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

YI ZHANG,¹ ACHIM BELL,² PHILIP S. PERLMAN,² and MICHAEL J. LEIBOWITZ¹

¹Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School and Cancer Institute of New Jersey, Piscataway, New Jersey 08854-5635, USA

²Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148, USA

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 in 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 *Sac-*

charomyces 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

Reprint requests to: Dr. Michael J. Leibowitz, Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854-5635, USA; e-mail: leibowit@umdnj.edu.

Abbreviation: mtDNA: mitochondrial DNA.

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 μ M (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 organellar 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 mitochondrially 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 *al3 α* , *al5 α* , and *bl5* 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 μ M.

Self-splicing experiments were also carried out with the group II introns, *al5 γ* and a shortened version of *al2*. As shown in Figure 3, the self-splicing of both introns is substantially inhibited by 100–250 μ M pentamidine and completely inhibited by 500 μ M (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 *al5 γ* 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 *al5 γ* construct used is more active in self-splicing under these conditions than is *al2*. The apparent first-order rate constant based on accumulation of intron lariat RNA is 0.17/min for *al5 γ* (with fraction active = 0.72 and R^2 = 0.975) and 0.009/min for *al2* (with active fraction = 0.33 and R^2 = 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 (Sraphin et al., 1987; Wenzlau, 1989; Wenzlau et al., 1989). These different mitochondrial genomes were cytoduced 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

FIGURE 1. Intron configuration in the *COX1* and *COB* genes of the four *S. cerevisiae* mitochondrial genomes (I^+II^+ , $I^+II^{5\gamma}$, I^+II° , and $I^\circ II^\circ$) used in this study. These genomes contain 12, 5, 4, and 0 introns, respectively, with group I and group II introns designated by different shading. None of these mtDNAs has the omega (ω) intron in its 21S rRNA gene. All DNA fragment probes were PCR amplified and are intron or exon specific (see Materials and Methods and Table 2). Their lengths and positions on the intron or exon sequences are indicated by solid black lines. DNA probes for introns are indicated on both intron-containing maps. Exon probes are indicated on the $I^\circ II^\circ$ map. The length and map position of the in vitro-transcribed precursors containing each self-splicing intron are indicated by dotted lines.

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^+II^+) showed much more growth inhibition than did the strains with fewer or no introns; the largest difference was demonstrated at 5 μ 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 in-

trons 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

FIGURE 2. Pentamidine inhibits the splicing of mitochondrial group I introns in vitro. Model pre-mRNAs containing aI3 α , aI5 α , or bI5 and flanking exons were prepared by in vitro transcription and analyzed for self-splicing as described in Materials and methods. Radiolabeled RNA ($\sim 5 \times 10^3$ cpm of lane 0* of bI5) or 100–200 ng of unlabeled RNA (all other lanes) were preincubated with or without the amount of pentamidine indicated for 20 min and then the reactions were initiated by addition of MgCl_2 , $(\text{NH}_4)_2\text{SO}_4$, and GTP (or $[\alpha\text{-}^{32}\text{P}]\text{-GTP}$). Samples were reacted for 2 h, terminated, and analyzed as described (see Materials and methods). Positions of major products are indicated. P: radiolabeled bI5 precursor; I-3'E: the splicing intermediate intron ligated with 3'E; 5'E-3'E: ligated exons; I: excised full-size intron; 5'I: the 5' portion of aI3 α generated by an internal cleavage of this intron; 3'I: the 3' portion of aI3 α generated by the same internal cleavage of this intron (Tabak et al., 1987); and I*: possibly a portion of bI5 intron.

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- μM 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 μM pentamidine, because there is still some detectable mRNA at this drug concentration. The level of *COB* mRNA was reduced less than that of *COX1*, perhaps because this *COB* gene has fewer introns.

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 α and aI5 β , 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

FIGURE 3. Pentamidine inhibits the self-splicing of two group II introns. Radiolabeled model pre-mRNA transcripts prepared as described in Materials and methods were preincubated for 5 min at 42°C in 60 mM Tris-Cl (pH 7.5) with the indicated concentrations of pentamidine, followed by reactions for the indicated times initiated by adding MgSO_4 and $(\text{NH}_4)_2\text{SO}_4$ to the standard concentrations. **A:** Data using al5 γ transcripts. **B:** Data using al2 transcripts. Alongside lane 1 in each panel the identities of the major signals are denoted by diagrams of the RNA species (from top to bottom: intron lariat, precursor RNA, excised linear or broken lariat, spliced exons, and free upstream exon [al2 only]). The length of each RNA species based on the known DNA sequence of the template is shown to the right of lane 12 in each panel.

