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Overexpression of DEAD box protein pMSS116 promotes ATP-dependent splicing of a yeast group II intron *in vitro*

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ABSTRACT

The group II intron bI1, the first intron of the mitochondrial cytochrome b gene in yeast is self-splicing *in vitro*. Genetic evidence suggests that *trans*-acting factors are required for *in vivo* splicing of this intron. In accordance with these findings, we present *in vitro* data showing that splicing of bI1 under physiological conditions depends upon the presence of proteins of a mitochondrial lysate. ATP is an essential component in this reaction. Overexpression of the nuclear-encoded DEAD box protein pMSS116 results in a marked increase in the ATP-dependent splicing activity of the extract, suggesting that pMSS116 may play an important role in splicing of bI1.

INTRODUCTION

In organisms with split genes the introns are removed from the primary transcript by RNA splicing. Splicing of nuclear pre-mRNA introns takes place on the spliceosome, a complex containing numerous proteins and five small nuclear ribonucleoprotein particles (snRNPs). Notably, several of the proteins participating in spliceosome assembly or the two transesterifications require ATP hydrolysis. Yet, for the chemical reaction itself energy input is dispensible, as group II introns undergo self-splicing without ATP (reviewed in 1). In contrast, protein-independent autocatalytic splicing of some group I and group II introns has been shown *in vitro* (2–5). Two lines of evidence, however, have led to the view that specific *trans*-acting factors, presumably proteins, are essential for *in vivo* splicing of most, if not all, group I and group II introns (reviewed in 6). First, self-splicing can only proceed under relatively non-physiological conditions, e.g. 60 mM Mg^{2+} and 45°C (2,5). Second, genetic analysis of splicing in fungal mitochondria has resulted in the characterization of numerous nuclear as well as organellar *trans*-acting mutants that impair splicing of one or more mitochondrial introns.

Two classes of proteins that participate in splicing of organellar introns have been identified by their different pattern of inheritence. Maturases are encoded by open reading frames located in the intron that they help to excise. Thus their synthesis is dependent upon mitochondrial translation (6–9). A second class of mutations that affects splicing of organellar introns is located on nuclear genes whose products are presumably imported into the mitochondria where they assist splicing. These genes include, for example, *CBP2*, *MSS18* and *MSS116* in yeast or *cyt-18* in *Neurospora* (10–13). The nuclear yeast gene *MSS116* was initially isolated via a genetic screen revealing that this gene can complement a nuclear mutant defective in splicing of several group II and possibly group I introns (12). Interestingly, the sequence of *MSS116* shows a remarkable degree of sequence homology with a new family of proteins, the DEAD box proteins, so called because they share the highly conserved motif Asp–Glu–Ala–Asp, together with six other conserved elements (14). Members of this and the related DEAH subgroup participate in a variety of RNA-associated functions, e.g. initiation of translation, spliceosomal splicing and ribosome assembly (15–19). Some members of the DEAD box family have been shown to possess an ATP-dependent RNA unwinding activitiy (20–22).

Most of the organellar splicing factors characterized to date are essential for excision of only one or of a few introns and exhibit no obvious sequence homologies amongst each other. This specificity of factors for their respective introns distinguishes organellar from spliceosomal splicing, where roughly the same set of factors process the majority of mRNAs. Consequently, it is widely believed that all introns with conserved secondary structures were originally self-splicing. According to this hypothesis, it was only later in evolution that protein-assisted splicing developed independently for each of these introns (6). Although numerous *trans*-acting factors affecting splicing of group I and group II introns have been defined by mutations in mitochondrial systems, biochemical evidence for such participation is still scarce and has been successfully demonstrated only for some proteins involved in group I intron splicing. One example is the CYT-18 protein in *Neurospora*, which is identical to mitochondrial tyrosyl-tRNA synthetase. The purified protein CYT-18 has been shown to facilitate splicing of the mitochondrial large rRNA intron (13,23). We were interested in identifying the *trans*-acting factors that promote splicing of group II intron bI1, the first intron of the cytochrome b gene in yeast mitochondria. The development of an assay comprising proteins of a mitochondrial lysate made it possible to demonstrate protein-assisted *in vitro* splicing of a group II intron. This reaction is ATP-dependent. One protein involved in splicing of bI1 is encoded by the nuclear gene

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MSS116. Overexpression of this gene increases the ATP-dependent splicing activity of the extract. Parameters of pMSS116-promoted splicing of bI1 have been characterized.

