Expression and engineering of hydrophobin genes
Scholtmeijer, Karin

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2000

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 4: Introns are necessary for mRNA accumulation in *Schizophyllum commune*

*Luis G. Lugones*, Karin Scholtmeijer*, Riko Klootwijk and Joseph G. H. Wessels

*Part of this work was published in Molecular Microbiology 32: 681-689 (1999).*

*These authors contributed equally to this work*
Abstract

The cDNA coding sequence of the Agaricus bisporus hydrophobin gene ABH1 under the regulation sequences of the Schizophyllum commune SC3 hydrophobin gene gave no expression in S. commune. In contrast, the genomic coding sequence (containing three introns) produced high levels of ABH1 mRNA when transformed to S. commune in the same configuration. Apparently, introns are needed for the accumulation of ABH1 mRNA. A similar effect of intron-dependent mRNA accumulation in S. commune was observed with the homologous SC3 and SC6 genes. Run-on analysis with nuclei harboring intron-containing and intron-less SC6 showed that this effect did not occur at the level of transcription initiation: genomic and cDNA sequences were equally active in this respect. A single intron was introduced at various positions and in the right orientation into the intron-less SC3 transcriptional unit. Accumulation of SC3 mRNA was restored in all cases. By PCR amplification no unspliced SC3 mRNA species could be detected. Furthermore, addition of an intron to a cDNA of the green fluorescent protein (GFP) gene effected clear fluorescence of the transgenic hyphae.

Introduction

In the basidiomycetous fungus S. commune four genes encoding different hydrophobins have been isolated: SC3, which is expressed in primary (monokaryotic, sterile) and secondary mycelium (dikaryotic, fertile) and SC1, SC4 (Schuren and Wessels, 1990) and SC6 (Lugones, 1998), which are only expressed in secondary mycelium (Wessels et al., 1995). The functions of the SC1 and SC6 hydrophobins are not yet known but SC3 has been shown to coat aerial hyphae with a hydrophobic rodlet layer (Wessels et al., 1991; Wösten et al., 1994a) and to have a role in the emergence of these hyphae from the growth medium (van Wetter et al., 1996; Wösten et al., 1999). Furthermore, it attaches hyphae to hydrophobic surfaces (Wösten et al., 1994a; van Wetter et al., 2000). SC4 was shown to line gas channels in fruiting bodies of S. commute (Wessels, 1993; 1996; Lugones et al., 1999b).

The hydrophobin ABH1 confers hydrophobicity to the outermost hyphae of A. bisporus fruiting bodies (Lugones et al., 1996) and also lines the gas channels that traverse the fruiting body tissue of this mushroom (Lugones et al., 1999b). Since no significant differences could be found in the biophysical properties of isolated ABH1 and SC3 (Lugones et al., 1996) we wondered whether ABH1 could functionally replace SC3. For this we tried to complement a S. commune mutant bearing a disruption in the SC3 gene (Wösten et al., 1994a; van Wetter et al., 1996) with a copy of the ABH1 gene placed under control of the SC3 regulatory sequences. Also placing SC6 under control of the SC3 regulatory sequences should allow a high expression of the SC6 hydrophobin in the primary mycelium.

Here we show that introns in the ABH1 gene are essential for its expression in S. commute. Also for the expression of the two S. commute hydrophobin genes SC3 and SC6 the presence of at least one intron was indispensable. Moreover, insertion of an intron in the
transcriptional unit of the \textit{GFP} gene elevates \textit{GFP} expression and allows for the use of this reporter gene in \textit{S. commune}. We further showed that the only intronic sequences specifically needed for mRNA accumulation were those at the splice and/or branch sites and that the event(s) affected by the presence of introns occur at the post-transcriptional level.

\textbf{Materials and methods}

\textit{Strains}

Cloning was done in \textit{E. coli} JM83. \textit{S. commune} strains 4-39 (CBS 341.18) and 72-3 were used. \textit{S. commune} strain 72-3 is identical to the wild-type strain 4-39 apart from a disrupted \textit{SC3} gene (\textit{\textDelta SC3} strain) (Wösten et al., 1994a; van Wetter et al., 1996).

