APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1996, p. 1951–1957 Vol. 62, No. 6
0099-2240/96/$04.00+0

Analysis of Heterologous Protein Production in Defined Recombinant *Aspergillus awamori* Strains

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Received 17 November 1995/Accepted 11 March 1996

A study was carried out to obtain more insight into the parameters that determine the secretion of heterologous proteins from filamentous fungi. A strategy was chosen in which the mRNA levels and protein levels of a number of heterologous genes of different origins were compared. All genes were under control of the *Aspergillus awamori* 1,4-β-endoxylanase A (*exlA*) expression signals and were integrated in a single copy at the *A. awamori pyrG* locus. A Northern (RNA) analysis showed that large differences occurred in the steady-state mRNA levels obtained with the various genes; those levels varied from high values for genes of fungal origin (*A. awamori* 1,4-β-endoxylanase A, *Aspergillus niger* glucoamylase, and *Thermomyces lanuginosa* lipase) to low values for genes of nonfungal origin (human interleukin 6 and *Cyamopsis tetragonoloba* [guar] α-galactosidase). With the *C. tetragonoloba* α-galactosidase wild-type gene full-length mRNA was even undetectable. Surprisingly, small amounts of full-length mRNA could be detected when a *C. tetragonoloba* α-galactosidase gene with an optimized *Saccharomyces cerevisiae* codon preference was expressed. In all cases except human interleukin 6, the protein levels corresponded to the amounts expected on basis of the mRNA levels. For human interleukin 6, very low protein levels were observed, whereas relatively high steady-state mRNA levels were obtained. Our data suggest that intracellular protein degradation is the most likely explanation for the low levels of secreted human interleukin 6.

In nature, filamentous fungi, including members of the genus *Aspergillus*, are able to use a great variety of carbon and nitrogen sources by secreting a wide range of different enzymes into their environment. This fact, together with the capacity of the filamentous fungi to secrete these enzymes in large amounts, has made these organisms attractive hosts for large-scale production of proteins (reviewed in references 14, 33, and 37).

A great deal of data concerning production of heterologous proteins in filamentous fungi has been reported previously (reviewed in references 14 and 33). From these data it is clear that, in general, the secreted yields of most heterologous proteins are low compared with the yields of homologous proteins and reach levels that in most cases do not exceed a few milligrams per liter of culture medium. Several strategies have been developed to improve these yields, including (i) the introduction of a large number of gene copies (37), (ii) the use of strong fungal promoters and efficient secretion signals (37), (iii) the use of gene fusions with a gene encoding a well-secreted protein (8, 10, 29, 38, 39), (iv) the use of protease-deficient host strains (8, 29), (v) medium development (2), and (vi) modification of the protein, random mutagenesis, and subsequent screening for higher levels of production (11). However, a more systematic study is necessary to identify and eliminate the factors that cause the low levels of expression of heterologous proteins.

The aim of this study was to obtain more insight into the parameters that determine the production and secretion of heterologous proteins. For this study, the filamentous fungus *Aspergillus awamori*, which has been shown to be an efficient producer of proteins (6, 13, 20), was chosen as the host strain. A strategy was developed to compare the levels of production of a homologous protein and different heterologous fungal and nonfungal proteins. To eliminate the possibility that differences in levels of production are caused by differences in the gene copy number or integration site, all expression cassettes were integrated in a single copy at a defined locus (17). To avoid differences in transcriptional regulation, all genes were controlled by the expression signals of the *A. awamori* 1,4-β-endoxylanase A (*exlA*) gene (20).

MATERIALS AND METHODS

Strains, media, and transformation. *A. awamori* AW15.7 (*exlA*::*uidA*$^+$ *pyrG*$^-$ Hm^R) (16), which was used as the recipient strain for transformation, is a derivative of *A. awamori* ATCC 11358 (= CBS 115.52). *Escherichia coli* JM109 (31) was used for propagation of plasmids. Induction of the *A. awamori exlA* promoter was carried out as described by Gouka et al. (16) by using a preculture medium containing 1% sucrose as the C source and an induction medium containing 5% D-xylose as the C source. For transformation of *A. awamori* AW15.7 2×10^6 spores were inoculated into *Aspergillus* minimal medium (MM) (4) supplemented with 10 mM uridine. After 20 to 24 h of agitated growth at 30°C, the mycelium was diluted 1:5, and the organism was cultivated for 16 to 20 h. Transformation of *A. awamori* was carried out as described by Punt and van den Hondel (28). *A. awamori* Pyr$^+$ transformants were selected on *Aspergillus* MM which was osmotically stabilized with 1.2 M sorbitol.

