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Glucoamylase Gene Fusions Alleviate Limitations for Protein Production in *Aspergillus awamori* at the Transcriptional and (Post)Translational Levels

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In this study we have analyzed the effects of a glucoamylase gene fusion on the mRNA levels and protein levels for the human interleukin-6 gene (*hil6*) and the guar α-galactosidase gene (*aglA*). Previously it was shown that production of nonfused α-galactosidase and hIL-6 in *Aspergillus awamori* was limited at transcriptional and (post)translational levels, respectively (R. J. Gouka, P. J. Punt, J. G. M. Hessing, and C. A. M. J. J. van den Hondel, Appl. Environ. Microbiol. 62:1951–1957, 1996). Vectors were constructed which contained either the *hil6* or *aglA* gene fused to the *Aspergillus niger* glucoamylase gene (*glaA*) under control of the efficient 1,4-β-endoxylanase A promoter and transcription terminator. For comparison, the vectors were integrated in a single copy at the *pyrG* locus of *A. awamori*. A *glaA* fusion to the 5′ end of the *hil6* gene resulted in a large increase in hIL-6 yield, whereas with a *glaA* fusion to the 3′ end of the *hil6* gene, almost no protein was produced. Nevertheless, the steady-state mRNA levels of both fusions were very similar and not clearly increased compared to those of a strain expressing nonfused hIL-6. Fusions of *glaA* to the 5′ end of the wild-type guar *aglA* gene resulted in truncated mRNA lacking almost 900 bases (>80%) of the *aglA* sequence. When the coding sequence of the wild-type *aglA* gene was replaced by a synthetic *aglA* gene with optimized *Saccharomyces cerevisiae* codon usage, full-length mRNA was obtained. Compared to a nonfused synthetic *aglA* gene, a *glaA* fusion with the synthetic *aglA* gene resulted in a 25-fold increase in the mRNA level and, as a consequence, a similar increase in the α-galactosidase protein level. The truncated transcripts derived from the wild-type *aglA* gene were further analyzed by nuclear run-on transcription assays. These experiments indicated that transcription elongation in the nucleus proceeded at least 400 bases downstream of the site where the truncation was determined, indicating that transcription elongation or premature termination was not the reason for the generation of truncated mRNAs. As the truncated mRNA also contained a poly(A) tail, truncation most likely occurs by incorrect processing of the *aglA* mRNA in the nucleus.

Filamentous fungi, especially members of the genus *Aspergillus*, have the ability to secrete large amounts of a wide range of different enzymes into their environment. This characteristic has favored the use of these organisms for large-scale production of commercially important enzymes (reviewed in references 9, 34, and 38). Although production of fungal proteins is usually quite efficient, nonfungal proteins are poorly produced and reach levels that often do not exceed a few milligrams per liter (9, 12, 34, 35). Therefore, several strategies have been developed to improve these yields. A successful strategy is based on the use of a well-secreted carrier protein, usually *Aspergillus niger* glucoamylase (GLA) (3, 6, 28, 39, 40) or *Trichoderma reesei* cellobiohydrolase I (23), which is fused to the N-terminal end of a heterologous protein. With this strategy a fusion protein is produced in which the N-terminal GLA is believed to improve the secretion efficiency of the heterologous protein by facilitating translocation and subsequent folding in the endoplasmic reticulum. Further along the pathway, in most cases the fusion protein is cleaved, resulting in the secretion of the separate proteins. Cleavage occurs either by autocatalytic processing of the heterologous protein (39), by an unknown fungal protease (22, 23, 28), or by a KEX2-like protease, for which a recognition site had been introduced in the fusion protein (3, 6, 40).

The aim of our study is to obtain more insight into the parameters determining the efficiency of the production and secretion of heterologous proteins in filamentous fungi. Recently, we showed that production of two different heterologous proteins, guar α-galactosidase (AGL) (encoded by *aglA*) and human interleukin-6 (hIL-6) (encoded by *hil6*), was limited at the transcriptional and posttranslational levels, respectively (12). In this study the effect of a *glA* gene fusion with the *hil6* and *aglA* genes on mRNA and protein levels was investigated. For our study, the filamentous fungus *Aspergillus awamori*, which has been shown to be an efficient producer of proteins (2, 8, 40) was used. Recently, a similar study was carried out with *T. reesei* for another fusion protein, cellobiohydrolase I (23). In contrast to those authors, we have chosen to analyze defined single-copy strains similar to those described previously (12), which would allow us to compare the effects of the gene fusions on the levels of production of the different target proteins more precisely.

MATERIALS AND METHODS

Strains, media, and growth conditions. *A. awamori* AW15.7 (*exlA*::*uidA*$^+$ *pyrG* HmB^r) (11), used as a recipient strain for transformation, is a derivative of *A. awamori* ATCC 11358 (CBS 115.52). In AW15.7 the genomic 1,4-β-endoxylanase (*exlA*) encoding sequence has been replaced by a DNA fragment containing an expression cassette with the gene encoding mature *Escherichia coli* β-glucuronidase under control of the *exlA* expression signals (11). *E. coli* JM109 (31) was

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TABLE 1. Strains and vectors used in this study

Strain[a]	Prepro-sequence	Gene or gene fusion	KEX2 site	Reference
AWGLA$_{KEX}$HIL6	*glaA*	*glaA$_{G2}$-hil6*	+	This paper
AWHIL6$_{KEX}$GLA	*exlA*	*hil6-glaA$_{G2}$*	+	This paper
AWGLA	*glaA*	*glaA$_{G2}$*	−	12
AWHIL6	*exlA*	*hil6*	−	12
AWGLA$_{KEX}$AGLS	*glaA*	*glaA$_{G2}$-aglA$_{syn}$*	+	This paper
AWGLA$_{KEX}$AGL	*glaA*	*glaA$_{G2}$-aglA*	+	This paper
AWGLAAGL	*glaA*	*glaA$_{G2}$-aglA*	−	This paper
AWAGLS	*exlA*	*aglA$_{syn}$*	−	12
AWAGL2	*exlA*	*aglA*	−	12
AW15.7-1			−	11

[a] The vectors correspond to the names of the strains preceded by the letter p. For example, strain AWGLA$_{KEX}$AGL corresponds to vector pAWGLA$_{KEX}$AGL.

used for propagation of plasmids. Induction of the *A. awamori exlA* promoter was carried out as described by Gouka et al. (11), using a transfer of mycelium from noninducing medium containing 1% sucrose as a C source to induction medium with 5% D-xylose as an inducer. D-Xylose concentrations were determined as described previously (11).

***Aspergillus* expression strains.** For construction of the five different expression strains, five expression vectors were made (Table 1), consisting of (i) an expression cassette, which includes *exlA* transcriptional regulatory sequences and DNA sequences encoding the truncated *A. niger* GLA G2 form (514 AA) (GLA$_{G2}$), thus lacking the starch-binding domain (3) fused to the coding sequence of either hIL-6 or guar AGL; (ii) a selection marker for transformation; and (iii) pUC19 sequences for cloning and amplification in *E. coli*.

In four of the five expression cassettes, the sequences encoding GLA$_{G2}$ and hIL-6, AGL (encoded by the wild-type gene), or AGL encoded by a synthetic gene (see below) (AGLS) were separated by a sequence encoding the amino acids NVISKR (identical to the *A. niger glaA* prosequence; referred to below as the KEX2 site). The C-terminal dibasic proteolytic cleavage site KR results in cleavage of the fusion protein into both separate proteins by a KEX2-like protease (3). All expression cassettes contained the *exlA* promoter and transcription terminator region. The DNA sequences of hIL-6 or AGL encoded the mature proteins, thus without the original pre- and prosequences.

