CONTROLLED EXPRESSION AND SECRETION OF BOVINE CHYMOSIN IN *ASPERGILLUS NIDULANS*

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To test the ability of the filamentous fungus *Aspergillus nidulans* to secrete bovine prochymosin, four plasmids were constructed in which the transcriptional, translational, and secretory control regions of the *A. niger* glucoamylase gene were functionally coupled to either prochymosin or preprochymosin cDNA. Three plasmid constructions involved the in-frame fusion of prochymosin coding sequences to glucoamylase sequences at 1) the glucoamylase signal peptide cleavage site, 2) the glucoamylase propeptide cleavage site, or 3) after 11 codons of the mature glucoamylase. In a fourth construction, preprochymosin was directly fused to the glucoamylase promoter. In all four constructions, the glucoamylase terminator was fused to the 3′ end of the prochymosin coding sequence. Secretion of polypeptides enzymatically and immunologically indistinguishable from bovine chymosin was achieved following transformation of *A. nidulans* with each of these plasmids. In all cases the primary translation product was partially processed to a polypeptide having a molecular weight similar to bovine chymosin. Synthesis of the chymosin polypeptides was induced in a medium that contained starch as the sole carbon source, whereas little or no expression was detected when xylose was the sole carbon source. Immunological assays indicated that the majority (>90%) of chymosin was extracellular. Hybridization analysis of genomic DNA from chymosin transformants showed chromosomal integration of prochymosin sequences, and for some transformants, multiple copies were observed.

Filamentous fungi are appealing organisms for the development of heterologous gene expression systems. In sharp contrast to other organisms such as *Escherichia coli* and *Saccharomyces cerevisiae,* which have been used for this purpose, many filamentous fungi secrete large quantities of protein into the culture medium. For example, certain *Aspergillus niger* strains secrete over five grams per liter of glucoamylase when grown under appropriate conditions (unpublished observations). Studies of heterologous gene expression in filamentous fungi have been limited to prokaryotic and lower eukaryotic genes. Bacterial genes which confer resistance to the antibiotics hygromycin B and G418 have been expressed in *Cephalosporium acremonium* [1] and *Neurospora crassa* [2], respectively. Several examples of heterologous genes complementing nutritional requirements in filamentous fungi are known[3,4]. Of relevance to the present study, the *N. crassa* gene encoding orotidine-5′-phosphate decarboxylase *(pyr* 4) complements pyr G auxotrophs of *A. nidulans* and forms the basis of a transformation system developed for *Aspergillus* [5]. In this paper, we describe the development of *A. nidulans* as a model system for expression and secretion of mammalian gene products in filamentous fungi. Although *A. nidulans* is not noted for producing copious amounts of extracellular protein, it is closely related to several commercially important species such as *A. niger, A. awamori,* and *A. oryzae,* which have this capacity. Thus, information obtained from the study of heterologous gene expression in *A. nidulans* may be applicable to the development of similar systems for industrially important *Aspergillus* species. Another important advantage of *A. nidulans* is that the organism is well characterized genetically. In addition, transformation systems for *A. nidulans* have been described[6]. To assess the usefulness of the *A. nidulans* system, we tested its ability to synthesize and secrete bovine chymosin (also known as rennin), an enzyme used in cheese manufacturing.

Chymosin is an aspartyl protease found in the fourth stomach of unweaned calves, where it cleaves k-casein in milk, resulting in clotting. Chymosin is secreted as a zymogen precursor (prochymosin), and its 42 amino acid NH_2-terminal propeptide is autocatalytically cleaved at low pH[7]. Comparison of the NH_2-terminal amino acid sequence inferred from cDNA[8] with that determined by prochymosin sequence analysis indicates that prochymosin is processed from a larger precursor (preprochymosin) containing a 16 amino acid signal peptide.

Expression of prochymosin by microorganisms has generated considerable interest because of its commercial application and limited availability. Strains of both *S. cerervisiae* and *E. coli* that produce prochymosin cytoplasmically have been described[9,10]. However, the prochymosin was largely insoluble and inactive, and laborious extraction and refolding procedures were required to restore enzyme activity[11]. Obviously, several advantages in fermentation and protein recovery could be obtained from secretion of prochymosin. Moir et al.[12] achieved secretion of prochymosin from *S. cerevisiae.* However, less than one percent of the amount produced was secreted into the medium. Recently, several factors have been identified

