Pentamidine inhibits mitochondrial intron splicing and translation in *Saccharomyces cerevisiae*

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**ABSTRACT**

Pentamidine inhibits in vitro splicing of nuclear group I introns from rRNA genes of some pathogenic fungi and is known to inhibit mitochondrial function in yeast. Here we report that pentamidine inhibits the self-splicing of three group I and two group II introns of yeast mitochondria. Comparison of yeast strains with different configurations of mitochondrial introns (12, 5, 4, or 0 introns) revealed that strains with the most introns were the most sensitive to growth inhibition by pentamidine on glycerol medium. Analysis of blots of RNA from yeast strains grown in raffinose medium in the presence or absence of pentamidine revealed that the splicing of seven group I and two group II introns that have intron reading frames was inhibited by the drug to varying extents. Three introns without reading frames were unaffected by the drug in vivo, and two of these were inhibited in vitro, implying that the drug affects splicing by acting directly on RNA in vitro, but on another target in vivo. Because the most sensitive introns in vivo are the ones whose splicing depends on a maturase encoded by the intron reading frames, we tested pentamidine for effects on mitochondrial translation. We found that the drug inhibits mitochondrial but not cytoplasmic translation in cells at concentrations that inhibit mitochondrial intron splicing. Therefore, pentamidine is a potent and specific inhibitor of mitochondrial translation, and this effect explains most or all of its effects on respiratory growth and on in vivo splicing of mitochondrial introns.

Keywords: intron splicing; mitochondria; pentamidine; translation; yeast

**INTRODUCTION**

Since its introduction, pentamidine has been used for the treatment and prophylaxis of infections by *Pneumocystis carinii* (Ivády & Páldy, 1957; Hughes, 1991) and other eukaryotic microbial pathogens. However, due to the inability to culture *P. carinii* from patients (Sloand et al., 1993), the mechanism of action of pentamidine on this organism remains unknown (reviewed by Queener, 1995). In vitro, pentamidine has been shown to inhibit topoisomerases from *P. carinii* (Dykstra & Tidwell, 1991). In addition, pentamidine is known to bind to the minor groove of duplex DNA (Cory et al., 1992; Nunn et al., 1994). Because gene sequence analysis has shown *P. carinii* to be a fungus related to *Saccharomyces cerevisiae* (Edman et al., 1988), this latter organism is a useful model for studying the mechanism of pentamidine antimicrobial activity (Ludewig et al., 1994). Pentamidine appears to act on a mitochondrial target in *S. cerevisiae*, because growth inhibition was observed at 200-fold lower pentamidine concentrations on nonfermentable glycerol medium than on a fermentable carbon source. However, the actual target of this inhibition was not mitochondrial DNA (mtDNA) maintenance, because petite mutants were generated only by much higher concentrations of pentamidine than are needed to inhibit growth on glycerol (Ludewig et al., 1994). Furthermore, pentamidine inhibits the growth of yeast at much lower concentrations than are required to uncouple oxidative phosphorylation (Ludewig et al., 1994). Such uncoupling has been noted in vitro in rat liver mitochondria, and may explain the toxicity of this drug to animals (Moreno, 1996).

*Pneumocystis carinii* harbors group I introns in its rRNA genes (Edman et al., 1988; Liu et al., 1992; Liu & Leibowitz, 1993). Model transcripts containing these...
introns self-splice in vitro (Sogin & Edman, 1989; Lin et al., 1992; Liu et al., 1992; Liu & Leibowitz, 1993), and their splicing is inhibited by pentamidine (Liu & Leibowitz, 1993, 1995; Liu et al., 1994). Pentamidine inhibits the in vitro splicing catalyzed by these ribozymes non-competitively relative to the guanosine cofactor, with total inhibition being achieved at concentrations of 250 μM (Liu et al., 1994). However, the inability to grow clinical isolates of P. carinii in culture has precluded determination of whether this inhibition accounts for the anti-Pneumocystis activity of the drug. Recently, pentamidine has been shown to inhibit the splicing of a nuclear group I intron in the 25S rRNA gene of another fungal pathogen, Candida albicans, in vitro and in living cells (Miletti & Leibowitz, 2000).

Aminoglycosides, another type of antibiotic, have been demonstrated to inhibit in vitro splicing of the td group I intron of the bacteriophage T4 gene encoding thymidylate synthase (von Ahsen et al., 1991, 1992; von Ahsen & Schroeder, 1991). Those aminoglycosides also inhibit the splicing of the td intron in vivo, but do so indirectly by interfering with translation of the precursor RNA containing this intron (Waldsich et al., 1998). It has been shown that a ribosome function is required to correctly fold the td intron to allow it to splice efficiently (Semrad & Schroeder, 1998).

Many group I introns of organelle genomes contain reading frames, and those introns are much larger in size than are those in eukaryotic nuclear rRNA genes and in prokaryotes (Michel & Westhof, 1990). It is not known if there are inhibitors capable of interfering with the self-splicing of these organelar introns. The yeast mitochondrial genome harbors multiple group I and group II introns, whose splicing is required for respiratory function, in the cytochrome oxidase subunit 1 (COX1) and cytochrome b (COB) genes (reviewed by Pon & Schatz, 1991). Some of these introns can self-splice in vitro (Van der Horst & Tabak, 1985; Gampel & Tzagoloff, 1987; Tabak et al., 1987; Winter et al., 1988; Partono & Lewin, 1988). Therefore, the effects of pentamidine on the splicing of group I and group II introns in vitro and in living cells were studied.

