Effect of Introns and AT-Rich Sequences on Expression of the Bacterial Hygromycin B Resistance Gene in the Basidiomycete *Schizophyllum commune*

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Previously, it was shown that introns are required for efficient mRNA accumulation in *Schizophyllum commune* and that the presence of AT-rich sequences in the coding region of genes can result in truncation of transcripts in this homobasidiomycete. Here we show that intron-dependent mRNA accumulation and truncation of transcripts are two independent events that both affect expression of the bacterial hygromycin B resistance gene in *S. commune*.

In the homobasidiomycete *Schizophyllum commune*, introns are needed for efficient expression of at least some homologous and heterologous genes (5). Accumulation of mRNA of the *SC3* and *SC6* genes of *S. commune*, the *ABH1* gene of *Agaricus bisporus*, and the *GFP* gene of *Aequorea victoria* did not occur in *S. commune* when cDNA coding sequences were introduced. In contrast, the mRNAs did accumulate when genomic sequences were used or when an intron was added to cDNA constructs. Run-on analysis with nuclei harboring intron-containing and intron-less transgenes showed that the introns affected a posttranscriptional event and that at least one intron was required for mRNA accumulation.

AT-rich stretches also hamper expression of heterologous genes in *S. commune*. When prokaryotic reporter genes (β-glucuronidase and β-galactosidase genes) or resistance genes (hygromycin B phosphotransferase and aminoglycoside phosphotransferase genes) (10, 11) were expressed in this fungus, no full-length transcripts were observed. Rather, they were truncated in the 5′ part of the coding sequence at the position of AT-rich stretches. Similar observations were made when the α-galactosidase (*aglA*) gene from *Cyamopsis tetragonoloba* or the hygromycin B resistance (*hph*) gene of *Escherichia coli* were expressed in *Aspergillus niger* (4) and *Cryptococcus curvatus* (J. Springer, unpublished data). In both cases the truncation was overcome by increasing the GC content of the AT-rich region.

In plants and animals, evidence exists that RNA splicing and 3′-end formation are coupled (12), especially when the sites of cleavage and polyadenylation are suboptimal (7). Analysis of 17 genes from homobasidiomycetes showed no conserved cleavage/polyadenylation sequence. In some cases a sequence resembling the eukaryotic consensus sequence for polyadenylation (AATAAA) was found (9), suggesting that coupling between RNA splicing and 3′-end formation may also occur in fungi.

To determine whether introns affect *hph* mRNA accumulation and play a role in 3′-end formation of mRNA, we constructed plasmids in which the *hph* gene of *E. coli* (8) was regulated by the *gpd* promoter and *SC3* terminator of *S. commune* (pHYB1.1) (Fig. 1). Alternatively, the *hph* gene was cloned in similar constructs that contained an artificial intron (as described in reference 5) directly downstream of the stop codon (pHYB1.2), the third intron of *SC6* (5) directly upstream of the start codon (pHYB2.1), or a combination thereof (pHYB2.2) (Fig. 1). These constructs also contained the phleomycin resistance cassette, allowing direct or indirect selection on hygromycin B. The *S. commune* ΔSC3 strain, containing a disrupted *SC3* gene (13, 14), was transformed with these constructs (10), and selection was carried out on phleomycin-containing medium. Northern blot analysis showed that none of the transformants (12 for each construct) accumulated *hph* mRNA (Fig. 2, lanes 17 to 20). Furthermore, the transformants were not resistant to hygromycin B. When the transformation mixture was plated directly on hygromycin B-containing medium no transformants could be selected. These results show that introduction of one or two introns in the *hph* expression cassette is not sufficient to effect accumulation of the *hph* mRNA transcripts.

Methylation of the *hph* gene also could prevent the expression of this gene in basidiomycetes (1, 6). However, it has been shown that the presence of homologous sequences in introduced constructs prevents extensive methylation of heterologous sequences (6). This result indicates that methylation of the *hph* gene in our system does not affect expression. Schuren et al. (11), however, suggest that low expression may be due to the presence of an AT-rich sequence in the *hph* gene.