Construction of the vectors was carried out as follows (Fig. 1). Vector pAWGLA1 or pAWGLA2 was used as the basis for all five vector constructions. These vectors contain the *A. awamori exlA* (15) promoter fused to the truncated *A. niger glaA* gene (encoding GLA$_{G2}$), followed by the *A. awamori exlA* transcription terminator in pUC19. The only difference between the vectors is located between the *Nar*I and the *Afl*II sites at the 3′ end of the *glaA* gene (Fig. 1).

For *glaA* fusions with the hIL-6 gene, pAN56-4 (3), containing a *glaA-hil6* fusion separated by a KEX2 site, was used. In pAN56-4 an *Afl*II site was introduced downstream of the stop codon of *hil6* by PCR, resulting in pAN56-4AflII. Simultaneously, the last two codons before the stop codon of *hil6* (CAA ATG) were changed into the sequence CAT ATG to introduce an *Nde*I site (this CAA-to-CAT transversion changes a glutamine into a histidine).

For construction of vector pAWGLA$_{KEX}$HIL6, in which *glaA* is fused to the 5′ end of the *hil6* gene, the *hil6* gene was isolated from vector pAN56-4AflII as a 0.6-kb *Eco*RV/*Afl*II fragment and introduced into pAWGLA2, which was also cut with *Eco*RV/*Afl*II, resulting in a correct in-frame fusion of *glaA* with *hil6*, separated by the KEX2-site. For construction of vector pAWHIL6$_{KEX}$GLA, in which *glaA* is fused to the 3′ end of *hil6*, a partial 1.8-kb *Eco*RI/*Nde*I fragment was isolated from pAWHIL6 (12). This fragment contains part of the *exlA* promoter and preprosequence, followed by the *hil6* gene up to the *Nde*I site. From pAWGLA1 a partial 7.9-kb *Bss*HII/*Eco*RI fragment was isolated, containing the *glaA* gene followed by the *exlA* transcription terminator, pUC19, and the remaining part of the *exlA* promoter. To obtain a correct fusion between the *Nde*I site at the 3′ end of *hil6* and the *Bss*HII site at the 5′ end of *glaA*, oligonucleotides (5′ TATGAATGTGATTTCCAAG 3′ and 5′ CGCGCTTGG AAATCACATTCA 3′) were used.

For the construction of the *Cyamopsis tetragonoloba aglA* fusion cassettes, two versions of the gene were used: the wild-type gene (24) and a synthetic gene with an optimized *Saccharomyces cerevisiae* codon bias (*aglA$_{syn}$*) (37) differing by 22% in nucleotide sequence from the cDNA clone. For construction of vector pAWGLA$_{KEX}$AGLS, in which *glaA* is fused to the 5′ end of the *aglA$_{syn}$* gene, first the 5′ end of the *aglA$_{syn}$* gene was adapted in vector pAWAGLS (12). In this vector the *aglA$_{syn}$* gene is under control of the *exlA* promoter and is preceded by the *exlA* preprosequence. At the 5′ end of *aglA$_{syn}$*, an *Eco*RV site was introduced by using an oligonucleotide linker (5′ GATCCGATATCCAAG 3′ and 5′ CTT GGATATCG 3′) which encodes part of the KEX2 site (amino acids ISK). The missing arginine (R) is obtained from the *exlA* prosequence. The *aglA$_{syn}$* gene was subsequently isolated from pAWAGLS as a partial 1.1-kb *Eco*RV/*Afl*II fragment and ligated with the 9.1-kb *Eco*RV/*Afl*II fragment from pAWGLA2.

The construction of pAWGLA$_{KEX}$AGL and pAWGLAAGL, in which the *glaA* gene is fused to the wild-type *aglA* gene, was identical for both vectors except for the linkers used to obtain the *glaA-aglA* fusion. These linkers were inserted into vector pUR2303AflII, a derivative of pUR2303 (37) in which an *Afl*II site was introduced almost immediately downstream of the stop codon of the *aglA* gene. pUR2303AflII was digested completely with *Nar*I, which cuts in the pBR322 sequence (four times), and with *Nco*I, which cuts after nucleotide 29 from the start of the mature coding sequence. For a GLA$_{KEX}$AGL fusion, the following oligonucleotide linker was inserted: 5′ CG CCA AAT GTG ATT TCC AAG CGC GCT GAA AAT GGA CTA GGC CAG ACA CCT CC 3′ and 5′ CAT GGG AGG TGT CTG GCC TAG TCC ATT TTC AGC GCG CTT GGA AAT CAC ATT TGG 3′, encoding the sequence PNVISKRAENGLGQTPP. For a GLAAGL fusion, the following oligonucleotide linker was used: 5′ CG CCA GCT GAA AAT GGA CTA GGC CAG ACA CCT CC 3′ and 5′ CAT GGG AGG TGT CTG GCC TAG TCC ATT TTC AGC TGG 3′, encoding the sequence PAENGLGQTPP. From both vectors the *aglA* gene was isolated as a 1.1-kb *Nar*I/*Afl*II fragment and ligated with a 9.1-kb *Nar*I/*Afl*II fragment from pAWGLA1, resulting in both cases in a correct in-frame fusion between *glaA* and *aglA*.

All relevant DNA sequences were verified by sequence analysis. As a selection marker, a mutant *A. awamori pyrG* gene (10) present on a 2.4-kb *Not*I fragment was inserted in all of the expression vectors. Transformants containing vectors integrated in a single copy at the *pyrG* locus in *A. awamori* AW15.7 (*exlA*::*uidA*$^+$ *pyrG* Hm^r) were identified by colony blot hybridization and subsequent Southern blot analysis.

To compare the effect of a GLA gene fusion of the heterologous genes with the corresponding nonfused genes, a number of control strains were included in the analysis (Table 1). Construction of these strains, AW15.7-1 (11) and AWGLA, AWHIL6, AWAGL2, and AWAGLS (12), has been described previously.

Recombinant DNA techniques. Standard recombinant DNA techniques were used for cloning procedures (31). *Aspergillus* chromosomal DNA and RNA were isolated as described by Kolar et al. (20). Colony blot hybridization of *A. awamori* was carried out as described by Kinsey (19). Transformation of *Aspergillus* and selection of transformants were carried out as described previously (11). For Northern blot analysis, as a probe a 400-bp DNA fragment that contained the 5′ and 3′ *exlA* untranslated sequences was used (12), which allowed a direct comparison of the amounts of specific mRNA in the different expression strains. With this probe, in each transformant the *uidA* mRNA provided with *exlA* expression signals could also be detected as a band of approximately 2.3 kb and used as a reference for the induction level.

For purification of polyadenylated mRNA, Dynabeads Oligo (dT)$_{25}$ (Dynal) were used.

Quantification of protein production levels. Quantification was carried out by Western blotting (31). For determination of protein concentrations, purified protein was used as a standard. Purified AGL and corresponding polyclonal antibodies were obtained from Unilever Research Laboratories. Purified GLA was obtained from Boehringer-Mannheim (lot 12005320-30). Monoclonal antibodies raised in mice against GLA were produced in our laboratory (21a). Purified hIL-6 (200-06) and polyclonal antibodies were supplied by Sanvertech. For detection, the ECL Western blot detection kit (Amersham) was used, based on peroxidase-labelled antibodies. Concentration of proteins was carried out as described by Broekhuijsen et al. (3). β-Glucuronidase assays were performed as described by Roberts et al. (27). AGL activity assays were carried out essentially as described by Overbeeke et al. (25).

For mycelial dry weight measurements, 25 ml of homogenous culture samples was filtered through Myracloth. The mycelium was squeezed, blotted dry, and dried overnight in a vacuum oven at 80°C.

Isolation of nuclei and nuclear run-on transcription assays. Isolation of nuclei and nuclear run-on transcription assays were carried out essentially as described by Schuren et al. (32).