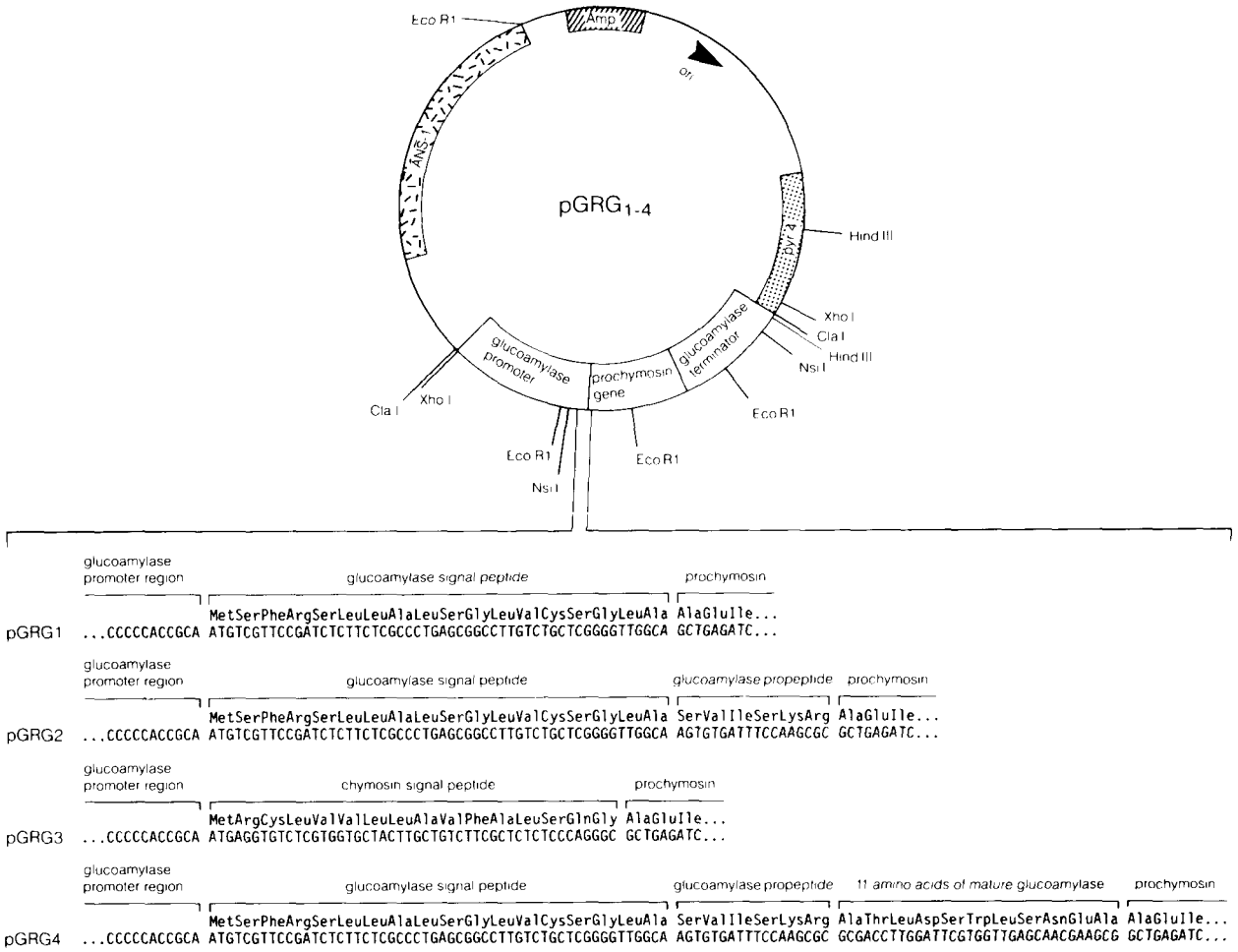

FIGURE 1 Restriction map of plasmids pGRG1-4 showing nucleotide and amino acid sequences of the glucoamylase-chymosin fusions. Precise fusions of the DNA sequences were derived by site-specific mutagenesis in bacteriophage M13mp19[26]. The origin of replication *(ori)* for *E. coli,* beta-lactamase gene *(amp), pyr* 4 gene and ASN-1 sequences were derived from pDJB3[20]. The *pyr* 4 gene from *Neurospora crassa* encodes orotidine-5′-phosphate decarboxylase, which relieves the auxotrophic requirement for uridine in *pyr* G mutants of *A. nidulans.* The ANS-1 sequences have been shown to increase transformation frequency[21].

which increase the efficiency of prochymosin secretion in yeast[12]. These include the use of yeast secretion signal peptides, integration of the prochymosin transcriptional unit into the nuclear genome, and mutations in a number of unidentified host cell genes which produce a "super secretor" *(ssc)* phenotype. Despite these improvements, the level of extracellular chymosin produced by yeast is somewhat low—20 mg per liter at a cell density of 25 grams per liter (dry weight)[13]. Filamentous fungi represent a possible alternative for expression and secretion of bovine chymosin. Several species produce large quantities of extracellular aspartyl proteases which have significant amino acid homology to chymosin[14-16].

Our strategy for the expression and secretion of prochymosin from *A. nidulans* involved the construction of plasmids containing a selectable marker *(pyr* 4) and various expression units composed of either prochymosin or preprochymosin cDNA flanked by transcriptional, translational, and secretory control regions of the *A. niger* glucoamylase gene. The complete nucleotide sequences of both *A. niger* and *A. awamori* glucoamylase genes have been reported[17,18] and are nearly identical. Glucoamylase production by these organisms appears to be induced by the presence of starch or maltose in the culture medium[17,19]. Analysis of glucoamylase mRNA levels indicates that glucoamylase gene expression is transcriptionally regulated[17]. We describe here the expression and regulation of several glucoamylase-prochymosin fusions when integrated into the *A. nidulans* genome.

RESULTS

To test whether *A. nidulans* could express and secrete a mammalian gene product, we constructed four expression vectors (designated pGRG1 through pGRG4 in which the transcriptional, translational, and secretory control regions of the *A. niger* glucoamylase gene were fused to either bovine prochymosin or preprochymosin coding sequences (Fig. 1). These vectors were used to transform *A. nidulans,* and six randomly chosen transformants derived from each vector were analyzed for production of extracellular polypeptides that reacted with chymosin-specific antibodies (Table 1). Extracellular chymosin polypeptides were produced by transformants derived from any of the four chymosin expression vectors. No chymosin polypeptides were detected in a culture filtrate of a pDJB3 negative control transformant. Like authentic bovine chymosin, the chymosin produced by *A. nidulans* was active in a milk clotting assay, was inhibited by the aspartyl protease inhibitor pepstatin, and was insensitive to the serine protease inhibitor, phenylmethylsulfonyl fluoride (data not shown).

