Expression of a reporter gene interrupted by the *Candida albicans* group I intron is inhibited by base analogs

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ABSTRACT

We previously reported the identification of an intron (CaLSU) in the 25S ribosomal RNA of some *Candida albicans* yeast strains. CaLSU was shown to self-splice and has the potential to adopt a secondary structure typical of group I introns. The presence of CaLSU in *C. albicans* strains correlates with a high degree of susceptibility to base analog antifungal agents, 5-fluorocytosine (5-FC) or 5-fluorouracil (5-FU). Cell death, resulting from addition of base analogs to growing cultures, precluded demonstration of a causal relationship between CaLSU presence and susceptibility to base analogs. In the present study, CaLSU was inserted in a non-essential lac Z reporter gene and expression was examined in *Saccharomyces cerevisiae*. Different mutations affecting in vitro self-splicing also had similar effects on reporter gene expression in vivo. This indicates that in vivo removal of CaLSU from the reporter gene occurs through the typical self-splicing mechanism of group I introns. Base analogs inhibited expression of the reporter gene product in a concentration-dependent manner upon their addition to the cultures. This supports a model in which disruption of intron secondary structure, consecutive to the incorporation of nucleotide analogs, is a major factor determining the susceptibility of *C. albicans* cells to base analogs.

INTRODUCTION

The discovery of catalytic RNA molecules has deeply modified our understanding of biological systems, genetic organisation and evolution (for reviews see among others: 1–6). Among catalytic RNAs, group I introns possess the capability of promoting their own removal from precursor RNA molecules to generate mature transcripts; this process is referred to as self-splicing. Despite the fact that we know >200 examples of group I introns, relatively few consequences of their presence have been observed at the phenotypic level. As an example, one ‘petite’ mutation in *Saccharomyces cerevisiae* has been mapped to a splicing-defective intron in the cytochrome b gene of mitochondria (7).

We have recently reported the discovery of a group I intron (CaLSU) in the ribosomal 25S rRNA precursor molecule of *Candida albicans* (8), an important opportunistic fungal pathogen of humans. The 379 base long sequence of CaLSU shares the usual putative secondary structure of group I introns, possesses the adequate nucleotides at specific positions known to be crucial for self-splicing, and was shown to catalyze its own excision in a standard in vitro self-splicing assay. However, CaLSU primary sequence is among the most divergent in the usual consensus sequences established for group I introns (see among others: 4,9–12).

In a previous study, phenotypes and genotypes of 120 clinical isolates of *C. albicans* were analyzed. In that regard, ~40% of the strains studied harbored CaLSU in their 25S rRNA coding sequences. In those strains with CaLSU, Southern blots and PCR analysis failed to reveal the presence of the rRNA-encoding gene devoid of CaLSU (unpublished data) in any of the 100–200 tandemly-arrayed copies encoding rRNA (rDNA) in the nucleus. This presence of CaLSU, in apparently every copy of the 25S rRNA-coding gene, was shown to correlate with susceptibility to base analogs exerting an antifungal activity, 5-fluorocytosine (5-FC) or 5-fluorouracil (5-FU). Intron-bearing *C. albicans* strains all exhibited a high degree of susceptibility to base analogs while strains lacking the intron differed widely in their susceptibility (8). Upon addition of 5-FC or 5-FU base analogs, these will be metabolically used to generate fluoro-substituted nucleotides that will then be incorporated into newly-synthesized RNA molecules. It has been shown that self-splicing of a *Tetrahymena* group I intron can be drastically reduced by the substitution of fluoronucleotides (13); this is likely due to disruption of intron secondary structure consecutive to weakening of base pairing. We postulated that a similar effect of base analogs can result from their addition to *C. albicans* cultures, disrupting the secondary structure essential for CaLSU splicing and resulting in an aberrant 25S rRNA and lack of normal functional ribosomes.

Addition of base analogs to cultures of *C. albicans* yeast cells harboring CaLSU results in cell death, preventing further study.