RT-PCR and cloning of a PCR fragment. For synthesis of cDNA from an mRNA template, Moloney murine leukemia virus reverse transcriptase (RT) (Gibco BRL) was used. cDNA synthesis, with poly(A)$^+$ RNA from strains AWGLAAGL and AWAGL2, was carried out according to the protocol of the supplier (Gibco BRL) (60 min, 37°C), with an oligo(dT) primer [5′ GGAATT CGCGGCCGC(T)$_{20}$(G/A/C) 3′]. Prior to PCR, the samples were extracted once with phenol-chloroform to remove the RT, and the cDNA was ethanol precipitated. PCR on the cDNA was performed with, as an upstream primer, an oligonucleotide specific for *aglA* compromising the first 18 nucleotides of the coding sequence of the mature protein and the oligo(dT) primers mentioned above. A specific DNA fragment of approximately 250 to 260 bp which contained the *aglA* gene as shown by Southern blot analysis was obtained (data not shown). The fragment was isolated by using two successive PCRs, since the first RT-PCR resulted in the amplification of a mixture of DNA products. The conditions for the first reaction were 1 min at 94°C, 1 min at 37°C, and 1 min at 72°C for 40 cycles. In the second reaction, which was performed with a fraction of the first PCR mixture, the conditions were changed to 30 cycles and an annealing temperature of 40°C. The 250- to 260-bp fragment obtained with AWGLAAGL cDNA was digested with *Nco*I and *Eco*RI, and the resulting 220-bp fragment was subcloned in pUC19 which was digested with *Afl*III and *Eco*RI.

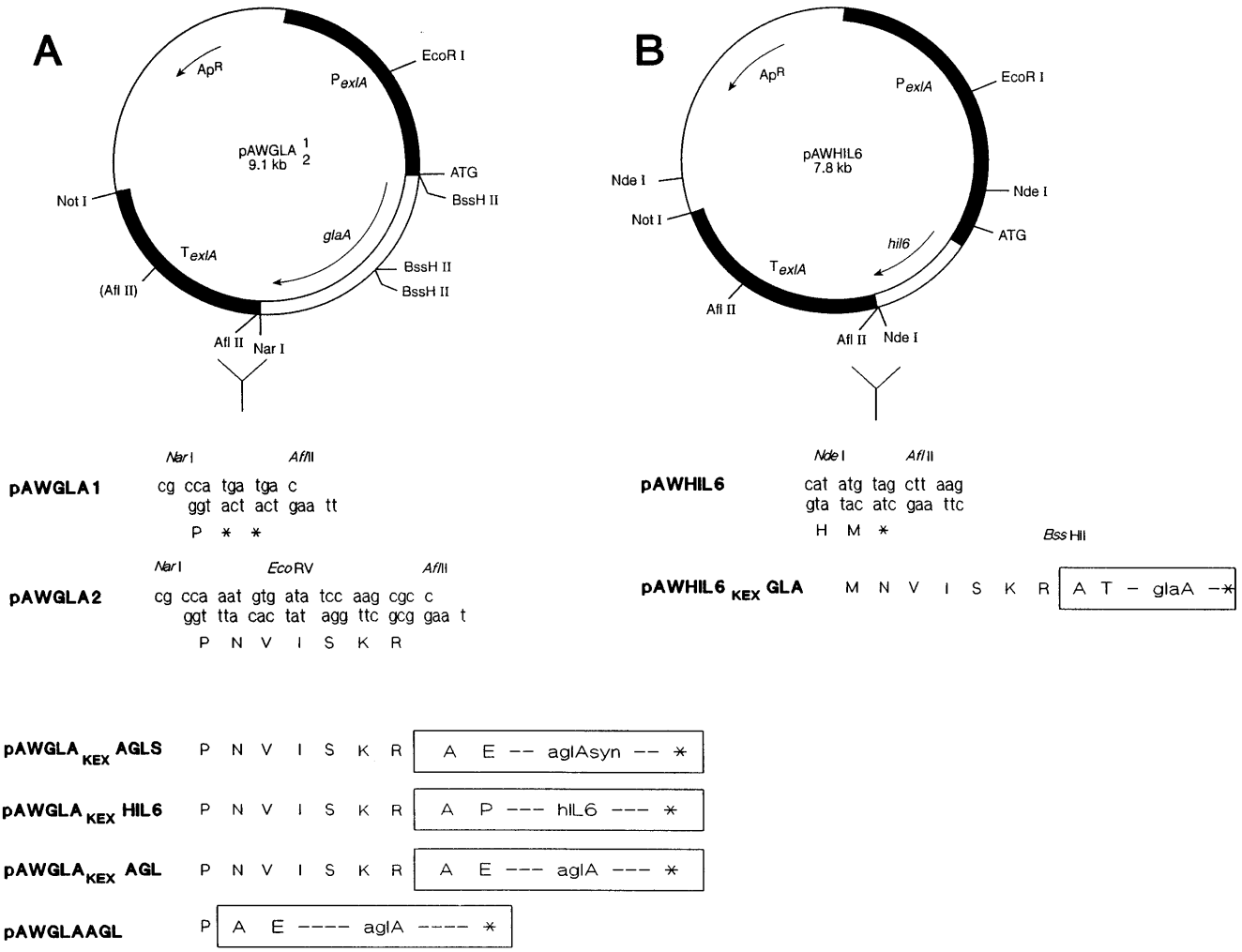

FIG. 1. Maps of plasmids pAWGLA1 and -2 (A) and pAWHIL6 (B), which were used as starting vectors for the construction of the gene fusions. Thin lines represent *E. coli* sequences, closed boxes represent *A. awamori exlA* 5′ and 3′ regulatory sequences, and open boxes represent either *A. niger glaA* (A) or *hil6* gene (B) sequences. Only relevant restriction sites are shown. The *Afl*II site in parentheses (A) has been removed in pAWGLA-1 and -2. Below the plasmid maps, the introduced linker sequences and the corresponding amino acid sequence are shown. The amino acid sequences of the fusion regions of the constructed fusion plasmids are indicated in the lower part. All fusions were obtained by using the restriction sites indicated in the figure (see Materials and Methods).

RESULTS

Construction of recombinant fungal strains and analysis of their growth and induction characteristics. Recently, we showed that production of guar AGL and hIL-6 was limited at the transcriptional and posttranslational levels, respectively (12). In this study, *glaA* gene fusions with the *hil6* and *aglA* genes were constructed to obtain more insight into which factors influence the expression of these genes and the production and secretion of the gene products. Five vectors containing expression cassettes in which *glaA* was fused to either *aglA*, $aglA_{syn}$, or *hil6* were constructed (see Materials and Methods) (Table 1). *A. awamori* AW15.7 (*exlA*::*uidA*$^+$ *pyrG* HmB^r) was transformed with the five vectors, and transformants with a single copy of the vector at the *pyrG* locus were identified by colony blot hybridization and subsequent Southern blot analysis. For each of the vectors, two single-copy transformants were used for further analysis. To compare the mRNA levels and the protein levels with those of strains containing the corresponding nonfused genes, these single-copy strains (12) were also included in the analysis (Table 1). As a control, AW15.7-1, a *pyrG*$^+$ derivative of AW15.7, was used (11). For analysis of mRNA levels and protein levels, each transformant was cultivated in duplicate according to the induction procedure described in Materials and Methods. Since mRNA and protein levels can be accurately compared only if no differences in growth and the level of induction occur, a number of control parameters were analyzed for each strain as described previously (12); growth of the cultures was checked by determination of culture morphology, dry weight, D-xylose consumption, and pH. With none of the parameters were significant differences observed, precluding any effects of them on the specific mRNA and protein levels. Measurements of the pH showed that it remained almost neutral (6.4 to 6.7) for all strains. In previous experiments (12) it was shown that hIL-6 and AGL were stable in this culture medium, excluding the effect of extracellular proteolytic degradation, a phenomenon that often influences the production of heterologous proteins in filamentous fungi (3, 28).