Chymosin expression varied considerably among transformants derived from a particular vector. For example, production by pGRG3 transformants varied between 12.5 and 216 µg/g of dry weight mycelia with a mean of 93 µg/g (Table 1). Growth rates of individual transformants differed somewhat, but appeared unrelated to the amount

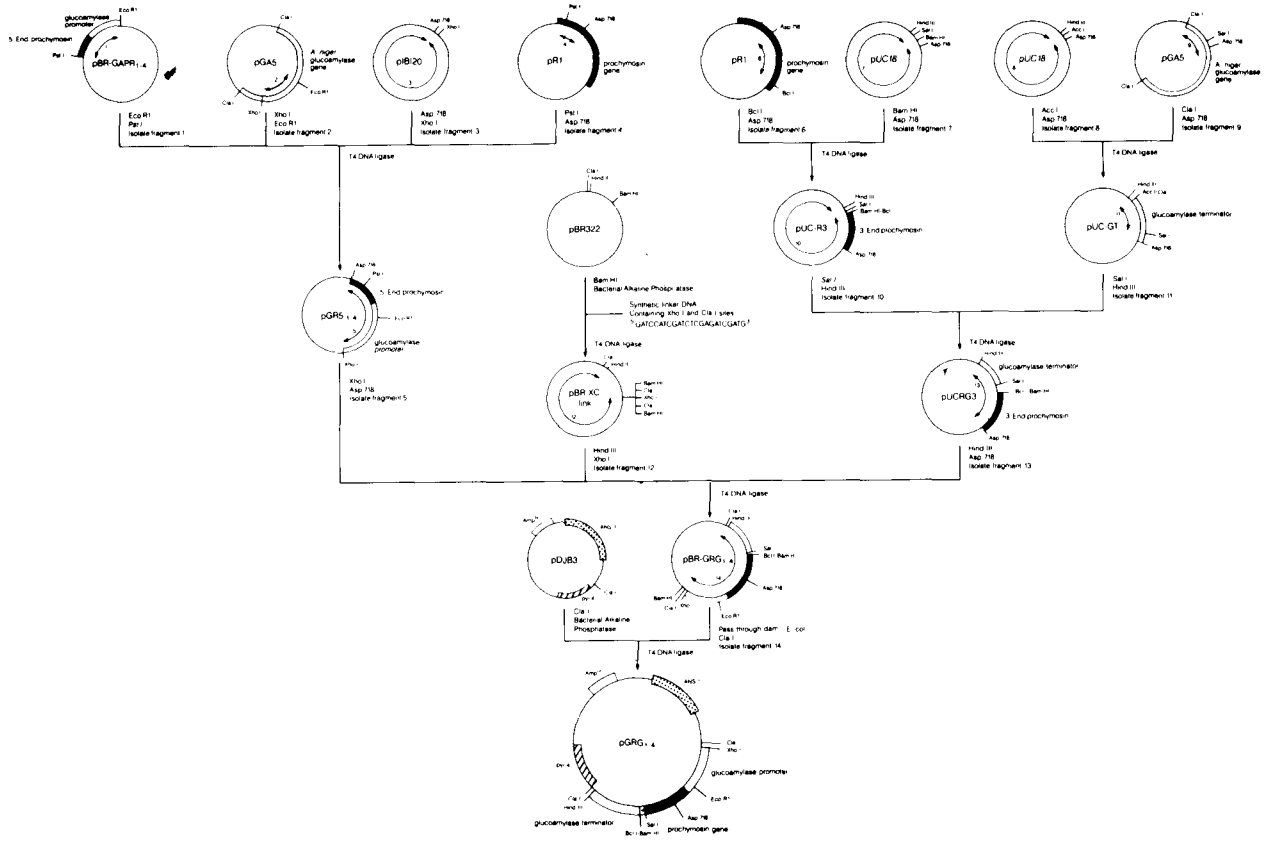

FIGURE 2 Construction of chymosin expression plasmids pGRG1-4. Glucoamylase-prochymosin fusion "cassettes" were assembled in the following manner: Restriction fragments containing approximately 1.9 kb of glucoamylase 5´-flanking DNA, each of the four glucoamylase-prochymosin fusion sequences, and a portion of the prochymosin cDNA extending to the unique Asp718 site were combined in plasmid pIBI20 to produce plasmids pGR5´1–4. Secondly, the glucoamylase terminator sequences and a portion of chymosin DNA from the Asp718 site to a BclI site near the stop codon were cloned in pUC18 to give plasmid pUCRG3´. Finally, the two halves of each cassette were combined in plasmid pBR-XClink producing plasmids pBR-GRGl-4. The completed cassettes were excised by ClaI digestion and recloned in pDJB3. Orientation of the ClaI cassettes was determined by restriction enzyme analysis of the plasmids.

of chymosin produced.

The levels of extracellular chymosin produced by *A. nidulans* transformants also varied among constructions which employed different types of secretion signals (i.e. signal peptide and/or propeptide) (Table 1). In general, pGRG1 transformants, which contained DNA sequences encoding the *A. niger* glucoamylase signal peptide fused directly to prochymosin, produced the highest levels of extracellular chymosin (mean = 146 µg/g dry weight). Cells transformed with pGRG4, which contained codons for the glucoamylase signal peptide, propeptide, and 11 amino acids of mature glucoamylase joined to prochymosin, also produced high levels of extracellular chymosin (mean = 119 µg/g dry weight). In contrast, cells transformed with pGRG2 which encoded the glucoamylase signal peptide and propeptide fused to prochymosin, produced much lower levels (mean = 23 µg/g dry weight), and one pGRG2 transformant produced no detectable chymosin. Interestingly, chymosin secretion was also obtained by expression of preprochymosin sequences in pGRG3 transformants (mean = 93 µg/g dry weight) suggesting that *A. nidulans* recognizes and processes the mammalian signal peptide.

The mitotic stability of transformants derived from each vector was assessed by measuring chymosin production and uridine prototrophy of 10 single spore isolates. No changes in chymosin levels or uridine prototrophy were observed.

Expression and secretion of chymosin. In order to study chymosin expression and secretion more closely, we selected one transformant derived from each vector that produced a high level of extracellular chymosin (compared to others derived from the same vector). The transformants were designated as GRG1-1, GRG2-1, GRG3-1, and GRG4-1. The concentration of extracellular chymosin produced by these transformants was measured by both an enzyme immunoassay (EIA) and by enzyme activity (Digiclot assay) (Table 2). A comparison of these assay shows that the amount of chymosin detected by enzyme activity is at least 80% of the immunologically active material, suggesting that most of the chymosin secreted by *A. nidulans* is enzymatically active. Furthermore, greater than 90% of the chymosin produced by *A. nidulans* is extracellular (Table 2), although the possibility of some insoluble chymosin bound to cellular debris which would not be detectable by the assay cannot be excluded. The total extracellular protein concentration of cultures grown in starch medium varied between 120 and 160 µg/ml, of which 0.4 to 1.5% was chymosin.