MATERIALS AND METHODS

Strains of *Saccharomyces cerevisiae*, growth conditions and preparation of mitochondrial matrix proteins

The wild-type strain used in this study was *S.cerevisiae* A237 (MATa, *trp1*, *ura3-52*, *rho*$^+$), which is devoid of mitochondrial DNase and RNase NUC1 (24). The host strain for transformations was A237, constructing A237/pGU (containing plasmid pGU) and the *MSS116*-overexpressing strain A237/pGU:*MSS116* (containing *MSS116* in plasmid pGU). Cultures of A237 were grown at 30°C in YP medium supplemented with 3% glycerol. Strains containing the pGU plasmid or a derivative thereof were grown in minimal medium supplemented with essential amino acids at 30°C. For preparation of mitochondrial matrix proteins cells were grown to log phase and harvested at a titer of 10^7 cells/ml. Mitochondria were prepared from spheroplasts by osmotic lysis and purified by differential centrifugation. Matrix proteins were obtained by sonication of the organelles and subsequent centrifugation at 25 000 *g* and 100 000 *g* to remove mitochondrial membranes and ribosomes, respectively. The collected extract (S100) was dialyzed and stored at –70°C in 20% glycerol, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 20 mM HEPES–KOH, pH 7.4, 100 mM NaCl.

Preparation of RNA

Transcripts were synthesized by *in vitro* transcription with T3 RNA polymerase. Transcription assays were carried out in a 20 µl reaction containing 5 µg template DNA, 40 U enzyme, 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 4 mM spermidine, 500 µM each rNTP for 2 h at 37°C. For generation of internally labeled transcripts 10 µCi [^{32}P]UTP were added to the assay. Following transcription preRNAs were electrophoresed on 5% polyacrylamide–8 M urea gels, autoradiographed, extracted from the gel and purified. Templates for synthesis of preRNA were plasmid BS/bI1/δ+24 (25), which harbors the complete intron bI1, 35 nt of the 5′ exon and 238 nt of the 3′ exon, and plasmid BS/aI5c (26), respectively.

In vitro splicing assays

In vitro self-splicing was performed in 20 µl Tris–HCl, pH 7.5, 60 mM $MgCl_2$, 2mM spermidine, 500 mM NH_4Cl at 45°C for 15 min. The reaction was stopped by ethanol precipitation. The resulting pellet was washed with 70% ethanol and dried under vacuum. Protein-dependent *in vitro* splicing was performed in 40 µl 10 mM HEPES–KOH, pH 7.5, 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 10 µg *Escherichia coli* tRNA and 10 U RNase inhibitor at 28°C for various times. The reaction was stopped with 60 µl 50 mM NaAc, pH 5.2, 50 mM EDTA, 0.1% SDS, 2.5 µl proteinase K (20 mg/ml). After phenol/chloroform extraction the reaction products were precipitated with ethanol and the resulting pellet washed and dried. The assays were done in the presence of 10–15 µg mitochondrial matrix proteins and in the presence of extracts previously digested with proteinase K respectively. The products of *in vitro* splicing reactions were separated by electrophoresis on denaturing 5% polyacrylamide gels.

Construction of plasmid pGU:*MSS116*

Plasmid CA7 (27) contains the complete sequence of *MSS116* with an additional 5′ 600 nt and 3′ 350 nt as a genomic *Hin*dIII fragment. The *Hin*dIII fragment of CA7 was cloned into Bluescript SK (Stratagene). The 5′ non-coding region of the *MSS116* sequence was then shortened to 60 nt by restriction of the plasmid with *Bal*I and *Sma*I. Restriction of the resulting plasmid with *Bam*HI and *Sal*I yielded a fragment containing the *MSS116* gene with 5′ and 3′ non-coding regions and adjacent Bluescript polylinker sequences. This fragment was cloned into the multicopy yeast 2µ plasmid pGU and the resulting plasmid designated pGU:*MSS116*. pGU was derived from plasmid pG1 (28) by replacing the *TRP1* marker gene with the *URA3* marker gene derived from pUC19. Transformation of A237 was carried out according to Klebe *et al.* (29). Transformants were selected on synthetic complete medium without uracil.

