97. Circadian Rhythm in *Neurospora* includes Oscillation of Cyclic 3', 5'-AMP Level

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Introduction. In *Neurospora crassa*, the rhythmic conidiation of band (*bd*) strain has been used to analyze the circadian clock system (Feldman and Dunlap, 1983). The rhythmic conidiation of *bd* exhibits three fundamental characteristics of circadian rhythms; (1) a free-running period of about 24 hr, (2) adjustment of the period length by light and (3) relative insensitivity of the period length to ambient temperature. However, the mechanism of the rhythmic conidiation in *bd* remains unknown.

The isolation of mutants in orthophosphate repressible cyclic phosphodiesterase (cPDase) (Hasunuma, 1983, 1984a), *cpd-1* and *cpd-2*, which show rhythmic growth in liquid media (Hasunuma, 1984b) and rhythmic conidiation on glycerol complete solid media (Hasunuma, 1984c), suggested the involvement of cyclic 3',5'-AMP in the generation of these rhythms. The levels of intracellular cPDase activity in *cpd-1*, *cpd-2* and *bd* were reduced when assayed at high (2 mM) substrate concentration. Intracellular levels of cyclic 3',5'-AMP in *cpd-1*, *cpd-2* and *bd* were reduced to 9.3–13%, 5.8–10% and 70% of wild type, respectively (Hasunuma, 1984b). Since the level of cyclic 3',5'-AMP has been identified as being a major factor in the morphogenesis of *Neurospora crassa* (Terenzi *et al.*, 1976), this work was undertaken to clarify the relationship between the level of cyclic 3',5'-AMP and rhythmic conidiation.

Materials and methods. Strains of *Neurospora crassa* used were *cpd-1*, *cpd-2* and *pho-2* (JFL 134), which is a mutant of alkaline phosphatase and is the progenitor strain for mutants in cPDase. The isolate of *cpd-1* used was KH190, and that of *cpd-2* was KH199. The isolation method of *cpd-1* and *cpd-2* was described by Hasunuma (1984a). Both of the *cpd-1* and *cpd-2* strains used were back-crossed twice to standard wild type, 74-OR23-1A, and contained *pho-2*. The band (*bd*) strain which has been used to study circadian rhythm of conidiation, was provided by Dr. H. Nakashima (National Institute for Basic Biology, Okazaki, Japan). Conidia from preculture (5 day-old) were suspended in sterilized water to give an optical density of 0.1 at 660 nm.

To assay the level of cyclic 3',5'-AMP, 0.2 ml of the conidial suspension from each strain was inoculated to 20 ml of liquid Fries minimal medium containing 1.5% sucrose (Davis and de Serres, 1970) in 100 ml of Erlenmeyer flasks. This was incubated at 25°C for 19 hr in darkness and then the culture was irradiated with white light (3.3 J/m²·sec) from 40 W fluorescent lights at 25°C for 6 hr. The culture was further incubated in darkness, and every three hours, mycelial pads were taken, push-dried by filter paper and immersed in 2 to 4 ml of ice cold 5% TCA. This procedure was carried out within 10 sec under a red safelight. The rest of the procedure was as described by Hasunuma (1984b). The amount of cyclic 3',5'-AMP was assayed by a radioimmuno-assay kit of Yamasa according to the supplier's procedure. To follow the conidiation rhythm, a drop of the conidial suspension was inoculated at one end of race tubes (12×250 mm) that held 10 ml of solid glycerol complete agar medium containing Fries's salts (Davis and de Serres, 1970).

Results and discussion. The nature of the banding growth of *cpd-1* and *cpd-2* in liquid media was analyzed by direct observation of sparse and dense parts of the submerged culture. A single colony, isolated by pasteur pipette and inoculated at the bottom of the liquid medium in small test tube, was incubated in darkness at 25°C for 7 days (Fig. 1-1). The submerged culture formed 7 clear bands. The culture was drawn out from the test tube onto a glass slide and was covered by cover slips (Fig. 1-2). Serial observation of dense

Fig. 1. Photographic representation of submerged mycelia and submerged conidia. 1: A single colony of *cpd-1* (KH190) was isolated by pasteur pipette and was inoculated at the bottom of a tube containing liquid Fries minimal medium. This was then incubated at 25°C for 7 days. 2: The culture was drawn out onto glass slide and covered by cover slips. Alternate appearance of dense part (a) and sparse part (b) can be observed. 3: Light microscopic observation of dense part (a) and sparse part (b) of the culture. Bars are 20μm long.

