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

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-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).

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.

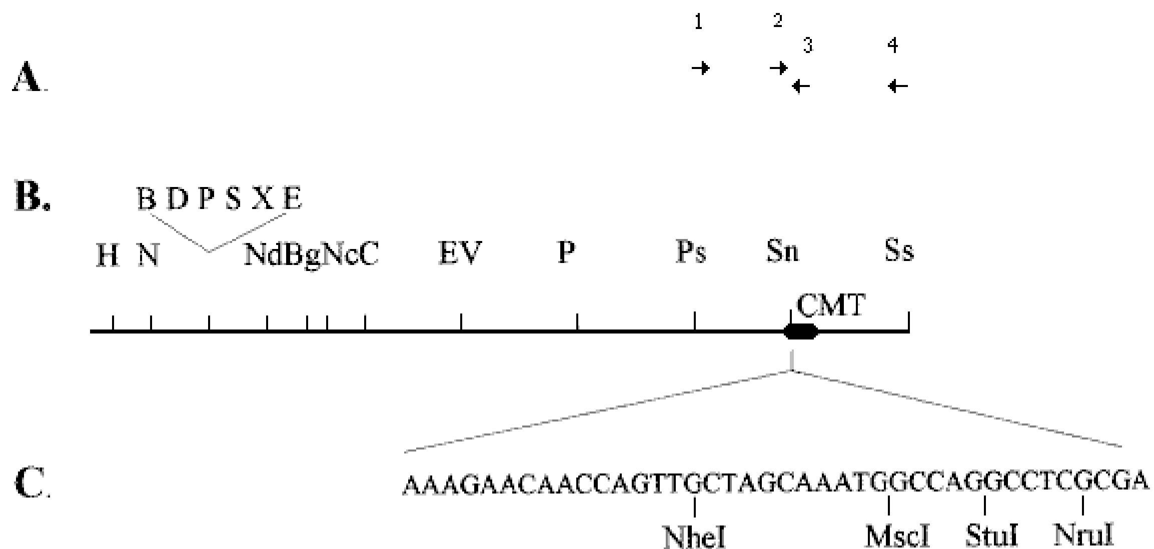

Figure 1. The copper-inducible metallothionein (cmt) gene.
A. Primers used to amplify the region from genomic DNA. Primers, 5 ->3 :

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, *Hin*dIII; N, *Not*I; B, *Bam*HI; D, *Dra*I; P, *Pvu*I; S, *Sph*I; X, *Xho*I; E, *Eco*RI; Nd, *Nde*I; Bg, *Bgl*II; Nc, *Nco*I; C, *Cla*I; EV, *Eco*RV; Ps, *Pst*I; Sn, *Sna*BI; Ss, *Ssp*I.
C. Insertion of a multicloning sequence at the 3 terminus of the inducible promoter region of the cmt gene. The region from the *Hin*dIII site to the indicated sequence was derived by PCR and cloned back into the *Hin*dIII/*Eco*RV sites of Bluescript SK. Inserts can be cloned into the *Nhe*I 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.

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RFLP type :
OMMMMMOMOOMMMMO--OOOOOOOMMOOO-MMMM-OMO
|         |          |         |
4450      4460       4470      4480
Isolate No.
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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 *Hin*dIII/*Ssp*I fragment in Bluescript SK using the *Hin*dIII and *Eco*RV sites. The identity of the clone was confirmed by sequencing into the *Ssp*I 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 contruct 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.

1985 EMBO J. 4:2665-8). 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 is 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.