DNA Methylation and Polyamines in Regulation of Development of the Fungus Mucor rouxii

CARMEN CANO,¹ LUIS HERRERA-ESTRELLA,² AND JOSE RUIZ-HERRERA¹*

Departamento de Genetica y Biologia Molecular, CINVESTAV, IPN, and Instituto de Investigacion en Biologia Experimental, Facultad de Quimica, Universidad de Guanajuato,¹ and Centro de Investigacion y Estudios Avanzados, Unidad Irapuato,² Mexico

Received 23 May 1988/Accepted 2 September 1988

DNA from intact or spherically growing spores of Mucor rouxii is highly methylated, whereas DNA from germlings has low levels of methylation. DNA from spores incubated with hydroxyurea or 1,4-diaminobutane is also highly methylated. The reversal of the effect of 1,4-diaminobutane by azacytidine correlated with DNA hypomethylation. These data suggest that the change in growth pattern from spherical to polarized correlates with the degree of DNA methylation and that this, in turn, may be controlled by polyamine levels.

*Molecular and Developmental Biology Laboratory, CINVESTAV-IPN, Mexico City, D.F., Mexico.

Mucor rouxii is a dimorphic fungus that grows either as a mycelium or like Saccharomyces cerevisiae depending on the environmental conditions (3, 21, 26). Irrespective of growth conditions, however, sporangiospores placed into an appropriate medium increase in volume by an isodiametric or spherical growth process termed phase I (4). After this phase, spores start a distinct phase of polarized growth termed phase II (4), emitting either germ tubes (mycelial growth) or buds, depending on the environmental conditions (3, 4, 21, 26). Syntheses of chitin (4) and proteins and RNA (7) occur exponentially with no appreciable change in rate through phases I and II of germination. In contrast, DNA synthesis is delayed and starts about 30 min before the onset of germination phase II, suggesting a possible correlation with the switch from spherical to polarized growth (7). Inhibition of DNA biosynthesis by the addition of hydroxyurea prevents the initiation of phase II of germination. In its presence cells remain almost spherical, attaining large volumes (7).

Ornithine decarboxylase activity and polyamine concentration are known to increase preceding growth phase II of Mucor racemosus and M. rouxii spores (6, 13). Exposure of germinating spores to 1,4-diaminobutane (DAB), an inhibitor of ornithine decarboxylase, inhibits polyamine formation and blocks the onset of growth phase II but not DNA synthesis (22). With this background we advanced the hypothesis (22) that spherical and polarized growth patterns of Mucor spores represented two different developmental programs.

In recent years it has become evident that DNA methylation regulates gene expression in eucaryotic organisms (19). An inverse correlation between 5-methylcytosine content and gene expression has been observed (12, 25), although more important than total amount seems to be their upstream location toward the particular gene (17, 28). In fungal DNA, the content of 5-methylcytosine is variable depending on the organism analyzed (1, 18) (one species contains N6-methyl adenine [20]), and the role of DNA methylation on gene regulation during fungal development has been suggested for several fungi (2, 16, 23, 24).

The role of polyamines in cell differentiation is well established (11, 27), although their mode of action remains subject to discussion. A plausible mechanism, according to our data with M. rouxii, would be that they were involved in gene expression through DNA demethylation. In this study we have investigated this hypothesis.

Different levels of DNA methylation in ungerminated and germinated sporangiospores. DNA from ungerminated spores, spherical (phase I) spores, or phase II spores (germlings) of M. rouxii IM80 (ATCC 29405) was isolated as described by Yelton et al. (29), followed by centrifugation in CsCl gradients. Samples (0.5 μg) were digested for 5 h at 37°C with the isoschizomer restriction enzymes MspI or HpaII (3 U) and subjected to agarose gel electrophoresis. Both enzymes recognize the sequence CCGG, but HpaII will not cut if the internal cytosine is methylated. The DNA samples from both ungerminated and spherical spores harvested before initiation of DNA synthesis contain a fraction resistant to HpaII (Fig. 1A, B, and H). A similar HpaII-resistant fraction was described in DNA from Phycomyces blakesleeanus spores (1). In contrast, DNA from germlings was found to be completely digestible by HpaII to polydisperse smaller fragments (Fig. 1C). Therefore, there is a clear difference in the levels of methylation of DNA from cells growing in a spherical fashion and those growing in a polarized way.