We made constructs with a modified *hph* gene in which eight changes in an AT-rich stretch at the 5′ end of the gene were made to increase the GC content without affecting the encoded amino acids (Fig. 3). The *S. commune* ΔSC3 strain (13, 14) was transformed with these constructs, pHYM1.1, pHYM1.2, pHYM2.1, and pHYM2.2 (Fig. 1), and selection took place on phleomycin-containing medium. Northern blot analysis of 15 transformants containing the modified *hph* gene (pHYM1.1)

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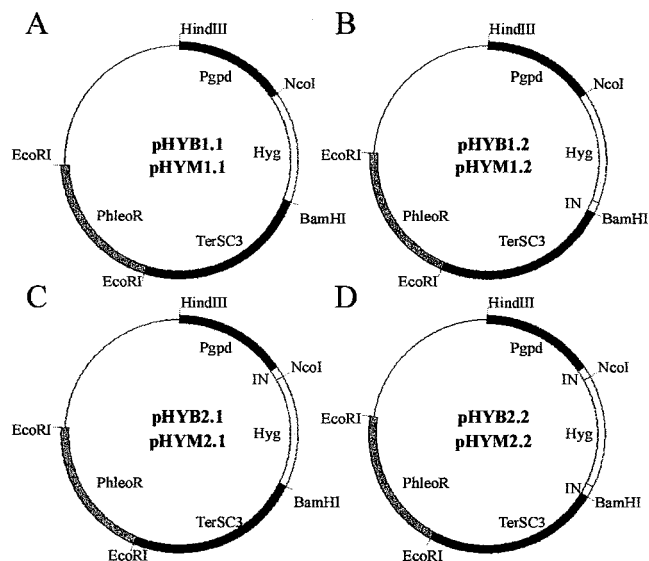
FIG. 1. Expression vectors used for transformation of *S. commune*. (A) Constructs pHYB1.1 and pHYM1.1, containing the unmodified and the modified hygromycin B gene, respectively, under regulation of the *gpd* promoter and *SC3* terminator of *S. commune*. (B) Constructs pHYB1.2 and pHYM1.2, as for panel A with the artificial intron directly downstream of the stop codon. (C) Constructs pHYB2.1 and pHYM2.1, as for panel A with the third intron of *SC6* directly upstream of the start codon. (D) Constructs pHYB2.2 and pHYM2.2, as for panel A with the artificial intron directly downstream of the stop codon and the third intron of *SC6* directly upstream of the start codon. The artificial intron was made by annealing two complementary oligonucleotides, resulting in a double-stranded DNA fragment of 50 bp with receded ends complementary to those of *Bam*HI. It contains consensus sequences for the splice and branch sites of *S. commune* introns. The sequences in between these three sites were randomly generated, although the average GC content for introns of *S. commune* (52%) was followed.

FIG. 2. Effects of introns and codon modification on hygromycin B mRNA accumulation. The recipient *S. commune* ΔSC3 strain (r, lane 16) was transformed with the modified hygromycin B gene (lanes 1 to 15) or the unmodified hygromycin B gene (lanes 17 to 20). Shown are hygromycin B constructs without introns (A and E), constructs containing the third intron of *SC6* directly upstream of the start codon (B and F), constructs containing an artificial intron directly downstream of the stop codon (C and G), and constructs containing both the third intron of *SC6* and the artificial intron (D and H). The mRNA was hybridized with a ^{32}P-labeled hygromycin B probe (A to D) or a ^{32}P-labeled ribosomal probe (E to H). The software used was Corel PhotoPaint 8.0 and CorelDRAW 8.0.

showed a low *hph* mRNA signal of the expected size (1,020 nucleotides) (Fig. 2A, lanes 1 to 15). These results suggest that increasing the GC content in an AT-rich sequence in the *hph* gene prevents truncation of transcripts in *S. commune*. Introduction of the third intron of the *SC6* gene directly upstream of the start codon or of the artificial intron directly downstream of the stop codon of the modified *hph* gene both increased the level of *hph* mRNA accumulation (Fig. 2B and C, lanes 1 to 15). Apparently, intron-dependent mRNA accumulation and truncation of transcripts at AT-rich stretches are independent processes that both affect expression of the *hph* gene in *S. commune*. When both introns were present in the *hph* construct, mRNA levels increased even further compared to those in the constructs containing a single intron (Fig. 2D, lanes 1 to 15). Previously, it was shown that addition of one intron outside the translational unit of *SC3* cDNA was sufficient to increase *SC3* mRNA accumulation to a level similar to that observed with the genomic *SC3* gene, which contains five introns (5). Because the *SC3* cDNA coding sequence is shorter (411 bp) than the *hph* gene (1,020 bp), we think that more than one intron may be needed for high mRNA accumulation for genes encoding longer transcripts.