Induction levels were verified by analysis of the expression of the β-glucuronidase gene, which was present on a P_{exlA}-*uidA*-

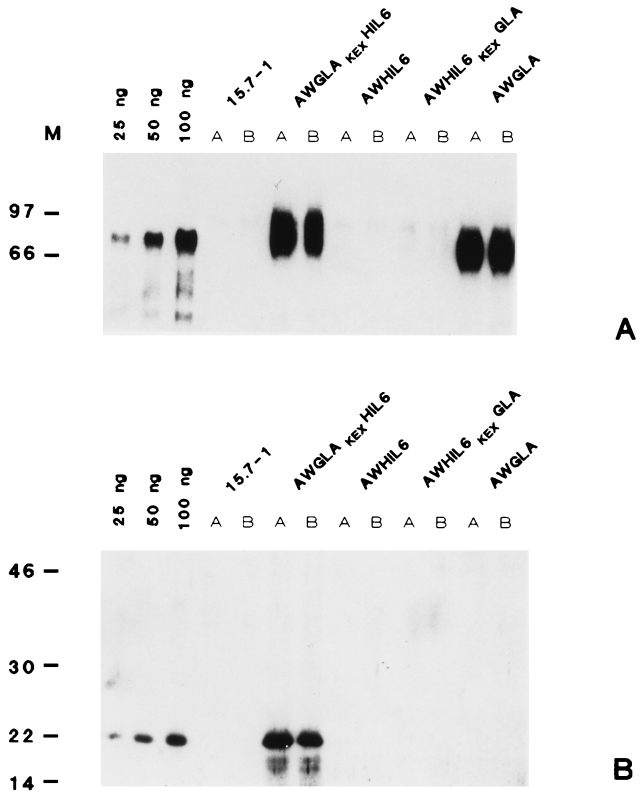

FIG. 2. Western blot analysis of strains harboring *glaA-hil6* constructs. Medium samples were taken 38 h after induction with D-xylose. Blots were incubated with antisera against GLA (A) and hIL-6 (B). The first three lanes of each panel contain purified protein; the remaining lanes contain 1-μl medium samples (A) or 15-μl medium samples (B). Purified GLA is a deglycosylated GLA_{G1} sample from *A. niger*; the amounts have been corrected for this deglycosylation. Protein levels were quantified more accurately by using serial dilutions (not shown). A and B above the lanes indicate samples of duplicate cultures. Lane M, molecular mass markers (in kilodaltons).

T_{exlA} reporter construct in all recombinant strains. In this case also, no significant differences were observed.

Previously it was shown that expression of genes driven by the *exlA* promoter is dependent on the presence of D-xylose in the medium. After approximately 48 h of cultivation, D-xylose was depleted in the medium, resulting in the degradation of mRNA and cessation of protein synthesis (11). Therefore, medium and mycelium samples were taken 22 and 38 h after induction. Since at 38 h the highest protein levels were obtained (about twice the amount of 22 h), only the results for the 38-h samples are reported.

Analysis of protein production in *hil6*-containing strains. The amounts of extracellular and intracellular hIL-6 for the relevant recombinant strains were quantified by Western blot analysis with purified hIL-6 as a standard. Only in medium samples of $AWGLA_{KEX}HIL6$ transformants, containing an expression cassette encoding GLA_{G2} fused to the N-terminal end of the hIL-6, could hIL-6 be detected, at levels of approximately 5 to 10 mg/liter (Fig. 2B). In medium samples of $AWHIL6_{KEX}GLA$ transformants, containing an expression cassette encoding GLA_{G2} fused to the C-terminal end of hIL-6, and of AWHIL6 transformants, no hIL-6 could be detected by Western blot analysis of nonconcentrated medium samples. After concentration of the medium, approximately 0.05 mg of hIL-6 per liter could be determined for both strains. As expected, in medium of the control strains AWGLA and AW15.7-1, hIL-6 could not be detected.

The GLA_{G2} levels in these strains were also determined by Western blot analysis (Fig. 2A). The amount of GLA_{G2} produced by $AWGLA_{KEX}HIL6$ transformants was slightly smaller than the amount produced by AWGLA transformants (125 to 175 mg/liter versus 150 to 200 mg/liter). In medium of $AWHIL6_{KEX}GLA$ strains, only 5 to 10 mg of GLA_{G2} per liter was present, which could be detected only when medium samples of 10 μl instead of 1 μl were applied to the gel (not visible in Fig. 2A). Upon induction with D-xylose, the endogenous *glaA* promoter is almost inactive. However, a small amount of endogenous GLA (approximately 10 mg/liter after 38 h) could be detected in all strains. Since endogenous (*A. awamori*) GLA is slightly larger in size than the (*A. niger*) GLA_{G2}, it could clearly be distinguished from GLA_{G2} on a protein gel.

As seen in Fig. 2A, the electrophoretic behaviors of GLA_{G2} and the processing product of $GLA_{KEX}HIL6$ are different. This difference cannot be explained by the presence of uncleaved $GLA_{KEX}HIL6$ fusion product, since the product was recognized only by a GLA antibody and not by an hIL-6 antibody (not shown in Fig. 2B). However, the addition of the amino acids NVISKR in the $GLA_{KEX}HIL6$ processing product (which is $GLA_{G2\text{-}KEX}$) may cause anomalous electrophoretic behavior, since two extra positively charged amino acids are introduced.

All protein levels are summarized in Table 2. To compare the amounts of protein independent of the molecular weights of the various proteins, the levels were calculated on molar basis and given relative to the amount of GLA_{G2} produced by the AWGLA strain (Table 2). It is clear that the GLA_{G2} production by $AWGLA_{KEX}HIL6$ is nearly as efficient (60 to 90%) as that by AWGLA. However, on molar basis the level of hIL-6 secreted by $AWGLA_{KEX}HIL6$ was only 10 to 20% of the GLA_{G2} level. Since GLA_{G2} and hIL-6 were initially produced as a fusion protein, this result would indicate that 60 to 90% of the hIL-6 is lost.

The hIL-6 level of $AWHIL6_{KEX}GLA$ was comparable to the hIL-6 level observed for AWHIL6, being only 0.1%, i.e., 100- to 200-fold lower than the hIL-6 yields observed for $AWGLA_{KEX}HIL6$. $AWHIL6_{KEX}GLA$ also produced less GLA_{G2} (3 to 6%), which was still higher than the hIL-6 level, indicating that more than 95% of hIL-6 is lost.

For all strains the presence of intracellular GLA_{G2} and hIL-6 was also analyzed, mainly to see whether accumulation of hIL-6 (and/or GLA_{G2}) had occurred, which could explain the low levels of hIL-6 in the medium. However, no significant accumulation of hIL-6 or GLA_{G2} was observed (data not shown).

Comparison of steady-state mRNA levels in *hil6*-containing strains. To investigate whether the differences in protein levels could be explained by variations in steady-state mRNA levels, Northern blot analysis was carried out (Fig. 3). As a probe a DNA fragment containing the 5′ and 3′ *exlA* untranslated regions was used (see Materials and Methods). In general, the mRNA level of a $GLA_{KEX}HIL6$ fusion was slightly higher than that of a $HIL6_{KEX}GLA$ fusion and nonfused hIL-6 but lower than that of nonfused GLA_{G2} (Table 2).