In this report, we show that pentamidine inhibits not only the in vitro splicing of all tested group I and group II intron ribozymes, but also the in vivo splicing of some group I and group II introns. However, the in vitro sensitivity does not correlate with the in vivo sensitivity. All of the group I and group II introns that are sensitive in vivo contain reading frames, most of which encode maturases required for intron splicing. Therefore, we investigated whether pentamidine inhibition of mitochondrial translation in S. cerevisiae might account for splicing inhibition. We report here that pentamidine is a potent and specific inhibitor of mitochondrial protein synthesis in yeast and that it has no detectable effect on the synthesis of nuclear encoded proteins. Thus, we propose that pentamidine acts on yeast mitochondria by interfering with mitochondrial translation and with intron splicing, with the latter effect probably resulting from reduced synthesis of mitochondrial encoded maturases.

**RESULTS**

**Pentamidine inhibits self-splicing of some group I and group II introns**

Mitochondrial DNA of yeast strains contains up to 12 group I introns and up to 4 group II introns (see Fig. 1 for definition of most of those introns). We tested the pentamidine sensitivity of splicing by representative yeast mitochondrial introns that can be assayed in vitro for ribozyme activity. We analyzed group I introns aI3α, aI5α, and bI5 using the self-splicing assay in which unlabeled pre-mRNA is incubated with [α-32P]-GTP and the transfer of label to the 5’ end of the intron–3’ exon intermediate and the linear excised intron RNA is measured (Cech et al., 1981; Zaug & Cech, 1982; Cech, 1994). Figure 2 shows that pentamidine inhibits the self-splicing of all three group I introns in a dose-dependent fashion, with complete inhibition being achieved at a drug concentration of 250 μM.

Self-splicing experiments were also carried out with the group II introns, aI5γ and a shortened version of aI2. As shown in Figure 3, the self-splicing of both introns is substantially inhibited by 100–250 μM pentamidine and completely inhibited by 500 μM (data not shown). These data, which are a subset of data from more extensive time-course experiments, illustrate that pentamidine reduces the rate of self-splicing of each intron in a concentration-dependent fashion. The aI5γ precursor preparation contains a low level of spliced products that was subtracted from each value for rate measurements. Under these incubation conditions, both introns self-splice mostly with branching and exon ligation. In very dark exposures several minor products not characterized previously were detected. Without added drug, the aI5γ construct used is more active in self-splicing under these conditions than is aI2. The apparent first-order rate constant based on accumulation of intron lariat RNA is 0.17/min for aI5γ (with fraction active = 0.72 and R² = 0.975) and 0.009/min for aI2 (with active fraction = 0.33 and R² = 0.99).

**Pentamidine sensitivity of growth correlates with mitochondrial intron content**

Yeast strains with varying intron contents in their mtDNAs (12, 5, 4, or 0 introns) are available (Séraphin et al., 1987; Wenzlau, 1989; Wenzlau et al., 1989). These different mitochondrial genomes were cytoderived into a161 nuclear background to generate isonuclear strains (Table 1). We determined the effects of the drug on cell growth of these strains on the nonfermentable
carbon source glycerol, on which mitochondrial respiratory function is essential. Figure 4 demonstrates that the growth on YPG agar of all of these strains harboring different mitochondrial introns was inhibited by pentamidine in a concentration-dependent fashion. However, the strain having 12 mitochondrial introns (I\*I\textsuperscript{+}I\textsuperscript{II}\textsuperscript{+}) showed much more growth inhibition than did the strains with fewer or no introns; the largest difference was demonstrated at 5 \(\mu\)M pentamidine. This selective growth inhibition of strains containing the most mitochondrial group I and group II introns indicates that the splicing of some or all of these introns is inhibited by the drug. However, the sensitivity of strains with fewer or no introns to higher levels of pentamidine indicates that the drug also inhibits yeast mitochondrial function via some other molecular target. Similar results were obtained in the a161 SUV3-1 nuclear background (Table 1), in which excised group I introns are more stable than in wild-type SUV3 cells (Zhu et al., 1989; Conrad-Webb et al., 1990; Stepien et al., 1992; Margossian et al., 1996).

**Pentamidine selectively inhibits the splicing of mitochondrial introns**

Northern blot analysis of RNA from raffinose grown cells was used to evaluate the effects of pentamidine...
on the levels of mitochondrial transcripts in strain a161 I\(^+\)II\(^+\) (Figs. 5 and 6). As controls for global effects of the drug on RNA metabolism, we determined mRNA levels of four mitochondrial genes that have no introns in this strain (OLI1, OLI2, COX3, and 21S rRNA) and one nuclear gene CYH2, which contains an intron. Drug treatment did not lead to the accumulation of unspliced CYH2 pre-mRNA, nor did it reduce the level of spliced CYH2 mRNA (Fig. 5). Furthermore, it did not obviously decrease the level of the control intronless mitochondrial transcripts (Fig. 5), suggesting that pentamidine is not a general inhibitor of mitochondrial transcription.