\textit{Growth conditions and media}

\textit{S. commune} was grown in minimal medium (MM, Dons et al., 1979) either or not solidified with 1.5\% agar. For transformation, the \textit{\textDelta SC3} strain was grown from a mycelial homogenate for two days at 24 °C and 225 rpm in 100 ml MM in 250 ml flasks. For RNA/DNA isolation, colonies were grown for 2-3 days on the surface of a perforated polycarbonate (PC) membrane (diameter 76 mm, 0.1 \mu m pores, Poretics, USA) that was positioned on solidified MM. For microscopic observations of GFP fluorescence, colonies were grown on microscope slides covered with a thin layer of MM (1.5\% agar). To prevent formation of aerial hyphae, showing strong autofluorescence, a PC membrane was placed on top of the inocula.

\textit{Construction of plasmids for transformation}

All constructs harbor a \textit{phleomycin} resistance cassette with the \textit{ble} gene from \textit{Streptoalloteichus hindustanus} between the promoter and terminator region from the \textit{S. commune} \textit{GPD} (Glyceraldehyde-3-phosphate dehydrogenase) gene derived from pGPHt (Schuren and Wessels, 1994). The constructs shown in Figure 1 were made starting from p3HNcI, which was kindly provided by Dr. T. Schuurs. This plasmid consists of a 1 kb genomic fragment from \textit{S. commune} that contains the promoter region of the \textit{SC3} gene and the 5' non translated region of the gene down to the translation start codon (where an \textit{NcoI} site was introduced by PCR), cloned in pUC20. The different coding sequences used, which were also provided with an \textit{NcoI} site at the translation start codon by PCR, were fused to the \textit{SC3} promoter by using \textit{NcoI} and a downstream restriction site from p3HNc. All these constructs thus bear the 70 bp non-translated 5' region from \textit{SC3}. The 3' non-translated sequences plus downstream sequences used (to which we will henceforth refer to as terminator region) were those from the \textit{ABH1} or \textit{SC3} genes. In pABH1g.1 the terminator region of \textit{ABH1} (450 bp downstream of the stop codon) was amplified by PCR together with the coding sequence. In pSC6g and pSC6c, a shorter \textit{ABH1} terminator region (425 bp) was cloned by using a restriction site present 425 bp downstream of the stop codon of \textit{ABH1}. In pABH1c.3 and pABH1g.3 a 1.6 kb \textit{BclI/PstI} fragment from the \textit{SC3} gene was used as terminator region. This fragment contained the coding sequence for the last 10 amino acids of \textit{SC3} and
downstream sequences. In all other constructs the SC3 terminator region was also used but the sequence encoding the last ten amino acids of SC3 was removed by PCR. pSC3ci6 and pGFPci6 were made by introducing a PCR fragment that contains the third intron from SC6 directly downstream of the SC3 stopcodon of pSC3c or the GFP stopcodon of pGFPc, respectively. pSC3cir5' and pSC3cir3' were constructed by cloning an artificial intron, in the proper or reversed orientation, directly behind the stopcodon of SC3 in pSC3c. The artificial intron consisted of two complementary oligonucleotides which were allowed to anneal giving a dsDNA fragment of 50 bp with receded ends complementary to those of BamHI receded ends. In order to facilitate screening, reconstitution of BamHI sites after cloning was prevented by manipulating the sequence at the downstream end of the fragment (a C was added before the receded end). The artificial intron contained the 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 obeyed. To determine the consensus splice and branch site sequences in S. commune introns, eighty introns from twenty genes from this organism were examined (5' splice site, G99T98G58A46G91T71; branch site, C85T99G49A100C68; and 3' site C54A100G100 (the numbers represent the percentage of occurrence of the indicated nucleotide at that position). The designed sequence is depicted below with the consensus splice sequences and branch site underlined:

5' gatccgtgagtagatctcagctatgagtgtctccctgtgacggtcgtacgtaacgcttatgagcgcgttacagc 3'
3' cactctacttagtgctccagacagacgggaggagaagggacgactgcagcaggtgctag 5'

Plasmids pSC3ciu, pSC3ci5', pSC3ci3' and pSC3cid were constructed by introducing the third intron of SC6 containing slight modifications of the 5' splice site (in bold) in order to make it conform to the consensus sequence for this site. The sequence of this intron is depicted below with the splice sequences and branch site underlined:

5' gtgagtccccacctttccaccaaaggtcgtacatatgtggctcaacgctcttagc 3'
3' cactcaggggtgaagaggtggttttccagaactgtgcatgtgctgtaggtgc 5'