Construction of *Aspergillus* expression vectors. Expression vectors (Table 1) carrying the genes coding for *A. awamori* 1,4-β-endoxylanase A (20), the truncated *Aspergillus niger* glucoamylase G-2 form lacking the starch-binding domain (8), *Thermomyces lanuginosa* lipase (7), human interleukin 6 (8), and *Cyamopsis tetragonoloba* α-galactosidase were constructed. For the last enzyme the following two gene versions were used: the wild-type gene (26) and a synthetic gene that had an optimized yeast codon bias (*aglA*$_{syn}$) (35) differing 22% in the nucleotide sequence from the cDNA clone. All of the genes were preceded by the *exlA* promoter and followed by the *exlA* transcription terminator. Furthermore, the genes were expressed with the *exlA* preprosequence and/or their own preprosequence (Table 1). All vectors were based on plasmid pAW14ANot, a vector derived from pAW14B (20) in which an *Eco*RI site that was present in the polylinker was changed into a *Not*I site by using a synthetic oligonucleotide. Two types of fusions were made, for which the following restriction sites were used: (i) for translation start fusions, the *Bsp*HI site (TCATGA), comprising the transla-

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TABLE 1. Expression strains

Strain	Transformant(s)	Copy no.	Prepro-sequence	Gene	Encoded protein	Origin	Origin of plasmid	Reference
AW15.7-1								
AW14A	2	1	*exlA*	*exlA*	1,4-β-Endoxylanase A	*A. awamori*	pAW14B	20
AWGLA	3, 4	1	*glaA*	$glaA_{G2}$	Glucoamylase G2	*A. niger*	pAN56-1[a]	8
AWLPL1	20	1	*lplA*	*lplA*	Lipase	*T. lanuginosa*	pTL-1[b]	7
AWLPL2	42	1	*exlA*	*lplA*	Lipase	*T. lanuginosa*	pTL-1[b]	7
AWHIL6	7, 12	1	*exlA*	hIL6	Interleukin 6	Human	pAN56-4	8
	2	2						
	11	5						
AWAGL1			*aglA*	*aglA*	α-Galactosidase	*C. tetragonoloba*	pUR2303	35
AWAGL2	69	>5	*exlA*	*aglA*	α-Galactosidase	*C. tetragonoloba*	pUR2303	35
AWAGLS	9, 11	1	*exlA*	$aglA_{syn}$	α-Galactosidase	*C. tetragonoloba*	pUR2746	35
	1	2						
	15	6–8						

[a] pAN56-1 is identical to pAN56-3 (8) except that pAN56-1 contains a shorter promoter fragment.
[b] pTL-1 contains a synthetically constructed *lplA* gene with a slightly modified DNA sequence to introduce restriction sites.

tion start codon ATG in pAW14ANot, was used (20); and (ii) for fusions of the *exlA* preprosequence with the part of a heterologous gene encoding the mature protein, the *Nru*I site (TCGCGA) that was present 3 nucleotides upstream of the cleavage site of the prosequence with the mature sequence (20) was used. In all cases fusion with the *exlA* transcription terminator was accomplished at an *Afl*II site (CTTAAG) that included the stop codon TAA. To create correct fusions, either a *Bsp*HI-*Afl*II fragment or an *Nru*I-*Afl*II fragment was isolated from pAW14ANot containing the 5′ and 3′ *exlA* regulatory sequences together with pUC19. The 5′ and 3′ end sequences of the heterologous genes were subsequently adapted to these sites to regenerate a correct fusion by using an appropriate restriction site in the coding sequence together with annealed synthetic oligonucleotides or fragments generated by PCR. The correct DNA sequence of each fusion was verified by sequence analysis. As a selection marker, a mutant *A. awamori pyrG* gene (17) present on a 2.4-kb *Not*I fragment was inserted into all expression vectors. All vectors were integrated at the *pyrG* locus in *A. awamori* AW15.7 (*exlA*::*uidA*$^+$ *pyrG*$^-$ Hm^R) by using the recently developed integration system of Gouka et al. (17).

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. (23). For Northern (RNA) blot analysis, a 400-bp DNA fragment was used as the probe, which permitted direct comparisons of the amounts of specific mRNAs in the different expression strains. This 400-bp DNA fragment was isolated by PCR from a vector derived from pAW14ANot, in which the *exlA* coding region was deleted and which contained only the 5′ and 3′ *exlA* noncoding sequences. With this probe, in each transformant the *uidA* mRNA provided with *exlA* expression signals could also be detected and used as a reference for the level of induction.