Analysis of protein production in *aglA*-containing strains. Previous experiments had shown that expression of the wild-type guar *aglA* gene resulted in undetectable mRNA levels, possibly due to truncation of the transcripts (12). Full-length mRNA, although at a low level, could be observed only when the wild-type guar *aglA* gene was replaced by a synthetic *aglA* gene with optimized yeast codon usage (12). As a result, AGL protein was detected only with strains containing the $aglA_{syn}$

TABLE 2. mRNA and protein levels

Strain	Relative mRNA level[a] (%)	Protein level[b] (mg/liter)			Relative protein level[c] (%)	
		GLA_{G2}	hIL-6	AGL	GLA_{G2}	Heterologous protein (hIL-6 or AGL)
AWGLA	100	150–200	—[d]	—	75–100	—
AWHIL6	25–50	—	0.05	—	—	0.1
$AWGLA_{KEX}HIL6$	50–75	125–175	5–10	—	60–90	10–20
$AWHIL6_{KEX}GLA$	25–50	5–10	0.05	—	3–6	0.1
AWAGL2	<1[e]	—	—	ND[f]	—	
AWAGLS	1	—	—	0.4	—	0.4
$AWGLA_{KEX}AGLS$	20–25	30–40	—	10–12	15–20	10–12
$AWGLA_{KEX}AGL$	<1[e]	ND	—	ND		
AWGLAAGL	<1[e]	ND	—	ND		

[a] Relative to the steady-state mRNA level of GLA_{G2}.
[b] Quantification of the protein levels was done by carrying out multiple Western blotting. GLA_{G2} levels were determined with pure, deglycosylated GLA (G1 form) from *A. niger* as a standard. The amounts of AGL were corrected for background values by using strain AW15.7-1 as a control.
[c] Protein levels (GLA_{G2}, hIL-6, and AGL) relative to the GLA_{G2} level of strain AWGLA (based on the following molecular masses: GLA_{G2}, 80 kDa; AGL, 45 kDa; hIL-6, 20 kDa). The GLA_{G2} level of 200 mg/liter was taken as 100%.
[d] —, not applicable.
[e] No full-length mRNA was detected (data based on comparison with $aglA_{syn}$ mRNA with an *aglA* probe).
[f] ND, not detectable.

gene. To further investigate these results and to see whether the limitations could be resolved, strains containing an expression cassette in which the *A. niger glaA* gene was fused to *aglA* or $aglA_{syn}$ were constructed.

The amounts of extracellular and intracellular AGL produced by the relevant strains (Table 1) were quantified by Western blot analysis with purified AGL protein as a standard. In medium samples of $AWGLA_{KEX}AGLS$, approximately 10 to 12 mg of AGL protein per liter could be detected (Fig. 4B), which was 25-fold more than the amount of AGL produced by the corresponding nonfused strain, AWAGLS (0.4 mg/liter). In medium of the latter strain, AGL was visible only after concentration of the sample (not visible in Fig. 4B) (12). AGL activity assays showed that all AGL, whether produced by $AWGLA_{KEX}AGLS$ or AWAGLS, was present as active protein (data not shown). In medium samples of transformants containing expression cassettes with *glaA* fused to the wild-type *aglA* gene, no AGL could be detected at all.

The GLA_{G2} levels in these strains also were determined by Western blot analysis (Fig. 4A). In medium of $AWGLA_{KEX}AGLS$ transformants, about 30 to 40 mg of GLA_{G2} per liter could be detected. In medium of $AWGLA_{KEX}AGL$ or AWGLAAGL, both containing the wild-type *aglA* gene, no GLA_{G2} could be detected. All strains also produced a small amount of the endogenous GLA in D-xylose medium (see above). The protein levels are summarized in Table 2.

In this case also, the protein levels were calculated on a molar basis and given relative to the amount of GLA_{G2} to compare GLA_{G2} levels with AGL levels (Table 2). The amount of GLA_{G2} in medium of $AWGLA_{KEX}AGLS$ was about 15 to 20% of the GLA_{G2} level in that of AWGLA. The level of AGL in medium of $AWGLA_{KEX}AGLS$ was more or less comparable (10 to 15%), suggesting that almost no intracellular degradation of AGL had occurred.

Analysis of intracellular protein levels showed only very low AGL levels, indicating that no accumulation had occurred (data not shown).

Analysis of steady-state mRNA levels in *aglA*-containing strains. To investigate whether fusion of *glaA* with *aglA* or $aglA_{syn}$ resulted in increased mRNA levels, Northern blot analysis was performed. The mRNA levels at both 22 and 38 h after induction were determined with as a probe the same DNA fragment as described in Materials and Methods and in reference 12. From Fig. 3 it is clear that a fusion of *glaA* with the $aglA_{syn}$ gene resulted in a large increase in the steady-state mRNA level. The level was approximately 25-fold higher than that observed for the nonfused $aglA_{syn}$ mRNA (not clearly visible in Fig. 3). However, this mRNA level was still about fourfold lower than that observed for nonfused *glaA* mRNA (AWGLA).

Fusions of *glaA* with the *aglA* wild-type gene, either in $AWGLA_{KEX}AGL$ or in AWGLAAGL, resulted in an mRNA molecule smaller than the expected size of 3.2 kb. Instead, a 2.2- to 2.3-kb mRNA was observed, which was at the same position on a Northern blot as the *uidA* mRNA, as could be shown by hybridization with an *aglA* (Fig. 3C)- or *glaA* (not shown)-specific probe. These experiments showed that the fusion mRNA was just a fraction larger than the *glaA* mRNA, indicating that a large part of the *aglA* gene was probably missing. Similar results (truncated *aglA* mRNA) had been observed for the nonfused *aglA* gene (12).

Analysis of truncated *glaA-aglA* or *aglA* mRNA. To determine which part(s) of the *glaA-aglA* mRNA was missing, Northern blot analysis was performed with probes derived from different parts of the *aglA* gene (Fig. 5). Strong hybridization was observed only when DNA fragment I, comprising the first 200 bp of the sequence encoding the mature AGL, was used as a probe. A much weaker hybridization signal was observed when fragment II (nucleotides 180 to 377) was used. With fragments III, IV, and V, comprising sequences downstream of nucleotide 378, no hybridization with the major truncated transcript was observed. However, with fragment III a faint hybridization signal was observed with a slightly larger mRNA. This hybridizing mRNA was also visible when fragments I and II were used, although the intensity of the signal was approximately 20- to 50-fold less than that of the major hybridization signal. Altogether, these results indicate that the majority of the truncated mRNAs lack at least 700 nucleotides of the 3′ part of the *aglA* coding sequence. In order to investigate whether a poly(A) tail was present in the *glaA-aglA* truncated mRNA molecule, poly(A) mRNA was isolated from strains containing *glaA* fusions with either the wild-type or the synthetic *aglA* gene and analyzed by Northern blotting with a DNA fragment comprising the *aglA* gene as a probe. With both strains, more than 90% of the hybridizing RNA was present in