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of their postulated effect on in vivo self-splicing. Another approach was required to allow a more direct demonstration of base analogs effect on splicing. In the present study, CaLSU was thus inserted in the coding sequence of a lacZ reporter gene and the resulting construct was expressed in S.cerevisiae. Site-directed mutagenesis was then used to further establish the importance of specific residues or sequence elements essential for catalytic activity of group I introns. The same sequences were shown to be important both for in vitro splicing of CaLSU and in vivo expression of active β-galactosidase encoded by the lacZ gene. In addition to providing definitive evidence for the assignment of CaLSU to group I introns, these results suggest that in vivo removal of CaLSU from lacZ occurs through a self-splicing mechanism. Addition of either 5-FU or 5-FC to the S.cerevisiae yeast cells prevented expression of β-galactosidase and this supports the idea that base analogs can interfere with in vivo self-splicing of CaLSU. The importance of these observations, in the context of using inhibitors of self-splicing as antimicrobial agents, is briefly discussed.

MATERIALS AND METHODS

Plasmids and yeast strains

The Bluescript II SK+ plasmid harboring the CaLSU intron flanked by ribosomal sequences has been previously described (8). The yeast plasmid pLGSD5 (2µ, URA3, GAL1::lacZ) and the S.cerevisiae strain MGD353-46D (MATa, ura3-52, trp1-289, leu2-3, 112 his3-D1, cyh2) were obtained from Dr Pierre Legrain (Institut Pasteur, Paris) and have been described elsewhere (14,15). The yeast actin clone was obtained from Dr Reginald K. Storms (Concordia University, Montréal).

DNA manipulations

Subcloning procedures and plasmid constructs were done essentially according to standard procedures (16). Polymerase chain reaction was performed using the Taq DNA polymerase (Promega). Site-directed mutagenesis using the unique site elimination procedure (17) was performed according to the manufacturer’s instructions (Pharmacia). All mutants were sequenced directly onto denatured double-stranded DNA using Sequenase, as recommended by the manufacturer (USB).

Plasmid constructs

The CaLSU intron was originally subcloned in pBluescript vector as a fragment flanked by 25S rRNA coding sequences (8); this plasmid was used as a template for PCR amplification. Oligonucleotides were designed to amplify a fragment of DNA encompassing CaLSU, short flanking rDNA sequences, and additional BamHI sites created at both ends. The conservation of flanking regions was essential since they participate in two separate pairings, P1 and P10, which enable accurate splicing and ligation of the exons in the classical model of group I intron splicing (4,18). The strategy for PCR amplification and sequences of the oligonucleotides used are presented in Figure 1A. The amplified fragment was digested with BamHI and subcloned at the unique BamHI site of pBluescript II KS+ vector (Stratagene). Site-directed mutagenesis of CaLSU was performed on this construct; oligonucleotides used were 5′-TTGCCCTCCGAATAGTTG-3′ for the 5′-IGS disruption, 5′-TTTCCGTGATCTACGA-3′ for the GTP binding site disruption and 5′-GATCCATTTCATGTGCTCC-3′ for 3′-exon disruption (mutations are underlined). The resulting wild-type and mutant introns were then used as a source of fragments for subcloning at the unique BamHI site located in the lacZ gene (see Figure 1B) of the yeast plasmid pLGSD5 for in vivo studies using lacZ as a reporter gene.

In vitro splicing of CaLSU

RNA substrates for self-splicing were produced by in vitro transcription using T3 RNA polymerase. The Bluescript II KS+ plasmids with CaLSU inserted at the BamHI site of the polylinker (wild-type, inverted and mutants) were linearized at the PvuII site, leaving flanking plasmid lacZ sequences on both sides of CaLSU. The original pBluescript clone of CaLSU flanked by ribosomal RNA sequences was used as a control (8).
previously described, transcription of this control was performed with T7 RNA polymerase after linearization at the NarI site of *C. albicans* 25S rDNA sequence, leaving ribosomal flanking sequences on both sides of CaLSU (8). All transcription reactions were performed in the presence of 0.1 mM of each ribonucleotide triphosphates; 50 µCi of radioactive UTP (800 Ci/mmol; ICN Biomedicals) was added to the reaction. The radioactive RNA products were analyzed onto denaturing 4% sequencing urea–polyacrylamide gels followed by autoradiography. Quantitation was performed using a Personal Laser Densitometer (Molecular Dynamics).