Quantification of protein production levels. β-Glucuronidase assays were performed as described by Roberts et al. (30). To quantify lipase activity, the formation of free fatty acids was measured (15). Glucoamylase activity was determined as described by Metwally et al. (24). α-Galactosidase activity assays were carried out essentially as described by Overbeeke et al. (26). Human interleukin 6 activity was measured by a biological assay, using human interleukin 6-dependent mouse hybridoma cells as described by Van Snick et al. (34).

Quantification by Western blotting (immunoblotting) was carried out as described by Sambrook et al. (31). Purified protein was used as a standard for determinations of protein concentrations. Purified 1,4-β-endoxylanase A, lipase, and α-galactosidase and the respective polyclonal antibodies were obtained from Unilever Research Laboratories. Purified glucoamylase was obtained from Boehringer-Mannheim (lot 12005320-30). Monoclonal antibodies raised against glucoamylase were produced in our laboratory. Purified human interleukin 6 that was produced by Peprotech (lot 4602) was supplied by Sanvertech. Polyclonal antibodies against human interleukin 6 that were produced by Endogen (lot 403015) were obtained from Sanvertech. Either phosphatase-labelled antibodies (Promega) or the enhanced chemiluminescence (ECL) Western blot detection kit (Amersham) based on peroxidase-labelled antibodies was used for detection. Amounts of total protein were determined with a Bio-Rad protein assay kit.

Dry weight measurements. Dry weight was determined by filtering 25-ml homogenous culture samples with a Myracloth filter. The resulting mycelium was squeezed, blotted dry, and dried overnight in a vacuum oven at 80°C.

Determination of D-xylose concentrations. D-Xylose concentrations were determined by measuring the reducing sugar content by the 2-hydroxy-3,5-dinitrobenzoic acid method used for the xylanase activity assays described by Hessing et al. (20). Appropriate amounts of medium samples were supplemented with distilled water until the total volume was 0.5 ml. A 0.5-ml portion of 2-hydroxy-3,5-dinitrobenzoic acid was added to each solution, and the preparation was mixed and incubated for 10 min at 100°C. The reducing sugar content was determined by measuring the optical density at 543 nm, and a D-xylose standard was used as a reference.

RESULTS

Construction of recombinant fungal strains and analysis of their growth and induction characteristics. *A. awamori* AW15.7 (*exlA*::*uidA*$^+$ *pyrG*$^-$ Hm^R) was transformed with the expression vectors described in Materials and Methods by using the recently developed integration system of Gouka et al. (17). Transformants that had a single copy of the vector at the *pyrG* locus were identified by a Southern blot analysis, and for each of the expression vectors two independent single-copy transformants were used for further analysis. In some cases multicopy transformants were also analyzed. Strain AW15.7-1, a *pyrG*$^+$ derivative of AW15.7, was used as a control (16).

To analyze mRNA and protein levels, each transformant was cultivated in duplicate by using the induction procedure described in Materials and Methods. Since mRNA and protein levels can be compared accurately only if there are no differences in growth and induction, a number of control parameters were analyzed for each strain.

The identical growth of the cultures was checked by analyzing the following three parameters: culture morphology, dry weight, and D-xylose consumption. All cultures consisted of small pellets that were similar morphologically. Dry weights were determined 38 h after induction, and an average value of 1.3 g of mycelium per 100 ml of medium was obtained (the standard error was 0.1 g/100 ml). In addition, D-xylose consumption in the culture medium was determined for all single-copy transformants as a measure of the growth rate. This analysis showed that after 22 h the D-xylose concentration had decreased from 5 to 3% and that after 38 h it had decreased further to about 1%. These results are consistent with the D-xylose consumption of a wild-type strain (16).

For efficient production of heterologous proteins the pH of the medium is also an important parameter, since an acidic pH might irreversibly inactivate heterologous proteins and/or activate acidic proteases which have been shown to degrade heterologous proteins, such as human interleukin 6 (8). Measurements of the pH showed that the pH remained almost neutral (pH 6.4 to 6.7) for all strains.