Isolation of *S.cerevisiae* RNA and Northern analysis

Cells were harvested in log phase, washed once in H_2O and the pellet frozen in liquid nitrogen. The pellet was resuspended in extraction buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5% SDS). Cells were broken by vortexing with glass beads and phenol/chloroform for 5 min. Nucleic acids were extracted three times with phenol/chloroform and precipitated from the liquid phase with ethanol, 0.3 M NaAc. Incubation for 3 h in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 3 M LiCl precipitated the RNA. Whole-cell RNA was separated on formaldehyde–agarose gels and transferred to nylon membranes (Amersham). DNA probes were ^{32}P-labeled by nick translation (30). The nick-translated hybridization probe for detection of *MSS116* sequences was the described *Hin*dIII fragment of plasmid CA7 (27).

Analysis of pMSS116 protein

Rabbit antibodies were raised against a 14 amino acid domain derived from amino acid positions 67–80 of pMSS116 (sequence S-R-P-R-T-R-S-R-E-D-D-D-E-V). SDS–PAGE was carried out by the method of Laemmli (31), using a 5% stacking gel and a 12% separating gel. Each lane was loaded with 30 µg mitochondrial proteins. After transferring the separated proteins to an Immobilon P membrane (Millipore) the products could be probed with antiserum P1, directed against the above-described amino acid domain. Immunoblots were stained with ECL (Amersham).

RESULTS

Protein-dependent splicing of bI1 depends on ATP

The group II intron bI1 has previously been shown to undergo self-splicing *in vitro*, a reaction identical with spliceosomal splicing in mechanism, resulting in the excision of an intron lariat via two subsequent transesterifications (5). Yet efficient self-splicing of bI1 requires high salt concentrations and high temperature, suggesting that *trans*-acting factors are essential for the *in vivo* excision of this intron. In order to characterize such factors, we established an *in vitro* system which allowed us to assay for protein-dependent splicing.

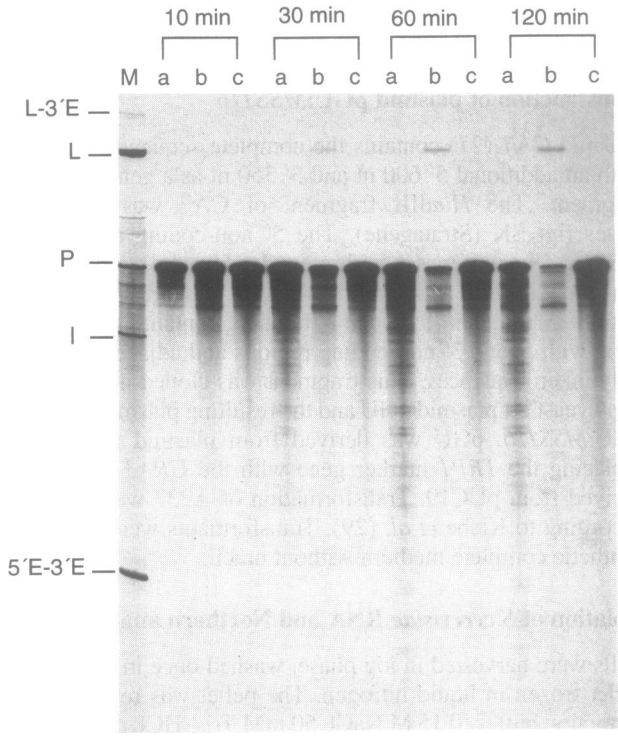

Figure 1. Protein-dependent splicing of bI1 in the presence of mitochondrial S100 extract. ^{32}P-Labeled bI1/δ+24 RNA ('preRNA') was incubated for various times in the presence of 15 µg mitochondrial matrix proteins pretreated with proteinase K (**a**), 15 µg native proteins (**b**) and in the absence of proteins (**c**). The products were gel electrophoresed on 5% denaturing polyacrylamide gels. Lane M shows the products of an autocatalytic reaction of preRNA. L-3′E, lariat with covalently bound 3′ exon; L, lariat; P, preRNA; I, linear intron, 5′E-3′E, ligated exons.

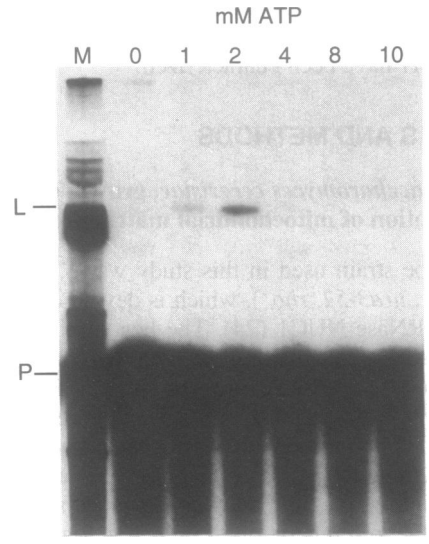

Figure 2. ATP dependence of splicing of bI1. Splicing assays in the presence of native mitochondrial proteins were carried out as described (see Materials and Methods) with increasing concentrations of ATP (0–10 mM).