**Candida albicans growth**

Strains of *C. albicans* harboring (strain 4F) or lacking (strain Lecocq) the CaLSU intron in their ribosomal RNA were grown 30 h at 30°C under gentle agitation in SC-uracil/glycerol/lactate medium [6.7 g/l yeast nitrogen base w/o amino acids (Difco), 5 g/l casamino acids (Difco), 20 mg/l tryptophan, 30 ml/l glycerol, 20 g/l lactate, 0.5 g/l glucose]. Cells were then centrifuged, washed twice in saline and resuspended in fresh medium at a concentration corresponding to an optical density of 0.25 at 600 nm. Base analog, 5-F, was then added at various concentrations and the cultures incubated in the same conditions for 24 h before measuring the optical density; a control culture was also incubated in the absence of 5-F.

**Saccharomyces cerevisiae strain manipulations**

*Saccharomyces cerevisiae* transformations were performed by the lithium acetate protocol (19). Cultures for β-galactosidase assays were prepared as follows. Transformants were plated on SC-uracil [6.7 g/l yeast nitrogen base w/o amino acids (Difco), 5 g/l casamino acids (Difco), 20 mg/l tryptophan] containing 20 g/l glucose and 20 g/l bacto-agar. Pools of at least 100 transformants were directly used to inoculate liquid SC-uracil medium with 3% glucose and 20 g/l bacto-agar. Cells were then centrifuged, washed in saline and resuspended in fresh medium at a concentration corresponding to an optical density of 0.25 at 600 nm. Base analog, 5-F, was then added at various concentrations and the cultures incubated in the same conditions for 24 h before measuring the optical density; a control culture was also incubated in the absence of 5-F.

**β-Galactosidase assay**

The assay was performed as previously described (20). Determination of the enzymatic activity, detection of the protein by immunoblotting and RNA levels analysis (Northern blots), were all performed from the same cultures. The β-galactosidase activity was expressed in standard Miller units.

**β-Galactosidase detection by immunoblotting**

Proteins were extracted from the yeast cells using the glass beads extraction procedure (21). Aliquots of 100 µg protein were loaded onto SDS–PAGE gels; following electrophoretic separation, proteins were electrotransferred onto nitrocellulose filters and analyzed by immunoblotting. A monoclonal anti-β-galactosidase antibody (Boehringer Mannheim) was used at a 1/2000 dilution and the secondary antibody was a rabbit anti-mouse alkaline phosphatase conjugate (BRL) used at the same dilution. Antigen–antibody complexes were detected using NBT-BCIP chromogenic substrates as recommended by the manufacturer (Gibco/BRL).

**Northern blot RNA analysis**

Total RNA extraction and Northern blots were performed as previously described (22). Polyadenylated RNA was isolated using Dynabeads mRNA Purification Kit according to the manufacturer's instructions (Dynal). Probes were prepared by random priming (Pharmacia). The CaLSU probe (401 nt) was gel-purified following a BamHI restriction endonuclease digest of the pBluescript construct (see Plasmid constructs). The lacZ probe is a BamHI–AccI DNA fragment (2834 nt) from pLGSD5. Finally, the plasmid bearing the complete yeast actin gene, including its intron, was used as a probe.

**RESULTS**

**Rationale for site-directed mutagenesis**

Three specific sequence elements or residues, known to promote accurate and efficient self-splicing of group I introns, were chosen as targets for site-directed mutagenesis. As a first mutant, a conserved guanosine residue in the P7 pairing of the catalytic core of group I introns was substituted for a uridine (‘G247U’ mutant). This residue was shown to be responsible for the binding of the GTP molecule required for catalytic cleavage of the 5′ intron–exon boundary during self-splicing of group I introns (23). Sequence elements, apparently required to position the two exons in close proximity before self-cleavage at the boundary between the intron and 3′-exon and ligation of the two exons, were used as our next targets. The internal guide sequence (IGS) lies in the P1 stem–loop and it can pair with a second element, consisting of the first few nucleotides of the 3′-exon, to form P10. We separately mutated either the IGS sequence (‘5′-disrupted’ mutant) or the complementary sequence in the flanking 3′-exon (‘3′-disrupted’ mutant). We also generated a double mutant in which both sequences were mutated (‘restored’ mutant). Although the new sequences are different from those of the wild-type intron, they potentially restore the P10 pairing. The nature and position of the different mutations are schematized in Figure 1B and C.

