ± 477 (49)	30	33
1b	Wild type	64,702 ± 3,976 (100)	6.6	6.1
	ΔDIVa	22,918 ± 447 (35)	21	27
	aI1-DIVa	53,588 ± 1,760 (83)	9	19
2a	Wild type	39,780 ± 8,020 (100)	8	ND
	ΔDIVa	15,463 ± 1,347 (39)	23	ND
	aI1-DIVa	21,496 ± 4,915 (54)	9	ND
2b	Wild type	45,768 ± 6,214 (100)	27	21
	ΔDIVa	29,282 ± 1,756 (64)	58	51
	aI1-DIVa	45,440 ± 4,173 (99)	35	34

[a] See Materials and Methods and reference 48 for details. Background was reaction mixtures lacking the dT_{18} primer. Values are shown as counts per minute − background ± standard deviation (percentage of wild-type value).
[b] RNase A (0.1 mg/ml) was excluded from the reaction buffer for these assays.
[c] Samples were incubated with RNase A for 5 min before the reaction was started by adding the primed substrate. ND, not determined.

target site in the recipient DNA to initiate double-strand break repair recombination with the donor allele (13, 14). In crosses with the wild-type intron, the proportion of events resulting from the two mechanisms is about equal (13). A different digest scoring a *Dde*I site at the 5′ end of exon 3 (see locations of *Dde*I sites in Fig. 4A) showed that the control cross using the donor strain with 3HA-tagged p62 also involves both RT-dependent and RT-independent events (data not shown).

The crosses using the ΔDIVa and aI1-DIVa donor strains have no detectable homing (Fig. 4B, lanes 6 and 7). These blots can detect residual homing at about 1% of the control level, so we conclude that these mutations inhibit homing at least 60-fold (i.e., 60% homing/1% detection limit). Because splicing appears to be inhibited by at most 30% in the ΔDIVa strain and by less than 5% in the aI1-DIVa strain, we conclude that aI2-DIVa plays a much more important role in homing than it does in splicing. Further, because RT-independent events are also abolished, we can conclude that DIVa is required for the formation of active RNP particles that cleave the recipient allele, as expected from the biochemical assays of Fig. 2.

Mature p62 is present in DIVa mutant strains. Because the results of Fig. 3C showed that these DIVa mutants accumulate excised intron RNA, we next tested whether the lack of active RNP particles might reflect the fact that they do not accumulate any p62. The outer membrane protein porin was used to balance the aliquots of mitochondrial protein in these experiments (lower gel in Fig. 5A). Tagged p62 is readily detected in Western blots of mitochondrial extracts from strain 1^02^{3HA} (Fig. 5A, lane 2), and there is no signal for strain 1^02^+ that lacks the HA tag (lane 1). The protein is present in mitochondria from strains ΔDIVa (lane 3) and aI1-DIVa (lane 5), though at about one-third the level in the wild-type strain (based on dilutions of the control sample [data not shown]). Stop codons in the RT domain of both strains eliminate detectable p62 (lane 4; also data not shown). It is likely that the