The expression of extracellular chymosin in *A. nidulans* was dependent upon the carbon source present in the medium (Table 2 and Fig. 3). In contrast to transformants grown in starch medium, little or no chymosin was detected by EIA (Table 2) or immunoblotting (Fig. 3) when cells were grown in xylose medium. The difference in chymosin expression levels between starch and xylose grown cells cannot be attributed to a difference in cell growth, because the mycelial growth on xylose as a sole carbon source was always as good or better than on starch medium.

Extracellular proteins of transformants grown in starch and xylose media were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and Western immunoblotting (Fig. 3). Both of these techniques revealed

polypeptides in culture filtrates of starch-grown transformants that comigrated with authentic bovine chymosin. In addition, the presence of a second, slower migrating polypeptide that appeared to comigrate with bovine prochymosin was revealed by immunoblotting (Fig. 3, Panel A). Little or no chymosin was detected by immunoblotting of extracellular proteins from xylose-grown cells.

In order to determine whether the starch-dependent expression of chymosin in *A. nidulans* was due to transcriptional control of the glucoamylase promoter, we compared steady-state levels of chymosin mRNA in starch- and xylose-grown transformants. Northern blot analysis of a transformant GRG-1 (Fig. 4) clearly shows the induction of a 1.2 kb mRNA in starch-grown mycelia, which hybridizes to a chymosin-specific probe. Only a small amount of this mRNA species was detected in xylose-grown cells. No chymosin-related mRNA was observed in a pDJB3 negative control transformant.

Integration of chymosin sequences. Stable transformants of *A. nidulans* are obtained only when vector sequences are integrated into the chromosome[20]. Autonomously replicating vectors have not been described for *Aspergillus*. One of the most striking features of transformation in *A. nidulans* is that integration often shows little dependence on extensive homology between the vector and the host genome[21]. In addition, transformation of *A. nidulans* with plasmids containing the ANS-1 sequence can result in integration of the vector at more than one site in the genome within a single transformant.[21]

In order to demonstrate the integrity of the chymosin DNA sequences integrated in *A. nidulans*, total genomic DNA was extracted from transformants, digested with XhoI and HindIII, and analyzed by the Southern method (Fig. 5). To determine whether a single copy or multiple copies of the vector had integrated, blots were hybridized simultaneously with a chymosin-specific probe (1.2 kb PstI fragment) and an *A. nidulans argB* gene probe (1.4 kb HindIII fragment). These probes were radiolabeled by nick translation to specific activities of 1.2×10^6 cpm/µg and 1.6×10^6 cpm/µg, respectively. The single-copy *arg* B gene was detected as a 1.4 kb band (Fig. 5) in both chymosin-producing (GRG1-1 through GRG4-1) and pDJB3 control transformants. As expected, chymosin-specific DNA sequences were detected only in GRG1-1 through GRG4-1 transformants. The sizes of the chymosin-specific bands (4.5 kb) were the same as for the intact glucoamylase-chymosin fragments in plasmids pGRG1 through pGRG4 (Fig. 1). This suggests that the glucoamylase-chymosin expression units were intact in the *A. nidulans* genome. Thus, integration of the chymosin

TABLE 1 Extracellular levels of chymosin antigen produced by *A. nidulans.*

	Extracellular Chymosin Concentration (µg/g)**	
Vector*	Mean	Standard Deviation
pDJB3	<3	–
pGRG1	146	52
pGRG2	22	17
pGRG3	93	69
pGRG4	119	48

*Plasmids pGRG1 through pGRG are described in Figures 1 and 2. *A. nidulans* transformed with pDJB3[21] served as a negative control for chymosin expression. The four expression vectors transformed *A. nidulans* at frequencies comparable to pDJB3 (approximately 100 transformants per microgram of DNA).

**Mean and standard deviation values were determined by analysis of chymosin expression from six randomly chosen transformants for each vector. Values are expressed in µg/g dry weight of mycelia. Culture filtrates were analyzed by EIA as follows: Microtiter assay plates were prepared by adsorbing urified rabbit antichymosin antibodies (6 µg/ml) to each well followed by washing with buffered saline and blocking with bovine serum albumin as previously described[33]. Aliquots of diluted culture filtrates or purified chymosin standards were added (100 µl per well) and incubated for one hour at 37°C. The wells were washed with buffered saline to remove unbound reactants. Horseradish peroxidase-labeled rabbit antichymosin reactants (100 µl of a 2.4 µg/ml solution) were added and the plates were incubated one hour at 37°C. After washing again to remove unbound antibodies, a chromogenic peroxidase substrate (*o*-phenylenediamine) was added and the absorbance at 490 nm was measured. Concentrations of chymosin were calculated from a standard curve constructed with authentic chymosin standards.

