SHORT PAPER

Suppression of the *cr-1* mutation in *Neurospora crassa*

Shin KORE-EDA, Tadako MURAYAMA* and Isao UNO**

*Institute of Applied Microbiology, University of Tokyo,
Bunkyo-ku, Tokyo 113*

**Kanto Gakuin University, Yokohama-shi,
Kanagawa 236*

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ABSTRACT

We have cloned a DNA fragment, which hybridized with the adenylate cyclase gene (*CYR1*) of *Saccharomyces cerevisiae*, from genomic DNA libraries of *Neurospora crassa*. The *cr-1* mutation was able to be suppressed by introducing this DNA fragment on a cosmid vector, judging from recovery of the adenylate cyclase activity and the abnormal morphology.

Cyclic AMP plays important roles of cellular functions in eukaryotic cells including fungi (Pall, 1981). In *Neurospora crassa*, cAMP is related to utilization of endogenous and exogenous carbon sources, morphology of mycelia, and formation of aerial hyphae and conidia. The *cr-1* mutants were originally isolated as morphological mutants characterized by abnormal colonial growth, formation of little or no aerial hyphae, and early, even conidiation over the surface of the colony. Later, biochemical analysis revealed that the *cr-1* mutants had a low adenylate cyclase activity and produced a small amount of cAMP (Terenzi et al., 1974, 1976; Flawia et al., 1977). All of these features are phenotypically corrected by growth of the mutant in the presence of exogenous cAMP (Terenzi et al. 1976; Rosenberg and Pall, 1979). We examined to clone a DNA fragment which suppresses the *cr-1* mutation in order to clarify the relationship between the *cr-1* mutation and adenylate cyclase.

The structural gene (*CYR1*) for adenylate cyclase was cloned from *S. cerevisiae* and sequenced (Kataoka et al., 1985). The DNA sequence of *CYR1* encodes a protein of 2026 amino acids. The catalytic domain is located at the C-terminal region of this gene product (Fig. 1). We have isolated a clone, which hybridized with the catalytic domain (*ScaI-ClaI*, 1.1 kbp) of the *CYR1* gene, from *N. crassa* genomic DNA libraries on lambda phage DNA obtained from Dr. Giles (unpublished data). To isolate a clone which hybridizes with the *CYR1* gene covering

** Corresponding author.

Fig. 1. A restriction map of the *CYR1* gene of *S. cerevisiae*. The hatched region represents the protein-coding sequence. The region used as a *CYR1* probe is indicated. Nucleic acid manipulations were described by Sambrook et al. (1989). Abbreviations used are as follows: Bg, *Bgl*II; C, *Cla*I; Sc, *Sca*I.

Fig. 2. Identification of Cos1 and Cos4. *E. coli* cells (DH1) carrying each cosmid DNA (3072 clones) were cultivated in each hole of microtiter plates, and transferred to Colony/Plaque Screen (NEN Research Product). Using these and the *Sac*I-*Sac*I (1.5 kbp) fragment probe, colony hybridization was carried out as described by Sambrook et al. (1989). Two clones, Cos1 and Cos4 were obtained by colony hybridization, which corresponded to pSV50-1:10G and pSV50-4:7C of genomic DNA libraries on cosmid (Vollmer and Yanofsky, 1986), respectively.

Fig. 3. Restriction maps of Cos1 and Cos4 and the probe fragment for Northern blot analysis. A filled box indicates the *SacI*-*SacI* (1.5 kbp) fragment which hybridized with the *CYR1* gene probe. An open box indicates the *PvuII*-*ClaI* (0.48 kbp) fragment used as a probe for the Northern blot analysis. Abbreviations used in this study are as follows: Cl, *ClaI*; E, *EcoRI*; Pv, *PvuII*; S, *SalI*; Sac, *SacI*.

the whole adenylate cyclase gene, *N. crassa* cosmid DNA libraries which carry large DNA fragments were used. The *SacI*-*SacI* (1.5 kbp) fragment, which hybridized with the catalytic domain, was obtained from this lambda clone and used as a probe for colony hybridization. The total of 3072 clones of cosmid libraries on pSV50, obtained from Dr. C. Yanofsky (Vollmer and Yanofsky, 1986), were tested for colony hybridization. Two clones (Cos1, and Cos4) hybridized with the probe, as shown in Fig. 2, and corresponded to pSV50-1:10G and pSV50-4:7C designated by Vollmer and Yanofsky (1986), respectively. The restriction map of these clones were constructed by using several restriction endonucleases. The restriction maps of these clones are shown in Fig. 3, and revealed that those two clones have an overlapped region. To examine the transcript of the cloned DNA, a Northern blot analysis was carried out. Poly [A]⁺ RNA was prepared from the wild type strain (St.L.74A), and the *PvuII*-*ClaI* fragment (0.48 kbp) prepared from the *SacI*-*SacI* (1.5 kbp) fragment (Fig. 3) was used as a probe. As shown in Fig. 4, only a 7.0 kbp band was observed, indicating that this DNA fragment was transcribed. The size of this transcript was similar to that of the *CYR1* gene of *S. cerevisiae* (Kataoka et al., 1985).

