Construction of two *Neurospora crassa* Lambda ZapII-cDNA libraries

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In the presence of the anti-microtubule agent benomyl, *N. crassa* synthesizes and secretes a major 60 kDa protein in the culture medium. This protein is absent in untreated cells (Rossier et al. 1989. Eur. J. Cell Biol. 50:333-339). To clone the gene coding for the 60 kDa protein and to unravel its function, we have constructed a cDNA library from benomyl-treated cells ("benomyl" cDNA library). A second cDNA library was constructed from untreated cells in order to clone genes coding for cytoskeletal proteins ("standard" cDNA library).

The two cDNA libraries have been constructed using the Lambda Uni-Zap II vector in the ZapTM cDNA synthesis kit (Stratagene, USA; Short et al. 1988. Nucleic Acids Res. 16:7583-7600). The advantage of this cloning vector is that each cDNA is in the proper orientation to facilitate expression of fusion proteins by the *lacZ* promoter in the Lambda Zap II vector for antibody screening. cDNA clones can also be identified by hybridization with a DNA probe. The pBluescript SK(-) plasmids containing the cDNA inserts can be excised from the purified Lambda Zap II clones with filamentous helper phage R408 for immediate restriction enzyme mapping, sequence analysis and in vitro mutagenesis. The pBluescript SK(-) plasmid is a 2958 base pair phagemid derived from pUC19 and carries an f1 origin of replication allowing single strand cDNA rescue, via filamentous helper phage infection, for site-specific mutagenesis or single strand sequencing. It possesses a *colE1* origin for replication in *E. coli* and the *AmpR* gene for selection on ampicillin medium.

The "standard" cDNA library has been made with polyA+ mRNAs from *N. crassa* (wild type, St. Lawrence 74A) cultivated in liquid Vogel medium for 19 hours and the "benomyl" cDNA library from *N. crassa* treated for 8 hours with 1.7 uM benomyl after 17 hours of growth in liquid Vogel medium. Total RNA from *N. crassa* was isolated by sedimentation through a CsCl gradient, solubilization in guanidine-HCl at 65 C and ethanol precipitation (Hoang-Van et al. 1989. Eur. J. Cell Biol. 49:42-47). 1.25 ug of poly A+ mRNAs were purified on oligo(dT)-cellulose columns and used as templates to construct the two cDNA libraries. First strand synthesis was primed with a poly(dT)-primer/linker containing a *Xho*I restriction site and performed with Moloney reverse transcriptase in the presence of 5-methyl-dCTP. The cDNAs were blunt ended using T4 DNA polymerase and ligated to *Eco*RI adaptors. These *Xho*I-*Eco*RI cDNAs were then unidirectionally inserted into the Lambda Zap II vector (1 æg), which has been predigested with *Xho*I and *Eco*RI and dephosphorylated with alkaline phosphatase. The 5' end of the mRNAs is then nearest to the *lacZ* promoter. The two *N. crassa* cDNA libraries were packaged with Gigapack Gold packaging extracts (Stratagene, USA). A total of 1.1 million recombinant phages for each library were obtained after the initial infection of PLK-F', a *mcrA*-, *mcrB*- *E. coli* strain, preventing digestion of hemi-methylated cDNA. Recombinant phages may be recognized by blue/white color identification when plated on lac- hosts in the presence of isopropyl- -D-thio-galacto-pyranoside (IPTG) and 5-bromo-4-chloro-3- indolyl- -D-galacto-pyranoside (X-Gal). A blue to white of <0.5% for the "standard" cDNA library and <1% for the "benomyl" cDNA library indicated that most of the resulting phages were recombinant. The titers before

amplification of the "standard" and "benomyl" cDNA libraries were 1.5 x 109 and 109 plaques/ml, respectively.

We have screened the fusion proteins from the "benomyl" cDNA library with rabbit polyclonal antibodies raised against the 60 kDa protein secreted by N. crassa in the presence of 1.7 uM benomyl. Fusion proteins expressed in the presence of 10 mM IPTG and then adsorbed onto nitro-cellulose filters were incubated with the anti-60 kDa antibodies (dilution 1:2000 in TBS containing 0.5% Tween-20). The peroxidase activity was revealed with 0.07 mg/ml diaminobenzidine-tetrahydro- chloride (DAB) in the presence of 0.03% H_2O_2 after incubation of the nitrocellulose filter with secondary antibodies (horseradish peroxidase-labeled donkey anti-rabbit IgG, Amersham, UK) (dilution 1:2000 in TBS containing 0.5% Tween-20). 175 positive clones were obtained after screening 280,000 plaques. 9 positive clones with insert of various sizes were analyzed by restriction enzyme mapping. Their sizes ranged from about 0.5 to 1.6 kb. Their fusion proteins overexpressed in the presence of 10 mM IPTG are shown in Fig. 1. The molecular weights of the fusion proteins were in agreement with the sizes of the inserts. Clones N65 and N121 (possibly full length cDNA clones) were inhibitory for *E. coli* growth (results not shown) and did not overexpress the fusion proteins in the presence of IPTG.