In contrast, both the 12.5- and 25-\(\mu\)M concentrations of pentamidine altered the pattern and levels of the COB and COX1 transcripts (Fig. 5). The levels of the fully-spliced mRNAs were reduced and the levels of partially spliced pre-mRNAs were increased, both in a dose-dependent fashion. For the COX1 gene, the level of the main pre-mRNA present in untreated cells barely changed with drug treatment, as even larger pre-mRNAs accumulated. The decrease in COX1 mRNA appears to be greater than the increase in its large pre-mRNAs, perhaps reflecting some instability of these large pre-mRNAs. For the COB gene, only a small amount of pre-mRNA was present in the control cells, and its level, plus that of several larger species increased with drug treatment. These pattern changes are interpreted as resulting from the inhibition of splicing by pentamidine treatment. In addition, the complexity of the pattern of partially spliced pre-mRNAs indicates that the drug inhibits the splicing of a number of introns in each gene. However, it is also evident that the splicing of no single intron is completely inhibited by up to 25 \(\mu\)M pentamidine, because there is still some detectable mRNA at this drug concentration.

Next we identified the specific introns affected by pentamidine using RNA blot analysis with intron-specific probes (Fig. 6). Except for low levels of aI5\(\alpha\) and aI5\(\beta\), most excised group I intron RNAs are not present in the RNA samples from this strain (Fig. 6A), but are readily detected in the untreated SUV3-1 sample (data not shown). Blots with all group I intron-specific probes...
except for bI5 show that pentamidine increases the level of overall intron-containing COX1 or COB pre-mRNAs in a dose-dependent manner (Fig 6A). The extent and pattern of increase vary significantly among these introns. The COX1 pre-mRNAs accumulated the most in response to pentamidine when probing for aI4α, with the larger pre-mRNA species showing the greatest degree of accumulation. The aI3α containing pre-mRNAs also markedly increased, but the level of two small pre-mRNAs decreased. On the other hand, the levels of aI5α or aI5β containing pre-mRNAs increased much less than did those of aI4α or the larger pre-mRNAs containing aI3α. The increase in the level of COB pre-mRNAs was greatest when probing for bI4, and the increase is also clearly demonstrated when probing for bI2 and bI3. Based on accumulation of overall pre-mRNAs in response to pentamidine, these data show that the splicing of those seven group I introns is inhibited by pentamidine, although to different extents.

Pre-mRNAs containing bI5 changed in a different way. Summing over all species, there is no increase in the level of the bI5-containing pre-mRNAs, but there is a marked shift towards larger pre-mRNA species. This shift is presumably due to inhibition of the splicing of other introns of that gene by pentamidine. This blot shows one RNA species (labeled Y in the bI5 panel of Fig. 6A) that is the size of the intron–3’ exon splicing intermediate; the level of this RNA barely changed in response to pentamidine. Therefore, we conclude that bI5 splicing is not sensitive to pentamidine in vivo.

COX1 intron 5α exhibits a different response to the drug. As noted above, the level of pre-mRNAs containing that intron does not increase much in the drug-treated samples. However, the level of a minor RNA in control samples, denoted as X on the aI5α blot (Fig. 6A), is increased markedly by the drug treatment. That RNA species is the size of the intron–3’ exon splicing intermediate; so the presence of that band suggests that the drug inhibits the second splicing reaction much more than it inhibits the first reaction.

Only two excised group I intron RNAs, aI5α and aI5β, were detectable in the suv3 wild-type strain. The level of the excised aI5α markedly decreased in response to pentamidine treatment, consistent with the inhibition of aI5α splicing. However, the level of the excised aI5β markedly increased in response to pentamidine. That increase may indicate that pentamidine stabilizes the...
excised aI5β, because the level of the aI5β containing pre-mRNAs was increased by the drug treatment.

Figure 6B shows the effects of pentamidine treatment on the splicing and excised intron RNA levels of the four group II introns present in the IⅡ+ strain. Splicing of aI1 and especially aI2 are inhibited by drug treatment, based on increased levels of intron-containing pre-mRNAs and decreased levels of excised intron RNAs. Splicing of aI5γ and bI1 was not affected by in vivo pentamidine treatment. Drug treatment somewhat increased the level of excised aI5γ and bI1 RNAs and did not lead to accumulation of pre-mRNAs containing those introns.

A strain carrying IⅡ+ mtDNA and the SUV3-1 mutation was analyzed in parallel, and the accumulation of COX1 and COB pre-mRNAs was similarly demonstrated in response to pentamidine (data not shown). The data obtained with strains containing fewer introns (5 or 4 shown in Fig. 1) confirmed that splicing of aI4α and bI4 is strongly inhibited by 25 μM pentamidine, that splicing of aI3α was less inhibited, and that splicing of aI5γ and bI5 is not inhibited (data not shown).

**Pentamidine inhibits mitochondrial translation**

We have demonstrated that pentamidine inhibits the splicing of group I and group II introns both in vitro and in vivo. All of the tested self-splicing introns were sensitive to pentamidine in vitro. The group I introns (aI3α, aI5α, and bI5) were quantitatively more sensitive than the group II introns (aI2 and aI5γ); introns of the same type showed similar sensitivity. However, the in vivo analysis showed that aI3α, aI5α, and aI2 are sensitive to pentamidine, whereas bI5 and aI5γ are resistant. Thus, the in vitro inhibition by pentamidine is not fully consistent with the in vivo findings. It has been reported that a ribosomal function is required for correct folding and efficient splicing of the group I td intron.