Effect of inhibition of DNA biosynthesis on its state of methylation. We have previously shown (7) that Mucor spores are in the premitotic phase (G2) of the cell cycle. During phase I of spore germination, nuclear division occurs previous to DNA duplication, which closely precedes the onset of germination phase II. The addition of hydroxyurea inhibits DNA biosynthesis and the phase of polarized growth but not the first round of nuclear division (7). In its presence, cells continue growing isodiametrically. We incubated Mucor sporangiospores in the presence of hydroxyurea for 18 h and analyzed the methylation levels of their DNA. Under these conditions DNA remained hypermethylated (Fig. 1E).

Effect of DAB on DNA hypomethylation. To determine whether inhibition of DNA synthesis or maintenance of its methylation level was responsible for the arrest in the transition from germination phase I to phase II, spores were germinated in the presence of the ornithine decarboxylase inhibitor DAB, which does not inhibit DNA biosynthesis (22) but maintains spores growing isodiametrically (22). DNA obtained from these cells showed the same high methylation level present in normal ungerminated or spherical spores, where no DNA synthesis had occurred, as
shown by the presence of the HpaII-resistant DNA fraction (Fig. 1F and H). Thus, when ornithine decarboxylase is inhibited and a polyamine shortage develops, newly formed DNA chains are hypermethylated.

**Antagonistic effect of 5-azacytidine.** 5-Azacytidine prevents DNA methylation (10). The analog is incorporated into newly synthesized DNA and thus inhibits DNA methylation, giving rise to gene activation (8, 9, 14, 15). The addition of 5-azacytidine to spores incubated in the presence of DAB permitted onset of polarized growth (Fig. 2) (albeit at a slower rate compared with control cells) with distorted morphology, since 5-azacytidine by itself decreased growth rate. DNA extracted from these cells had low levels of methylation, since it could be completely digested by HpaII (Fig. 1G).

In conclusion, our data suggest that the level of DNA methylation determines the establishment of the polarized phase of growth in *M. rouxii*, probably through the regulation of the genes involved in the synthesis of products necessary for apical growth of the hyphae. High levels of methylation lead to spherical growth, and low levels of methylation are required to activate polarized growth. It is known that DNA hypomethylation occurs during its replication (19). Our evidence obtained with DAB and 5-azacytidine suggests that polyamines regulate the level of DNA methylation during replication and in this way may regulate cell differentiation. Whether polyamines act directly on the methylation reaction or indirectly by regulation of methyl transferases remains to be determined. We suggest that

---

**FIG. 2.** Reversal of DAB effect by 5-azacytidine. (A) Spores were streaked on plates of YPG medium (5) containing 0.5 mM DAB and filter papers containing (a) 0.2, (b) 0.4, (c) 2, (d) 4, (e) 8, or (f) 20 mM azacytidine were placed over the plates. Photographs were taken after 72 h. (B) Higher magnification of paper circle e. Notice mycelium growing out from the filter paper. Swollen spores are visible over the plate (arrows).
spore germination of Mucor species is a simple model for revealing the intimate mechanism by which DNA methylation and polyamines regulate differentiation in eucaryotic cells.

This work was partially supported by DIGICYSA of the SEP and the Consejo Nacional de Ciencia y Tecnología, Mexico.

We thank S. Bartnicki-Garcia, University of California, Riverside, for critical reading of the manuscript. Luis Herrera-Estrella and Jose Ruiz-Herrera are National Investigators, Mexico.

