Cloning of the copper-inducible metallothionein (cmt) promoter from Neurospora crassa.

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Abstract
There are only a limited number of vectors with inducible promoters that are convenient for in vivo expression in Neurospora crassa. Promoters have been identified and cloned that are induced with blue-light (bli-4; Pietschmann et al 1991 Fungal Genetics Newsl. 38:85-6) or by quinic acid (qa-2; Campbell et al 1994 Fungal Genetics Newsl. 41:20-1). Constitutive promoters have also been used, derived from the beta-tubulin gene bml (Nakano et al 1993 Fungal Genetics Newsl. 40:54-6). The glucose-repressible promoter of grg-1 has also been used (Nakano ibid; Pall and Brunelli 1994 Fungal Genetics Newsl. 41:63-4). The promoter of the N. crassa copper metallothionein gene (cmt) is capable of induction levels of at least 100-fold (Munger et al. 1987 J. Biol. Chem. 262:7363-7) and has been used to express tyrosinase and laccase (Kupper et al. 1990 Curr. Genet. 18:331-5; Schilling et al. 1992 Curr. Genet. 22:197-203).

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Cloning of the copper-inducible metallothionein (cmt) promoter from *Neurospora crassa*.

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There are only a limited number of vectors with inducible promoters that are convenient for in vivo expression in *Neurospora crassa*. Promoters have been identified and cloned that are induced with blue-light (*bli-4*; Pietschmann et al 1991 Fungal Genetics Newsl. 38:85-6) or by quinic acid (*qa-2*; Campbell et al 1994 Fungal Genetics Newsl. 41:20-1). Constitutive promoters have also been used, derived from the beta-tubulin gene *bml* (Nakano et al 1993 Fungal Genetics Newsl. 40:54-6). The glucose-repressible promoter of *grg-I* has also been used (Nakano ibid; Pall and Brunelli 1994 Fungal Genetics Newsl. 41:63-4). The promoter of the *N. crassa* copper metallothionein gene (*cmt*) is capable of induction levels of at least 100-fold (Munger et al. 1987 J. Biol. Chem. 262:7363-7) and has been used to express tyrosinase and laccase (Kupper et al. 1990 Curr. Genet. 18:331-5; Schilling et al. 1992 Curr. Genet. 22:197-203).

The use of the *cmt* promoter could enhance studies where selective expression is essential. Unfortunately, published vectors are no longer available. Our laboratory has undertaken the reisolation of the promoter in a convenient construct. A partial sequence of the *cmt* loci was published and deposited in EMBL database (#X03009). From this, four primers were constructed (Figure 1A) to generate PCR fragments 1 kb (primers 1 to 4), 700 bp (primers 1 to 3), and 400 bp (primers 2 to 4) using genomic DNA from wild-type strain 74A as a template. Each fragment cross-hybridized with the others, suggesting that each derived from overlapping chromosomal regions.

![Figure 1](Image)

**Figure 1.** The copper-inducible metallothionein (cmt) gene.  
A. Primers used to amplify the region from genomic DNA. Primers, 5 ‒ 3 :

```plaintext
1 2 3 4
```

B.  
```
BDPSEX
HN NdBGNC EV P Ps Sn Ss
```

C.  
```
AAAGAACAACCAGTGCTAGCAATGGCCAGCGCTCAGCGA
NheI Mscl StuI NruI
```
1, GACATCACATGAACATTGCA; 2, GGCGCTTCTTCCTGCAACTGCGGCTCTGGC; 3, AAGATGGGATCGGACAGGCG; 4, CGGGCGTTGTCATCACAG. Primers are positioned relative to the restriction fragment in panel B corresponding to the relevant region of genomic DNA.

B. Restriction map of a 4.1 kb region of cosmid G9:12G. Restriction enzymes: H, HindIII; N, NotI; B, BamHI; D, Drai; P, PvuI; S, SphiI; X, XhoI; E, EcoRI; Nd, NdelI; Bg, BglII; Ne, NcoI; C, ClaI; EV, EcoRV; Ps, PstI; Sn, SnaBI; Ss, SspI.

C. Insertion of a multicloning sequence at the 3' terminus of the inducible promoter region of the cmt gene. The region from the HindIII site to the indicated sequence was derived by PCR and cloned back into the HindIII/EcoRV sites of Bluescript SK. Inserts can be cloned into the NheI site to use their own ATG codon, or into the three other restriction enzyme sites to use the encoded ATG sequence in any of the three reading frames.

The Orbach/Sachs library and the Vollmer/Yanofsky N. crassa genomic libraries (FGSC) were screened with a random-hexamer labeled probe derived by gel-purifying the 1 kb PCR fragment generated from primers 1 and 4 (Figure 1A). The Vollmer/Yanofsky library did not yield any hybridizing signals, even upon repeated probing. In contrast, the Orbach/Sachs library had six strongly hybridizing colonies. Four were isolated and the specific hybridization was confirmed by Southern analysis. Cosmids G4:1H, G9:12G, G10:10C, and X16:10H, hybridized to all PCR fragments recovered from the genomic PCR. Results of RFLP mapping (Metzenberg et al. 1984 Fungal Genetics Newsl. 31: 35-42) indicated that cosmid G9:12G contains sequences from LGVI, as would be expected of the cmt gene (Fig. 2). PCR fragments were generated from this cosmid that were identical to those deriving from genomic DNA.

RFLP type:
OMMMMMOMOMOMOM--OMOMMOMOMOMOMOMOM
|        |          |         |
4450     4460       4470      4480
Isolate No.

Figure 2. Sequences of cosmid G9:12G map to LGVI by RFLP. The RFLP was detected using PstI on parental strain FGSC 4488 (O) and FGSC 2225 (M). Progeny from a cross of these two strains, the MultiCentII kit (FGSC) were scored for the RFLP as indicated. - : not scorable; M: Mauriceville pattern; O: Oak Ridge pattern. Underlined letters: difference in RFLP with the closest existing probe, RAPD marker R15.7 (Metzenberg and Grotelueschuen 1994 Fungal Genetics Newsl. 40:130-133).

A segment of cosmid G9:12G was subcloned as a 4.1 kb HindIII/SspI fragment in Bluescript SK using the HindIII and EcoRV sites. The identity of the clone was confirmed by sequencing into the SspI end with the primer T7/T3(Gibco-BRL). This sequence was identical to that reported by Munger et al. (1985 EMBO J. 4:2665-8). A restriction map is provided (Figure 1B).

We have taken two approaches to construct more useful promoter derivatives. First, a multicloning sequence has been inserted into the full length promoter (Figure 1C). Secondly, the entire 4 kb upstream region is reportedly required for a functional inducible promoter. It is known that large deletions in the upstream region render the promoter constitutive (Munger et al.
Deletion analysis is being done from the 5' end of the current promoter to generate smaller clones that still retain inducibility.

Preliminary experiments using the coding region of the *hph* gene cloned behind the full-length promoter fragment indicated that 0.5 mM CuSO$_4$ could induce high level resistance to hygromycin B. These experiments were hindered by interference of the CuSO$_4$ with hygromycin B. A precipitate formed that raised the level of background growth and made the assay difficult to interpret in some tests. A new construct with a different reporter is being made to confirm and quantitate the level of inducibility of the promoter with CuSO$_4$ and to facilitate further deletion analysis. All constructs will be made available to the FGSC upon verification.