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Expression vectors for Neurospora crassa and expression of a bovine preprochymosin cDNA

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Abstract
The filamentous fungi, owing to their ability to secrete high levels of proteins, are attractive organisms for the expression and secretion of heterologous proteins of commercial and medical value. We report the construction of three expression vectors for the production of heterologous proteins in *Neurospora crassa* and demonstrate their utility by expression of a bovine preprochymosin cDNA and secretion of processed, enzymatically active bovine chymosin.

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Expression vectors for *Neurospora crassa* and expression of a bovine preprochymosin cDNA

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The filamentous fungi, owing to their ability to secrete high levels of proteins, are attractive organisms for the expression and secretion of heterologous proteins of commercial and medical value. We report the construction of three expression vectors for the production of heterologous proteins in *Neurospora crassa* and demonstrate their utility by expression of a bovine preprochymosin cDNA and secretion of processed, enzymatically active bovine chymosin. One of the three vectors is based on the constitutive transcriptional promoter and terminator of the beta-tubulin gene (Orbach et al. 1988. Mol. Cell. Biol. 8:2111-2118), and the other two incorporate the glucose repressible promoter and the terminator of *grg-1* (McNally and Free 1989. Curr. Genet. 14:545-551). Results on the different levels of chymosin expression are presented.

The beta-tubulin promoter vector, pTPT1, was constructed in pTZ18R (Pharmacia), in which several of the multiple cloning sites were deleted. The beta-tubulin promoter and terminator fragments were subcloned from pSV50 (Vollmer and Yanofsky 1986. Proc. Natl. Acad. Sci. USA 83:4869-4873), which expresses a mutant, benomyl resistant beta-tubulin allele. A 350 base pair *Sal*I-*SfaN*I promoter fragment, ending five nucleotides upstream of the translation initiating ATG, and a 380 bp beta-tubulin terminator fragment, from an *Exo*III-generated end 73 nucleotides upstream of the beta-tubulin stop codon to the downstream genomic HindIII site, were combined with *Kpn*I, *Sma*I and *Bam*HI sites between them.

The two *grg-1* promoter vectors are based on a genomic clone of *grg-1* into which an *Xho*I linker had been inserted (pMTF52, gift of S. Free, State University of New York at Buffalo) 67 nucleotides downstream of the primary site of transcriptional initiation (22 nucleotides upstream of the first ATG codon). A second *Xho*I linker was inserted into an *Nae*I site downstream of the last *grg-1* exon and upstream of the polyadenylation signal, and all sequences between the two *Xho*I sites were deleted. One version of the *grg-1* promoter expression vector, pGRGS, has an 837 bp promoter fragment measured from the first site of transcription initiation, and the other, pGRGL, has a 1563 bp promoter fragment.

A bovine preprochymosin cDNA (pBC8, gift of M. McCaman, Berlex) was subcloned into the three expression vectors and pMTF52. After initially subcloning the cDNA into pTZ18R, the cDNA was inserted into pTPT1 between the *Kpn*I and *Bam*HI sites and into pGRGS, pGRGL, and pMTF52 at the unique *Xho*I site generating pTCT1, pGRGSC, pGRGLC and pGRC52 (Figure 1), respectively. Chymosin, an aspartyl protease, is secreted as a zymogen, prochymosin, which is autocatalytically activated to chymosin at low pH. The enzyme is naturally found in the

fourth stomach of the calf and cleaves kappa-casein. We took advantage of this milk clotting activity to rapidly screen transformants for expression and secretion of chymosin.