All transformants (25 each) containing the modified *hph* gene in both the absence (pHYM1.1) and presence (pHYM1.2, pHYM2.1, and pHYM2.2) of introns were able to grow on
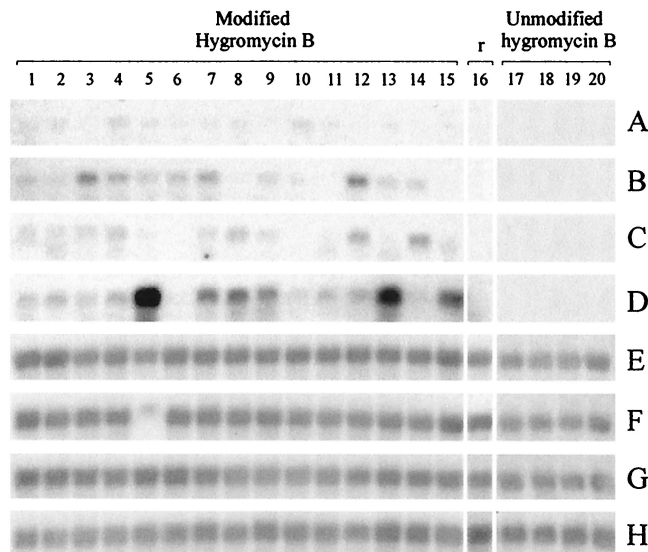
medium containing up to 100 μg of hygromycin B ml^{−1}. Transformants containing the third intron of *SC6* upstream of the start codon (pHYM2.1) grew more slowly, indicating that they were less resistant. Yet, *hph* mRNA levels in transformants with an intron cloned upstream of the start codon or downstream of the stop codon were similar, showing that the differences in resistance were not due to differences in transcription level. The intron directly upstream of the start codon may be prone to incorrect splicing, resulting in part of the protein molecules being inactive and thus in reduced resistance.
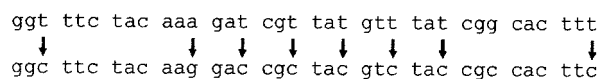

FIG. 3. The GC content of the AT-rich region between nucleotides 160 and 195 of the hygromycin B gene was increased by changing all A and T residues at the third position of the codons to C or G without affecting the encoded amino acid. The substitutions were introduced into the hygromycin B sequence by PCR. The 197 bp at the 5′ end of the coding sequence of *hph* were amplified by using an oligonucleotide primer corresponding to sense nucleotides 1 to 22 (accession no. Z32698) containing an *Nco*I site and to antisense nucleotides 158 to 197 containing the modified nucleotides and an *Acc*I site. The remaining 865 bp of the coding sequence of *hph* were amplified by using oligonucleotide primers corresponding to sense nucleotides 158 to 197 containing the modified nucleotides and an *Acc*I site and to antisense nucleotides 1001 to 1022 containing an additional *Bam*HI site. Ligation of both fragments resulted in a 1,062-bp *Nco*I/*Bam*HI fragment encompassing the complete modified *hph* gene. Note that these substitutions were introduced in an *hph* gene that had a C instead of a G at position 189.

We used plasmid pHYM1.1 to transform *S. commune* for direct selection on hygromycin B. In contrast to what occurred after selection on phleomycin, many false positives were obtained when hygromycin was used as a selection marker, as described earlier (6). No false positives were observed when 1 M sorbitol instead of 1 M MgSO$_4$ was used for regeneration of protoplasts, and similar numbers of transformants (five [phyleomycin selection] and three [hygromycin B selection] per microgram of DNA) were obtained on hygromycin B-and phleomycin-containing media. All transformants selected on phleomycin grew on fresh phleomycin-containing plates, while 21 of 25 transformants also grew on hygromycin B-containing medium. Similarly, of 38 transformants selected on hygromycin B, 38 and 31 grew on fresh hygromycin B- and phleomycin-containing plates, respectively. Southern blot analysis of 10 transformants selected on hygromycin B confirmed the presence of one or more copies of the *hph* gene in the genome (data not shown).

Until now only the phleomycin resistance cassette could be used in *S. commune* as a selectable marker. The modified *hph* gene is now also available and is as efficient for transformant selection as the phleomycin resistance cassette. These results suggest that other inefficiently expressed prokaryotic genes (e.g., β-glucuronidase, β-galactosidase, and aminoglycoside phosphotransferase genes [11]) can also be successfully expressed in *S. commune* by replacing AT-rich sequences in the coding sequence and introduction of one or more introns. Although hygromycin B resistance has recently been observed in *A. bisporus* following transformation with an unmodified *hph* gene, using an *Agrobacterium tumefaciens* DNA transfer system (2), commercially important homobasidiomycetes like *A. bisporus* and *Pleurotus ostreatus* (oyster mushroom) have generally been difficult to transform using prokaryotic antibiotic resistance genes. Replacement of AT-rich sequences and introduction of one or more introns might increase levels of expression of these genes and may result in more efficient transformation.

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