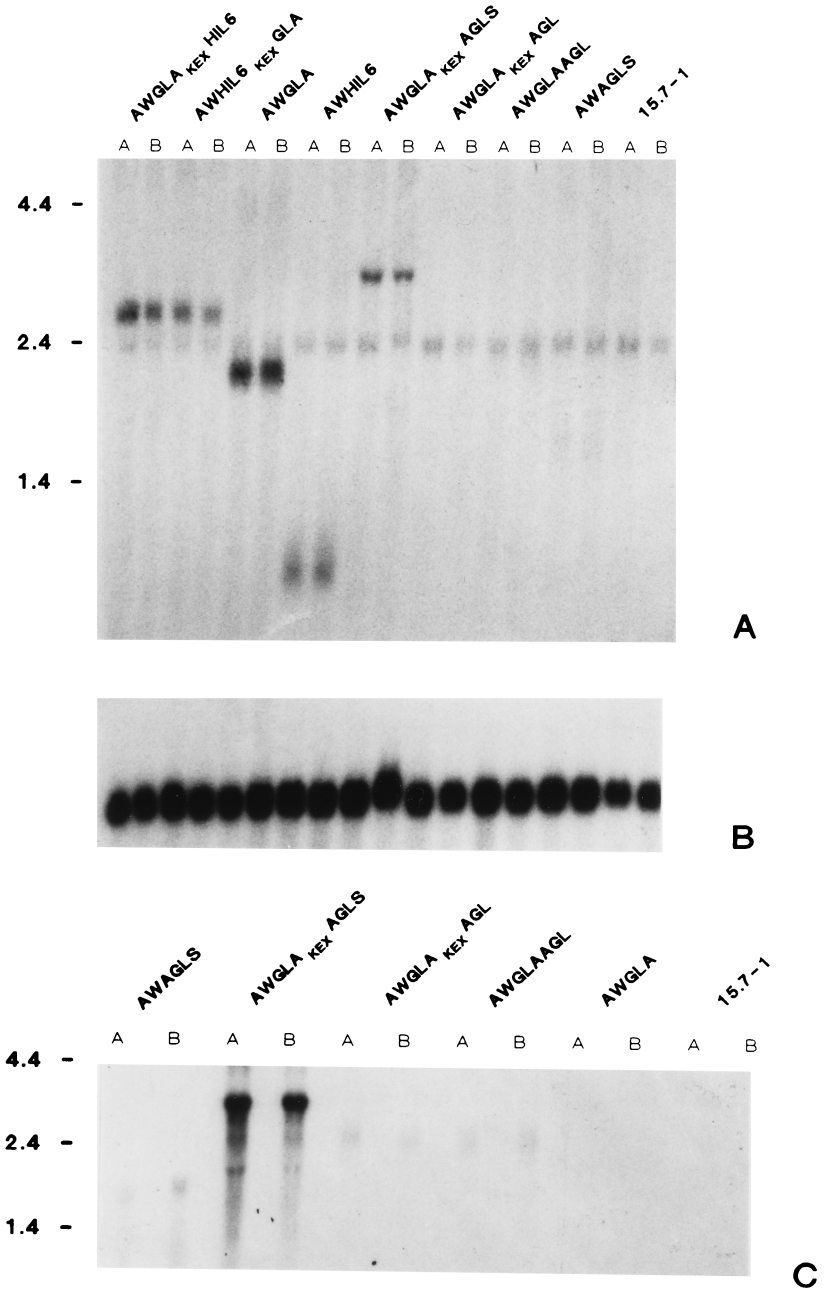

FIG. 3. Northern blot analysis of total RNA isolated from all expression strains after 38 h of induction. Probes were DNA fragments that contained both the 5′ and 3′ *exlA* noncoding regions (A), a 1.4-kb DNA fragment from pAB5-2 containing the *A. niger gpdA* gene (B), and a 1.1-kb DNA fragment from pAWAGL2 containing the *aglA* gene (C). A and B above the lanes indicate samples of duplicate cultures. The hybridizing 2.3-kb band in all lanes of panel A is derived from the *uidA* mRNA containing the *exlA* promoter and transcription terminator. Molecular size markers (in kilobases) are indicated on the left.

the poly(A)$^+$ fraction, which clearly indicated that the truncated mRNA also was polyadenylated (data not shown).

To further identify the 3′ end of the truncated mRNA, RT-PCR was carried out with poly(A) mRNA isolated from multicopy strains of AWGLAAGL and AWAGL2 (see Materials and Methods). A 220-bp fragment was obtained and cloned in pUC19. Restriction enzyme analysis of the vectors showed that more than 95% had an insert of approximately 220 bp, whereas a small percentage (<5%) of the inserts were slightly larger. Based on this restriction enzyme analysis, four plasmids containing the 220-bp fragment were analyzed by DNA sequence analysis. This analysis showed that the four cDNAs contained the first 196 to 200 bases of the AGL-encoding sequence, followed by a poly(A) tract (Fig. 6). Nucleotide sequence analysis of some plasmids containing a larger cDNA insert showed that these fragments indeed corresponded to a slightly larger *aglA* mRNA.

To determine whether the truncation of the *glaA-aglA* mRNA was due to premature termination, nuclear run-on transcription assays were carried out. Since the specific mRNA levels of the single-copy strains were too low to carry out reliable run-on assays (data not shown), multicopy strains

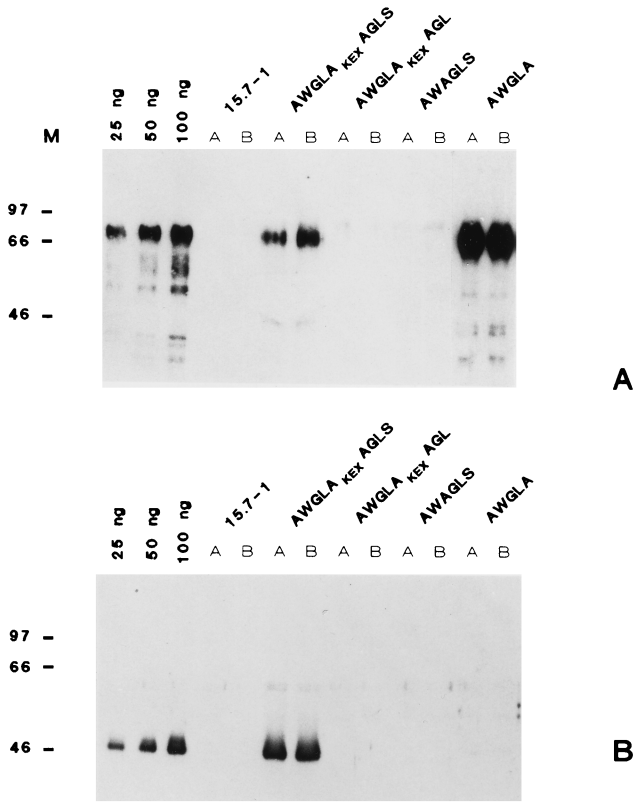

FIG. 4. Western blot analysis of strains harboring *glaA-aglA*$_{(syn)}$ constructs. Medium samples were taken 38 h after induction with D-xylose. Blots were incubated with antisera against GLA (A) and AGL (B). The first three lanes of each panel contain purified protein; the remaining lanes contain 1-μl medium samples (A) or 15-μl medium samples (B). Purified GLA is a deglycosylated GLA$_{G1}$ sample from *A. niger*; the amounts have been corrected for this deglycosylation. Protein levels were quantified more accurately by using serial dilutions (not shown). A and B above the lanes indicate samples of duplicate cultures. Lane M, molecular mass markers (in kilodaltons).

which had a higher *aglA*-specific mRNA level were used. Different fragments of the *aglA* gene, generated by PCR, were slot blotted and hybridized with radioactively synthesized RNA. Figure 7 shows the results of run-on transcription assays of multicopy strains of AWGLAAGL, AWAGL2, and AWAGLS and, as a control, AWGLA, which contained a single copy of the *glaA* gene at the *pyrG* locus. Radioactive mRNA isolated from AWGLAAGL, AWAGL2, and AWAGLS hybridized with all *aglA* DNA fragments tested, indicating that in all three strains transcription proceeds at least 600 bases from the start of the mature AGL-encoding sequence. Comparison of the signal intensities observed for the different hybridizing DNA fragments with those in a similar blot hybridized with a ^{32}P-labelled *aglA* DNA fragment showed an identical pattern, indicating that the differences observed were due to the decreasing length of the DNA fragments used on the blot. No *aglA*-specific signal was observed when RNA from AWGLA, which did not contain the *aglA* gene, was used as a probe. From the results shown in Fig. 7, it can also be seen that in AWGLAAGL a high level of *glaA* mRNA, most likely as part of the *glaA-aglA* fusion, is present, whereas this level is much lower in the strains that do not contain the *glaA* fusion. Also, in AWGLA the *glaA* level was low, probably due to the presence of a single copy of the *glaA* gene only. In all strains there is a low level of *uidA* transcription and a higher level of *gpdA* transcription. Since all strains lack the *exlA* gene, this gene was used as a negative control. In most of the strains a signal is absent; however, some faint background signal is sometimes visible.