This intron was also synthesized in vitro as two complementary oligonucleotides, which were allowed to anneal giving a dsDNA fragment of 51 bp. Plasmids pSC3ci5' and pSC3ci3' were constructed by introducing this intron in the SmaI site in the coding sequence of the SC3 cDNA (pSC3c) in the 5'-3' and 3'-5' orientation, respectively. pSC3ciu was constructed by cloning a similar intron containing NcoI receded ends, directly upstream of the startcodon of SC3 in pSC3c. pSC3cid was constructed by cloning a similar intron containing BamHI receded ends, directly behind the stopcodon of SC3 in pSC3c.
Transformation of *S. commune*

*S. commune* strain 72-3 (∆SC3) was transformed essentially as described by Schuren and Wessels (1994), except that it was protoplasted in 1 M MgSO₄ containing 1 mg ml⁻¹ Lysing enzymes from *Trichoderma harzianum* (Sigma, USA). 5-10 µg of DNA was added to 3·10⁷ protoplasts in 100 µl 1 M sorbitol. Transformants were selected on MM-plates containing 40 µg ml⁻¹ phleomycin (Cayla, France) and 500 µg ml⁻¹ caffeine (Sigma, USA), allowing selection of transformants despite the presence of another phleomycin resistance cassette already contained in the ∆SC3 strain (Wösten et al., 1994a; van Wetter et al., 1996).

*Figure 1.* Constructs used for transformation of *S. commune*. The hatched boxes represent various introns, the third intron of *SC6* ( ), the third intron of *SC6* with modifications of the 5’ splicing sequence ( ), and an artificial intron ( ). The black boxes ( ) represent native introns. In the column Expr., the level of expression of the different constructs is given. (++) represents the level of mRNA accumulation as reached by the SC3 gene in the wild-type strain or higher. (-) represents low or undetectable levels of mRNA.

*Transformation of S. commune*

*S. commune* strain 72-3 (∆SC3) was transformed essentially as described by Schuren and Wessels (1994), except that it was protoplasted in 1 M MgSO₄ containing 1 mg ml⁻¹ Lysing enzymes from *Trichoderma harzianum* (Sigma, USA). 5-10 µg of DNA was added to 3·10⁷ protoplasts in 100 µl 1 M sorbitol. Transformants were selected on MM-plates containing 40 µg ml⁻¹ phleomycin (Cayla, France) and 500 µg ml⁻¹ caffeine (Sigma, USA), allowing selection of transformants despite the presence of another phleomycin resistance cassette already contained in the ∆SC3 strain (Wösten et al., 1994a; van Wetter et al., 1996).
Chapter 4

Immunodetection at the colony level
Up to 30 colonies were grown on one polycarbonate (PC) membrane positioned on MM-agar medium. The PC-membrane containing the colonies was transferred to a PVDF (polyvinylidene difluoride) membrane overlying a fresh MM-agar plate. After 3 h incubation at 30 °C the PVDF membrane was removed. Secreted ABH1 immobilized at the PVDF membrane was monitored by immunodetection using antibodies raised against ABH1 (Lugones et al., 1996).

Fluorescence Microscopy
To monitor GFP fluorescence, colonies grown on microscope slides were observed in an Axiophot microscope by epifluorescence (Carl Zeiss, Germany) using FITC filters (#K3, L4) with excitation and suppression ranges of 450-490 nm and 515-560 nm, respectively.

RNA and DNA isolation, Blot Hybridization and Run-on analysis
Four colonies per plate were grown on a PC-membrane until a colony diameter of 2 cm was reached (about three days). They were then harvested, put in 1.5 ml conical tubes and immediately frozen in liquid N2. They were ground in the tubes for 15 s by using a loosely fitting metal pestle cooled in liquid nitrogen and turning at 1000 rpm. Nucleic acids were then isolated following the DNA CTAB extraction protocol described by Murray and Thompson (1980). To isolate both RNA and DNA, nucleic acids were not precipitated directly with isopropanol after the high salt TE buffer step, but 1/3 volume 8 M LiCl was added to the samples which were then held on ice for 3 h. After centrifugation, the pellet containing the RNA was treated and analyzed as described in Schuren et al. (1993a). Re-hybridizing membranes with an 18S rRNA probe quantified RNA on gels. The genomic DNA in the supernatant was precipitated by adding 2/3 volume isopropanol, washed with 70% ethanol and further analyzed as described by Schuren et al. (1993a). Run-on experiments with isolated nuclei were performed as described by Schuren et al. (1993b).