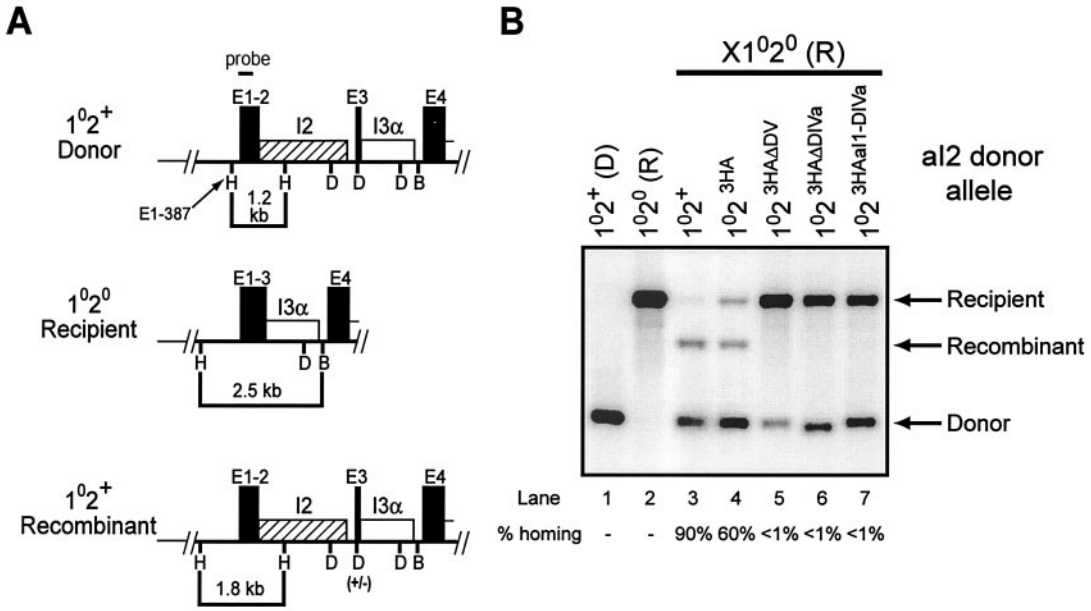

FIG. 4. DIVa mutations block homing. (A) Diagram of donor, recipient, and recombinant *COXI* alleles. The donor strain contains a short G+C-rich insertion that introduces an *Hpa*II (H) site 387 nt upstream of the 3′ end of exon 1, which provides the upstream marker E1-387. Relevant fragments of *Hpa*II (H)-*Bam*HI (B) digests detected with an exon 1-specific probe are indicated. *Dde*I (D) sites that distinguish products of RT-dependent homing from RT-independent homing products and donor alleles are shown (see Materials and Methods for further details). (B) Southern blot-RFLP analysis of mtDNA from crosses. The mtDNA is digested with *Hpa*II-*Bam*HI and probed with an exon 1-specific oligonucleotide that distinguishes the three alleles as shown in panel A. Inputs of mtDNA from both parental strains were determined as described in Materials and Methods (data not shown, but see reference 33 for an example) and used to calculate the extent of homing in each cross, reported below the gel.

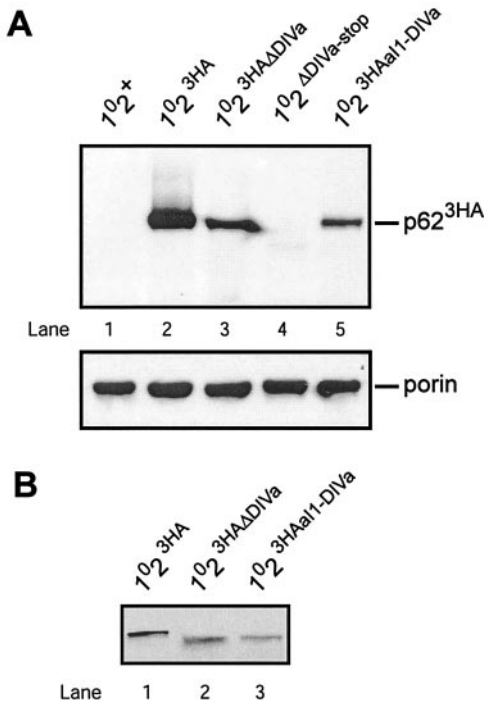

FIG. 5. Western blots of p62 levels. (A) p62 is present in DIVa mutant strains. Approximately 30 μg of protein from crude mitochondrial fractions of the indicated strains was separated by electrophoresis in an SDS–7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and subjected to Western blot analysis. p62-3HA is detected with the 6B12 anti-HA monoclonal antibody. An antibody to porin, a nucleus-encoded mitochondrial outer membrane protein, is used as a control for the loading of mitochondrial proteins. (B) Deletion of DIVa shortens p62. Whole-cell extracts of the strains shown were balanced for equal input of p62 and fractionated on the above gel system, but for a longer time of electrophoresis.

lower amounts of protein in the ΔDIVa and aI1-DIVa mutant strains result from different stabilities rather than from less synthesis.