To analyze the induction levels in the host strain used for transformation, AW15.7, the endogenous *exlA* coding sequence was replaced by a DNA fragment containing an expression cassette with the gene encoding mature *E. coli* β-gluc-

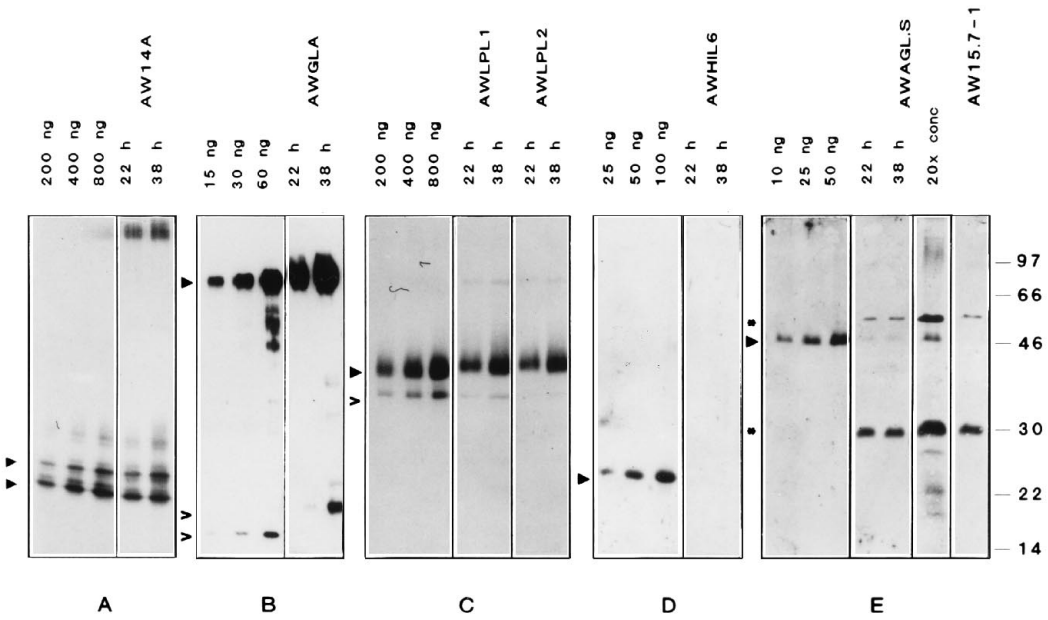

FIG. 1. Western blot of medium samples taken after induction with D-xylose. Blots were incubated with antisera against 1,4-β-endoxylanase (A), glucoamylase (B), lipase (C), human interleukin 6 (D), and α-galactosidase (E). Each panel contained a range of concentrations of purified protein and 15-μl medium samples from 22- and 38-h cultures, except for panel B, which contained 1-μl medium samples. The purified glucoamylase protein was a deglycosylated sample. Panel E also contained 10 μl of a 20×-concentrated sample of 38-h medium. The positions of the specific proteins are indicated by solid arrowheads. The positions of specific degradation products are indicated by open arrowheads, and the positions of background signals identified with control strain AW15.7-1 are indicated by asterisks. The positions of molecular weight markers (10^3) are indicated on the right.

uronidase under control of the *exlA* expression signals (16). The presence of this reporter construction in all of the different recombinant strains permitted the use of β-glucuronidase activity as a control for the induction of the *exlA* promoter. Both after 22 h of induction (average β-glucuronidase activity, 660 U/mg; standard error, 88 U/mg) and after 38 h of induction (average β-glucuronidase activity, 884 U/mg; standard error, 107 U/mg), no significant differences in β-glucuronidase activity were observed in the various single-copy strains, indicating that the levels of induction were similar in all strains.

From the results of the growth and induction analysis it can be concluded that the growth and induction parameters for all single-copy strains were similar, indicating that these parameters had no effect on the specific mRNA and protein levels of the cultures.

Analysis of protein production. The extracellular and intracellular protein levels of the different expression strains were quantified by performing a Western blot analysis and enzyme activity assays.

When the Western blot analysis was used, 1,4-β-endoxylanase A, glucoamylase, lipase (in AWLPL1 and AWLPL2), and synthetic α-galactosidase were detected in medium samples taken 22 and 38 h after induction. The results obtained with samples of the strains are shown in Fig. 1. Human interleukin 6 was detected in only very low amounts after the medium samples were concentrated 20 times. No wild-type α-galactosidase was detected in medium samples obtained from strains containing the wild-type guar *aglA* gene (AWAGL1 or AWAGL2) (data not shown). Figure 1 shows that the amount of protein present at 22 h was always about one-half the amount present at 38 h.