LITERATURE CITED
1. Antequera, F., M. Tamame, J. R. Villaneuva, and T. Santos. 1984. DNA methylation in the fungi. J. Biol. Chem. 259:8033–8036.
2. Antequera, F., M. Tamame, J. R. Villaneuva, and T. Santos. 1985. Developmental modulation of DNA methylation in the fungus Phycomyces blakesleeanus. Nucleic Acids Res. 13:6545–6558.
3. Bartnicki-Garcia, S. 1963. Mold-yeast dimorphism of Mucor. Bacteriol. Rev. 27:293–304.
4. Bartnicki-Garcia, S., and E. Lippman. 1977. Polarization of cell wall synthesis during spor germination. Exp. Mycol. 1:230–240.
5. Bartnicki-Garcia, S., and W. J. Nickerson. 1962. Induction of yeastlike development in Mucor by carbon dioxide. J. Bacteriol. 84:829–840.
6. Calvo-Mendez, C., M. Martinez-Pacheco, and J. Ruiz-Herrera. 1987. Regulation of ornithine decarboxylase activity in Mucor bacilliformis and Mucor rouxii. Exp. Mycol. 11:270–277.
7. Cano, C., and J. Ruiz-Herrera. 1988. Developmental stages during the germination of Mucor sporangiospores. Exp. Mycol. 12:47–59.
8. Chiu, C. P., and H. M. Blau. 1985. 5-Azacytidine permits gene activation in a previously noninducible cell type. Cell 40:417–424.
9. Cresnot, F., and J. K. Christman. 1982. Inhibition of DNA methyl transferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2′-deoxycytidine. J. Biol. Chem. 257:2041–2048.
10. Friedman, S. 1979. The effect of azacytidine on E. coli DNA methylase. Biochem. Biophys. Res. Commun. 89:1324–1333.
11. Heby, O. 1981. Role of polyamines in the control of cell proliferation and differentiation. Differentiation 14:1–20.
12. Hoffman, R. M. 1984. Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. Biochim. Biophys. Acta 738:49–87.
13. Inderlied, C. B., R. L. Chilhar, and P. S. Sypherd. 1980. Regulation of ornithine decarboxylase during morphogenesis of Mucor racemosus. J. Bacteriol. 141:699–706.
14. Jaenisch, R., A. Schnieke, and K. Harkers. 1985. Treatment of

mice with 5-azacytidine efficiently activates silent retroviral genomes in different tissues. Proc. Natl. Acad. Sci. USA 82:1451–1455.
15. Jones, P. A. 1984. Gene activation by 5-azacytidine, p. 165–187. In A. Rizin, H. Cedar, and D. A. Riggs (ed.), DNA methylation. Biochemistry and significance. Springer Verlag, New York.
16. Jupe, E. R., J. M. Magill, and C. W. Magill. 1986. Stage-specific DNA methylation in a fungal plant pathogen. J. Bacteriol. 165:420–423.
17. Murray, E. J., and F. Grosfeld. 1987. Site specific demethylation in the promoter of human-globin gene does not alleviate methylation mediated suppression. EMBO J. 6:229–2335.
18. Proffit, J. H., J. R. Davie, D. Swinton, and S. Hattman. 1984. 5-Methylcytosine is not detectable in Saccharomyces DNA. Mol. Cell. Biol. 4:985–988.
19. Razin, A., H. Cedar, and A. D. Riggs (ed.). 1984. DNA methylation. Biochemistry and biological significance. Springer Verlag, New York.
20. Rogers, S. D., M. E. Rogers, G. Saunders, and G. Holt. 1986. Isolation of mutants sensitive to 2-amino purine and alkylating agents and evidence for the role of DNA methylation in Penicillium chrysogenum. Curr. Genet. 10:557–560.
21. Ruiz-Herrera, J. 1985. Dimorphism in Mucor species with emphasis on M. rouxi and M. bacilliformis, p. 361–384. In P. J. Szaniszlo (ed.), Fungal dimorphism. Plenum Publishing Corp., New York.
22. Ruiz-Herrera, J., and C. Calvo-Mendez. 1987. Effect of ornithine decarboxylase inhibitors on the germination of sporangiospores of Mucorales. Exp. Mycol. 11:287–296.
23. Russel, P. J., K. D. Rodland, E. M. Rachlin, and J. A. McClosey. 1987. Differential DNA methylation during the vegetative life cycle of Neurospora crassa. J. Bacteriol. 169:2902–2905.
24. Russel, P. J., J. A. Welsch, E. M. Rachlin, and J. A. McClosey. 1987. Different levels of DNA methylation in yeast and mycelial forms of Candida albicans. J. Bacteriol. 169:4393–4395.
25. Sellem, C. H., M. C. Weiss, and D. Cassio. 1985. Activation of a silent gene is accompanied by its demethylation. J. Mol. Biol. 181:363–371.
26. Sypherd, P., P. T. Borgia, and J. L. Paznokas. 1978. Biochemistry of dimorphism in the fungus Mucor. Adv. Microb. Physiol. 18:67–104.
27. Tabor, C. W., and H. Tabor. 1984. Polyamines. Annu. Rev. Biochem. 53:749–790.
28. Word, C., A. Bolden, C. M. Nalin, and A. Weissbach. 1987. In vitro methylation of the 5′-flanking regions of the mouse-β-globin gene. J. Biol. Chem. 262:11057–11063.
29. Yelton, M. M., J. E. Hamer, and W. E. Timberlake. 1984. Transformation of Aspergillus nidulans by using a trp C plasmid. Proc. Natl. Acad. Sci. USA 81:1470–1474.