Results

Heterologous expression of ABH1 in S. commune requires introns
Because we were unable to target genes to defined positions in the genome, we cloned genes to be expressed and the phleomycin resistance cassette for selection of transformants in the same plasmid. This ensured that the phleomycin resistant colonies had inserted the plasmid at a location permitting gene activity. Initially, we compared the expression of the genomic sequence of ABH1 containing its own terminator region (pABH1g.1) and the cDNA sequence of ABH1 provided with the terminator region of SC3 (pABH1c.3), both under control of the SC3 promoter in the ∆SC3 strain of S. commune (Figure 1). The ∆SC3 strain was used because we attempted functional complementation of the SC3 gene by introducing the ABH1 sequence. Immunodetection at the colony level showed that colonies transformed with pABH1g.1 produced ABH1 while those transformed with pABH1c.3 gave no reaction with
the antibody (results not shown). Since the ABHI constructs contained the SC3 5’ non-translated region it was very simple to accurately compare the ABHI mRNA levels in the ∆SC3 strain to that of SC3 in the wild type strain. Some pABH1g.1 transformants reached an ABHI mRNA level similar to that of SC3 mRNA in the wild type strain (results not shown). This showed that the A. bisporus termination, polyadenylation, and splicing signals were fully recognized by S. commune. On the other hand the lack of expression from the cDNA construct could be a consequence of an incompatibility of the SC3 terminator with the heterologous sequence or the lack of introns in the ABHI coding region. To discriminate between these two possibilities the construct pABH1g.3 was made which only differed from pABH1c.3 with respect to the presence of the three introns of the ABHI gene (Figure 1). After transformation to S. commune wild-type strain 4-39, five colonies from each type were randomly selected and grown for RNA and DNA extraction. Northern blotting and hybridization to an ABHI probe showed that three out of five pABH1g.3-transformed colonies had a high level of ABHI mRNA while none of five pABH1c.3 transformants showed expression except one with a very low level of ABHI mRNA (Figure 2). Southern blots confirmed that all colonies contained the ABHI construct and that there were no obvious differences in ABHI copy number between the pABH1g.3 and the pABH1c.3 transformants (results not shown). Since the only difference between the transformed constructs was the presence or absence of introns we concluded that the presence of introns is necessary for the expression of ABHI in S. commune. Somewhat unexpected, ABHI did not phenotypically complement the SC3 mutation.

Figure 2. Northern analysis of five randomly chosen transformants containing genomic or cDNA sequences encoding ABHI. ABHI cDNA served as a probe. (A) pABH1g.3 transformants containing genomic ABHI (1-5). (B) pABH1c.3 transformants containing ABHI cDNA (6-10). A similar amount of RNA isolated from A. bisporus fruiting bodies serving as a control (Ab).

Homologous expression of SC3 and SC6 in S. commune requires introns
To investigate whether introns were also needed for the expression of homologous genes a similar set of constructs to those used for ABHI was made for SC3 (Figure 1). pSC3g contains the genomic coding sequence from SC3 and pSC3c encompasses the SC3 cDNA, both stretching between start and stopcodon. A modification was made to the terminator region, which in the ABHI expression vectors had been cloned by restriction of the gene and still carried the sequence that codes for the last 10 amino acids of SC3. To remove this coding sequence, a fragment containing the sequences downstream of the stopcodon of SC3 was amplified by PCR and used as terminator region. The constructs were introduced in the ∆SC3 strain. Northern analysis showed a high SC3 mRNA level in most of the transformants bearing a SC3 genomic coding region (Figure 3A) and a low one for all transformants that contained the SC3 cDNA (Figure 3B).
The *SC6* gene, which is transcriptionally silent in monokaryotic strains (Schuren et al., 1993b), was also tested in this way. Two constructs were made (Figure 1): pSC6g that contains the genomic coding sequence from *SC6* and pSC6c encompassing the *SC6* cDNA, both stretching between start-and stopcodon. Both constructs contained the promoter region of the *SC3* gene and terminator region of the *ABH1* gene. The constructs were used to transform the wild type strain 4-39 and 10 randomly chosen phleomycin resistant colonies from each type were screened for *SC6* mRNA accumulation. It was found that 7 out of 10 pSC6g transformants showed high *SC6* expression (Figure 3C) while all 10 pSC6c transformants failed to give any expression (Figure 3D). Southern analysis of 5 colonies of each type showed that all colonies contained extra *SC6* copies in addition to the endogenous copy, and no obvious differences were found in the number of integrated copies of the two plasmids (result not shown). In spite of the forced expression of *SC6* in the monokaryon, this gene could not complement the phenotype caused by the mutation in the *SC3* gene.