In comparing lanes 2 and 3 of the gel shown in Fig. 5A, the IEP present in strain ΔDIVa appears to migrate somewhat faster than the control p62. This inference is confirmed by the analysis of their mobilities on a more highly resolving gel (Fig. 5B, lanes 1 and 2), where we analyzed extracts of whole cells to avoid possible alteration of the protein during spheroplasting and fractionation of cell extracts. Lane 3 of this gel shows that the protein in strain aI1-DIVa is somewhat larger than that in strain ΔDIVa and does not comigrate with the control protein. These data suggest that the N terminus of p62 is encoded upstream of DIVa with the deletion of DIVa resulting in loss of 11 amino acid residues and substitution of aI1-DIVa affecting 13 codons, 10 of which are changed. We show below that the p62 proteins with these altered N termini still have RT activity.

p62 binds to mitochondrial RNA with reduced affinity. Next we tested whether the p62 in mitochondria from strain ΔDIVa sediments to the RNP particle fraction. The standard protocol for preparing active RNP particle fractions from the wild-strain entails lysing flotation-purified mitochondria with NP-40 in the presence of 25 mM $CaCl_2$ and 500 mM KCl, layering the lysate on top of a 1.85 M sucrose cushion containing those same salts, and sedimenting for 17 h. The resulting pellet fractions contain p62 and most large mitochondrial transcripts, including *COXI* mRNA, pre-mRNA, and excised intron RNA (23, 48). In preliminary experiments with the HA-tagged wild-type strain, we observed that the sum of all fractions from the standard protocol accounts for only about half of the p62 present in the initial mitochondrial lysate. With lysates from strain ΔDIVa, none of the p62 pelleted and little of the loaded p62 was detected in the fractions. We infer that the sedimentation step separates intact RNP particles from proteases that are present in the lysate but that any free p62 remaining in the supernatant is degraded during the long centrifugation.

For that reason, we devised a faster fractionation scheme in which all of the p62 is recovered from both mutant and wild-type strains. As diagrammed in Fig. 6A, mitochondria from the 3HA-tagged wild-type and ΔDIVa strains were purified and lysed as described above and then fractionated by differential centrifugation (see Materials and Methods). The 20,000 × *g* centrifugation removes most of the mtDNA nucleoids, and the 100,000 × *g* centrifugation was expected to pellet ribosome subunits and other RNP complexes. Each pellet fraction was suspended in the same volume of SDS buffer as the supernatant fraction, and identical aliquots were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting with the antisera indicated (Fig. 6B). As a control for proteolytic degradation, an aliquot of lysed mitochondria (M2) was held on ice for the duration of the fractionation steps, and its p62 level was compared with that in a balanced sample of mitochondria lysed with SDS to inactivate proteases (M1). As shown in Fig. 6B, lanes 1 and 2, the amounts of p62 in the M1 and M2 fractions are indistinguishable, showing that p62 is not lost during this fractionation.

By this protocol, most of the p62 in lysed wild-type mitochondria remains in the low-speed supernatant fraction (S20) (Fig. 6B, lanes 3 and 4) and then about 50% of it pellets at 100,000 × *g* (Fig. 6B, lanes 5 and 6). For mitochondria from strain ΔDIVa, nearly all of the p62 is in the S20 fraction and then remains in the S100 fraction after the higher-speed centrifugation (lanes 11 and 12). Importantly, *COXI* transcripts are recovered in the P100 fraction to the same extent from both strains (data not shown). Adding a cocktail of protease inhibitors to the NP-40 lysis buffer did not alter the recovery or distribution of p62 in this fractionation for either strain (data not shown). These data show that p62 has reduced binding affinity to ΔDIVa RNA under the standard fractionation conditions with 500 mM KCl (compare lanes 6 and 12).

To learn whether the accumulated p62 is bound to endogenous RNA at all, we repeated this fractionation using a lower concentration of KCl in the lysis buffer (150 mM) so as to permit more weakly bound proteins to pellet with the RNA. As shown in Fig. 6C, essentially all of the p62 remains in the S20 from both strains (lanes 3 and 9) and ~90% of the p62 pellets remain in the 100,000 × *g* fraction from both strains (lanes 6 and 12). These data suggest that the binding of p62 to endogenous RNAs in mitochondria of strain ΔDIVa is weaker than in the wild-type strain. The maturase present in mitochondria of strain aI1-DIVa fractionates in the same way as that from strain ΔDIVa (data not shown).