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**TABLE 1. S. cerevisiae strains.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/Description</th>
<th>Mitochondrial genome</th>
<th>Nuclear genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID41-6/161a</td>
<td>MATa ade1 lys1</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161</td>
</tr>
<tr>
<td>a161U7</td>
<td>MATa ade1 lys1 ura3</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161-U7</td>
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<tr>
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<td>MATa ade1</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161-lys+</td>
</tr>
<tr>
<td>a161GII-5yc</td>
<td>MATa ade1 lys1</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161</td>
</tr>
<tr>
<td>a161 SUV3-1</td>
<td>MATa ade1 lys1 SUV3-1</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161 SUV3-1</td>
</tr>
<tr>
<td>a161</td>
<td>EtBr cured a161GII-5y</td>
<td>ρ0</td>
<td>a161</td>
</tr>
<tr>
<td>a161 SUV3-1</td>
<td>EtBr cured a161 SUV3-1</td>
<td>ρ0</td>
<td>a161</td>
</tr>
<tr>
<td>a161 IⅡ+</td>
<td>a161 ← M497 IⅡ+</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161</td>
</tr>
<tr>
<td>a161 IⅡ5y</td>
<td>a161 ← M497 IⅡ5y</td>
<td>ρ+(l’Ⅱ)+</td>
<td>a161</td>
</tr>
<tr>
<td>a161 IⅡ0</td>
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<td>ρ+(l’Ⅱ)+</td>
<td>a161</td>
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<tr>
<td>a161 IⅡ0</td>
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<td>ρ+(l’Ⅱ)+</td>
<td>a161 SUV3-1</td>
</tr>
</tbody>
</table>

References for the original yeast strains:
- *Wenzlau et al., 1989;*  
- *Conrad-Webb & Butow, 1995;*  
- *Seraphin et al., 1987;*  
- *Podar et al., 1998;*  
- *Wenzlau, 1989;*  
- *Wenzlau et al., 1997;*  
- *Podar, 1997;*  

<sup>a</sup> indicates the direction of the mitochondrial transfer by cytoduction.
and in vivo inhibition of td intron splicing by various aminoglycoside antibiotics occurs secondarily to translation inhibition by these drugs (Waldsich et al., 1998). Among 12 group I and group II introns analyzed in this study, only the three mitochondrial introns containing no ORF (aI5g, bI1, and bI5) are resistant to pentamidine inhibition in vivo. This result suggests that in vivo inhibition of intron splicing by pentamidine may be secondary to an effect of this drug on translation.

To avoid possible secondary effects of splicing inhibition on translation, the intronless strain a161 I1 II1 was used to investigate whether pentamidine inhibits mitochondrial translation. First, western blot analysis was used to determine the effect of pentamidine on the levels of some proteins in raffinose grown yeast cells. Figure 7 shows that 12.5–25 μM pentamidine treatment during 4–5 cell doublings resulted in almost complete depletion of the steady-state levels of the mitochondrial CO2 and CO3, but had no effect on the levels of the nuclear gene encoded proteins S4 and L32 (ribosomal proteins), and porin (mitochondrial outer membrane protein).

Selective reduction of mitochondrial CO2 and CO3 levels by pentamidine could result from inhibition of translation or from accelerated protein degradation in mitochondria. These two choices were then discriminated by 35S incorporation experiments. The effect of
pentamidine on mitochondrial protein synthesis was determined in yeast cells treated with cycloheximide to eliminate the interfering signal of cytoplasmic translation. Erythromycin, a known inhibitor of yeast mitochondrial translation, was included as a control. Figure 8A, lanes 1–4, shows that in the absence of cycloheximide, nearly all $^{35}$S incorporation was into nuclear encoded proteins and up to 250 $\mu$M pentamidine had no effect.
Pentamidine inhibits RNA splicing and translation

**FIGURE 7.** Pentamidine specifically reduces mitochondrial protein levels. Cells of intronless strain a161 Pil 
were grown in the absence or presence of 12.5 and 25 μM pentamidine as in Figure 4. Six hours of growth resulted in four doublings, and 8 h resulted in five doublings. A nitrocellulose blot of a denaturing polyacrylamide gel of whole-cell extracts was prepared as described in Material and methods, and was probed with primary antibodies to nuclear encoded proteins L32 and porin (A), and to mitochondrial encoded proteins CO2 and CO3 (B), sequentially. CO2 and CO3 correspond to the products of the COX2 and COX3 genes, respectively. The polyclonal antibody to ribosomal protein L32 is known to cross-react with ribosomal protein S4 (Vilardell & Warner, 1997).

DISCUSSION

**Pentamidine inhibits self-splicing of mitochondrial introns**

This report demonstrates that pentamidine inhibits the self-splicing of both group I and group II mitochondrial intron ribozymes. Pentamidine is the first chemical agent that affects the self-splicing of group II introns. Sensitivity to pentamidine was measured following preincubation with the drug at the reaction temperature followed by starting the reactions by adding the cations Mg2+ and NH4+ as indicated. The group I introns al3+α, al5α, and bl5 were all comparably sensitive to pentamidine in vitro with complete inhibition achieved at 125–250 μM. Complete inhibition of the self-splicing of the group II introns al5γ and al2 was achieved at 500 μM. We have confirmed that preincubation without cations increases the extent of drug sensitivity of all the tested introns up to tenfold (Zhang, 1999; data not shown). The previous report that self-splicing of al5γ was resistant to 500 μM pentamidine (Liu et al., 1994) was based on assays in which the RNAs were not preincubated with the drug.