**In vitro splicing of CaLSU mutants**

The different mutant introns with flanking lacZ sequences in pBluescript were transcribed in vitro and their capacity to self-splice was examined and compared with the same intron flanked by ribosomal RNA sequences in the original plasmid construct (Fig. 2, CaLSU/25S rRNA). RNA products, resulting from self-splicing reaction occurring during the transcription reaction containing adequate magnesium and GTP concentrations required for splicing, were analyzed; we have been unable to achieve splicing of lacZ–CaLSU RNA species after gel purification. It is likely that, in this case, splicing has to be initiated concomitantly with transcription; this point will be further discussed. Nevertheless, splicing products generated in the transcription reaction were easily analyzed. The wild-type intron in lacZ (Fig. 2, CaLSU construct wild type) was able to self-splice, generating free intron (379 bases long) and religated exons (263 bases long) from the 642 bases long precursor. Other minor intermediates
Figure 2. In vitro self-splicing of CaLSU mutants. In vitro transcription of RNA precursors encoded in pBluescript was allowed to proceed at 37°C for 1 h as described in Materials and Methods. Radioactive RNA products, generated by splicing occurring during the transcription reaction, were then analyzed by denaturing polyacrylamide gel electrophoresis. The first lane shows the result obtained with the original clone of CaLSU inserted in rRNA, while the other lanes are the different constructs of CaLSU inserted in the multiple cloning site of pBluescript as indicated. Schematic representations of the linearized RNA transcription templates are presented. NarI and PvuII restriction sites used for linearization; 5′SS and 3′SS, 5′ and 3′ splicing sites; T7 and T3, origins of transcription for the RNA polymerase used; 5′E, 5′-exon; IVS, Ca.SU intervening sequence; 3′E, 3′-exon; dashed lines indicate intron (CaLSU) sequences removed by splicing, and ligation of the exons. The different bands were identified by their electrophoretic mobility, as compared with radiolabeled 1 kb ladder, and are indicated alongside the gel. The asterisk denotes a band observed in all CaLSU constructs which likely results from a premature transcriptional arrest. Due to their small amounts, the free 5′ and 3′ exons are not visible on the photograph.

products were detected; this was also previously observed in the reaction of CaLSU self-splicing in its original ribosomal context (free 5′-exon, intron–3′-exon and free 3′-exon). The CaLSU intron has thus kept the capability of self-excision even though it is inserted in a lacZ gene different from its initial location in ribosomal RNA. However, the efficiency of self-splicing in the lacZ context is reduced compared to the efficiency observed in the original ribosomal context, as shown by a decreased percentage of precursor spliced to its products from 75% in the original ribosomal context down to 25% in the lacZ context. A more important accumulation of the intermediate ‘intron–3′-exon’ species was also observed in the lacZ context (Fig. 2).

We then examined the different mutants for their ability to self-splice. As expected, insertion of the intron in the opposite orientation (Fig. 2, CaLSU construct inverted) and the mutation in the GTP binding site (Fig. 2, CaLSU construct G247U) essentially prevented intron removal and exons ligation. Disruption of the IGS (Fig. 2, CaLSU construct 5′-disrupted) or flanking 3′-exon (Fig. 2, CaLSU construct 3′-disrupted) sequences resulted in an accumulation of the ‘intron–3′-exon’ intermediate as well as a loss of self-splicing and exons ligation ability. The double mutant, restoring base pairing between a modified IGS and modified 3′-flanking region, had a good self-splicing ability since ~15% of the precursor was spliced to its products (Fig. 2, CaLSU construct restored).

Effect of CaLSU on in vivo expression of a reporter gene

Following the analysis of in vitro self-splicing, we proceeded to determine the in vivo activity of the same constructs (wild-type and mutants). CaLSU was inserted at the beginning of the lacZ gene in the yeast vector pLGSD5 (Fig. 1A), and transformed in S.cerevisiae yeast cells. In the absence of CaLSU removal, its presence should prevent production of active β-galactosidase since there are multiple termination codons in all three reading frames of CaLSU; levels of β-galactosidase activity should thus be a good indication of the efficiency of CaLSU splicing in vivo.