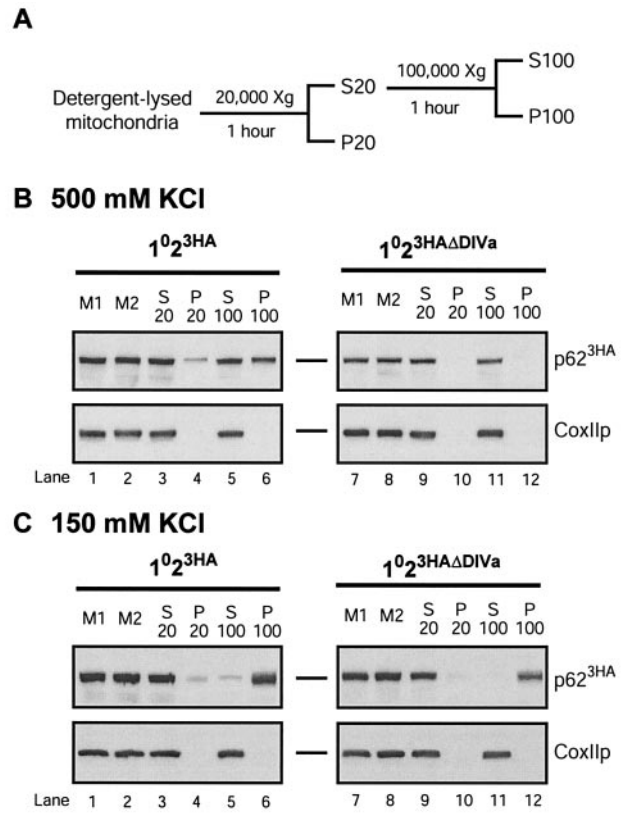

FIG. 6. Fractionation experiments. (A) Diagram of the protocol for fractionating lysed mitochondria. Flotation-gradient-purified mitochondria were isolated and lysed as described in Materials and Methods. The lysate was fractionated by differential centrifugation to yield supernatant and pellet fractions as indicated. Pellet fractions were dissolved in SDS buffer, and SDS was added to aliquots of the supernatant fractions prior to gel analysis. (B) Fractionation of p62 from lysates containing 500 mM KCl. Equal aliquots of each fraction from strains with 3HA-tagged wild-type or ΔDIVa alleles of aI2 were analyzed in SDS–7.5% polyacrylamide gels, as described for Fig. 5A. M1 is a sample of mitochondria lysed by SDS and boiled. M2 is a sample of mitochondria lysed with NP-40 and held on ice until the fractionation steps were completed, with SDS added just prior to gel electrophoresis. The lower gel for each strain shows the distribution of the inner membrane protein CoxIIp, demonstrating that the NP-40 lysis completely dissolved the inner membrane under these conditions. Proteins that represent the outer membrane (porin) and matrix (Ilv5p) fractionated the same as CoxIIp for both strains. (C) Fractionation of p62 from lysates containing 150 mM KCl. This experiment has the same format as that in panel B, except that the lysis buffer contained 150 mM KCl.

p62 in the mutant strains has RT activity. Using an exogenous poly(rA) template primed with oligo(dT)$_{18}$, we have reported previously that aI2 RNP particle fractions from wild-type and some mutant strains have RT activity (33, 48). For this assay the RT is released from the endogenous RNA by RNase A treatment. If RNase A is left out of the reaction, RT activity is much lower, showing that binding to endogenous RNA substantially inhibits transfer of p62 to the exogenous primed substrate. Further, if the RNase A treatment is carried out before the template-primer complex is added, the RT is substantially inactivated, indicating that this enzyme needs to be bound to RNA to remain active (48).

RNP particle fractions were prepared from these three strains by lysing mitochondria in the buffer containing 150 mM KCl and 25 mM $CaCl_2$ and sedimenting them through a 1.85 M sucrose cushion containing the same salts. Aliquots of RNP particle fractions containing comparable amounts of p62 (based on Western blots) were then assayed for RT activity. Table 2 summarizes four experiments done with two different RNP preparations from each strain. The data show that RNP particles from strain ΔDIVa have 35 to 64% of the wild-type activity, while aI1-DIVa has 49 to 99% of wild-type activity. If RNase A is omitted from the reaction, less activity is observed in each case (compare columns for "full system" and "no RNase A"), showing that the p62 needs to be released from endogenous RNA to obtain maximal cDNA synthesis by using the exogenous primed template. We note that the mutant preparations are less dependent on RNase A digestion than is the control, consistent with the above finding that the mutant proteins are more weakly associated with endogenous RNAs. Pretreatment with RNase A to digest endogenous RNA before adding the exogenous substrate reduces the activity in all three preparations (compare columns for "complete assay" and "pre-RNase"), indicating that p62 in each sample is stabilized by binding to endogenous RNA. These results indicate that the mutant proteins retain substantial RT activity and are associated with endogenous mitochondrial RNAs.