It is likely that pentamidine and cations may compete for binding sites on the introns, because we notice an inverse relationship between the sensitivity to pentamidine and the concentration of cations in the self-splicing buffers. For example, the nuclear group I introns, which splice optimally in 0.4 mM spermidine and 1.25–5 mM Mg2+ (Liu et al., 1994; Milette & Leibowitz, 2000) are more sensitive to pentamidine than is any of the three yeast group I introns, which need higher levels of cations (50 mM Mg2+ and 200 mM NH4+) (Zhang, 1999). The group II introns require the highest level of pentamidine for complete inhibition of self-splicing and they are usually assayed with even higher cation levels (100 mM Mg2+ and 1–2 M NH4+) (this article; A. Bell, unpubl.).

**Pentamidine inhibits mitochondrial function and intron splicing in vivo**

This report demonstrates that a low concentration of pentamidine selectively inhibits the growth on glycerol medium of yeast strains containing 12 mitochondrial introns relative to strains with fewer or no introns. However, somewhat higher concentrations of the drug inhibit respiratory growth of the strain with no mitochondrial introns, indicating that the drug may have several molecular targets. RNA blot experiments showed that pentamidine treatment inhibits splicing of all group I introns except for bl5, although the extent of inhibition greatly varies among these introns. Splicing of group II introns al1 and al2 was inhibited, but introns al5γ and b11 were completely unaffected. These in vivo data contrast strikingly with the in vitro data, where no differential sensitivity to pentamidine was observed for
group I introns (al3α, al5α, and bl5) or group II introns (al5γ and al2), indicating that the in vitro and in vivo mechanisms of inhibition are different. Although most of the group I introns analyzed in vivo do not self-splice, and so could not be analyzed under both conditions, we noticed that the most pentamidine-sensitive introns were those that are known to depend on an intron-encoded maturase for their splicing (Lambowitz et al., 1999). This pattern of inhibition and sensitivity suggested that the in vivo splicing defects could be a secondary consequence of pentamidine interfering with either the maturase proteins or with mitochondrial translation.

Pentamidine inhibition of mitochondrial translation explains most of its inhibition of mitochondrial intron splicing

Direct experiments determined that mitochondrial translation is a sensitive target for pentamidine and that most of the in vivo effects of the drug on both group I and group II intron splicing are explained by the drug inhibiting the synthesis of intron-encoded maturases. Assays of translation with 35S-labeled methionine and cysteine showed that the concentrations of drug used in the RNA blot experiments strongly inhibit mitochondrial translation. No inhibition of translation on cytoplas-
mic ribosomes was detected even at a much higher level of the drug (250 μM). We show that pentamidine at 1 μM inhibited mitochondrial translation to about the same extent as did 2.7 mM (2 mg/mL) erythromycin, a well-known drug that binds mitochondrial ribosomes and inhibits mitochondrial translation in these strains. The present study identifies the major target of pentamidine inhibition of mitochondrial function under these conditions, but our findings do not identify a specific mechanism of translation inhibition. It is interesting to note that many of the known inhibitors of group I intron self-splicing (von Ahlsen & Schroeder, 1991; von Ahlsen et al., 1991, 1992; Liu et al., 1994; Wank et al., 1994) and now pentamidine are translation inhibitors. Mitochondrial translation is a much more sensitive target of this drug than is mitochondrial intron splicing. Translation is strongly inhibited after only 35 min of drug treatment, whereas splicing was only partially inhibited after hours of treatment. The smaller or delayed effect on splicing indicates that the maturases present at the time of drug addition are probably turned over relatively slowly.

However, inhibition of synthesis of maturases may not completely explain the different levels of inhibition of splicing of introns with reading frames. It has been demonstrated that ribosomal function is required for the correct folding of the tI intron of phage T4 (Semrad & Schroeder, 1998). It is possible that a similar phenomenon may be present in the yeast system. The best candidate for such an effect is aI3x, because its reading frame does not code for a maturase (Guo et al., 1995), but its splicing was partially inhibited by the drug in vivo. Splicing of intron aI5b was only slightly inhibited by the drug and it does not code for a maturase either (Johnson & McEwen, 1997).

It was surprising that pentamidine inhibits the second step of aI5α splicing much more than the first step, leading to the in vivo accumulation of the intron–downstream exon splicing intermediate. That intron has an open reading frame that is known to code for a DNA endonuclease of the LagLI-DADG family (Moran et al., 1992; Séraphin et al., 1992) and indirect evidence suggests that it does not code for a maturase (Moran et al., 1992). The inhibition by pentamidine of only half of the aI5α splicing reaction is not likely due to a direct effect of the drug on aI5α RNA, because we did not observe a strong second-step defect in the self-splicing experiments. Instead, it is possible that the aI5α-encoded protein is a splicing factor that, unlike the other group I intron maturases, is mainly needed for the second splicing reaction. If so, then the accumulation of the splicing intermediate would result from the translation defect caused by the drug.