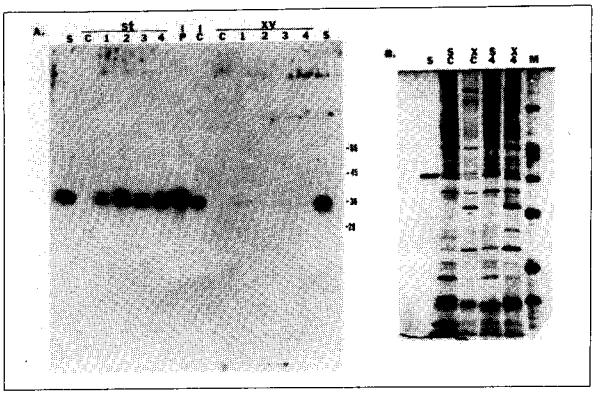

FIGURE 3 Panel A. Western immunoblotting analysis of culture filtrates from *A. nidulans* transformants. A 2.5 ml aliquot of each culture filtrate was desalted on a PD-10 column (Pharmacia, Inc., Piscataway, NJ) equilibrated with 50 mM sodium phosphate buffer, pH 6.0. Samples were then analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting as described by Towbin et al.[35] Chymosin bands were identified using rabbit antichymosin antibodies and ^{125}I-protein A (Amersham, Arlington Heights, IL). Regulated expression of chymosin was demonstrated by comparing starch medium cultures (marked st) to xylose medium cultures (marked xy). Lanes 1, 2, 3, and 4 represent 22 µl of culture filtrate from transformants GRG1-1, GRG2-1, GRG3-1, and GRG4-1, respectively. Lanes containing a pDJB3 negative control filtrate are marked C. Lanes marked S shows 50 ng of authentic chymosin standard. Lane IP shows a mixture of prochymosin and chymosin standards combined with 22 µl of culture filtrate from a pDJB3 negative control transformant. Lane IC shows purified chymosin mixed with 22 µl of culture filtrate from a pDJB3 transformant. These controls were included in order to test whether other proteins produced by *A. nidulans* affected the mobility of the expressed chymosin polypeptides. The positions of molecular weight markers are indicated at the right. Sizes are given in kilodaltons. Panel B. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of extracellular proteins from pDJB3 and pGRG4 transformants. Culture filtrates were desalted by chromatography on PD-10 columns (Pharmacia Inc., Piscataway, NJ) and the proteins were precipitated with an equal volume of 20% trichloroacetic acid. The protein pellets were washed with acetone, dried, and resuspended in loading buffer (2% sodium dodecylsulfate (w/v), 10% glycerol (w/v), 0.016 M Tris-HCl, pH 6.8, and 0.0025% bromphenol blue). Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and visualized by the silver staining method of Morrissey[36]. Lane (S): 250 ng of purified chymosin; lane (SC): culture filtrate from a pDJB3 negative control transformant grown in starch medium; lane (XC): culture filtrate from a pDJB3 transformant grown in xylose medium; lane (S4): culture filtrate from chymosin-producing transformant GRG4-1 grown in starch medium; lane (X4): culture filtrate of GRG4-1 grown in xylose medium. Approximately 10 µg of protein was loaded in each lane. Lane M shows the positions of molecular weight markers (BioRad Laboratories, Richmond, CA). Sizes are given in kilodaltons.

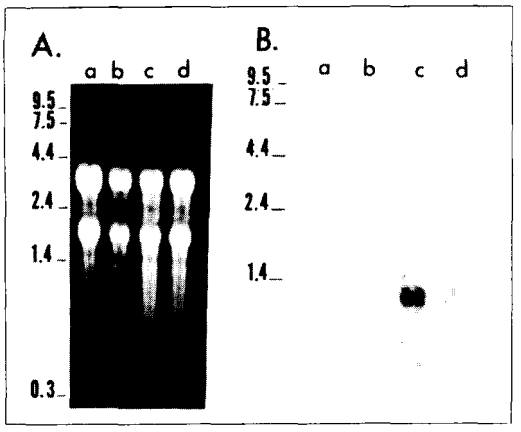

FIGURE 4 Effect of carbon source on chymosin mRNA synthesis. Total cellular RNA (~10 µg) was fractionated by electrophoresis on 1.5% formaldehyde-agarose gels and blotted to a Nytran membrane (Schleicher & Schuell, Keene, NH). Panel A shows the ethidium bromide stained gel of total RNA extracted from *A. nidulans* transformants. Panel B shows the autoradiogram of the Northern blot hybridized with radiolabeled chymosin DNA. Lanes (a) starch-grown pDJB3 transformant, (b) xylose-grown pDJB3 transformant, (c) starch-grown transformant GRG1-1, (d) xylose-grown GRG1-1. The positions of RNA size markers (Bethesda Research Laboratories) are at the left of each panel. Sizes are given in kilobases.

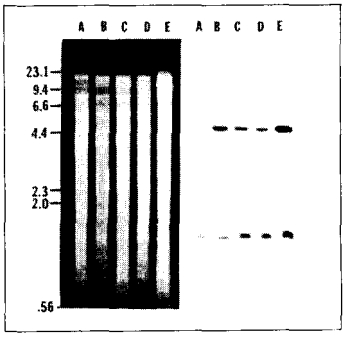

Figure 5 Hybridization analysis of total DNA extracted from *A. nidulans* transformants. Left panel shows a 0.65% agarose gel with 5 µg of total DNA from each transformant digested with XhoI and HindIII (ethidium bromide stain). Right panel shows the autoradiogram obtained by blotting and hybridization of the same gel. The blot was probed simultaneously with a chymosin-specific fragment (1.2 kb PstI fragment from pR1) and an *A. nidulans argB* gene probe (1.4 kb HindIII fragment from pBB116[37]. The probes were radiolabeled by nick translation[32] to specific activities of 1.2×10^6 cpm/µg (chymosin probe) and 1.6×10^6 cpm/µg (argB probe). Hybridization and washing conditions were essentially as described Singh and Jones[38]. Lanes (A), pDJB3 transformant; (B), GRG1-1; (C), GRG2-l; (D), GRG3-1; (E), GRG4-1. Molecular weights of bacteriophage lambda Hind III fragments are indicated at the left in kilobases.

expression vectors probably occurred by recombination outside the expression units.

For some transformants, notably GRG1-1 and GRG4-1, it was apparent that the chymosin-specific hybridization signal was much stronger than that of the argB signal (Fig. 5). Since the length and specific activities of the probes for these two sequences were nearly equal, it may be inferred that some transformants contain multiple integrations and/or local amplification of the chymosin expression units.