and sparse parts (Fig. 1-2, a, b) revealed that dense parts contained predominantly submerged conidia (Fig. 1-3a) and sparse parts contained submerged mycelia (Fig. 1-3b). There is no previous report of submerged conidia, since it was thought that conidia were only formed on aerial hyphae. However, these results clearly show that submerged conidia are formed under very reduced levels of cyclic 3',5'-AMP. Thus *cpd-1* and *cpd-2* exhibited rhythmic conidiation both in liquid media and on solid media. These strains were shown to have reduced levels of cyclic 3',5'-AMP (Hasunuma, 1984b). If the level of cyclic 3',5'-AMP oscillates according to the operation of the clock system, the oscillation may be reflected in the morphogenesis of these strains. In the case of wild type, the level of cyclic 3',5'-AMP is so high that thick mycelia with sparse conidia are evenly formed on solid media (Hasunuma, 1984c), whereas *cpd-1*, *cpd-2* and *bd* will form specific morphologies (conidia or mycelia) (Hasunuma, 1984c) depending on the levels of cyclic 3',5'-AMP. To test this possibility, the level of cyclic 3',5'-AMP in cultures grown in liquid Fries minimal medium were assayed; the results in Fig. 2 clearly show rhythmic oscillations in cyclic 3',5'-AMP levels. The four strains showed a minor peak of cyclic 3',5'-AMP after 15 hr from light off and a

Fig. 2. The levels of cyclic 3',5'-AMP in the liquid culture of wild type (*pho-2*), *cpd-1*, *cpd-2* and *bd*. The experimental procedure was as described in Materials and methods. a: Amount of growth (mg dry weight/culture) of wild type (—○—), *cpd-1* (—●—), *cpd-2* (—▲—) and *bd* (—△—). b: Amount of cyclic 3', 5'-AMP (p mol/culture). Symbols are the same as in (a). Each point is the mean of duplicate assays. The vertical bars indicate the standard error of the mean. The first arrow indicates time of light on and the second arrow time of light off. Horizontal bars indicate the time of conidiation on solid glycerol complete media in a parallel experiment.

major peak after 24 hr. However the levels of cyclic 3',5'-AMP in *cpd-1* and *cpd-2* were very low and their peaks were not so prominent. A second minor peak appeared 39 to 42 hr after light off and a second major peak appeared 48–50 hr after light off. Thus the levels of cyclic 3',5'-AMP in these four strains showed oscillations with a period length of about 24 hr. As indicated by bars in Fig. 2b, conidiation of *bd* on solid media in a parallel experiment occurred 2–3 hr after a major peak of cyclic 3',5'-AMP. In the case of *cpd-1* and *cpd-2* the first band was obscure, with very sparse conidiation, but the peak of the second band appeared at 52 hr after light off, corresponding to a major peak of cyclic 3',5'-AMP. The conidiation of *bd* on solid media was much denser than in *cpd-1* and *cpd-2*, which may be the result of a higher level of cyclic 3',5'-AMP in *bd* than in *cpd-1* and *cpd-2*. On solid media the delay of conidiation from the peak of cyclic 3',5'-AMP in *bd* may reflect the time required for the morphogenesis of aerial hyphae and conidia to develop or may be due to differences in the experimental condition. However, it is noticeable that conidiation on solid media in *bd*, *cpd-1* and *cpd-2* took place after or coincident with major peaks of cyclic 3',5'-AMP levels.

Summary. Banding growth in liquid media exhibited by mutants in orthophosphate repressible cyclic phosphodiesterase, *cpd-1* and *cpd-2*, was found to be due to the alternate appearance of submerged mycelia and submerged conidia. Thus *cpd-1* and *cpd-2* exhibited rhythmic conidiation both in liquid and on solid media, which indicated the persistence of circadian rhythm in liquid media. The examination of the levels of cyclic 3',5'-AMP in wild type, *cpd-1*, *cpd-2* and band (*bd*) strain revealed rhythmic oscillations in cyclic 3',5'-AMP levels with period lengths of about 24 hr. Conidiation on solid media took place 2–3 hr after the appearance of a major peak of cyclic 3',5'-AMP.

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References

- Davis, R. H., and de Serres, F. J. (1970): *Methods in Enzymol.*, **27A**, 79–143.
Feldman, J. F., and Dunlap, J. C. (1983): *Photochem. Photobiol. Rev.*, **4**, 319–368.
Hasunuma, K. (1983): *J. Bacteriol.*, **156**, 291–300.
—— (1984a): *Neurospora Newsl.*, **31**, 33–34.
—— (1984b): (submitted to *Current Genetics*).
—— (1984c): *Proc. Japan. Acad.*, **60B**, 260–264.
Terenzi, H. F. *et al.* (1976): *J. Bacteriol.*, **126**, 91–99.