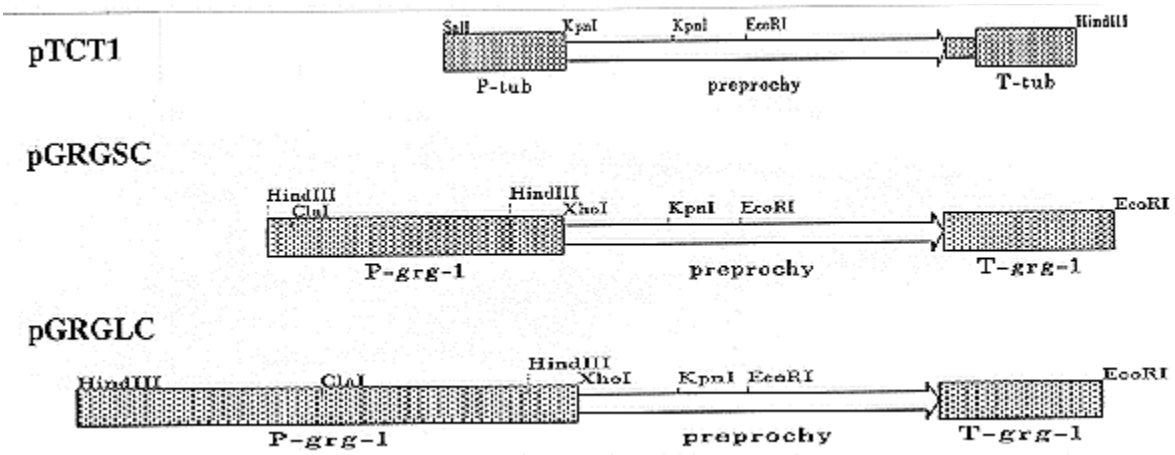

Figure 1. *Neurospora crassa* expression vectors TCT1, pGRGSC, and pGRGLC. Steps in the construction of the chymosin expression plasmids are described in the text. Presented are the expression cassettes for each plasmid. Stippled boxes represent the respective promoter and terminator sequences (beta-tubulin or *grg-1*). Open arrow represents preprochymosin sequences.

We co-transformed the *his-2 mtr* strain of *N. crassa* (Stuart and Koo 1988. Genome 30:198-203) with either pTCT1, pGRGSC, pGRGLC, or pGRC52 and pSV50- cosmid 6:11E, bearing *his-2*+, from the ordered cosmid library of Vollmer and Yanofsky (ibid). Histidine prototrophs were selected on minimal medium, and transformants were transferred to agar slants. Conidia from isolated transformants were inoculated into 5 ml of Vogel's + 2% sucrose liquid medium. Following three to four days incubation, the medium was screened for presence of milk clotting activity (Ward et al. 1990. Bio/Techniques 8:436-440).

Quantitation of chymosin levels from selected transformants was determined by comparison of bovine chymosin (Sigma) and recombinant chymosin on Western transfers (Figure 2). Secreted proteins from four-to-five-day-old cultures were concentrated by ultrafiltration (Centricon 30, Amicon), and Western transfers were probed with a rabbit anti-prochymosin antiserum (gift of M. McCaman, Berlex). An immunoreactive protein comigrating with bovine chymosin was observed in the culture medium of most milk- clotting transformants (Fig. 2). In some instances, a higher molecular weight, immunoreactive protein was observed (e.g. see Fig. 2A, lanes 3 and 4) that might represent prochymosin or pseudochymosin.

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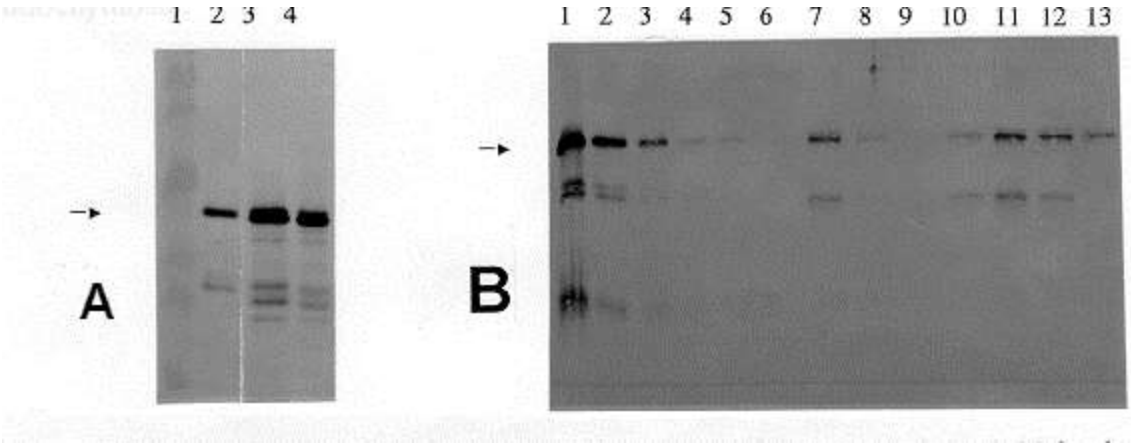