except for bl5 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 al4 α , with the larger pre-mRNA species showing the greatest degree of accumulation. The al3 α containing pre-mRNAs also markedly increased, but the level of two small pre-mRNAs decreased. On the other hand, the levels of al5 α or al5 β containing pre-mRNAs increased much less than did those of al4 α or the larger pre-mRNAs containing al3 α . The increase in the level of *COB* pre-mRNAs was greatest when probing for bl4, and the increase is also clearly demonstrated when probing for bl2 and bl3. 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 bl5 changed in a different way. Summing over all species, there is no increase in the level of the bl5-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 bl5 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 bl5 splicing is not sensitive to pentamidine in vivo.

COX1 intron 5 α 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 al5 α 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, al5 α and al5 β , were detectable in the *suv3* wild-type strain. The level of the excised al5 α markedly decreased in response to pentamidine treatment, consistent with the inhibition of al5 α splicing. However, the level of the excised al5 β markedly increased in response to pentamidine. That increase may indicate that pentamidine stabilizes the

TABLE 1. *S. cerevisiae* strains.

Strain	Genotype/Description	Mitochondrial genome	Nuclear genome
ID41-6/161 ^a	<i>MATa ade1 lys1</i>	$\rho^+(I^+II^+)^a$	a161
a161U7 ^b	<i>MATa ade1 lys1 ura3</i>	$\rho^+(I^0II^0)^c$	a161-U7
a161GII-0 ^a	<i>MATa ade1</i>	$\rho^+(I^+II^0)^a$	a161-lys+
a161GII-5 γ ^d	<i>MATa ade1 lys1</i>	$\rho^+(I^+II^{5\gamma})^e$	a161
a161 <i>SUV3-1</i> GII-5 γ ^f	<i>MATa ade1 lys1 SUV3-1</i>	$\rho^+(I^+II^{5\gamma})^e$	a161 <i>SUV3-1</i>
a161	EtBr cured a161GII-5 γ	ρ^0	a161
a161 <i>SUV3-1</i>	EtBr cured a161 <i>SUV3-1</i> GII-5 γ	ρ^0	a161 <i>SUV3-1</i>
a161 I^+II^+	a161 $\leftarrow^g$ M497 I^+II^+	$\rho^+(I^+II^+)$	a161
a161 $I^+II^{5\gamma}$	a161 $\leftarrow$ M497 $I^+II^{5\gamma}$	$\rho^+(I^+II^{5\gamma})$	a161
a161 I^+II^0	a161 $\leftarrow$ M497 I^+II^0	$\rho^+(I^+II^0)$	a161
a161 I^0II^0	a161 $\leftarrow$ M497 I^0II^0	$\rho^+(I^0II^0)$	a161
a161 <i>SUV3-1</i> I^+II^+	a161 <i>SUV3-1</i> $\leftarrow$ M497 I^+II^+	$\rho^+(I^+II^+)$	a161 <i>SUV3-1</i>
a161 <i>SUV3-1</i> $I^+II^{5\gamma}$	a161 <i>SUV3-1</i> $\leftarrow$ M497 $I^+II^{5\gamma}$	$\rho^+(I^+II^{5\gamma})$	a161 <i>SUV3-1</i>
a161 <i>SUV3-1</i> I^+II^0	a161 <i>SUV3-1</i> $\leftarrow$ M497 I^+II^0	$\rho^+(I^+II^0)$	a161 <i>SUV3-1</i>
a161 <i>SUV3-1</i> I^0II^0	a161 <i>SUV3-1</i> $\leftarrow$ M497 I^0II^0	$\rho^+(I^0II^0)$	a161 <i>SUV3-1</i>

References for the original yeast strains:

^aWenzlau et al., 1989;

^bConrad-Webb & Butow, 1995;

^cSeraphin et al., 1987;

^dPodar et al., 1998;

^eWenzlau, 1989;

^fPodar, 1997.

^g $\leftarrow$ indicates the direction of the mitochondrial transfer by cytoduction.

excised $\text{al}5\beta$, because the level of the $\text{al}5\beta$ 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 I^+II^+ strain. Splicing of $\text{al}1$ and especially $\text{al}2$ are inhibited by drug treatment, based on increased levels of intron-containing

pre-mRNAs and decreased levels of excised intron RNAs. Splicing of $\text{al}5\gamma$ and $\text{bl}1$ was not affected by in vivo pentamidine treatment. Drug treatment somewhat increased the level of excised $\text{al}5\gamma$ and $\text{bl}1$ RNAs and did not lead to accumulation of pre-mRNAs containing those introns.