The construct harboring wild-type CaLSU allowed production of active β-galactosidase while intron insertion in the inverted orientation resulted in complete inactivation of the lacZ gene (Table 1). However, the level of activity was much lower in the intron-containing construct (~15% compared to the original intronless pLGSD5 plasmid). The GTP binding site mutant (G247U) was inactive while mutants disrupted in either 3′-flanking (3′-disrupted) or 5′-IGS region (5′-disrupted) exhibited barely detectable levels of activity (<0.1% of wild-type). About 10% of activity, compared to the wild-type value, was observed with the double mutant restoring base pairing between IGS and the 3′-flanking sequence (restored, Table 1). Altogether, these data indicate that production of β-galactosidase activity, when CaLSU is inserted in the lacZ gene, is largely dependent on the same
sequences that affect in vitro self-splicing. This suggests that in vivo splicing of CaLSU occurs through autocatalytic activity of the intron since production of β-galactosidase is affected by the same mutations as in vitro self-splicing.

Table 1. β-galactosidase activity of various lacZ constructs in pLGSD5

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<thead>
<tr>
<th>LacZ Constructs (in pLGSD5)</th>
<th>β-galactosidase activitya (Miller units)</th>
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<tbody>
<tr>
<td>Intronless</td>
<td>411</td>
</tr>
<tr>
<td>Wild-type CaLSU</td>
<td>32</td>
</tr>
<tr>
<td>Inverted CaLSU</td>
<td>&lt;1</td>
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<tr>
<td>Mutants of CaLSU</td>
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<tr>
<td>G247U (GTP binding)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5' disrupted</td>
<td>&lt;1</td>
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<tr>
<td>3' disrupted</td>
<td>&lt;1</td>
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<td>Restored (5'−3')</td>
<td>3</td>
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aPools of yeast cells obtained by transformation of the different plasmid constructs were analyzed. Intronless and the different plasmids harboring wild-type, inverted, or mutants of CaLSU are identified as described in the text.

We also verified that the amount of protein correlates with the results of enzymatic activity dosage. This is of importance because, in the strategy for plasmid construct, it was impossible to avoid addition of a few extra amino acids; in the event of correct splicing, there will be addition of six amino acids close to the amino-terminal end of the β-galactosidase protein (Fig. 1A). Immunoblotting analysis was performed with a monoclonal anti-β-galactosidase antibody; a major protein species was detected with smaller species likely corresponding to degradation products. The amount of both the full-length species or total immunoreactive products was significantly decreased by insertion of CaLSU in the lacZ coding region (Fig. 4A compare first lane to fourth lane). This experiment showed that the reduction of enzyme activity does reflect a reduced amount of β-galactosidase enzyme produced by the cells harboring the lacZ gene interrupted by CaLSU, compared to the original intronless plasmid. There is thus no evidence of a detrimental effect on the enzymatic activity due to the presence of the six additional amino acids.

Effect of base analogs on in vivo β-galactosidase expression

We next applied the reporter gene system to study the in vivo effect of base analog antifungal agents. This is of increasing concentrations of either 5-FC or 5-FU were added to cultures of S.cerevisiae harboring the pLGSD5 plasmid with the lacZ gene interrupted by wild-type CaLSU. In the range of concentration used, the growth of C.albicans strains, harboring the intron in their ribosomal RNA-coding genes, was strongly inhibited by base analogs (Fig. 3, and data not shown); as discussed later, the susceptibility of strains lacking the intron was much more variable but they tend to be significantly more resistant. In the same concentration range, the base analogs do not affect the growth of the S.cerevisiae strain used in this study (data not shown). Similarly, the level of β-galactosidase activity encoded by the native pLGSD5 plasmid vector was only barely affected, with 80% activity remaining at high concentration of either base analogs, 25 µg/ml 5-FC or 250 µg/ml 5-FU (Fig. 4). In contrast, when we analyzed the production of active β-galactosidase from the plasmid harboring CaLSU in the lacZ gene, we observed a rapid decrease at low doses of either 5-FC (2–5 µg/ml) or 5-FU (50 µg/ml) (Fig. 4).

We further used immunoblotting analysis to verify if reduction in the levels of β-galactosidase activity observed in the presence of base analogs was due to an actual decrease in the amount of enzyme produced. The experiment confirmed that increasing concentrations of 5-FC results in progressive decrease in the amount of β-galactosidase protein in yeast cells harboring the plasmid with CaLSU inserted in the lacZ gene (Fig. 5A).