Previous studies of mutations of several introns that inhibit the second step of splicing showed that the downstream product accumulates in vivo, whereas the upstream product is degraded (Lamb et al., 1983; Peebles et al., 1993). That situation is present here as well, with the downstream product of partial splicing of aI5α being readily detected, whereas the signal for RNAs containing the upstream sequences is very weak. In the RNA blot experiments we had noted that pentamidine reduces the level of fully spliced COXI mRNA much more than it elevates the level of pre-mRNAs. We suggest that the inhibition of aI5α second-step splicing by pentamidine may be the cause of the relative instability of those RNAs unspliced for introns upstream of aI5α.

It has previously been demonstrated that pentamidine acts on an unidentified mitochondrial target to inhibit the glycerol growth of S. cerevisiae (Ludwig et al., 1994). Our results show that pentamidine inhibits yeast mitochondrial translation potently and specifically. This effect and inhibition of nuclear group I intron splicing in C. albicans (Miletti & Leibowitz, 2000) are two apparent direct effects of pentamidine on yeast cells. Because P. carinii is a pathogenic fungus related to S. cerevisiae and C. albicans, the mechanism of pentamidine action on P. carinii may also involve interference with mitochondrial translation and nuclear group I intron splicing (Edman et al., 1988; Sogin & Edman, 1989; Lin et al., 1992; Liu et al., 1994; Liu & Leibowitz, 1993).

MATERIALS AND METHODS

Yeast strains and growth conditions

The S. cerevisiae strains used for these studies are listed in Table 1. Figure 1 shows a diagram of the COX1 and COB genes of the strains used here. The figure indicates exons, introns, and the open reading frames found within some of the introns, as well as the locations of DNA probes and in vitro RNA transcripts used in this work. Note that the T-I10 mitochondrial genome is a hybrid of mitochondrial DNA from three other yeast species and none of its sequences originated from the T-I1-I2 strain (Wenzlau et al., 1989). The T-I1-I2 is identical to the T-I10 DNA except for the addition of aI5γ (derived from strain D273; Wenzlau, 1989). The T-I10 mitochondrial genome is also from a different source than the other three mitochondrial genomes (Séraphin et al., 1987).

For RNA preparation, cells were grown in liquid YPR medium (1% yeast extract, 2% peptone, 2% raffinose) culture at 240 rpm overnight at 30°C, and then diluted to 0.1 OD600 with fresh YPR medium and grown at 30°C to mid-log phase (about four doubling times) for each experiment. Pentamidine isethionate (Sigma Chemical Co.) was added to the flasks at the time of dilution, and growth was continued as indicated. Aliquots were removed at indicated times for preparation of RNA. Pentamidine sensitivity assays for all isonuclear strains were performed on solid YPG medium (1% yeast extract, 2% peptone, 3% glycerol, 2% agar) containing the indicated concentrations of pentamidine isethionate.

Cytoduction

Isonuclear strains containing mitochondrial genomes with different intron configurations were generated by cytoduction
(Kaiser et al., 1994). The \( p^\beta \) strains were made by growing the \( p^\beta \) parental strains in rich dextrose medium containing ethidium bromide (30 \( \mu \)g/mL) in the dark for 3 days. Four types of mtDNA with different introns (Table 1) were first cytotoxically introduced into strain M497 (\( M\Delta T \)a his4–15 karl–1 can1 \( p^\beta \)), then cytotoxically introduced into \( M\Delta T \)a nuclear backgrounds a161 and a161 SUV3-1 (Table 1).

**RNA preparation and analysis**

Cell pellets were obtained by centrifugation (3,000 \( \times \) g, 10 min, room temperature) and stored at \(-20^\circ\)C for RNA extraction. Total yeast RNA was isolated as described previously (Hagen et al., 1995), and extracted RNA was dissolved in 40 \( \mu \)L DEPC-treated water and stored at \(-70^\circ\)C. RNA concentration was determined by OD \( 260 \) from yeast cultures that were denatured at 65 \( ^\circ\)C for 5 min and fractionated on a 1.2\% agarose-6\% formaldehyde gel for each experiment. RNA integrity and quantity were monitored by the intensity of cytoplasmic rRNA bands detected by ultraviolet fluorescence after gel electrophoresis with ethidium bromide in the loading buffer. RNA was transferred onto Hybrid Bond N\(^+\) nylon membranes by the capillary transfer method and fixed onto membranes by UV crosslinking. Each new RNA membrane was prewashed in 0.1\% SDS, 0.1\% SSC at 55 \( ^\circ\)C for 15 min, whereas stripped membranes (see below) were used for hybridization directly. Hybridization of DNA probes (10\(^7\) cpm, see below) was carried out either with or without formamide (Sambrook et al., 1989). The method without formamide gave less carry-over of probe after stripping. In the formamide method, 5\( \times \) SSPE, 2\( \times \) Denhardt's reagent, 50\% formamide, 1\% SDS, 5\% dextran sulfate and salmon sperm DNA (100 \( \mu \)g/mL) were included in the prehybridization and hybridization buffers, and at least 4-h prehybridization and overnight hybridization were carried out at 42 \( ^\circ\)C. The prehybridization and hybridization conditions in the method without formamide were exactly as described by Sambrook et al. (1989). Hybridized membranes were exposed to Kodak X-Omat or DuPont NEN Reflection film at \(-40^\circ\)C.