**Intron-dependent mRNA accumulation in S. commune is a post-transcriptional event**

To examine whether the absence of introns in some way caused transcriptional inactivity, run-on analysis was performed on nuclei isolated from four transformants from the *SC6*

**Figure 3.** Northern analysis of transformants of *S. commune* containing integrated *SC3* and *SC6* gene constructs with and without introns. Numbered lanes contain RNA from transformants. Lanes marked wt and r contain RNA from the recipient wild-type strain 4-39 and the 72-3 strain, respectively. Strain 72-3 transformed with (A) the intron-containing construct pSC3g and (B) the intron-less construct pSC3c. The RNA was probed with *SC3* cDNA. Strain 4-39 transformed with (C) the intron containing construct pSC6g and (D) the intron-less construct pSC6c. RNA was probed with *SC6* cDNA. Equal amounts of RNA were loaded, as verified by hybridization with an 18S rRNA probe.

**Figure 4.** (A) Nuclear run-on analysis. Nuclei were isolated from two transformants (4 and 8) containing pSC6g (*SC6* gene with introns) and two transformants (22 and 26) containing pSC6c (*SC6* cDNA). Run-on transcription was performed in the presence of [32P]-UTP. Radioactive transcripts were hybridized to specific DNAs patterned onto hybond N membranes, as indicated on the left bottom panel. (B) *SC6* mRNA levels of the same transformants at the time of isolation of the nuclei.
series (two containing pSC6c and two containing pSC6g), which showed similar copy numbers (1 to 3). As observed earlier (Schuren et al., 1993b), SC7 and SC4 were found not to be transcribed in the monokaryon while SC3 was active (Figure 4). SC6 was found to be similarly active in all four transformants. These results show that the effect of introns on the SC6 mRNA levels takes place at the post-transcriptional level.

Introduction of an intron restores mRNA accumulation

The third intron from SC6 was cloned directly behind the SC3 stop codon in the cDNA construct pSC3c (pSC3ci6, Figure 1) and this construct was introduced into the ΔSC3 strain. The SC3 mRNA level in transformant colonies was found to increase to up to 100% of that of the wild type strain 4-39 (Figure 5A). This was also the case when the third intron from SC6 was inserted within the coding sequence of the SC3 cDNA (pSC3ci5’, Figure 1; Figure 5B). When the same intron was inserted in the 3’-5’ orientation (pSC3ci3’, Figure 1), the SC3

mRNA level in transformant colonies was not elevated (Figure 5C). This indicated that the effect of the intron on mRNA levels was not due to the presence of specific sequences (e.g. enhancers) apart from the splicing consensus sequences. This was tested further by using an artificial intron, which was cloned behind the stop codon of the SC3 cDNA-coding region in pSC3c in two orientations (pSC3cir5’ and pSC3cir3’, Figure 1). Northern analysis of ten randomly chosen phleomycin resistant colonies of each type showed that colonies transformed with the construct bearing the intron in ‘splicing orientation’ (pSC3cir5’) accumulated levels of SC3 mRNA up to 100% of the wild type expression level (Figure 6A). Unexpectedly, also the transformants with the artificial intron in the ‘non-splicing orientation’ (pSC3cir3’) showed a higher SC3 mRNA level than transformants containing pSC3c (SC3 cDNA without added intron, not shown), although this level was on average somewhat lower than that of strains transformed with pSC3ci5’ (Figure 6B). When the sequence of the inverted intron in the integration region was examined in detail, it became clear that a new intron-like element had arisen which could use splice and branch sites fortuitously occurring in the intron itself and the terminator region of SC3:

Figure 5. Northern analysis of strains with SC3 cDNA constructs containing an SC6 intron. (A) 72-3 transformed with pSC3ci6 (intron behind the stop codon of the SC3 cDNA sequence in the 5’-3’ orientation, lane 1-5). (B) 72-3 transformed with pSC3ci5’ (intron within the SC3 cDNA sequence in the 5’-3’ orientation, lane 1-5). (C) 72-3 transformed with pSC3ci3’ (intron within the SC3 cDNA sequence in the 3’-5’ orientation, lane 1-5). Lanes marked wt and r correspond to the wild-type strain 4-39 and recipient strain 72-3, respectively, serving as controls. Equal amounts of RNA were loaded. SC3 cDNA served as probe.
To see whether this putative new intron was indeed spliced and also to confirm splicing in the case of the artificial intron in the 5'-3' orientation, cDNA was synthesized from mRNA of both types of transformants using a poly dT oligonucleotide as primer. PCR was performed using both RT mixtures as template and the construct pSC3cir5' (SC3 cDNA construct plus artificial intron) as a control. As primers, oligonucleotides were synthesized corresponding to sequences at both sides of the inserted intron. The amplification product that arose from each RT mixture was 350 bp long, which is 50 bp shorter than the PCR product using pSC3cir5' DNA (containing the artificial intron) as target (Figure 7). This showed that the artificial intron was spliced in both cases. In the case of the amplification product from the RT mixture of the transformant containing the pSC3cir3' construct (inverted intron) also a band at the height of the unspliced mRNA was observed. However, this band was less intense, suggesting that some mRNA still contained the inverted intron. This was confirmed by the fact that the cDNA corresponding to this mRNA could be cut at a BglII restriction site present in the artificial intron (not shown).