The results of these experiments indicate that transcription elongation had occurred at least 400 bases downstream of the *aglA* mRNA truncation, as was determined by Northern blot analysis (Fig. 5) and RT-PCR (Fig. 6). Whether transcription termination was correct could not be observed in this way, but the similar results obtained with both *aglA* and *aglA*$_{syn}$ in a run-on transcription assay suggest that termination is correct.

In conclusion, the results with the nuclear run-on transcription assays, showing identical results for both wild-type and synthetic *aglA*, indicate that truncation of *aglA* mRNA is a result of incorrect processing.

DISCUSSION

The use of a gene fusion strategy for the production of heterologous proteins has been the subject of several studies. Although precise data are sparse, it is generally believed that a gene fusion has its beneficial effect mainly on the (post)translational level, e.g., by enhanced stabilization, increased efficiency of secretion, or prevention of proteolytic degradation.

Effect of gene fusion on hIL-6 production. In a previous study we showed that although relatively high hIL-6 mRNA levels were obtained, very small amounts of hIL-6 protein were produced (12). In other members of the genus *Aspergillus*, production of hIL-6 was also low (3, 5). Inefficient intracellular transport and/or extracellular proteolytic degradation have been proposed as explanations for this phenomenon. As has

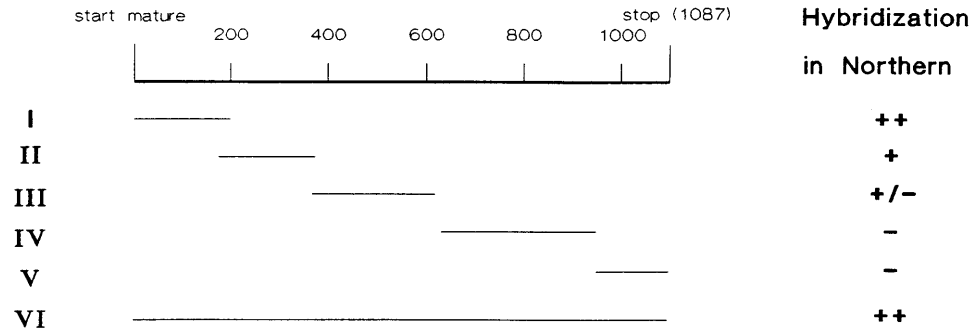

FIG. 5. Schematic representation of the DNA sequence encoding mature guar AGL. Roman numerals correspond to the DNA fragments used for hybridization on a Northern blot. ++, strong hybridization; +, weak hybridization, +/−, weak hybridization with a larger mRNA transcript but not with the major truncated transcript; −, no hybridization.

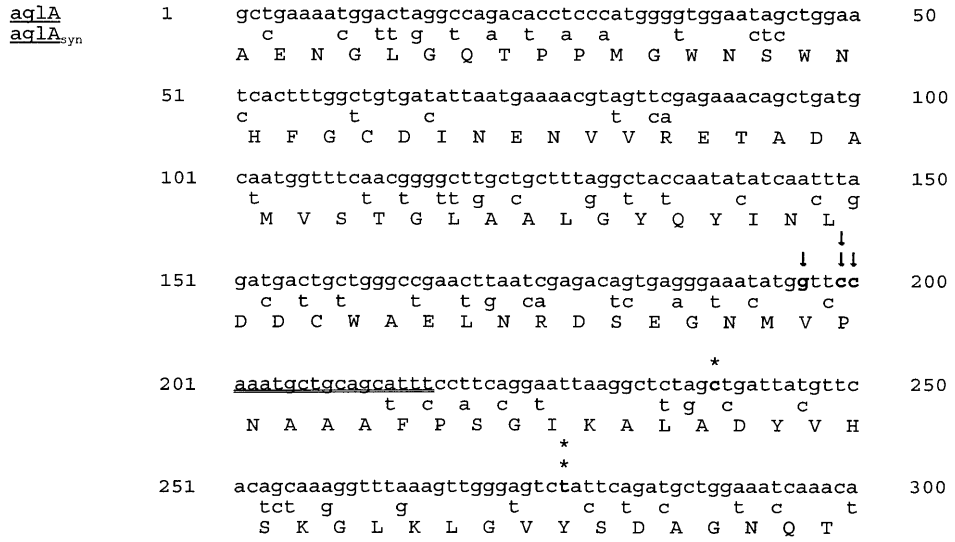

FIG. 6. Part of the DNA sequence encoding mature guar AGL (start = position 1). The upper row shows the wild-type *aglA* gene; the middle row shows the synthetic ($aglA_{syn}$) gene (only differences are indicated). The lower row shows the amino acid sequence. Arrows indicate the last nucleotide prior to the poly(A) tract of the major transcripts (>95%). An asterisk marks the last nucleotide prior to the poly(A) tract of some of the minor transcripts (<5%). The double arrow and double asterisk indicate nucleotides which have been found in two independent cDNA clones. The 16-bp inverted repeat is underlined.

been shown in previous experiments (12), hIL-6 is stable in our culture medium, excluding extracellular proteolytic degradation as the reason for the absence of hIL-6 in the growth medium. By fusing GLA_{G2} to the N-terminal end of hIL-6, we showed that, like in *A. niger* (3) and *Aspergillus nidulans* (6), in *A. awamori* hIL-6 production could also be highly improved. Interestingly, in our case levels of 5 to 10 mg of secreted hIL-6 per liter were obtained with a single-copy strain, whereas in *A. niger* and *A. nidulans* these amounts were obtained with strains containing multiple copies of the fusion construct, which illustrates that *A. awamori* can be used for efficient production of heterologous proteins.

The steady-state *glaA-hil6* fusion mRNA level was still lower than that of nonfused *glaA* mRNA, suggesting that *hil6* contains motifs which influence the stability of the mRNA.

To investigate whether inefficient protein synthesis or secretion could be an explanation for the initial low hIL-6 levels in nonfused *hil6*-containing strains, a $glaA_{G2}$ fusion to the 3′ end of *hil6* was constructed. With this strain, very low levels not only of hIL-6 but also of GLA_{G2} were obtained. Northern blot analysis showed that the *hil6-glaA* steady-state mRNA levels were similar to the levels of *glaA-hil6* mRNA, excluding the possibility that differences were at the transcriptional level. These results indicate that the main bottleneck in hIL-6 production probably occurs at the (post)translational level, either by inefficient translation, inefficient translocation, or rapid degradation, which could be due to improper folding.