An internally ^{32}P-labeled transcript harboring bI1 was incubated under various conditions with an S100 extract prepared from a crude mitochondrial lysate. To minimize non-specific endogeneous nuclease activity, mitochondria were isolated from yeast strain A237, which is deficient in the extremely active mitochondrial NUC1 exo-endonuclease (24). After incubation of the transcript with the mitochondrial S100 extract under conditions resembling the physiological state and in the presence of ATP the RNA was analyzed by polyacrylamide gel electrophoresis.

To monitor the efficiency of splicing we screened for the presence of an intron lariat, which is easy to detect due to its low electrophoretic mobility. As can be inferred from the time course shown in Figure 1, splicing under physiological conditions only proceeded in the presence of mitochondrial S100 extract (lanes b), whereas no lariat was formed if the RNA was incubated in reaction buffer alone (lanes c). Preincubation with proteinase K resulted in a marked reduction in splicing activity, indicating the participation of proteins in the reaction (lanes a). The time course shows that splicing increases linearly for 2 h and lariat formation plateaus at ~5% of the input preRNA (Fig. 1). In comparison with optimized *in vitro* self-splicing, where >50% of the intron was excised from the precursor after 30 min (see lane M in Fig. 1), the protein-dependent *in vitro* reaction is relatively slow.

Lysate-dependent splicing of group II intron bI1 can only proceed in the presence of ATP and Mg^{2+}. According to the experiment shown in Figure 2, there is an optimum splicing activity at 2 mM ATP. This activity drops at higher ATP concentrations, however, when the Mg^{2+} concentration is increased concomitantly with ATP, protein-dependent splicing activity remains unchanged (not shown). The apparent decline in splicing activity at higher ATP concentrations thus seems to be due to the complexing of ATP with Mg^{2+}, rather than to an inhibitory effect of high ATP concentrations. As Mg^{2+} concentrations >10 mM also promote autocatalytic splicing, all subsequent experiments were carried out at 10 mM Mg^{2+} and 2 mM ATP. The ATP dependence of the splicing activity observed in these experiments with mitochondrial lysates clearly distinguishes protein-dependent splicing of bI1 from autocatalytic splicing of group II introns, a pathway that has been shown to be intrinsically independent of a nucleotide co-factor (3–5).

Overexpression of *MSS116*

Having established that protein-assisted splicing of bI1 depends on ATP, we further investigated the nature of this splicing activity. As shown in lanes a of Figure 1, preincubation of the extract with proteinase K resulted in a clear reduction in splicing activity. The faint band in lanes a after 60 and 120 min respectively could be due to incomplete digestion of lysate proteins by proteinase K. Another possible explanation could be that proteinase K-generated peptide fragments may have a certain stabilizing effect on the catalytically competent conformation of this group II intron. Peptides with high positive charge have previously been shown to stimulate catalysis of the hammerhead ribozyme (40). In contrast, splicing activity proved insensitive to micrococcal nuclease and thus seems to consist of one or several protein component(s) lacking an essential RNA component (not shown).

At the time of our initial experiments it was shown that the yeast gene *MSS116* can complement a nuclear mutant deficient in splicing of bI1, another group II intron and possibly several group I introns in yeast mitochondria (12). In the same study the gene *MSS116* was shown to be located on a 2.9 kb genomic *Hin*dIII fragment with an ORF coding for a protein with 664 amino acids.

The derived protein sequence of pMSS116 shares a remarkable homology with members of the DEAD/H box family, a protein family some members of which have a demonstrated ATP-dependent RNA unwinding activity (14,20–22). Compared with the prototypes of this family, p68 and eIF4a, pMSS116 possesses an additional stretch of 30 mainly positively charged amino acids at its N-terminus, as expected of a presequence required for targeting proteins into mitochondria (12,32).