To analyze whether the position of an introduced intron influences mRNA accumulation, the third intron of SC6 with a modification in the 5' splice site was introduced directly upstream of the startcodon (pSC3ciu), directly downstream of the stopcodon (pSC3cid) or within the SC3 cDNA sequence (pSC3ci5'). The constructs were transformed into the ∆SC3 strain and SC3 mRNA accumulation was monitored by Northern blot analysis (Figure 8). When the intron was placed either upstream of the startcodon or downstream of the stopcodon, SC3 mRNA accumulated on average to a similar level (30% and 38% of the

![Figure 6. Northern analysis of colonies transformed with SC3 cDNA constructs provided with an artificial intron. (A) pSC3cir3' containing the artificial intron in the 5'-3' orientation behind the stopcodon of SC3 cDNA. (B) pSC3cir3', as (A) but containing the artificial intron in the 3'-5' orientation. Lanes marked wt and r correspond to the wild-type strain 4-39 and recipient strain 72-3, respectively. Numbered lanes (1-5) correspond to 5 randomly selected transformants. Equal amounts of RNA were loaded. The probe used was a SC3 cDNA.

![Figure 7. PCR on (1) pSC3cir5' DNA containing the artificial intron and on a reverse transcriptase mixture of (2) RNA from strain 72-3 transformed with pSC3cir5' and (3) transformed with pSC3cir3'.

![Artificial intron (reversed) SC3 Terminator]

5' gatcgaacctgagcatgatctgctgctg
new intron
3' gaagctcactgacggtcagcaggtacg

61
WT SC3 mRNA level, respectively). Transformants containing the construct with the intron in the SC3 cDNA sequence showed an average accumulation of SC3 mRNA to 60% of the wild-type SC3 mRNA level. These results indicate that the presence of an intron in the SC3 cDNA coding sequence restores SC3 mRNA accumulation to a higher level compared to introns placed outside the translational unit. However, the position of the intron is not crucial in effecting an increase in mRNA accumulation.

**Figure 8.** Northern analysis of colonies transformed with (A) pSC3ciu, containing the modified third intron of SC6 in front of the start codon of the SC3 cDNA sequence, (B) pSC3ci5’, containing the same intron within the coding sequence of SC3 cDNA and (C) pSC3cid, containing the same intron behind the stop codon of SC3 cDNA. Numbered lanes contain RNA from transformants. Lanes marked wt and r contain RNA from the wild-type strain 4-39 and the recipient strain 72-3, respectively. Lanes marked c and g contain RNA from colonies transformed with pSC3c (cDNA) and pSC3g (genomic DNA), respectively. Lanes marked a1 and a2 contain RNA from two colonies transformed with pSC3cir5’. The blot was probed with SC3 cDNA and the relative amount of RNA loaded on gel was determined by hybridization with an 18S rRNA probe.

*Introduction of an intron in GFP elevates accumulation of its mRNA*

To determine whether introns are also required for efficient expression of genes other than those for hydrophobins, we compared the expression of the cDNA of the gene encoding Green Fluorescent Protein (pGFPe, Figure 1), which does not contain introns, with expression of this sequence with an intron (the third intron of SC6) cloned directly behind the stopcodon (pGFPei6, Figure 1). In both cases the GFP sequence was placed under regulation of the SC3 promoter and terminator region and transformed to S. commune wild-type stain 4-39. Northern analysis showed a higher GFP mRNA level in transformants bearing the intron-containing GFP construct than those bearing the intron-less construct (results not shown). Microscopical observation showed that transformants bearing the intron-containing GFP construct were clearly fluorescent, in contrast to transformants bearing the intron-less construct (Figure 9).
In the present study we show that gene expression in *S. commune* requires introns, at least in the case of the four genes analyzed. After introducing the coding sequences of *ABH1* (*A. bisporus*), *SC3*, *SC6* (*S. commune*) and *GFP* (*Aequorea victoria*), without introns no or very low expression levels were observed contrasting with the expression of intron-containing sequences. All experiments were carried out using the *SC3* promoter, but similar results were obtained when genomic *SC3* coding sequences, *SC3* cDNA coding sequences and *SC3* cDNA coding sequences with an intron directly behind the stop codon were expressed using the *gpd* promoter of *S. commune* (not shown). Placement of one intron outside the translational unit enhanced expression of *SC3* to levels similar to those found in transformants containing an *SC3* coding region with all native introns. This was true for both introns tested, *i.e.* the third intron from *SC6* and an artificial intron. Apparently, the nature of the intron is not important.