Although the amounts of GLA_{G2} produced by AW-GLA_{KEX}HIL6 were almost as large as those produced by a strain containing nonfused GLA_{G2}, much less hIL-6 was produced (Table 2). Since separation of both proteins by cleavage of the KEX2 site is thought to occur in the late Golgi, part of

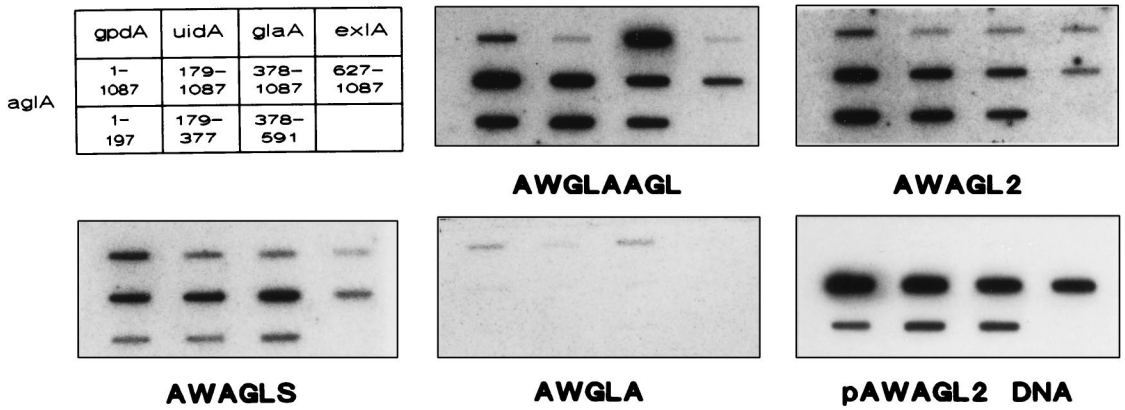

FIG. 7. Run-on transcription assay with nuclei from induced cultures of the multicopy strains AWGLAAGL 24, AWAGL2 69, and AWAGLS 15 and the single-copy strain AWGLA 3. One microgram of several PCR-derived DNA fragments was slot blotted on membranes and hybridized with labelled RNA isolated from the nuclei after run-on transcription in the presence of [^{32}P]UTP. The upper left panel shows the DNA fragments which were slot blotted. DNA fragments used for the genes shown in the upper row comprised the total coding region. The middle and lower rows contain DNA fragments obtained from the *aglA* gene. The numbers refer to the corresponding nucleotide sequence in the *aglA* DNA sequence counted from the start of the mature protein (start = position 1; stop = position 1087). The panel with pAWAGL2 DNA shows a filter that was hybridized with a ^{32}P-labelled DNA fragment containing the *aglA* gene as a control for the radioactive signal.

the hIL-6 protein (60 to 90%) must have been degraded (either intracellularly or by mycelium-associated proteases).

Effect of gene fusion on AGL production. The few data reported in the literature concerning mRNA levels of fused and nonfused genes indicate that the effect of a gene fusion on steady-state mRNA levels can be either advantageous or disadvantageous. Van Hartingsveldt et al. (36) showed that when chymosin was fused to the first 71 amino acids of GLA, compared with fusions to the first 18 or 24 amino acids, the mRNA level dropped by a factor 2.5, suggesting a disadvantageous effect. Interestingly, the mRNA levels for nonfused chymosin were already high. A decrease was also suggested when the total coding region of GLA was fused to prochymosin (39). In contrast, increases in mRNA levels were reported for a fusion of GLA to hen egg white lysozyme (18) and fusion of CBHI to Fab (23).

Our study showed that fusion of GLA to the N-terminal end of guar AGL (encoded by $aglA_{syn}$) resulted in a 25-fold increase in AGL protein levels compared to those with a nonfused $aglA_{syn}$ strain. Northern blot analysis showed that, in contrast to the results obtained with *hil6*, this increase was mostly due to higher steady-state mRNA levels. These results clearly show that a GLA fusion not only has an influence at the (post-)translational level but also can have a drastic effect at the transcriptional level. Since the mRNA level observed for the fusion mRNA was still about fourfold lower than the level of nonfused $glaA_{G2}$ mRNA, it is clear that, similar to results found for fusions of the *hil6* gene with *glaA*, the chimeric mRNA is less stable.

Strains containing expression cassettes with the wild-type *aglA* gene revealed truncated *aglA* mRNA molecules which only contained the first 200 bases of the *aglA* transcript. Nuclear run-on transcription assays showed that transcription elongation had proceeded at least 400 bases downstream of the truncation, indicating that premature termination was not the reason for the truncation.

The presence of a defined, although truncated, specific mRNA signal and the presence of a poly(A) tail imply that the truncation occurs posttranscriptionally, probably due to incorrect processing by the polyadenylation complex. This processing involves recognition of a specific DNA sequence. In higher eukaryotes this signal is a highly conserved hexanucleotide sequence (AAUAAA), which is present approximately 15 to 25 nucleotides upstream of the poly(A) addition site (16, 21, 41). In addition, a less conserved U- or GU-rich sequence is usually present downstream of the polyadenylation site (16, 21, 41). In contrast, in the yeast *S. cerevisiae* a diffuse AU-rich element is usually found instead of the mammalian consensus poly(A) signal (26). None of these motifs is sufficient to direct 3′ end processing, and therefore, several structural motifs have to act in concert for efficient 3′ end formation (13, 17).

For filamentous fungi, no experimental data about 3′ end formation of mRNAs are available. In some genes an AAU AAA sequence has been found, whereas in others it is absent (4, 14), indicating that also in fungi other motifs or combinations of motifs might be necessary to act as a polyadenylation signal. When a consensus polyadenylation signal has been identified, its functionality has never been proven experimentally.

In the case of the truncated *aglA* gene, an AAUAAA sequence upstream of the poly(A) tract is absent. Analysis of the *aglA* sequence and subsequent comparison with the $aglA_{syn}$ gene, which does produce full-length *aglA* mRNA, reveal some putative AT-rich regions upstream of the poly(A) tail which might be involved in incorrect processing. This signal is not present in the $aglA_{syn}$ gene.

Similar results have been reported for a number of heterologous genes which were expressed in yeasts. Also in that case the corresponding proteins were not detectable, which was due to the presence of putative internal yeast transcriptional terminator sequences resulting in truncated mRNA. The problem was first described for the highly AT-rich *Clostridium tetani* tetanus toxin fragment C gene in *S. cerevisiae* and was solved by synthesis of a gene with increased GC content (from 29 to 47%) (29). Identical problems have been observed for at least four other genes (30). With one of these genes, encoding human immunodeficiency virus (HIV) type 1 envelope protein (33), truncated mRNA was produced in *Pichia pastoris*, whereas in *S. cerevisiae* full-length mRNA was found. In contrast to tetanus toxin fragment C, HIV Env was only slightly AT rich; however, it contains AT-rich runs which might have caused the problem in this case. Full-length mRNA could be obtained by construction of a synthetic HIV Env gene with increased GC content, especially by eliminating AT-rich runs. However, in these studies the authors performed no experiments to discriminate between premature termination of RNA polymerase II and incorrect processing as the reason for the truncation.

Remarkably, in *C. tetragonoloba aglA* there is a perfect inverted repeat of 16 bases (AAATGCTGCAGCATTT) immediately downstream of the aberrant polyadenylation site of the *aglA* gene which might be involved in incorrect processing. In the $aglA_{syn}$ gene this repeat is reduced to only 8 bases. Finally, it is interesting that truncation of *aglA* mRNA was also observed when the wild-type *aglA* gene was expressed in *A. niger* and *A. nidulans* (26a). This would suggest that the problems observed with expression of the guar *aglA* gene are specific for *Aspergillus* species (if not for more fungal species), since for all other species reported (*Bacillus subtilis* [25], *Hansenula polymorpha* [7], *S. cerevisiae* [37], and *Kluyveromyces lactis* [1]), this was not the case. This might indicate that transcription termination and/or correct 3′ end formation for fungal mRNAs might be different from that for other organisms, as was also suggested for the yeasts *S. cerevisiae* and *P. pastoris* (33).

In conclusion, our results have clearly shown that production of heterologous proteins is limited both at transcriptional and at (post)translational levels. Limitations at the mRNA level are determined by the coding region of the heterologous gene, resulting either in low mRNA stability or in truncated mRNA, possibly due to AT-rich regions. At the (post)translational level, intracellular degradation is the major factor. Degradation can occur at least at two locations: first, during or immediately upon translation, as was observed for hIL-6 or an hIL-6–GLA fusion, preventing the synthesis of the fusion protein, and second, after cleavage of hIL-6 from GLA in a GLA–hIL-6 fusion protein. As this cleavage is thought to occur in the Golgi, degradation occurs afterwards.

Finally, we showed that GLA gene fusions can largely alleviate limitations at the transcriptional level and at the (post)-translational level.

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