We inferred that the gene product of *MSS116* could be a possible candidate for the ATP-dependent splicing activity observed in mitochondrial lysates. Therefore, we have compared the splicing activity of an extract from an *MSS116*-overexpressing strain with that of a strain carrying the chromosomal copy of this gene only. *MSS116* was overexpressed under transcriptional control of the constitutive *GPD* promotor (33). To remove possible endogeneous expression signals, the sequence 5′ of the *MSS116* initiation codon was shortened to a length of 60 nt. The resulting 2.3 kb fragment containing *MSS116* flanked by some non-coding sequences was inserted into the 2μ plasmid pGU behind the *GPD* promotor. Vector pGU is derived from pG1 (28) by replacing the selection marker *TRP1* with *URA3*. Subsequently the construct pGU:*MSS116* was transformed into yeast strain A237. As a negative control we used extracts from wild-type strain A237, which contains a single copy of *MSS116* compared with the overexpressing strain. To ensure similar growth conditions A237 was transformed with plasmid pGU lacking the *MSS116* insert (A237/pGU).

Analysis of the expression levels of *MSS116* was performed by Northern and Western blots of A237/pGU:*MSS116* and A237/pGU respectively. A RNA blot probed with an *MSS116*-specific DNA fragment showed a signal of 2.1 kb that was present in A237/pGU:*MSS116* at high concentration, while the transcript from the chromosomal gene was hardly detectable in A237/pGU (Fig. 3a). Thus *MSS116* is transcribed with high efficiency into stable mRNA in the overexpressing strain. As a next step an antibody was raised against the 14mer peptide P1 (S-R-P-R-T-R-S-R-E-D-D-D-E-V), representing an N-terminal pMSS116 epitope (amino acid positions 67–80) of potentially high immunodominance as predicted by the program DNA STAR (34,35). The anti-P1 serum, but not pre-immune serum, recognized a protein in the mitochondrial lysate with an apparent molecular weight of 72 kDa. This coincides with the size predicted for pMSS116 lacking the ~30 amino acids of a putative import sequence at its N-terminus (Fig. 3b). While the silver stained SDS–PAGE gel showed no difference in the expression pattern of mitochondrial proteins between A237/pGU:*MSS116* and A237/pGU (not shown), the Western blot in Figure 3b revealed that the concentration of protein pMSS116 is at least 30-fold higher in A237/pGU:*MSS116* than in A237/pGU. The cellular localization of pMSS116 was determined by immunodecoration of the mitochondrial matrix fraction, as compared with the membrane fraction. Anti-P1 only recognized a band in the matrix fraction. This experiment confirmed accumulation of pMSS116 in the mitochondrial matrix (Fig. 3c).