In order to determine whether the level of chymosin expression was correlated with the number of integrated gene copies, five pGRG2 transformants and five pGRG4 transformants which produce different amounts of chymosin were analyzed by Southern hybridization. The five pGRG2 transformants produce from 0–42 µg chymosin per gram of mycelia (dry weight), whereas the pGRG4 transformants give yields of 30–145 µg per gram of mycelia. The results shown in Figure 6 suggest that chymosin expression levels among transformants derived from a single vector are not directly correlated with gene copy number (as reflected by the intensities of hybridization signals in total cellular DNA). For example, transformant GRG2-5 produces more extracellular chymosin than GRG2-1 (40 µg/g versus 28 µg/g), yet GRG2-5 appears to have fewer intact copies of the chymosin expression unit. Interestingly, several transformants have one or more bands of hybridization which have higher mobility than the original 4.5 kb ClaI fragment. These may represent either intramolecular rearrangements of the expression unit or integration events which have occurred by nonhomologous recombination within the 4.5 Kb ClaI fragment. Although these fragments are large enough to allow for chymosin expression, the Southern analysis does not reveal whether the chymosin coding sequences are uninterrupted and functional. Although pGRG4 transfor-

TABLE 2 Expression, regulation, and localization of chymosin from selected transformants.

	Chymosin Concentration					
	Digiclot*		EIA**			
Transformant	Starch Medium (µg)	Xylose Medium (µg)	Starch Medium (µg)	Xylose Medium (µg)	Intracellular (% of total)	Extracellular (% of total)
Control (pDJB3 transformant)	<16	<16	<1	<1	—	—
GRG1-1	159	<16	189	<1	6	94
GRG2-1	53	<16	39	<1	6	94
GRG3-1	56	<16	64	<1	7	93
GRG4-1	54	<16	61	<1	4	96

*Digiclot assays of culture filtrates from *A. nidulans* transformants, representing each expression vector, and a pDJB3 negative control transformant were done as follows: To 200 µl of clotting substrate (0.25% skim milk, 40 mM calcium chloride, 3% PEG 8000 in 50 mM sodium acetate, pH 6.0) at 37°C, 100 µl of sample diluted in 50 mM sodium phosphate, pH 6.0, was added. Clotting time was measured using a Digiclot ELVI 818 (Logos Scientific, Henderson, NV). Authentic bovine chymosin gave a linear standard curve at concentrations between 50 and 400 ng/ml when plotted as clotting time (seconds) versus the reciprocal of chymosin concentration. The assay is a modification of a previously published procedure[34].

**EIA methods are described in Table 1. Intracellular chymosin levels were estimated by disrupting the mycelial contents of a starch medium culture with a Braun homogenizer in 20 ml of 50 mM sodium phosphate, pH 5.5, 10 mM EDTA, 10 µg/ml bovine serum albumin, 0.01% Mazu, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenized mycelia were filtered through Miracloth, centrifuged at 30,000 × g for 30 minutes, and the supernatants were collected and stored at −70°C until they were analyzed. Control experiments in which authentic chymosin was added before homogenization showed no appreciable chymosin degradation.

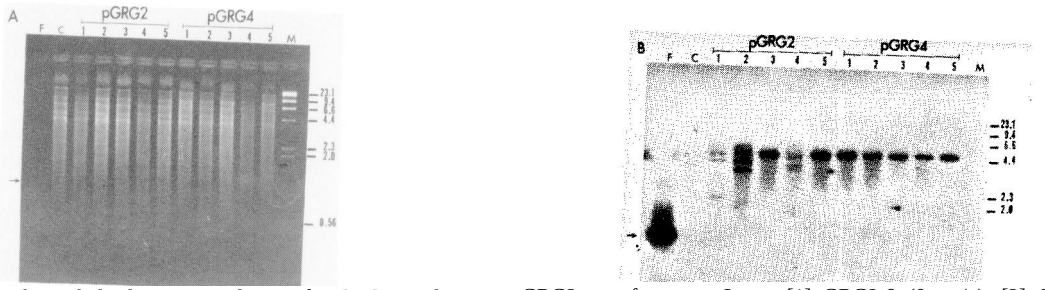

FIGURE 6 Southern hybridization analysis of pGRG2 and pGRG4 transformants which produce different levels of extracellular chymosin. Total cellular DNA (~10 µg) was digested with ClaI and fractionated by electrophoresis on 1% agarose gels. Following electrophoresis the DNA was transferred to Nytran (Schleicher & Schuell, Keene, NJ), and probed with a chymosin DNA probe. Panel A shows the ethidium bromide stained gel of digested genomic DNAs. Panel B shows the autoradiograph of the Southern blot, Samples are loaded in order of increasing chymosin production (given in parentheses in µg/g dry weight mycelia). pGRG2 transformants: Lanes [1] GRG2-9 (0 µg/g), [2] GRG2-18 (13 µg/g), [3] GRG2-11111 (28 µg/g), [4] GRG2-5 (40 µg/g), [5] GRG2-6 (42 µg/g). pGRG4 transformants: Lanes [1] pGRG4-26 (30 µg/g), [2] GRG4-6 (55 µg/g), [3] GRG4-21 (58 µg/g), [4] GRG4-17 82 µg/g), [5] GRG4-1 (145 µg/g). Lanes [F] and [C] contain 6 ng of a purified chymosin DNA fragment (indicated by arrow) and 10 µg of genomic DNA from a pDJB3 negative control transformant, respectively. Lane M contains HindIII digested bacteriophage lambda DNA markers. The sizes of DNA markers are given at the right in kilobases.

mants generally produce more chymosin than pGRG2 transformants, there is no appreciable difference in the number of integrated expression units between these two classes. Thus, the disparity in chymosin production between pGRG2 and pGRG4 transformants does not appear to be related to a difference in copy number.