A strain carrying I^+II^+ 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 $\text{al}4\alpha$ and $\text{bl}4$ is strongly inhibited by 25 μM pentamidine, that splicing of $\text{al}3\alpha$ was less inhibited, and that splicing of $\text{al}5\gamma$ and $\text{bl}5$ 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 ($\text{al}3\alpha$, $\text{al}5\alpha$, and $\text{bl}5$) were quantitatively more sensitive than the group II introns ($\text{al}2$ and $\text{al}5\gamma$); introns of the same type showed similar sensitivity. However, the in vivo analysis showed that $\text{al}3\alpha$, $\text{al}5\alpha$, and $\text{al}2$ are sensitive to pentamidine, whereas $\text{bl}5$ and $\text{al}5\gamma$ 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

FIGURE 4. Pentamidine sensitivity of growth relates to mitochondrial intron content. Four mtDNAs with different intron configurations (I^+II^+ , $I^+II^{5\gamma}$, I^+II^0 , I^0II^0) were cytoduced into nuclear genome a161 to generate four *S. cerevisiae* strains, as described in Materials and methods and Table 1. About 1×10^5 cells of each strain were inoculated on YPG agar plates or plates containing various concentrations of pentamidine and incubated at 30 °C for 5 days.

FIGURE 5. Pentamidine-induced accumulation of pre-mRNAs of mitochondrial intron-containing genes (*COX1* and *COB*). Pentamidine (12.5 and 25 μ M) was added to cultures of strain a161 I⁺II⁺ when OD₆₀₀ was 0.1 in a final volume of 30 mL; control cultures received no pentamidine. When the cultures reached mid-log phase, total yeast RNA was prepared, and four sets of 15 μ g of each RNA preparation were fractionated on a 1.2% agarose-6% formaldehyde gel and transferred to four Hybond N⁺ nylon membranes. Mitochondrial 21S rRNA was used as the loading control. The exon probes for the mitochondrial *COX1*, *COB*, *OLI2*, *OLI1*, *COX3*, and 21S rRNAs, and the chromosomal *CYH2* RNA were sequentially hybridized to the RNA membranes. The positions of pre-mRNAs and RNAs are indicated. *OLI2* probe hybridization showed two bands (marked with L and S) representing two partially processed RNA species containing *OLI2* mRNA sequence (Pelissier et al., 1992). Mature *OLI2* RNA was not detected in these strains with the a161 nuclear genome. The bands on Northern blots were quantitated as described in Materials and methods. Numbers below each lane indicate quantitation of mature RNA levels (both L and S RNAs for *OLI2*) in that lane. Levels of various mature RNAs in each sample were normalized with that of 21S rRNA, and then the values of each mature RNA were normalized with the untreated samples set at 1.

(Semrad & Schroeder, 1998), 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 (a15 γ , b11, and b15) 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 I⁰II⁰ 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 μ M pentamidine treatment during 4–5 cell doublings resulted in almost complete depletion of the steady-state levels of the mitochondrial translation products 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

A. Group I introns

B. Group II introns

FIGURE 6. Specificity of pentamidine inhibition of intron splicing. The same four RNA membranes used in Figure 5 were hybridized with different intron probes. **A:** group I introns; **B:** group II introns. The position of every excised intron is indicated by the arrow with its size indicated. The pre-mRNAs and the structural subgroup (Michel & Westhof, 1990) to which each intron belongs are also indicated. RNA species marked with X and Y are discussed in the text.

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 mitochond-

rial 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 μ M pentamidine had no effect

FIGURE 7. Pentamidine specifically reduces mitochondrial protein levels. Cells of intronless strain a161 I⁰II⁰ were grown in the absence or presence of 12.5 and 25 μ M 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).

on protein synthesis by cytoplasmic ribosomes. Addition of cycloheximide resulted in nearly complete inhibition of translation of nuclear encoded proteins, allowing the preferential labeling of the mitochondrial translation products. As shown in Figure 8A, lanes 7–9, pentamidine is a potent inhibitor of mitochondrial protein synthesis. Pentamidine at 12.5–25 μ M was more inhibitory than 2.7 mM erythromycin (2 mg/mL) (Fig. 8A, lane 6); 1 μ M pentamidine (Fig. 8A, lane 9) was as inhibitory as 2.7 mM erythromycin. As loading controls, western blot analysis was performed for the same whole-cell extracts to determine the steady-state protein levels in the drug-treated and -untreated cells. Figure 8B shows that the steady-state levels of both the nuclear encoded protein porin and mitochondrial encoded proteins CO2 and CO3 are equivalent in all the samples. The minimal change in steady-state CO2 and CO3 levels during these 35-min pentamidine treatments indicates that pentamidine does not dramatically increase mitochondrial protein turnover. Thus, the reduction of mitochondrial-encoded CO2 and CO3 levels by pentamidine treatment of cells for 4–5 doubling times resulted from translation inhibition rather than from accelerated protein degradation in mitochondria.