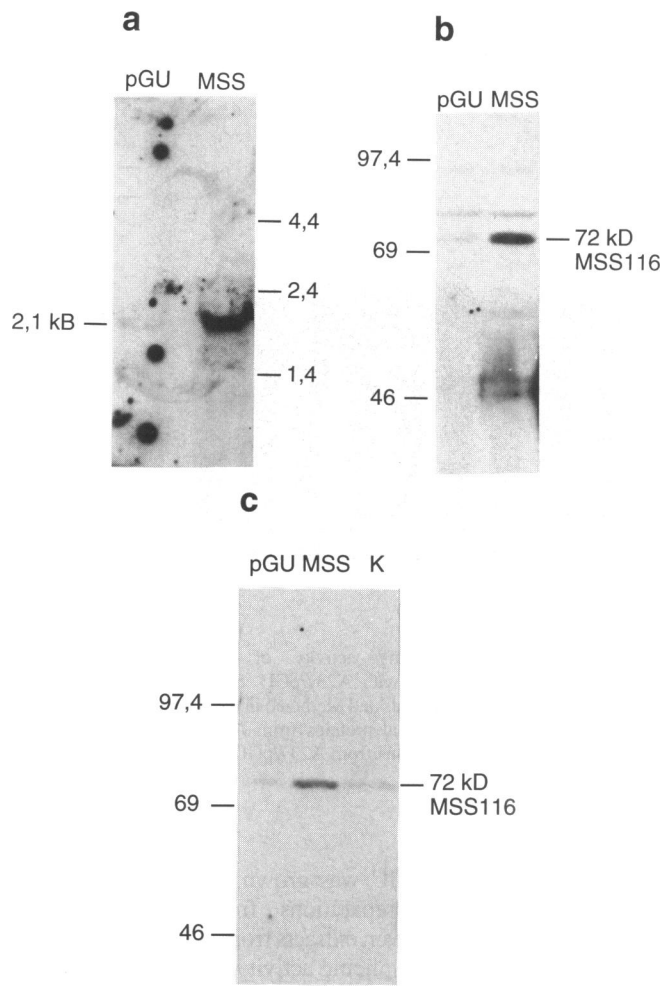

Figure 3. Overexpression of *MSS116* in a yeast strain transformed with plasmid pGU:*MSS116*. (**a**) Northern analysis of whole-cell RNA from A237/pGU (pGU) compared with RNA from A237/pGU:*MSS116* (MSS). The RNA was separated on a formaldehyde–agarose gel, transferred to a nylon membrane and probed with a nick-translated *MSS116*-specific DNA fragment. The hybridization probe detects a 2.1 kb transcript, specifically overexpressed in A237/pGU:*MSS116*. (**b**) Immunoblot analysis of mitochondrial proteins. Mitochondrial matrix proteins from the strains indicated were separated on 12% SDS–polyacrylamide gels with a 5% stacking gel. In the immunoblot shown anti-pMSS116 antiserum P1 was used for decoration. The molecular weight of pMSS116 was estimated from comparison with a standard protein marker, indicated on the left side of the blot. (**c**) Immunoblot analysis of proteins shows the localization of pMSS116 in the mitochondrial matrix. Identical amounts of proteins (20 μg) were separated by SDS–PAGE and decorated according to (b). Matrix proteins are from A237/pGU (pGU) and A237/pGU:MSS116 (MSS). Lane K shows mitochondrial membrane proteins from A237/pGU:MSS116 for comparison.

In vitro splicing of bI1 is promoted by overexpression of *MSS116*

In order to investigate whether the level of *MSS116* expression correlates with the splicing activity found in mitochondrial lysates we incubated bI1/δ+24 preRNA (25) with the two respective extracts. As can be seen in the time course shown in Figure 4, the level of lariat formation was significantly increased in the A237/pGU:*MSS116* extract (lanes c) compared with the A237/pGU extract (lanes b). The difference in splicing activities is especially apparent after 60 min and correlates roughly with the level of overexpression observed in the Western blot. According to the experiment shown in Figure 4, lariat formation seems to have a lag period in the lysate from the overexpressing strain. However, this observation was not consistent and was not investigated further. We also observed that extracts from strain A237/pGU exhibited a reduced splicing activity than those from non-transformed A237. This is probably due to the difference in

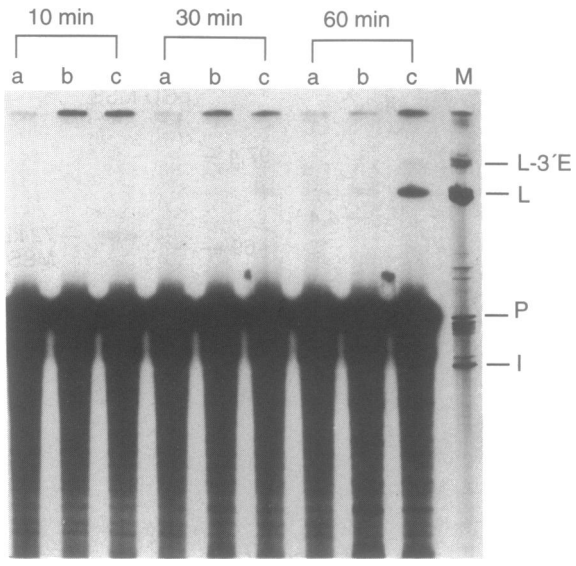

Figure 4. Enhanced splicing activity of extracts derived from A237/pGU:*MSS116* compared with A237/pGU. Splicing assays were performed as described (see Materials and Methods) in the absence of proteins (**a**), in the presence of mitochondrial proteins from A237/pGU (**b**) and in the presence of mitochondrial proteins from A237/pGU:*MSS116* (**c**).

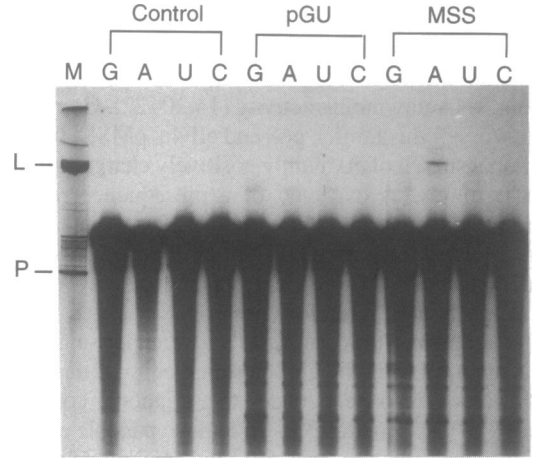

Figure 5. The splicing reaction specifically requires ATP. Splicing assays were carried out in the absence of proteins (control), with A237/pGU extracts (pGU) and with protein extracts from A237/pGU:*MSS116* (MSS). Where indicated, 2 mM ATP in the reaction buffer was substituted by 2 mM GTP (G), CTP (C) or UTP (U) respectively. Incubation was performed for 45 min.