RNA membranes generated from a given RNA preparation were hybridized with different probes to obtain directly comparable results. After each hybridization, probe was removed by pouring boiling 0.1\% SDS solution onto the membrane and allowing it to cool to room temperature prior to the next hybridization. Although the stripping was not always 100\% efficient, the hybridization order of different probes was designed so that carry-over did not affect the visualization of the newly probed bands.

RNA bands on Northern blots (exposures not saturated) were scanned using GS-670 Imaging Densitometer (BioRad), and the quantitation of RNA bands was based on these images analyzed with the Molecular Analyst program.

**Preparation of radioactive probes**

DNA fragments were generated either by restriction digestion or PCR amplification, and \([\alpha-\text{\( ^{32} \)}\text{P}]-\text{dCTP} (3,000 \( \text{Ci/mmole DuPont Inc.} \)) was incorporated by the random primer method (Boehringer Mannheim), following the manufacturer’s instructions or by a PCR labeling method according to a protocol from Boehringer Mannheim. The PGK1 and CYH2 probes were prepared by the random primer DNA labeling method. The PGK1 probe was a 0.7-kb \text{Styl}-digested DNA fragment and CYH2 probe was a 0.6-kb EcoRI- and HindIII-digested DNA fragment of the respective genes. PCR products used as DNA probes were labeled by either random priming or PCR labeling. All PCR-generated probes were as indicated in Table 2. Radioactive DNA fragments were purified by Quick Spin Column (Sephadex G-50 (fine)) (Boehringer Mannheim) according to the manufacturer’s instructions and quantitated by liquid scintillation spectrometry.

**In vitro splicing assays**

**Self-splicing of group I introns**

PCR amplified DNA fragments (represented by dotted lines in Fig. 1) with a T7 promoter at their 5’ ends were transcribed (Liu et al., 1994) by T7 RNA polymerase (Promega) in the presence of either 500 \( \mu \)M rNTPs for nonradioactive precursors or 500 \( \mu \)M of rATP, rCTP, and rGTP, 200 \( \mu \)M of rUTP, and 10 \( \mu \)Ci [\( \alpha-\text{\( ^{32} \)}\text{P}]-\text{UTP} (3,000 Ci/mmole, NEN-DuPont Inc.) for radioactive precursor preparation, under recommended conditions. Primers were selected with coordinates 4964–7177 for \( \alpha_{3\alpha} \) (Bonitz et al., 1980), 7964 (Bonitz et al., 1980) –1718 (Hensgens et al., 1983) for \( \alpha_{5\alpha} \), and 1769–3297 for \( b_{15} \) (Nobrega & Tzagoloff, 1980). The precursors generated from PCR templates with these primers contain

<table>
<thead>
<tr>
<th>Probe*</th>
<th>Length (bp)</th>
<th>Coordinates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLI1</td>
<td>201</td>
<td>195–395</td>
<td>Hefta et al., 1987</td>
</tr>
<tr>
<td>COX3</td>
<td>595</td>
<td>413–819</td>
<td>Thalenfeld &amp; Tzagoloff, 1980</td>
</tr>
<tr>
<td>21S rRNA</td>
<td>351</td>
<td>3330 ... 4822(^b)</td>
<td>Sor &amp; Fukuhara, 1983</td>
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<td>960</td>
<td>6858 ... 9701(^b)</td>
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<tr>
<td>COB</td>
<td>793</td>
<td>371 ... 3297(^b)</td>
<td>Nobrega &amp; Tzagoloff, 1980</td>
</tr>
<tr>
<td>aI1</td>
<td>390</td>
<td>175–564</td>
<td>Bonitz et al., 1980</td>
</tr>
<tr>
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<td>548</td>
<td>2659–3206</td>
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<td>296</td>
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<td>858</td>
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<td>456</td>
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<td>753</td>
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<td>504</td>
<td>8805–9308</td>
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<td>744</td>
<td>–1624 ... (–881)</td>
<td>Lazowska et al., 1980</td>
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<td>646</td>
<td>–580–65</td>
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<td>494</td>
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<td>644</td>
<td>802–1445</td>
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<tr>
<td>bI5</td>
<td>592</td>
<td>2314–2905</td>
<td>Nobrega &amp; Tzagoloff, 1980</td>
</tr>
</tbody>
</table>

*Gene probes are exons; intron probes do not contain exon sequences.