DISCUSSION

Using an in vitro reverse-splicing complementation assay, we found that DIVa is required for p62 to bind to aI2 intron lariat and reconstitute reverse-splicing activity. This finding extends an earlier study of the bacterial group II intron Ll.LtrB, in which it was found that deletion of DIVa abolished the high-affinity binding of its maturase LtrA and decreased the rate of in vitro maturase-assisted forward splicing by 13- to 24-fold (45). We also found that a small segment of aI2-DIVb is needed for reconstituting reverse-splicing activity, suggesting that this substructure may play a greater role in maturase binding for the yeast intron than for the bacterial intron (45). It is not known whether aI2-DIVb plays this role by interacting directly with p62 or by influencing the configuration of DIVa or LIV. Despite this difference in detail, we conclude that DIVa is a comparably important part of the binding site for both group II IEPs, despite having different roles in translation initiation (see the introduction).

Deletion of DIVa from aI2 resulted in only moderate inhibition of in vivo splicing. This splicing in the absence of DIVa was blocked by stop codons in the intron ORF, showing that it still depends on p62. The degree of inhibition of in vivo forward splicing caused by deleting DIVa is considerably less for the yeast aI2 intron than it is for the *L. lactis* Ll.LtrB intron (~30 and ~80%, respectively [X. Cui, M. Matsuura, Q. Wang, H. Ma, and A. M. Lambowitz, unpublished data]). Although different assays were used to measure these residual levels of splicing in the two systems, taken at face value the yeast intron appears to be less dependent on DIVa for forward splicing in vivo than is the bacterial intron. We will show elsewhere that in vivo splicing of aI2 strongly depends on the DEAD-box protein Mss116p, a potential RNA helicase (H.-R. Huang and P. S.

Perlman, unpublished data), and so differences in accessory proteins used by the two introns may be a factor.

A model was proposed for the bacterial intron in which the maturase first binds to the high-affinity DIVa binding site and then makes weaker contacts in the catalytic core that lead to efficient splicing (27, 45). Chemical modification-protection mapping indicates that the bacterial maturase-RT protects sites in intron domains I, II, and VI (27). The residual in vivo splicing in ΔDIVa mutants of both the bacterial and yeast introns is maturase dependent, showing that their maturases can support significant levels of splicing via interactions with sites in pre-mRNA besides DIVa. It will be interesting to learn whether the DIVa-independent contacts are the same for the two introns.

In contrast to the modest effect on aI2 splicing, deletion of DIVa from aI2 strongly inhibits homing. A similar differential effect on homing was reported recently for the *L. lactis* intron ΔDIVa mutant (12). These findings shows that DIVa is much more important for mobility than it is for splicing. We show that the yeast ΔDIVa strain accumulates excised intron RNA and p62, though both are present at somewhat reduced levels. Because aI2 splicing by the ΔDIVa strain depends on synthesis of p62, it is evident that the p62 that is made binds to ΔDIVa pre-mRNA in a way that supports splicing. However, we show here that the binding of p62 to endogenous RNAs in the ΔDIVa strain is qualitatively weaker than in the wild-type strain, as judged by failure of the protein to sediment from a 500 mM KCl lysate. Lowering the ionic strength of the mitochondrial lysis buffer restored p62 sedimentation to the RNP particle fraction. Those mutant RNP fractions are shown to have RT activity that is stabilized by its binding to endogenous RNAs. Because the ΔDIVa strain accumulates similar amounts of pre-mRNA and excised aI2 RNA (Fig. 3C, lane 4), it is possible that the p62 is mainly bound to pre-mRNA. If so, then those pre-mRNA RNP particles would not be active for the reverse-splicing reaction that initiates homing. Alternatively, if some of the protein is bound to ΔDIVa lariat RNA, then it must be bound in a way that is not compatible with homing. Thus, the mutant strains probably lack the reverse splicing and/or endonuclease activities needed for the initial steps in homing so that both the RT-dependent and RT-independent homing events that occur in the control cross are inhibited in crosses using the mutant strains. The finding that reverse splicing was not reconstituted in vitro with ΔDIVa lariat RNA also shows that DIVa is required for the functional binding of the maturase to lariat RNA.