To determine whether the proteins found in the Western blot analysis were enzymatically active, enzyme activity tests were performed. When it was assumed that the specific activities of the proteins were similar to the specific activities in the original organism, it was concluded that the amounts were indeed comparable to the amounts determined on the basis of the Western blot analysis results (Table 2), indicating that the proteins were enzymatically active. Specific 1,4-β-endoxylanase A activity assays could not be carried out since 1,4-β-endoxylanase A makes only a minor contribution to the total xylanase activity (16).

In order to compare the levels of production of the different proteins, all protein levels were calculated on a molar basis and expressed relative to the 1,4-β-endoxylanase A protein level at 38 h. As shown in Table 2, the production of heterologous fungal proteins is very efficient. The amount of glucoamylase produced was even higher than the amount of 1,4-β-endoxylanase A produced, whereas the levels observed for the lipase-producing strains were comparable to the 1,4-β-endoxylanase A protein level. Expression of the *lplA* gene with the *lplA* preprosequence and expression of the *lplA* gene with the *exlA* preprosequence resulted in similar amounts of lipase, suggesting that the different preprosequences did not influence protein secretion.

For all of the cases tested, the protein levels obtained with the nonfungal genes were always clearly lower than those with the fungal genes. For human interleukin 6 protein amounts of a few tenths of a microgram per liter could be detected by a biological activity assay and on a Western blot after the medium samples were concentrated 20 times (data not shown). Guar α-galactosidase could not be detected in the culture medium, even after medium samples were concentrated. The same was true when the medium of a multicopy strain was analyzed. Surprisingly, α-galactosidase encoded by a synthetic gene that differed only in codon usage from the wild-type clone was produced at levels of 0.2 to 0.4 mg $liter^{-1}$ (approximately 1% of the 1,4-β-endoxylanase A protein level). The intracellular protein levels of all of the strains were also determined. 1,4-β-Endoxylanase A and lipase were present in the soluble

TABLE 2. Relative mRNA and extracellular protein levels at 38 h[a]

Strain	Gene	Prepro-sequence	RNA level[b]	Protein level (mg liter^{-1}) as determined by:		Mol wt of protein (10^3)	Relative amt of protein[d]
				Western blot analysis	Enzyme activity assay[c]		
AW14A	*exlA*	*exlA*	++++	20–26		23	100
AWGLA	*glaA*	*glaA*	+++	>100	100–150	80	200
AWLPL1	*lplA*	*lplA*	+++	26–35	30	38	90
AWLPL2	*lplA*	*exlA*	+++	26–35	30	38	90
AWHIL6	hlL6	*exlA*	+	<0.1	<0.1	23	<1
AWAGL1	*aglA*	*aglA*	−	ND[e]	ND	46	ND
AWAGL2	*aglA*	*exlA*	−	ND	ND	46	ND
AWAGLS	$aglA_{syn}$	*exlA*	+/−	0.2–0.4	0.4	46	1

[a] When necessary, the protein amounts were corrected for background values by using strain AW15.7-1.
[b] mRNA level compared with the level of *exlA* mRNA. −, no mRNA detected; +/−, 1 to 5% of the *exlA* level; +, 6 to 25% of the *exlA* level; ++, 26 to 50% of the *exlA* level; +++, 51 to 75% of the *exlA* level; ++++, 76 to 100% of the *exlA* level.
[c] Protein levels were determined by enzyme activity assays by assuming that the specific activity was identical to the specific activity of the protein in the original organism. Glucoamylase levels were determined by using pure glucoamylase (G1 form) as the standard.
[d] Protein levels were calculated on a molar basis and were expressed relative to the level of 1,4-β-endoxylanase A protein at 38 h.
[e] ND, not detected.

fractions of the extracts at levels that were about 5% of the extracellular levels and at lower levels in the insoluble fractions. The intracellular glucoamylase levels were higher than the 1,4-β-endoxylanase A and lipase levels but still markedly lower than the glucoamylase levels observed in the medium, suggesting that the high level of production of glucoamylase results in high intracellular glucoamylase levels. Synthetic α-galactosidase, wild-type α-galactosidase, and human interleukin 6, which could not be detected or were hardly detected in the culture fluid, also were not detected in the mycelial extracts in either the soluble fraction or the insoluble fraction.