Finally, we performed Northern blot analysis to determine levels of lacZ RNA. We observed that the total amount of lacZ RNA is not decreased by the presence of the intron; as expected, transcription of the intron-bearing β-galactosidase gene produced a higher molecular weight precursor lacZ RNA than the intronless gene (Fig. 5B). Base analogs treatment resulted only in a slight reduction in the amount of lacZ mRNA in cells harboring either intronless or intron-bearing lacZ from pLGSD5 (Fig. 5B and C). This small decrease appears to be non-specific since it was also observed with the two species of mRNA detected with the actin probe (Fig. 5D), despite the fact that similar amounts of total RNA, detectable by ethidium bromide staining, was present in each lane. Identical results were observed with either total RNA or purified polyadenylated RNA (data not shown) and are thus unlikely to be due to the purification procedure. The lacZ RNA species observed in the cells harboring CaLSU also hybridized with the intron probe and this confirms that this band corresponds to precursor unspliced RNA; as expected, there was no hybridization with the intron probe in yeast cells transformed with pLGSD5 alone (Fig. 5C, left-hand panel).

DISCUSSION

In the present study, we devised a system to examine in vivo splicing of CaLSU in an effort to gather more evidence supporting our hypothesis that base analogs can exert an antifungal activity through inhibition of self-splicing. The rationale was to introduce CaLSU in a non-essential reporter lacZ gene, an approach used with success by previous investigators (24,25). These earlier
Figure 4. Effects of base analogs on β-galactosidase production in S. cerevisiae. Pools of transformants harboring pLGSD5 vector (intronless lacZ) or the same plasmid with CaLSU inserted in the lacZ gene (lacZ with CaLSU) were used to inoculate liquid cultures with increasing concentrations of either 5-fluorocytosine or 5-fluorouracil. The results of β-galactosidase dosage are presented as the percentage of activity compared to the value observed in the absence of base analogs. The results are the average of two independent experiments with independent pools of transformants.

studies used E. coli as a model microorganism and the extent of self-splicing was visually estimated using the blue phenotype conferred by expression of the lacZ gene in bacterial colonies grown on adequate indicator plates. However, we have failed to adapt this bacterial system to a quantitative assay of CaLSU splicing (data not shown). We thus devised a similar system using S. cerevisiae; this new original system also proved adequate to study the role of base analogs since cells from the chosen strain can incorporate and use base analogs for nucleic acid synthesis while being resistant to their lethal effect.

The predicted secondary structure of CaLSU, the presence of consensus sequence elements, and the occurrence of magnesium and GTP-dependent in vitro splicing, initially allowed us to assign CaLSU to group I introns. In addition to these standard criteria, in vitro splicing analysis of CaLSU mutants, reported in the present study, further supports the idea that this initial assignment to classical group I self-splicing introns was justified and that prediction of GTP binding site and sequence elements involved in the P10 pairing were correct. As previously reported, the accumulation of the ‘intron–3′-exon’ intermediate in the single P10 mutants suggests that disruption of this particular pairing results in a loss of 3′ splice site selection ability (18). As could be expected, such an accumulation was eliminated after restoration of the P10 pairing by complementary mutations in both the IGS and 3′-exon. The variation observed in the amount of ligated exons with the different constructs is comparable to results obtained by Michel et al. (23) after similar mutagenesis of the Tetrahymena intron. Mutants exhibiting reduced self-splicing proved to be similarly affected in vivo. However, in vivo splicing of the CaLSU constructs in yeast was quite inefficient even for the wild-type intron. The low efficiency of splicing is also consistent with RNA analysis since, despite our repeated attempts, the mature spliced form of the lacZ mRNA resulting from CaLSU removal could not be detected on Northern blots. The presence of intermediate or aberrant splicing products may be partly responsible for the difficulties in pursuing further characterization by either RNase mapping or reverse transcriptase polymerase chain reaction (RT–PCR) procedures (data not shown).