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 Mg^{2+} and NH_4^+ as indicated. The group I introns al3 α , al5 α , and bl5 were all comparably sensitive to pentamidine in vitro with complete inhibition achieved at 125–250 μ M. Complete inhibition of the self-splicing of the group II introns al5 γ and al2 was achieved at 500 μ M. 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 al5 γ was resistant to 500 μ M 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 Mg^{2+} (Liu et al., 1994; Miletti & 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 Mg^{2+} and 200 mM NH_4^+) (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 Mg^{2+} and 1–2 M NH_4^+) (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 al5 γ and bl1 were completely unaffected. These in vivo data contrast strikingly with the in vitro data, where no differential sensitivity to pentamidine was observed for

A. ³⁵S labeling

B. Western

FIGURE 8. Pentamidine inhibits mitochondrial translation. Cells of intronless strain a161 I⁰II⁰ were grown, and treated with the indicated concentrations of drugs (Pent: pentamidine; Ery: erythromycin; Cyh: cycloheximide), and labeled with L-[³⁵S] methionine and L-[³⁵S] cysteine as described in Methods and materials. **A:** In vivo translation products after 10 min labeling. TCA-precipitated whole-cell extracts (60 μL) were analyzed by SDS-PAGE followed by autoradiography. Samples from cells without cycloheximide treatment (lanes 1–4) incorporated much more radioactivity than did those from cells treated with cycloheximide (lanes 5–9), so one-tenth volume of those samples was loaded for analysis after precipitation. According to the previous study by Manthey and McEwen (1995), the ³⁵S incorporated mitochondrial proteins separated on this SDS-PAGE should be Var1p, CO1, CO2, CO3, and Cytb as indicated. Var1p corresponds to the product of *VAR1* gene, CO1 to the product of *COX1* gene, and Cytb to the product of *COB* gene. The small translation products ATPase8/9 ran off this gel and were not seen. **B:** Steady-state protein levels in the labeled cells. Whole-cell extracts (4 μL) were analyzed by SDS-PAGE for western analysis with antibodies to nuclear-encoded protein porin, and to mitochondrial-encoded proteins CO3 and CO2.

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 μ M). We show that pentamidine at 1 μ M 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 Ahsen & Schroeder, 1991; von Ahsen 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 *td* 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 *al3 α* , 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 *al5 β* 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 *al5 α* 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 *al5 α* splicing reaction is not likely due to a direct effect of the drug on *al5 α* RNA, because we did not observe a strong second-step defect in the self-splicing experiments. Instead, it is possible that the *al5 α* -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 *al5 α* 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 *al5 α* second-step splicing by pentamidine may be the cause of the relative instability of those RNAs unspliced for introns upstream of *al5 α* .

It has previously been demonstrated that pentamidine acts on an unidentified mitochondrial target to inhibit the glycerol growth of *S. cerevisiae* (Ludewig 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 I^+II^0 mitochondrial genome is a hybrid of mitochondrial DNA from three other yeast species and none of its sequences originated from the I^+II^+ strain (Wenzlau et al., 1989). The $I^+II^{5\gamma}$ is identical to the I^+II^0 DNA except for the addition of *al5 γ* (derived from strain D273; Wenzlau, 1989). The I^0II^0 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 ρ^0 strains were made by growing the ρ^+ parental strains in rich dextrose medium containing ethidium bromide (30 $\mu\text{g}/\text{mL}$) in the dark for 3 days. Four types of mtDNA with different introns (Table 1) were first cytotoduced into strain M497 (*MAT α his4-15 karl-1 can1 ρ^0*), then cytotoduced into *MAT α* 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°C for RNA extraction. Total yeast RNA was isolated as described previously (Hagen et al., 1995), and extracted RNA was dissolved in 40 μL DEPC-treated water and stored at -70°C . RNA concentration was determined by OD₂₆₀. RNA blotting and hybridization were performed as described previously (Thomas, 1980; Moran et al., 1994) with minor modifications. The same amount of total RNA (12 or 15 μg , based on OD₂₆₀) from yeast cultures was denatured at 65°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 Hybond 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 $\times$ SSC at 55°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 $\mu\text{g}/\text{mL}$) were included in the prehybridization and hybridization buffers, and at least 4-h prehybridization and overnight hybridization were carried out at 42°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°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 (Bio-Rad), 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 [α - ^{32}P]-dCTP (3,000 Ci/mmol; DuPont Inc.) was incorporated by the random primer method (Boehringer Mannheim), following the manufacturer's instruc-