One possible explanation for the undetectable levels of α-galactosidase and the low levels of human interleukin 6 in the medium is proteolytic degradation. Although no proteolytic activity toward a mixture of marker proteins was detected in the culture medium of our *A. awamori* strain (16), it is possible that both α-galactosidase and human interleukin 6 were degraded specifically. To check this, purified α-galactosidase (10 μg) and human interleukin 6 (50 ng) were added to 22- and 38-h AW15.7-1 medium samples, and the preparations were incubated at 30°C. At times up to 24 h after incubation began, samples were removed, and these samples were analyzed by performing activity assays (α-galactosidase) or a Western blot analysis (human interleukin 6). Both proteins appeared to be stable for at least 24 h, indicating that degradation by extracellular proteases was not the reason for the undetectable or low levels of protein.

Analysis of mRNA synthesis. To investigate whether the results obtained in the protein analysis could be explained by differences at the transcriptional level, the specific mRNA levels at 22 and 38 h after induction were determined by Northern blot analysis. A DNA fragment comprising the 5′ and 3′ untranslated *exlA* sequences (see Materials and Methods) was used as the probe. As a control for the amount of RNA on the blot, hybridization was carried out with the *A. niger gpdA* gene (36) as a probe. Figure 2 shows the results obtained with the 38-h samples. Similar results were obtained with the 22-h samples (data not shown). It is clear from Fig. 2 that there were considerable differences at the steady-state mRNA level for the different genes. The highest mRNA levels were obtained with the fungal genes encoding *exlA*, *glaA*, and *lplA*. No differences in mRNA levels were observed with the *lplA* gene expressed with either the *lplA* preprosequence (AWLPL1) or the *exlA* preprosequence (AWLPL2), indicating that the difference in preprosequence did not influence transcription efficiency and/or mRNA stability.

The steady-state mRNA levels observed for the nonfungal genes were remarkably lower. The human interleukin 6 steady-state mRNA levels were approximately four- to eightfold lower than the fungal gene levels. No full-length guar *aglA* mRNA was detected when the *aglA* or the *exlA* preprosequence was used (data not shown). When the total *aglA* coding region was used as the probe, a faint hybridization signal which might have represented a truncated mRNA was visible (Fig. 3). This truncated mRNA probably could not hybridize efficiently with the 5′ and 3′ *exlA* probe. In a multicopy strain this signal was stronger. Interestingly, low but significant levels of full-length mRNA were detected when the $aglA_{syn}$ gene was expressed. As expected, in the human interleukin 6 and $aglA_{syn}$ multicopy strains the mRNA levels were higher than the levels in a single-copy strain.

Considering the results obtained with the guar *aglA* expression vectors, it was important to eliminate the possibility that unwanted sequence alterations in the *aglA* coding sequence were introduced during the construction of the vectors. Therefore, the *aglA* coding region from both *aglA*-containing vectors (pAWAGL1 and pAWAGL2, corresponding to strains AWAGL1 and AWAGL2 [Table 1]) was placed in a yeast expression vector and expressed in *Saccharomyces cerevisiae*. With both vectors transformants which produced high levels of full-length α-galactosidase mRNA and enzymatically active α-galactosidase were obtained, indicating that no mutations had occurred in the sequence.

DISCUSSION

Heterologous protein production. The objective of this study was to obtain more insight into the parameters that determine the differences in heterologous and homologous protein production in filamentous fungi. Since it is clear from previously published data that, in general, levels of protein production can be roughly divided into two groups, high levels for fungal proteins and low levels for nonfungal proteins (33, 37), we chose to express three fungal genes, the genes for 1,4-β-endoxylanase A (*exlA*), glucoamylase (*glaA*), and lipase (*lplA*), and two nonfungal genes, the genes for human interleukin 6 (*hil6*) and guar α-galactosidase (*aglA*). The last gene was avail-