DISCUSSION

We have described the development of *A. nidulans* as a model system for the synthesis and secretion of mammalian gene products in filamentous fungi. To illustrate the usefulness of the system, we demonstrated the expression and secretion of bovine chymosin. Like authentic bovine chymosin the enzyme produced by *A. nidulans* was active in milk clotting, reacted with chymosin-specific antibodies in both EIA and Western immunoblotting experiments, was inhibited by pepstatin, and was partially processed to a polypeptide with an NH_2-terminus identical to that of authentic chymosin (see below). Surprisingly, expression and secretion of chymosin was obtained following transformation with any of four expression vectors, which differed only in the secretory control sequences that were fused to prochymosin DNA. The pGRG3 transformants, which employed sequences encoding the 16 amino acid mammalian signal peptide of preprochymosin (Fig. 1), secreted chymosin at levels comparable to those obtained by using the signal peptide from *A. niger* glucoamylase. This suggests that *A. nidulans* is rather permissive with respect to secretion signals. In *S. cerevisiae* the nature of the secretion signal appears to be much more critical for secretion of chymosin, because the native chymosin signal peptide is about one-fourth as efficient as the yeast signal peptide from invertase[12]. The lower level expression from pGRG2 transformants relative to pGRG4 transformants was somewhat surprising, because the vectors differed only by the presence of 11 codons of mature glucoamylase in pGRG4 (Fig. 1). As shown by Southern blotting experiments (Fig. 6), the difference in chymosin expression between pGRG2 and pGRG4 transformants is apparently not the result of a difference in integrated gene copy number between the two vectors. Although the reason underlying this marked difference in expression are unknown, the disparity may reflect a difference in mRNA stability, translation efficiency, or processing.

The secretion of chymosin by *A. nidulans* represents a dramatic improvement over other microbial systems such as yeast[12,13] and *E. coli*[9] that have been used for this purpose. In all *A. nidulans* transformants tested more than 90% of the chymosin synthesized was secreted into the culture medium and the majority of this material was enzymatically active. Using immunoblotting techniques, we demonstrated the presence of some prochymosin or glucoamylase-prochymosin fusion polypeptides as indicated by bands that comigrated with authentic prochymosin. The identity of these zymogens was supported by acid catalyzed activation to a "pseudochymosin" intermediate and subsequently to fully processed chymosin as described by Pedersen et al.[22] It seems likely that prochymosin is actually the secreted product, and activation of the zymogen subsequently occurs in the acidic environment of the culture medium (pH 4.6). However, we cannot formally exclude the possibility of prochymosin cleavage by an *A. nidulans* protease. In this regard, we have found that the sequence of the NH_2-terminus of the polypeptide purified from GRG3-1 culture filtrate which comigrates with authentic chymosin was identical to NH_2-terminus of chymosin (unpublished data). Since the corresponding polypeptides produced from GRG1-1, GRG2-1, and GRG4-1 also comigrate with chymosin, it is expected that they also will have the correctly processed NH_2-terminus.

Interestingly, chymosin expression in *A. nidulans* appeared to be regulated by the *A. niger* glucoamylase promoter. The *A. nidulans* transformants secreted little or no chymosin in xylose medium relative to starch medium. Northern blot analysis revealed that synthesis of chymosin-specific mRNA was induced by culturing cells in starch medium, and little was synthesized in xylose-grown cells (Fig. 4). Glucoamylase expression in *A. niger* group fungi exhibits similar regulation by carbon source[17,19] in that transcription of the glucoamylase gene is induced in the presence of starch[19]. Similarly, we found that the complete *A. niger* glucoamylase gene was expressed in *A. nidulans* when starch was used as the sole carbon source, but little activity was detected in xylose medium (unpublished data). Transcript mapping experiments will be required to determine whether exactly the same transcriptional start and end points are used in *A. nidulans* and *A. niger*. However, the regulated synthesis of only one chymosin mRNA species with expected size in Northern blotting experiments suggests that the transcriptional boundaries may be the same in both species.

Typically, transformation of *A. nidulans* with vectors containing the *N. crassa pyr4* gene as a selectable marker results in integration of the vector at more than one site on different linkage groups[21]. By Southern hybridization analysis with two probes of approximately the same length and specific activity, we demonstrated that some chymosin-producing transformants contain multiple copies of the glucoamylase-chymosin expression unit. However, we did not observe a direct correlation between the number of integrated copies of the gene and the level of its expression in *A. nidulans* as has been demonstrated for *S.*

cerevisiae[13]. Differences in the level of chymosin expression among transformants derived from a single vector may reflect different chromosomal locations of the integrated expression unit. Higher expression could possibly result from juxtaposition of the chymosin expression unit with genomic sequences that improve expression. In this regard, Southern hybridization analysis suggests that integrations at multiple sites in the genome as well as tandem duplications of the transforming DNA occur in many of the transformants (data not shown). This was also noted by Ballance and Turner[21], who first studied transformation of *A. nidulans* with vectors containing the *pyr* 4 and ANS-1 sequences.

Although chymosin secretion was accomplished in a fungal species which characteristically secretes little extracellular protein, *A. nidulans* is taxonomically related to commercially important species such as *A. niger* and *A. awamori*. Transformation of the *A. niger* group fungi with *A. nidulans* genes[3,4] and the regulated expression of the *A. niger* glucoamylase promoter, described herein, provide additional evidence of relatedness among these *Aspergillus* species.

EXPERIMENTAL PROTOCOL

Bacterial strains and cloning vectors. *E. coli* 294 (ATCC 31446) was used for propagation of plasmids. When required, the adenine methylase-deficient *E. coli* strain GM48 (ATCC 39099) was used. *E. coli* JM101[23] was used as the host for bacteriophages M13mp18 and M13mp19[24] as well as for plasmid pUC18[24]. The *A. nidulans* cloning vector pDJB3 was described by Ballance and Turner[21]. Plasmid pIBI20 was purchased from International Biotechnologies, Inc. (New Haven, CT), and pBR322 has been described[25]. Plasmid pGA5 contains a 6.5 kb ClaI restriction fragment of *A. niger* DNA which includes the entire glucoamylase coding sequence as well as 2.7 kb of 5′-flanking DNA and 1.7 kb of 3′-flanking sequences (unpublished data). Plasmids pRl and pR3 (gifts of Brad Snedecor, Genentech, Inc.) are pBR322-derivatives that encode prochymosin cDNA and preprochymosin cDNA, respectively.