Many explanations come to mind for the apparently very low splicing efficiency observed with CaLSU in the reporter gene. First of all, the intron is inserted in a sequence context differing from its original location, and there is evidence that the presence of certain flanking sequences can affect self-splicing of group I introns (26). This is also consistent with the fact that all our efforts to achieve in vitro self-splicing of the gel-purified lacZ–CaLSU precursor were unsuccessful, despite the fact that this was readily achieved when CaLSU was in its original ribosomal sequence context (8, data not shown). We suspect that a 3′-flanking region inhibits self-splicing of complete transcripts; the spliced products observed in the transcription reaction may then result from splicing initiated during transcription, and before synthesis of the

Figure 5. Protein and mRNA analysis of the effects of base analogs. Yeast cells harboring either the intronless lacZ gene in pLGSD5, or the same plasmid with the CaLSU-interrupted lacZ gene, were grown in the presence of increasing concentrations of 5-fluorocytosine. (A) Proteins were extracted and the same amount of total protein was analyzed by SDS–PAGE and immunoblotting with the anti-β-galactosidase antibody. (B, C and D) Polyadenylated RNAs were extracted and analyzed by Northern blot using a lacZ probe (B), CaLSU probe (C), or yeast actin probe (D) as a control.
transcript is completed. It should also be noted that, in vivo, lacZ mRNA synthesis will be the result of RNA polymerase II activity while rRNA precursors are polymerase I transcripts; this, as well as the normally nucleolar location of the ribosomal transcript, may also affect the efficiency of in vivo splicing. Another possible explanation is the participation of accessory proteins during in vivo splicing of so-called self-splicing introns; the S.cerevisiae CBP2 protein and Neurospora CYT-18 have been conclusively identified as such accessory proteins involved in the splicing of some group I introns (27–30). The absence of the adequate protein in the heterologous S.cerevisiae host, or non-nucleolar localization of the transcript, may prevent access of CalSU to the adequate protein cofactors.

It is likely that the lacZ reporter gene system will be useful for the study of splicing inhibitors as well as various studies dealing with in vivo self-splicing in the context of an eucaryotic cell. Despite the limitations due to a low splicing efficiency, this system was still well-suited to study the effect of base analogs on CalSU splicing. Addition of increasing concentrations of base analogs to the yeast cultures progressively reduced expression of β-galactosidase; this effect was observed only when the lacZ gene was interrupted by CalSU, indicating that the intron is responsible for the effect. The level of protein produced was reduced although unspliced mRNA was present at a level similar to the lacZ mRNA normally formed in the absence of CalSU. Altogether, these results strongly suggest that base analogs can block in vivo self-splicing of CalSU when used at doses similar to the ones that are toxic to Calbicins strains harboring CalSU in their 25S rRNA. We believe that the inhibition is due to perturbations of CalSU secondary structure resulting from incorporation of fluoro-substituted nucleotides derived from the base analogs. This is consistent with the inhibition of in vitro self-splicing observed for the Tetrahymena group I intron upon substitution of uracil by 5-fluorouracil residues (13). Candida albicans strains devoid of CalSU vary widely in susceptibility to base analogs. This is due to the pleiotropic effect of base analogs resulting from incorporation of fluoro-substituted nucleotides into various nuclear acids: chromosomal DNA, ribosomal RNA and messenger RNA (31,32). Levels of metabolic incorporation vary from strains to strains and is likely responsible for variations in susceptibility. In contrast, yeast strains harboring CalSU in their rRNA are uniformly very sensitive to the effect of base analogs (8, and data not shown). Altogether, these observations support a model in which self-splicing relying on adoption of a precise secondary structure by the intron, can be more easily affected by base analogs than are other molecular targets; as a result, intron presence is a major base analogs susceptibility factor in Calbicins.

It is interesting to notice that some antibiotics and antifungal agents have been previously shown to inhibit in vitro self-splicing of group I introns (33–37). It has even been suggested that susceptibility to antimicrobial agents could be used to detect new group I introns (34); our discovery of CalSU is particularly significant in this regard. Another interesting observation is the presence of group I introns in the chromosomally-encoded rRNA of Pneumocystis carinii, a fungal pathogen of increasing medical importance. It has been recently observed that pentamidine, an anti-Pneumocystis agent, can inhibit in vitro self-splicing (37); there is, however, no other evidence that this inhibitory effect is responsible for the in vivo effect of the agent. The reporter gene system described in the present study could be applied to the study of such other introns and antifungal agents, ultimately leading to new antimicrobial therapeutic approaches directed against self-splicing introns.

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