growth medium (A237/pGU was grown on selective minimal medium). In parallel preparations from A237/pGU and A237/pGU:*MSS116*, however, extracts from A237/pGU:*MSS116* always exhibited a higher splicing activity than A237/pGU. Our results thus demonstrate that overexpression of nuclear gene *MSS116* alone is sufficient to increase ATP-dependent splicing of group II intron bI1 in a mitochondrial lysate.

In order to elucidate this process further, the specificity of pMSS116-promoted splicing with respect to co-factors and the RNA substrate was investigated. According to the results shown in Figure 5, pMSS116-promoted splicing of bI1 proceeds only in the presence of ATP, while none of the other standard rNTPs can be used. A similiar specificity for ATP has also been shown for other DEAD box porteins, including DbpA (19) and eIF4a (21). To learn more about the substrate specificity of pMSS116-promoted splicing we tested whether A237/pGU:*MSS116* lysate can also enhance splicing of other group II introns. Intron aI5c, the last intron of the *coxI* gene in yeast mitochondria is closely related to bI1 in secondary structure and primary sequence, both introns belonging to the same subgroup of group II introns (36). *In vitro* self-splicing of aI5c has been observed under conditions similiar to those of bI1 (3,4). However, neither the extract from A237/pGU nor that from A237/pGU:*MSS116* had any detectable effect on splicing of aI5c, i.e. no lariat formation could be observed under conditions optimized for bI1 splicing (not shown). Our findings are consistent with results from the genetic screen, showing that *MSS116* is not essential for *in vivo* excision of aI5c (12). The observation that overexpression of *MSS116* is not sufficient to generally enhance splicing of group II introns seems to indicate that pMSS116 does not affect bI1 splicing as a sequence-non-specific RNA helicase, an activity observed for some DEAD box proteins (20–22).

DISCUSSION

In this work we have analyzed protein-dependent splicing of mitochondrial yeast intron bI1, an *in vitro* autocatalytic group II intron. We established an *in vitro* system that allows splicing of bI1 preRNA only in the presence of a mitochondrial S100 extract. ATP is an essential component in this reaction. The fact that the splicing activity of the extract is significantly increased by overexpression of DEAD box protein pMSS116 suggests that this protein may play an important role in splicing of bI1.

In vitro splicing of bI1 under physiological conditions depends on a protein lysate and ATP

Splicing of group II introns proceeds via two transesterifications, resulting in the excision of a branched structure, the intron lariat (3–5). Extensive research with mutants of autocatalytic group II introns has provided a relatively clear concept of the function of conserved secondary structure domains 1–6 for the splicing reaction (36,37). These data imply that all catalytic activities required for splicing are inherent in the conserved structure of the intron RNA. However, at physiological Mg^{2+} concentrations and at low temperature no autocatalytic activity could be observed. Our results show that under these conditions efficient lariat formation (and consequently formation of the functional mRNA) depends upon the presence of one or several protein(s) from a mitochondrial S100 extract.

This finding supports the hypothesis that the autocatalytic reaction, which is hardly detectable under physiological *in vitro* conditions, can be enhanced considerably by the action of *trans*-acting factors. The observation that proteinase K, but not micrococcal nuclease, reduces splicing and that ATP is an essential cofactor, which cannot be substituted by other ribonucleotide triphosphates, suggests that DEAD box protein pMSS116 could be the candidate protein. The specificity for ATP is a major characteristic of the DEAD box proteins investigated

to date (19–21). The dependence on ATP distinguishes the protein-dependent pathway from *in vitro* self-splicing of group II introns, which does not require an external energy source. In both the protein-dependent and the autocatalytic pathways the intron is excised as a lariat. It therefore seems reasonable to assume that protein-dependent and autocatalytic splicing of bI1 proceed via the same mechanism, despite the fact that protein-dependent splicing relies on the presence of ATP.