tions 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 *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 μM rNTPs for nonradioactive precursor preparation or 500 μM of rATP, rCTP, and rGTP, 200 μM of rUTP, and 10 μCi [α - ^{32}P]-UTP (3,000 Ci/mmol, NEN-DuPont Inc.) for radioactive precursor preparation, under recommended conditions. Primers were selected with coordinates 4964–7177 for al3 α (Bonitz et al., 1980), 7964 (Bonitz et al., 1980) –1718 (Hensgens et al., 1983) for al5 α , and 1769–3297 for bl5 (Nobrega & Tzagoloff, 1980). The precursors generated from PCR templates with these primers contain

TABLE 2. PCR-generated probes.

Probe ^a	Length (bp)	Coordinates	Reference
OLI1	201	195–395	Hefta et al., 1987
OLI2	295	193–487	Macino & Tzagoloff, 1980
COX3	595	413–819	Thalenfeld & Tzagoloff, 1980
21S rRNA	351	3330 ... 4822 ^b	Sor & Fukuhara, 1983
COX1	960	6858 ... 9701 ^b	Bonitz et al., 1980
COB	793	371 ... 3297 ^b	Nobrega & Tzagoloff, 1980
al1	390	175–564	Bonitz et al., 1980
al2	548	2659–3206	Bonitz et al., 1980
al3 α	296	5284–5579	Bonitz et al., 1980
al4 α	858	7239–8096	Bonitz et al., 1980
al5 α	456	230–685	Hensgens et al., 1983
al5 β	753	1785–2537	Hensgens et al., 1983
al5 γ	504	8805–9308	Bonitz et al., 1980
bl1	744	–1624–(–881)	Lazowska et al., 1980
bl2	646	–580–65	Lazowska et al., 1980
bl3	494	2980–3473	Lazowska et al., 1989
bl4	644	802–1445	Nobrega & Tzagoloff, 1980
bl5	592	2314–2905	Nobrega & Tzagoloff, 1980

^aGene probes are exons; intron probes do not contain exon sequences.

^bThese probes do not include intron sequences within the listed coordinates and were generated from templates from intronless strains.

each full-length intron (al3 α : 1514 nt, al5 α : 1366 nt, and bl5: 730 nt) and 5' and 3' exons: 244 and 454 nt for al3 α , 514 and 353 nt for al5 α , 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-8 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 μ L of 62.5 mM Tris-HCl (pH 7.5) containing 10 U of RNasin (Promega) at 42 °C (al3 α and al5 α) or 37 °C (bl5) for 20 min. Then the splicing was started by addition of 4 μ L of a reaction mix containing splicing components MgCl₂, (NH₄)₂SO₄, and 10 μ Ci (al3 α and al5 α) or 30 μ Ci (bl5) of [α -³²P]-GTP (3,000 Ci/mmol, NEN-Dupont Inc.). The cation concentrations in splicing reactions are 50 mM MgCl₂ and 100 mM (NH₄)₂SO₄. Incubation at 42 °C (al3 α and al5 α) 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 (5×10^3 cpm per reaction) was preincubated and assayed for self-splicing, as above, except that the preincubation was in 8 μ L Tris buffer and 2 μ L of reaction mix containing nonradioactive GTP (2 mM) was added after preincubation. The splicing products were fractionated on a 3.5% PAGE-8 M urea gel that was dried and visualized as described (Liu & Leibowitz, 1993).

Self-splicing of group II introns

Model pre-mRNAs containing al5 γ or al2 were obtained as T7 RNA polymerase (Boehringer Mannheim) transcripts of plasmid pJD20, following digestion with *Hind*III, and T3 RNA polymerase (Boehringer Mannheim) transcripts of plasmid pSZD2 following digestion with *Bst*EII, respectively. The al5 γ 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 μ Ci [³²P]-UTP (3,000 Ci/mmol, 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 μ L 60 mM Tris-HCl (pH 7.5) with various concentrations of pentamidine for a 5-min preincubation at 42 °C before adding 5 μ L of MgSO₄ and (NH₄)₂SO₄ solution to start the reaction. The reacted samples were fractionated on a 4% PAGE-8 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 I⁰II⁰ 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 μ Ci 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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