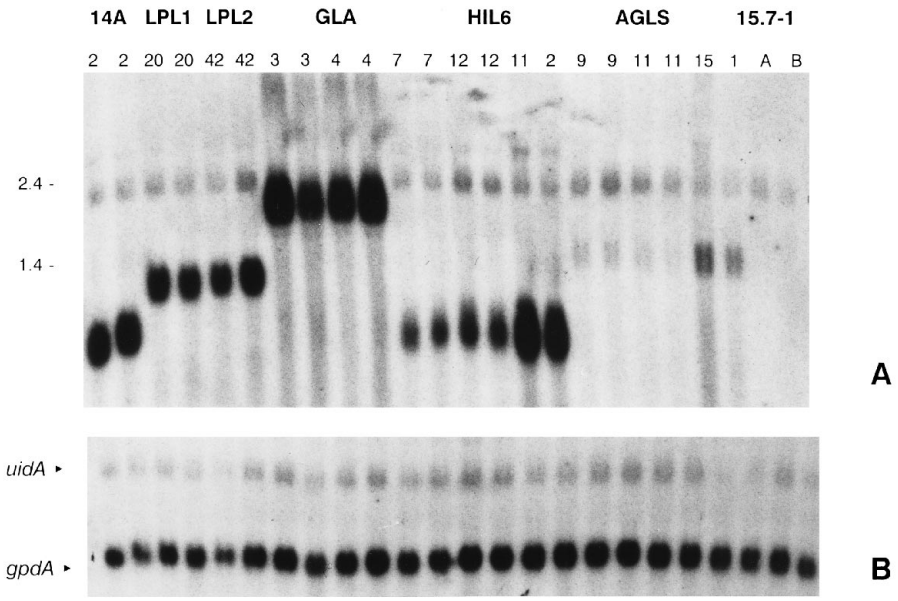

FIG. 2. Northern blot analysis of total RNAs isolated from expression strains after 38 h of induction. The probes used were a DNA fragment that contained both the 5′ and 3′ *exlA* noncoding regions (A) and a mixture of a 1.4-kb *Hin*dIII DNA fragment from vector pAN5-2 (36), containing the *A. niger gpdA* gene, and a 1.9-kb *Nco*I fragment from pNOM102 (30), containing the *uidA* gene (B). The numbers above the lanes correspond to the transformant numbers shown in Table 1. Identical numbers indicate that RNAs from duplicate cultures of the same transformant were used. Duplicate cultures of AW15.7-1 were designated A and B. The positions of molecular size markers are indicated on the left. Abbreviations: 14A, strain AW14A; LPL1, strain AWLPL1; LPL2, strain AWLPL2; GLA, strain AWGLA; HIL6, strain AWHIL6; AGLS, strain AWAGLS; 15.7-1, strain AW15.7-1.

able in two versions, a plant cDNA clone and a synthetic gene with optimized yeast codon bias. High extracellular protein levels were obtained with the fungal genes, which showed that the fungal genes were expressed and that their protein products were secreted almost equally efficiently. These results are in agreement with the results obtained with other homologous and heterologous fungal proteins which showed that production of fungal proteins is usually efficient (37).

High levels of production of nonfungal proteins are generally very difficult to obtain without optimization procedures (e.g., gene fusions or random mutagenesis and selection for strains producing higher levels of protein), and the initial levels that are produced often do not exceed a few milligrams per liter (reviewed in reference 33). The results which were obtained in this study with human interleukin 6 and plant α-galactosidase are consistent with these observations. Production of human interleukin 6 in other *Aspergillus* species is also very inefficient. In *Aspergillus nidulans* initial levels of a few tenths of a microgram per liter were found (9), whereas in *A. niger* human interleukin 6 could not even be detected (8). Transport efficiency and extracellular proteolytic degradation have been suggested as possible explanations for the initially low levels of human interleukin 6. Our results show that in the strain which we used other factors are involved (see below).

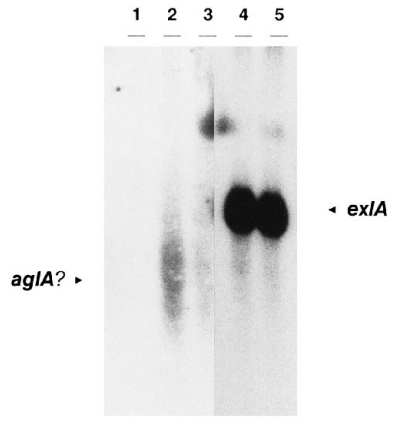

FIG. 3. Northern blot analysis of total RNAs isolated from strain AWAGL2 transformant 69 after 0 (lane 1), 4 (lane 2), and 6 (lane 3) h of induction and from strain AW14A after 4 (lane 4) and 6 (lane 5) h of induction. The probe used was a DNA fragment containing the 0.7-kb *exlA* coding region (for AW14A) or the 1.2-kb *aglA* coding region (for AWAGL2).

The total absence of guar α-galactosidase was even more remarkable since production of this protein is possible in a range of organisms, including *Bacillus subtilis* (27), *Hansenula polymorpha* (12), *S. cerevisiae* (35), and *Kluyveromyces lactis* (5), although the efficiency of production varies. Data obtained from a Northern analysis showed that α-galactosidase was absent because full-length *aglA* mRNA was absent (see below).