The *N. crassa* beta-tubulin cDNA has also been cloned by screening fusion proteins of the "standard" cDNA library with rabbit polyclonal antibodies raised against *N. crassa* beta-tubulin and monoclonal antibodies raised against chick brain beta-tubulin (Amersham, UK) (Hoang-Van, preparation).

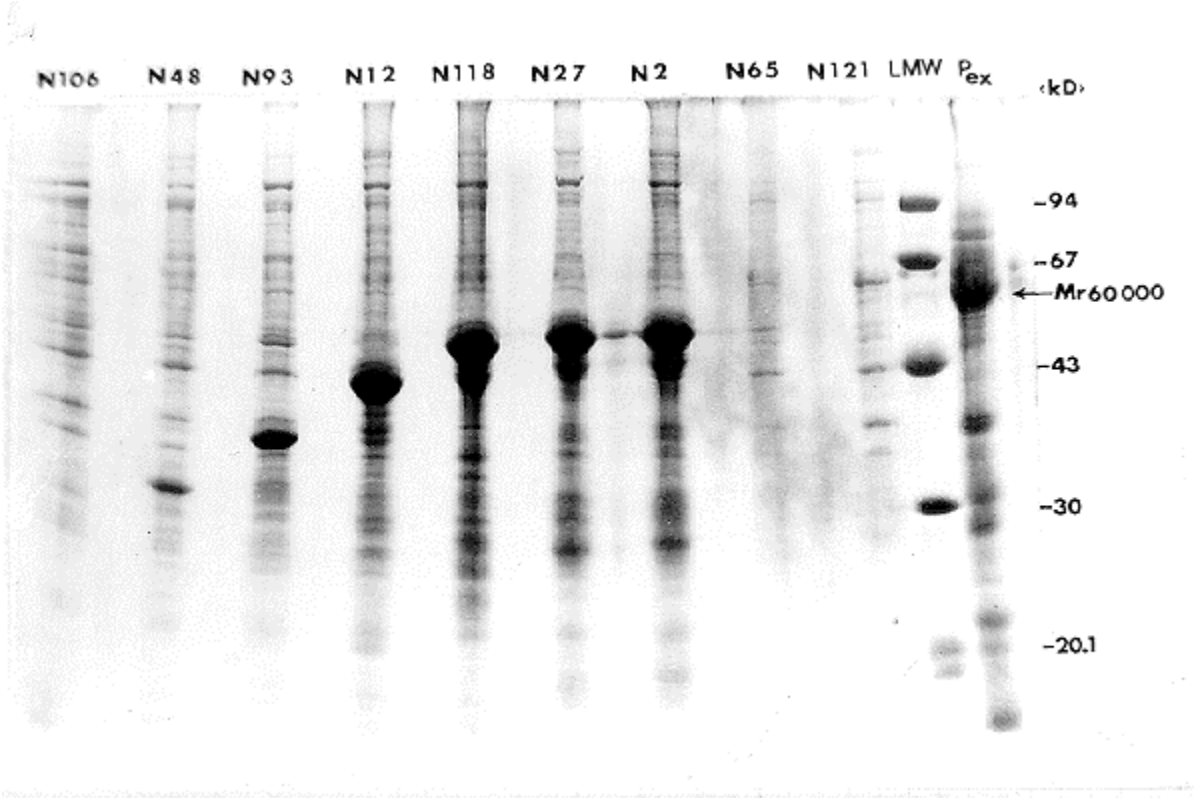

Figure 1. Fusion proteins overexpressed in *E. coli* by cDNA inserts of various sizes of the gene coding for the 60 kDa protein secreted by benomyl-treated cells of *N. crassa*. Overexpression

was induced in the presence of 10 mM IPTG. These fusion proteins in the form of inclusion bodies were purified according to Marston (1986. Biochem. J. 240:1-12), dissolved in 8 M urea and separated by SDS-PAGE (10% acrylamide). LMW: Low molecular weight standards. Pex: extracellular 60 kDa protein (arrow) from culture medium