Transformation procedure for *Aspergillus nidulans.* *A. nidulans* strain G191 *(pyr* G89, *paba* A1; *yaY9, fwA1)* was transformed by a modification of the procedure of Ballance et al.[5,22]. Conidia were incubated at 37°C in yeast extract glucose (YEG) medium which is 2% glucose and 0.5% yeast extract. Approximately 1×10^8 germlings were harvested by centrifugation, washed with YEG, and incubated at 30°C in 50% YEG containing 0.6 M KCl, 0.5% Novozyme 234 (Novo Industries, Copenhagen, DK), 0.5% $MgSO_4 \cdot 7H_2O$ and 0.05% bovine serum albumin. After 90 min of incubation, the suspension was filtered through Miracloth (Calbiochem-Behring Corp., La Jolla, CA), washed, and transformed as previously described[5]. Transformants were selected on osmotically stabilized minimal medium[5] supplemented with 10 µg/ml *p*-aminobenzoic acid (PABA). Following incubation at 37°C for 2-3 days, the transformants were transferred to fresh minimal medium plates.

Culture conditions. *A. nidulans* transformants were grown on minimal medium dates supplemented with PABA and incubated at 37°C for 4–5 days. Conidia were washed from plates and approximately 1×10^5 spores were used to inoculate 50 ml of modified minimal medium in a 250 ml baffled flask. To test for induction of prochymosin synthesis, the medium modification involved addition of 5% maltodextrin M-040 (Grain Processing Corp., Muscatine, IA) as the sole carbon source. To measure uninduced levels of prochymosin 5% xylose was used as the sole carbon source. Both media contained 68 mg/ml potassium phosphate (pH 4.6), 1 mg/ml uridine, 10 µg/ml PABA, 50 µg/ml streptomycin sulfate, and 0.01% Mazu DF-60P antifoam (Mazer Chemicals, Inc., Gurnee, IL). Duplicate flasks were incubated at 37°C on a rotary shaker (150 rpm) for 3–5 days. Under these conditions, the pH of both starch and xylose media remained constant at pH 4.6. A plasmid pDJB3-derived transformant served as a negative control for chymosin production. Cultures were harvested by filtration through Whatman No. 1 filter paper, and the filtrates were stored at –70°C until they were analyzed. Filters were dried and weighed to determine mycelial dry weight. Preliminary time course experiments showed that cultures were in mid to late exponential-phase growth at 3.5 days (data not shown). Microscopic examination revealed no visible lysis at that time.

Analysis of chymosin. Purified calf chymosin and rabbit antichymosin antiserum were gifts of Marianne Harboe (Christian Hansen Laboratories, Copenhagen, DK). Enzyme immunoassay (EIA) and Western immunoblotting procedures are described in Table 1 and Figure 3, respectively. The Digiclot assay for chymosin activity is described in Table 2.

Construction of vectors for expression and secretion of prochymosin. Expression vectors were constructed encoding either the natural precursor of bovine chymosin (preprochymosin) or a fusion precursor in which DNA sequences for *A. niger* glucoamylase and prochymosin were precisely fused (Fig. 1). The strategy for construction of these vectors involved the following steps: First, a 337 bp EcoRI-RsaI DNA fragment containing a portion of the glucoamylase 5′-coding region was cloned in M13mp19 upstream of a small XbaI-PstI DNA fragment corresponding to the NH_2-terminal portion of preprochymosin isolated from plasmid pR3. Next, nucleotides between the DNA fragments were deleted by site-specific mutagenesis of the bacteriophage template[26] using specific primer sequences to derive the following fusions of glucoamylase and either prochymosin or preprochymosin: Primer 1 (5' GCTCGGGGTTGGCAGCTGAGATCACCAG 3') was used to join the glucoamylase signal peptide codons to the first codon of prochymosin. Primer 2 (5' TGATTTCCAAGCGCGCTGAGATCACCAG 3') was used to fuse sequences of the glucoamylase signal peptide and propeptide to the first codon of prochymosin. (The glucoamylase propeptide comprises six amino acids following the signal peptide and ends with the sequence Lys-Arg[17].) Primer 3 (5' ACTCCCCCACCGCAATGAGGTGTCTCGT 3') joined the nucleotides immediately preceding the initiation codon of glucoamylase to the ATG start codon of preprochymosin. Primer 4 (5' TGAGCAACGAAGCGGCTGAGATCACCAG 3') fused codons of the glucoamylase signal peptide, propeptide, and 11 amino acids of mature glucoamylase to the first codon of prochymosin. Plaques containing the desired deletions were identified by plaque hybridization[26] and the nucleotide sequence of each fusion was confirmed by DNA sequencing[27]. DNA segments containing the fused sequences were subcloned into a pBR322-derivative to produce plasmids pBR-GAPR1-4. Finally, sequences from plasmids pBR-GAPR1-4 were incorporated into expression plasmids pGRG1-4, which employed the 5′- and 3′-transcriptional and translational control sequences of the*A. niger* glycoamylase gene (Fig. 2). DNA fragments were isolated by polyacrylamide gel electrophoresis or agarose gel electrophoresis[28]. Restriction endonucleases, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, DNA polymerase I (Klenow fragment), and T4 DNA ligase were purchased and used according to the manufacturer's instructions (Boehringer-Mannheim Biochemicals, Indianapolis, IN or New England Biolabs, Beverly, MA).

Isolation and analysis of nucleic acids. *A. nidulans* DNA and RNA were isolated as described previously[29]. Genomic DNA from transformants was digested with an appropriate restriction enzyme, fractionated on 1% agarose gels, and analyzed for the presence of glucoamylase or chymosin specific fragments by the method of Southern[30]. Total RNA from selected transformants was fractionated by formaldehyde-agarose gel electrophoresis and analyzed for glucoamylase or chymosin mRNA by the Northern blot method[31]. Hybridization probes were radiolabeled by nick translation[32].

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