**Discussion**

Figure 9. Hyphae from *S. commune* transformed with (A, B) pGFPc (*GFP* construct without an intron) and (C, D) with pGFPci6 (*GFP* construct with the third intron of *SC6* behind the stop codon). Pictures were taken with (A, C) phase contrast and (B, D) with UV.
By chance, introduction of an artificial intron behind the stopcodon of the SC3 cDNA coding sequence in inverted orientation gave rise to a new putative intron, which contained all the sequences known to be required for splicing. As 3′ splice site the sequence CAG present in the SC3 terminator region (6 bp downstream of the stopcodon of SC3) was probably used. By PCR it was shown that the introns in both orientations were spliced. However, a faint band at the height of the non-spliced mRNA was observed after amplification of the mRNA of the gene with the artificial intron in 3′-5′ orientation. This could indicate that the inverted intron was less efficiently spliced than the intron in the normal orientation.

The position of an introduced intron seems to influence mRNA accumulation. When an intron was placed within the SC3 cDNA coding sequence the average SC3 mRNA accumulation was increased two times compared to situations where the intron was placed outside the translational unit (before the startcodon or behind the stopcodon). In plants it was shown that the splicing efficiency correlates with the context in which an intron was placed (Carle-Urioste et al., 1994), which may also hold for S. commune. The context outside of the transcriptional unit of SC3 is different from that of the coding sequence. The GC content of coding sequences of S. commune is higher (64%) than that of non-translated regions (52%) (Schuren, 1992).

For the SC6 mRNA it was shown that the presence of introns only affected accumulation of mRNA and not the rate of transcription, excluding a role of the inserted sequences as enhancers. The findings that an artificial intron can substitute for a native intron, and that a native intron only affects mRNA accumulation when inserted in the right orientation, also argues against a role as enhancers.

The phenomenon of intron-dependent mRNA accumulation was previously shown to occur in mammalian and plant cells (Simpson and Filipowicz, 1996; Koziel et al., 1996 and references therein). The results presented here are in agreement with the general idea that dependency of mRNA accumulation on introns is a post-transcriptional event (Simpson and Filipowicz, 1996; Koziel et al., 1996 and references therein). However, its mechanism is not clear. Not all genes of plant or mammalian origin contain or require introns for efficient mRNA accumulation and the requirement of introns in plants is species dependent. It was suggested (Buchman and Berg, 1988) that genes that can be expressed in the absence of an intron contain sequences that are recognized by a constituent of the splicing system without the assembly of an active spliceosome but allowing mRNA accumulation in a similar way as intron-containing sequences. In addition, it was suggested (Koziel et al., 1996 and references therein, Xu and Hall, 1994) that differences in the degree of mRNA accumulation in plants are related to the splicing efficiency of the intron used. This may explain the fact that not all introns affect mRNA accumulation equally.