Figure 2. Western transfer of medium from various *N. crassa* transformants. A. Lane 1. Molecular weight markers. Phosphorylase B, 106 kD; Bovine serum albumin 80 kD; Ovalbumin 49.5 kD; Carbonic anhydrase 32.5 kD; Soybean trypsin inhibitor 27.5 kD; Lysozyme 18.5 kD. Lane 2. 100 ng authentic bovine chymosin standard. Lane 3. Concentrated medium (equivalent to 210 l of unconcentrated medium) from a pTCT1 transformant. B. Lanes 1-5: authentic bovine chymosin standards, 100 ng, 33.3 ng, 11.1 ng, 3.7 ng, 1.2 ng, respectively. Lanes 6-13: 12 l medium from individual transformants. Lanes 6-10: pGRGSC transformants. Lanes 11-13: pGRGLC transformants. (arrow) - mature chymosin

A range of secreted chymosin levels as assessed by milk clotting activity was observed among the various sets of transformants. As determined by Western transfer analysis for the highest expressors from each set, the non-regulated beta-tubulin promoter vector and pGRGS expressed approximately the same level of chymosin, while the longer *grg-1* promoter vector, pGRGL, produced more. We estimated that the highest expressing pTCT1 transformant expresses between 0.3-0.5 ug/ml of enzymatically active chymosin. The highest expressing pGRGLC transformant secretes between 0.9-1.2 ug/ml enzymatically active recombinant bovine chymosin.

The authenticity of the *N. crassa*-expressed chymosin was evaluated by immunoprecipitation. The anti-prochymosin serum was added to 1 ml of culture medium from a five day culture of a chymosin transformant, the mixture was incubated overnight at 4 C, and fixed Staphylococcus aureus cells were added to remove the antibody-antigen complexes. This treatment removed essentially all milk clotting activity. In addition, milk clotting was not inhibited by the serine protease inhibitor phenylmethysulfonyl fluoride.

Summary: We have constructed three expression vectors based on the constitutive beta-tubulin promoter and the regulatable *grg-1* promoter. We have confirmed the effectiveness of these vectors by the expression and secretion of the enzymatically active mammalian protein bovine chymosin. The chymosin was expressed from a bovine preprochymosin cDNA, and chymosin was secreted under the direction of its own secretion signal. Very little chymosin was detected in cellular protein extracts, indicating the efficiency of this heterologous secretion signal peptide in Neurospora. This is in contrast to its poor effectiveness in directing secretion of chymosin from *Saccharomyces cerevisiae* (Smith et al. 1985. Science 229:1219-1224) and Aspergillus (Ward et al. 1990. Bio/Techniques 8:436-440). However, secretion signal efficiency is highly variable and often appears to be protein specific. We might therefore expect a homologous fungal secretion

signal sequence to direct secretion of some proteins better than a heterologous secretion signal sequence. To that end, we are cloning cDNAs of selected Neurospora proteins.

These expression vectors may be useful for the overexpression and study of homologous proteins and for the expression of heterologous proteins in *N. crassa. Neurospora crassa* naturally secretes few proteins, which may simplify purification of heterologous proteins targeted for secretion and engineered for overexpression. *Neurospora crassa*, therefore, has potential for development as a safe and well understood production organism.

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