Previous research indicated that aI1 and aI2 maturases are specific for their respective introns (1, 7, 34), and our findings here suggest that differences in DIVa contribute to the specificity of the aI2 maturase-intron RNA interaction. When we substituted DIVa from the related yeast intron aI1, wild-type p62 had only a low level of reverse-splicing activity in vitro, suggesting that binding is either weaker or incorrect. The aI1-DIVa strain splices aI2 at wild-type levels, providing in vivo evidence that the heterologous DIVa has partial function in the aI2 context. Interestingly, despite having the control levels of spliced mRNA and excised intron RNA, the aI1-DIVa strain has about the same reduced amount of p62 as in strain ΔDIVa, and its binding to endogenous RNAs is similarly weakened. Further, the heterologous DIVa did not restore detectable homing, indicating that requirements for functional binding of p62 to pre-mRNA and lariat RNA must differ somewhat. Because aI1 and aI2 are closely related, additional comparisons may provide clues about maturase specificity and the modularity of both RNA and protein components in group II introns.

Although translation of the Ll.LtrB ORF is initiated at a start codon within DIVa (30), p62 appears to be translated as a precursor protein initiated at the start codon of exon 1 in *COXI* pre-mRNA. The preprotein is evidently cleaved to yield the active protein that accumulates, plus an N-terminal fragment that is presumably degraded. This situation is thought to reflect that the yeast intron evolved from an ancestral intron whose ORF was encoded within DIV but was then extended upstream to be translated in frame with the upstream exon, providing an alternate mechanism of translational regulation. Our finding that the p62-related protein that accumulates in the ΔDIVa mutant strain is slightly smaller than wild-type p62 (Fig. 5B) indicates that the protein-processing site is upstream of DIVa, perhaps even outside of DIV. Although it is possible that the changes near the N terminus of the protein contribute to the phenotypes of the ΔDIVa and aI1-DIVa mutants, we show that both mutants have substantial RT activity, a sensitive indicator of the integrity of the protein. Further, our in vitro experiments show that the deletion of DIVa RNA strongly inhibits the functionally relevant binding of wild-type p62 protein that accumulates in the ΔDV mutant strain. If unbound p62 is more susceptible to degradation in vivo, then the weakened binding detected here may well explain the reduced level of p62 in both mutant strains.

Finally, our results may have implications for group II intron splicing and mobility in other organisms. Mitochondrial and chloroplast genomes of plants have a number of group II introns, only a few of which can encode a maturase (17). It has been suggested that those few maturase-like proteins might assist the splicing of multiple group II introns in these organelles (10, 44). A recent study showed that putative genes encoding proteins related to group II intron maturases are present in the nuclear genomes of higher plants, suggesting that a "master maturase" might be nucleus encoded (31). Our findings that DIVa is not essential for forward splicing of either intron tested to date further focus attention on the other contacts that the maturase makes with pre-mRNA. In order for a single maturase to splice multiple introns, those non-DIVa contacts would have to be conserved, and it is encouraging that the weaker contacts identified in Ll.LtrB are all in the conserved core of the intron (27). On the other hand, binding of the yeast aI2 maturase to DIVa in lariat RNA appears to be critical for homing, making it unlikely that a master maturase would efficiently mobilize multiple introns that do not have closely related DIVa structures.

ACKNOWLEDGMENTS

We thank Huatao Guo and Jian Yang for their helpful technical advice, Steven Zimmerly for providing plasmid pSZD2, and Stuart Connell for technical assistance.

This research was supported by research grants from the National Institutes of Health (GM31480 to P.S.P. and GM37951 to A.M.L.) and the Robert A. Welch Foundation (I-1211 to P.S.P.). During part of this research, Michael Chao was an NIH predoctoral trainee (T32-HL07360).

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