It is not yet clear whether the splicing reaction itself is required for the increase in mRNA accumulation. For instance, unsplicable derivatives of the Hsp82 intron, the Adh1 intron in maize (Sinibaldi and Mettler, 1992; Luehrsen and Walbot, 1994) or the IVS2 intron in mammalian cells (Buchman and Berg, 1988) were unable to increase mRNA
accumulation, while unsplicable derivatives of a PAT1-GUS fusion in Arabidopsis still increased mRNA levels (Rose and Beliakoff, 2000). Two possible mechanisms for intron dependent mRNA accumulation are generally accepted. One is that introns protect the pre-mRNA from degradation (Kurachi et al., 1995), either by adopting a stable secondary structure or by providing binding sites for factors protecting the pre-mRNA, such as oligo-U-binding proteins (Gniadkowski et al., 1996) or heterogeneous nuclear ribonucleoproteins (Krecic and Swanson, 1999). An alternative model is that spliceosome assembly onto the introns in pre-mRNA facilitates an association with enzymes involved in other aspects of RNA maturation (such as polyadenylation) and transport of the mRNA to the cytoplasm. Using an intron-containing and intron-less CAT-gene in mammalian cells, it was shown that intron-mediated mRNA accumulation most likely was caused by a mechanism, which favored polyadenylation and transport of mRNA’s (Huang and Gorman, 1990). Furthermore, for the human β-globin gene it was shown that the second intron, but not the first, was necessary for accumulation of its cytoplasmic mRNA and that the presence of this intron and not its splicing promotes efficient polyadenylation (Antoniou et al., 1998). On the other hand, a correlation between splicing and mRNA transport was observed when intron-containing and intron-less mRNA’s were introduced in nuclei of Xenopus oocytes (Luo and Reed, 2000). mRNA’s containing introns were, after removal of the introns (spliced mRNA), much more rapidly and efficiently exported than the intron-less mRNA’s (non-spliced mRNA). Moreover, it was shown that different ribonucleoprotein complexes assembled on the spliced and the non-spliced mRNA’s. From this it was concluded that splicing generates a specific nucleoprotein complex that targets mRNA for transport.

In conclusion, subtle differences in the specificity of association of ribonucleoproteins or spliceosomal proteins with the mRNA and the influence of splicing or spliceosome assembly on other events like capping, polyadenylation, turnover and transport into the cytoplasm may account, in a complex way that is not yet understood, for the various effects observed in intron-dependent mRNA accumulation.

Even less is known about intron-dependent mRNA accumulation in fungi. Only in one other fungus, Podospora anserina, the phenomenon was reported to occur (Dequard-Chablat and Rötig, 1997). In the ascomycetous yeast Saccharomyces cerevisiae only 2-5% of the genes contain introns and no requirement for introns has been observed when genes from plants or vertebrates were expressed (Hiraiwa et al., 1997; Lin et al., 1997). However, characteristics and splicing mechanisms of yeast introns are different from those of filamentous fungi (Gurr et al., 1987). In contrast to introns in genes from animals, S. cerevisiae and plants, introns of filamentous fungi are characteristically short (≤100 bp). With respect to the degree of degeneracy of the splice and branch sites they resemble more the introns from animals and plants than those of S. cerevisiae (Gurr et al., 1987).

In filamentous fungi the proportion of genes containing introns is higher. A survey of the EMBL nucleotide databank showed that in the filamentous ascomycetes Aspergillus niger, Aspergillus nidulans, Neurospora crassa, Trichoderma reesei and Podospora anserina 68, 82, 78, 80 and 81% of the nuclear, protein encoding genes contain introns, respectively
(210 genes were analyzed in total). cDNA sequences are often used to express heterologous genes in *A. niger* and *T. reesei* (Carrez *et al.*, 1990; Ward *et al.*, 1992; Arundhati *et al.*, 1995) and no data are available on requirement of introns. However, in the ascomycete *P. anserina* it was shown that the presence of at least one intron was required for expression of the gene encoding the ribosomal protein S12 (Dequard-Chablat and Rötig, 1997). For basidiomycetes, a survey of the EMBL nucleotide databank (Lugones, 1998) showed that all analyzed nuclear, protein encoding genes (85 genes in total) of the homobasidiomycetes *S. commune*, *A. bisporus*, *Coprinus cinereus*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Lentinus edodes* contained introns. In contrast, analysis of 36 genes of the heterobasidiomycete *Ustilago maydis* showed that only 14% of the genes contained introns.

It is conceivable that this phenomenon of intron-dependent mRNA accumulation only plays a role in organisms that generally contain introns in their genes. The results presented for *S. commune* may therefore be relevant for other homobasidiomycetes, such as the commercially important species *Agaricus bisporus* (common mushroom) and *Pleurotus ostreatus* (oyster mushroom), in establishing efficient transformation of protoplasts with plasmids carrying bacterial selection or reporter genes. They also indicate that introducing introns may increase expression levels of heterologous genes in ascomycetes such as *A. niger* and *T. reesei*.

**Acknowledgements**

We thank Professor R. Kahmann (Ludwig-Maximilian-Universität München, Institute of Genetics and Microbiology) for kindly providing us with the synthetic GFP-gene (S65T), Kasia Karwacka for making some of the constructs, Lourens Bosch for performing some of the transformations and RNA isolations and Dr. Han Wösten for discussions and comments on the manuscript. K. Scholtmeijer was financially supported by The Netherlands Technology Foundation (STW), coordinated by the Life Sciences Foundation (SLW).