*These probes do not include intron sequences within the listed coordinates and were generated from templates from intronless strains.
each full-length intron (al3α: 1514 nt, al5α: 1366 nt, and bl5: 730 nt) and 5′ and 3′ exons: 244 and 454 nt for al3α, 514 and 353 nt for al5α, and 459 and 240 nt for bl5. Unincorporated nucleotides and salts were removed from RNA samples by passage through Sephadex G-50 columns, as described above, following the transcription reaction. The radioactive RNA transcripts were fractionated on a 3.5% PAGE-M urea gel and the homogeneous precursor band was recovered from the gel (Liu & Leibowitz, 1993). Prior to the self-splicing reaction, 100–200 ng of nonradioactive precursor was preincubated with the indicated concentrations of pentamidine in 16 μL of 62.5 mM Tris-HCl (pH 7.5) containing 10 U of RNAse (Promega) at 42 °C (al3α and al5α) or 37 °C (bl5) for 20 min. Then the splicing was started by addition of 4 μL of a reaction mix containing splicing components MgCl2, (NH4)2SO4, and 10 μCi (al3α and al5α) or 30 μCi (bl5) of [α-32P]-GTP (3,000 Ci/mm, NEN-Dupont Inc.). The cation concentrations in splicing reactions are 50 mM MgCl2 and 100 mM (NH4)2SO4. Incubation at 42 °C (al3α and al5α) or 37 °C (bl5) was continued for 2 h and the unincorporated [α-32P]-GTP in reactions with nonradioactive precursor was removed by passing the samples over a Sephadex G-50 column. The excluded volume, containing the RNAs, was concentrated prior to electrophoretic analysis. The radio-labeled RNA precursor containing bl5 (5 × 103 cpm per reaction) was preincubated and assayed for self-splicing, as above, except that the preincubation was in 8 μL Tris buffer and 2 μL of reaction mix containing nonradioactive GTP (2 mM) was added after preincubation. The splicing products were fractionated on a 3.5% PAGE-M urea gel that was dried and visualized as described (Liu & Leibowitz, 1993).

Self-splicing of group II introns

Model pre-mRNAs containing al5γ or al2 were obtained as T7 RNA polymerase (Boehringer Mannheim) transcripts of plasmid pJD20, following digestion with HindIII, and T3 RNA polymerase (Boehringer Mannheim) transcripts of plasmid pSZD2 following digestion with BstEII, respectively. The al5γ transcript is 1,497 nt long and contains the entire 887-nt-long intron plus 5′ and 3′ exons of 292 and 318 nt, respectively (see Jarrell et al., 1988). The al2 transcript is 1,678 nt long and contains a 1,204-nt-long intron (deleted for 1,313 nt of the intron reading frame of domain 4) and 5′ and 3′ exons of 441 and 33 nt respectively. Plasmid pSZD2 was constructed along the lines described for pSZD1 (which was deleted for 1.55 kb of the intron) (Zimmerly et al., 1995) and was provided by Steven Zimmerly and Alan Lambowitz. Transcriptions contained 3 mM rCTP, rGTP, rATP, and rUTP including 0.2 μCi [32P]-UTP (3,000 Ci/mm, Amersham). Transcripts were gel purified and stored in pH 7.5 buffer containing 40 mM Tris-HCl and 20 mM EGTA until used. Self-splicing was carried out in 40 mM Tris-HCl, 100 mM MgSO4, and 1 M (NH4)2SO4 at pH 7.5 and 42 °C. RNA samples were diluted in 10 μL 60 mM Tris-HCl (pH 7.5) with various concentrations of pentamidine for a 5-min preincubation at 42 °C before addition of 5 μL of MgSO4 and (NH4)2SO4 solution to start the reaction. The reacted samples were fractionated on a 4% PAGE-M urea gel, analyzed by PhosphorImager scanning, and the effects of the inhibitor on the reaction kinetics determined as described by Franzen et al. (1994).

Western blot analysis

Cells were collected by centrifugation for 5 min at 5,000 rpm. Whole-cell extracts were prepared by glass bead lysis and separated by SDS-PAGE and transferred to nitrocellulose membranes as described (Carr-Schmid et al., 1999). Membranes were probed with monoclonal antibodies to porin, CO2 and CO3 (Molecular Probes Inc.), and polyclonal antibody to L32 (1:2,000 dilution; Vilardell & Warner, 1997), followed by detection with ECL chemiluminescence kit (Amersham Inc.). For each experiment, the same membrane was treated for reprobing following manufacturer’s instruction (Amersham Inc.).

In vivo labeling of mitochondrial translation products

Cells of the intronless strain a161 I′II′ were grown at 30 °C to mid-log phase as described above. Cells harvested from a 10-mL culture were washed with distilled water and then with 40 mM potassium phosphate buffer, pH 7.4, containing 0.45% raffinose. Cells were then resuspended in 2 mL of 40 mM potassium phosphate buffer, pH 7.4, containing 0.45% raffinose (McKee et al., 1984). Incubation at 30 °C was begun with no treatment or with addition of erythromycin or pentamidine, and was continued for 15 min, and then cycloheximide was added followed by a 10-min incubation. Then 100 μCi of Easy Tag Expression-[35S] protein labeling mix containing 73% L-[35S] methionine and 22% L-[35S] cysteine (1,175.0 Ci/mmol, Dupont Inc.) was added and incubation was continued for 10 min. Incorporation of label was terminated by the addition of 0.1 vol of cold 0.2 M methionine. Whole-cell extracts were prepared by glass bead lysis as for western analysis (Carr-Schmid et al., 1999), except that the labeled cells were washed once with distilled water before lysis. The same whole-cell extracts were either analyzed directly on 12% SDS-PAGE for western blot analysis of the steady-state protein levels or concentrated by TCA precipitation (Rosenberg, 1996). TCA-precipitated whole-cell extracts were then analyzed on 12% SDS-PAGE, dried, and exposed against X-ray film to detect the labeled translation products.

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Y. Zhang et al.

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