To examine the relationship between *cr-1* mutation and the DNA fragments isolated, Cos1 and Cos4 were introduced into a CR1-3 strain (*a cr-1*). This strain was constructed as follows; FGSC4009 (*a cr-1*) was obtained from Fungal Genetics Stock Center, and had been backcrossed several times to the St.L.74A strain to obtain an isogenic strain, and a *cr-1* progeny (CR1-3) was used in this study. Because *cr-1* mutants periodically accumulate modifier mutations that change the morphology of the mutant (Garnjobst and Tatum, 1970), we frequently returned to a silica gel-preserved stock for the source of our conidia to be sure of working with a modifier-free *cr-1* stock. The transformation was carried out as described by Vollmer and Yanofsky (1986). As Cos1 and Cos4 carry the benomyl resistant gene, 15 and 20 transformants indicating benomyl-resistance were isolated at

Fig. 4. Northern blot analysis of the DNA fragment hybridized with the *CYR1* gene probe. Poly[A]⁺ RNA (5 μ g) was prepared from the wild type strain (St.L.74A) as described by Sherman et al. (1986). It was separated by formaldehyde-agarose gel electrophoresis, transferred to Hybond-N⁺ nylon membrane (Amersham), and hybridized with a ³²P-labeled probe, the *Pvu*II-*Cla*I (0.48 kbp) fragment (see text), as described in the instruction manuals. Position and length in kilobases of molecular weight markers of denatured DNA fragments are indicated on the left side of the panel. The symbols 25S and 18S on the right side refer to the *Neurospora* ribosomal RNA

random, respectively. To establish stable transformant strains, these transformants were each screened three times by single colony isolations. Eleven of the 15 transformants carrying *Cos1*, and 19 of the 20 transformants carrying *Cos4*, indicated normal morphology. Most of the benomyl-resistant strains showed normal morphology, but a few showed the *crisp* phenotype. This might be due to the disruption of the wild type gene carried by donor DNA when it was integrated into genome DNA. To confirm further whether these transformants carry the *cr-1* mutation, 10 transformants from each group were crossed with the wild type

Fig. 5. Morphologies of the *cr-1*, transformant and wild type strains on an agar medium in the absence (–) or presence (+) of cAMP. The conidia of *cr-1* (CR1-3), transformant (TC1-23, TC1-39, TC4-106, TC4-117, and TC4-146), and wild type (St.L.74A) strains were inoculated into the Vogel agar medium containing 1.5% sucrose (Vogel, 1956) without cAMP or with 2.5 mM cAMP, and incubated at 25°C for 7 days.

strain (St.L.74A), and were subjected to a random spore isolation analysis. The *crisp* progeny clones were observed in all crosses tested, indicating that they still had the *cr-1* mutation. The morphologies of the *cr-1* mutant and of some transformants are shown in Fig. 5. The *cr-1* parental strain shows abnormal morphology in the absence of cAMP, and became almost normal in the presence of cAMP. When transformants were grown in the absence of cAMP, their morphology was normal, and not affected by the addition of cAMP. The result suggested that the suppression was due to the introduction of *Cos1* or *Cos4*.

We further measured the elongation rate of the hyphal tip of the wild type (St. L.74A), *cr-1* (CR1-3), and two transformants (TC4-106 and TC4-117) (Table 1). The elongation rate of the wild type strain was not affected by adding cAMP. The growth rate of the *cr-1* mutant was increased about 5-folds by adding cAMP, but the growth rate of a *cr-1* strain in the presence of cAMP was less than 20% of that of the wild type strain. However, the growth rate of two transformants was more than 50% of the wild type strain even in the absence of cAMP.

The adenylate cyclase activity in *cr-1* mutant strains was not detectable in this study, although the wild type strain indicated the normal level of this activity. Two transformants (TC4-106 and TC4-117) indicated more than 30% of that of wild

Table 1. Growth of the *cr-1* mutant, transformant, and wild type strains in the absence or presence of cAMP

Strain	Genotype	The addition of 5 mM cAMP	The rate of the hyphal elongation (mm/hr)
St. L.74A	+	—	3.4
		+	3.1
CR1-3	<i>cr-1</i>	—	0.11
		+	0.58
TC4-106	<i>cr-1/Cos4</i>	—	2.9
		+	2.3
TC4-117	<i>cr-1/Cos4</i>	—	1.9
		+	1.5

Growth was measured as the hyphal elongation rate in growth tubes containing the Vogel agar medium (1.5% sucrose) with or without 5 mM cAMP.

Table 2. The adenylate cyclase activity in the *cr-1* mutant, transformant, and wild type strains

Strain	Genotype	Adenylate cyclase activity (units/mg protein)
St.L.74A	+	43.9
CR1-3	<i>cr-1</i>	<0.5
TC4-106	<i>cr-1/Cos4</i>	14.0
TC4-117	<i>cr-1/Cos4</i>	17.3

The adenylate cyclase activity was measured at least two times in crude extracts, as described by Murayama et al. (1987), and the mean values are presented.

type strain, as shown in Table 2. These results indicate that the cloned DNA fragment was able to suppress the *cr-1* mutation, and suggest that this fragment carries the adenylate cyclase gene of *N. crassa*. The subcloning and sequencing of this DNA fragment is in progress.

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