The role of pMSS116 in splicing of bI1

A genetic approach has previously shown that bI1 cannot be excised from the primary transcript in yeast strains carrying a mutation in the nuclear gene *MSS116* (12). We have constructed a strain that overexpresses DEAD box protein pMSS116. Northern analysis shows that this gene is transcribed in strain A237/pGU:*MSS116* with considerable efficiency into a stable mRNA. It seems, therefore, that the low abundance of *MSS116* mRNA in the wild-type is due to a low level of transcription, rather than to the instability of the transcript, as discussed earlier (27). The translation product pMSS116 is present in the mitochondrial matrix of strain A237/pGU:*MSS116* as a soluble compound at a considerably increased concentration as compared with the wild-type strain. Data from our *in vitro* splicing assay show that overexpression of *MSS116* significantly enhances protein-dependent splicing of bI1. As in the wild-type lysate, the reaction is dependent on ATP.

A plausible interpretation of these results is that the ATP-dependent splicing activity can be attributed to DEAD box protein pMSS116. This is supported by at least two lines of evidence. First, the splicing activity present in mitochondrial extracts exhibits all the biochemical characteristics observed for DEAD box proteins, namely dependence on ATP and Mg^{2+} (17–20). Secondly, Western analysis has provided evidence that overexpressed pMSS116 is accumulated in the mitochondrial matrix, as already suggested by the putative import sequence at the N-terminus of the protein (12). An indirect influence of pMSS116 on bI1 splicing via mitochondrial translation can be excluded, since splicing of bI1 does not require a mitochondrially encoded maturase (38). Our results, of course, do not preclude the possibility that pMSS116 could possibly be a cytoplasmic translation factor that induces the synthesis of a splicing factor specific for bI1 and other mitochondrial introns. Thus we would like to emphasize that a conclusive elucidation of the role of pMSS116 in splicing of bI1 will require purification of this protein. Addition of the purified component to the mitochondrial lysate should promote splicing of bI1 similarly to the overexpression described in this work.

A model for the interaction between pMSS116 and bI1

Apparently, after transcription only a certain subset of preRNA molecules has the proper tertiary structure required for RNA catalyzed splicing. It has been speculated that several cycles of unfolding and refolding promoted by an RNA helicase activity could increase the proportion of reactive preRNA molecules and thereby the efficiency of the overall reaction. A previous model suggested that pMSS116 might promote splicing of, at least, three mitochondrial group II and group I introns via such an RNA unwinding activity (12). Unwinding of synthetic RNA substrates has been observed for several DEAD box proteins (20–22). However, no increased RNA helicase activity could be detected in extract A237/pGU:*MSS116* as compared with A237/pGU using conditions under which p68 unwinds small synthetic RNA molecules (data not shown). It thus seems unlikely that pMSS116 has a sequence-non-specific RNA unwinding activity.

Further examination of the model included testing the effect of *MSS116* overexpression on *in vitro* splicing of group II intron aI5c. This intron is closely related to bI1 and requires almost identical conditions for self-splicing. Yet we could not detect any effect of pMSS116 overexpression on *in vitro* splicing of aI5c. This result, which is consistent with the genetic data (12), may provide some insight into the mechanism of pMSS116-dependent splicing. Although group II introns bI1 and aI5c have a very similiar secondary (and maybe tertiary) structure, this common feature is obviously not sufficient for interaction with pMSS116, as can be concluded from our experimental results. One rather unlikely explanation could be that pMSS116 acts as an RNA helicase specific for the unwinding of an unknown subdomain of bI1. Such a primary target would have to be common to all group I and group II introns which cannot splice in an *MSS116* mutant (12), but absent from aI5c. Thus a more plausible model would be that pMSS116 can only interact with its RNA substrate(s) due to accessory factors specific for the respective introns. This would be consistent with the hypothesis of an independent development of protein-assisted splicing in different group II introns.

Evolutionary implications

Splicing of group II introns and spliceosomal splicing proceed via the same molecular mechanism. This observation has prompted speculation that group II introns may be the evolutionary ancestors of spliceosomal introns. According to this model, spliceosomal introns gradually developed from group II introns by: (i) becoming increasingly dependent upon *trans*-acting protein factors; (ii) transposition of several formerly *cis*-acting RNA structures into *trans*-acting snRNAs (39). Until now no similarities with respect to *trans*-acting factors have been described. Our results show that splicing of a group II intron depends on ATP and at least one protein belonging to the DEAD box family. While spliceosomal splicing may require far more factors than group II intron splicing, future analysis will answer the intriguing question of whether pMSS116 and PRP proteins belonging to the DEAD/H box family share a similiar function in group II and spliceosomal splicing, respectively.

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