Heterologous steady-state mRNA levels. The results of mRNA analyses showed that mRNA stability is an important reason for the low or undetectable protein levels (especially α-galactosidase levels) of heterologous genes, since considerable differences in the steady-state mRNA levels were observed. The mRNA levels in the strains expressing the three fungal genes were clearly higher than mRNA levels in the strains expressing the nonfungal genes. In particular, in transformants containing the plant α-galactosidase expression cassette, no detectable levels of full-length α-galactosidase mRNA were found. A similar result was observed previously when the gene was expressed in *A. nidulans* and *A. niger* under control of the *A. nidulans gpdA* promoter (27a). Surprisingly, changing the codon usage of the gene resulted in low, but detectable levels of full-length *aglA* mRNA.

In the $aglA_{syn}$ gene 173 minor codons out of 364 codons, according to the yeast codon bias, had been modified in major

codons in such a way that the occurrence of a strong secondary structure was avoided (a total of 192 codons were changed). These modifications resulted in an overall change of 22% in the nucleotide sequence with no effect on the amino acid sequence (35).

Preliminary results obtained with nuclear run-on transcription assays performed with strain AWAGL2 indicated that transcription initiation occurred. These results support our finding that the mRNA that was observed in the Northern blot analysis might indeed be a truncated version of the *aglA* mRNA. Experiments are now in progress to determine why no detectable levels of full-length mRNA were obtained.

Since transcription initiation of the guar *aglA* gene occurs, the absence of full-length mRNA must have been caused by factors other than transcription initiation per se. These factors include problems in transcription elongation, posttranscriptional maturation events like the addition of a 3′ poly(A) tail or a 5′ cap, transport to the cytoplasm, and/or cytoplasmic degradation. Considering the results obtained, it is still not known at which of these levels the problems occur. The fact that changing the codon sequence of the gene results in full-length mRNA might suggest that the coding region might contain sequences that affect mRNA synthesis and/or mRNA instability, as observed previously for other heterologous genes (21, 25). In higher eukaryotes several factors which determine the stability of an mRNA molecule have been described (reviewed in references 3 and 19). From these experiments, which were carried out in mammalian cells, yeast cells, and cell-free systems, at least the following five structural components of the mRNA have been shown to strongly influence stability: (i) the 5′ 7-methylguanosine triphosphate cap and (ii) the 3′-poly (A) tail, both of which have a protective function on mRNA stability; (iii) the mRNA length, which sometimes negatively regulates mRNA stability; (iv) posttranscriptional base modifications, such as methylation of adenine residues or the conversion of adenines to inosines; and (v) mRNA-stabilizing or -destabilizing sequences. Experiments are currently in progress to determine which of these components affects *aglA*.

Proteolytic degradation. A low level of protein in the medium could also be due to extracellular proteolytic degradation, as has been suggested previously in some studies (1, 8). In our study no human interleukin 6 protein was observed, whereas relatively high levels of human interleukin 6 mRNA were found. Since extracellular proteolytic degradation of human interleukin 6 did not occur, it is very likely that human interleukin 6 was degraded before it entered the culture medium. One reason for this degradation could be that the protein was improperly folded. A protein which has been shown to be involved in folding is the immunoglobulin heavy-chain binding protein BiP (18). BiP has also been shown to interact with misfolded proteins, and it is believed that it plays a role in degradation of these misfolded proteins (22). Recently, the *A. niger* BiP-encoding gene (*bipA*) was isolated in our laboratory (28a). An analysis of *A. niger* strains which overproduce certain proteins showed that BiP mRNA is induced (28a). This induction is probably triggered by a so-called unfolded protein response (32), as has been observed in protein overproduction in other systems. To investigate whether in our case the expression of heterologous genes also resulted in increases in *bipA* mRNA levels, a Northern blot analysis of all expression strains in which the *A. niger bipA* gene was used as a probe was carried out. This analysis showed that in all strains the BiP mRNA levels were similar to the level observed in control strain AW15.7-1, which contained no expression cassette (data not shown). These observations indicated that expression of the homologous and heterologous genes did not lead to *bipA* induction. Also, human interleukin 6 and *aglA* multicopy strains did not have increased *bipA* mRNA levels. From these data we concluded that protein degradation through an unfolded protein response does not occur in our expression strains.

ACKNOWLEDGMENTS

This work was supported in part by Unilever Research Laboratories, Gistbrocades, Solvay Duphar, and the Dutch State Mines.

We thank R. Contreras for carrying out the human interleukin 6 biological activity assays, C. T. Verrips for critically reading the manuscript, and W. Musters, G. C. M. Selten, C. Groeneveld, and D. Carrez for fruitful discussions.

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