Genetic Engineering

Principles and Methods

Volume 8

Edited by

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and

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Washington, D.C.

Plenum Press New York and London

The Library of Congress cataloged the first volume of this title as follows:

Genetic engineering: principles and methods, v. 1-
New York, Plenum Press [1979–
v. ill. 26 cm.
Editors: 1979– J. K. Setlow and A. Hollaender.
Key title: Genetic engineering, ISSN 0196-3716.

1. Genetic engineering—Collected works. I. Setlow, Jane K. II. Hollaender, Alexander, date.
QH442.G454 575.1 79-644807
MARC-S

ISBN 0-306-42277-8

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PREFACE TO VOLUME 1

This volume is the first of a series concerning a new technology which is revolutionizing the study of biology, perhaps as profoundly as the discovery of the gene. As pointed out in the introductory chapter, we look forward to the future impact of the technology, but we cannot see where it might take us. The purpose of these volumes is to follow closely the explosion of new techniques and information that is occurring as a result of the newly-acquired ability to make particular kinds of precise cuts in DNA molecules. Thus we are particularly committed to rapid publication.

Jane K. Setlow

Alexander Hollaender

ACKNOWLEDGMENT

The Editors are most grateful to Elizabeth Seubert and Donna Lange, who did all the processing of the manuscripts.

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REGULATION OF GENE ACTIVITY DURING CONIDIOPHORE DEVELOPMENT IN *ASPERGILLUS NIDULANS*

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INTRODUCTION

The fungi have proven to be exceedingly useful for furthering our understanding of the nature and organization of eukaryotic structural genes and for increasing our knowledge of the elements and processes regulating their expression. Particularly noteworthy are several species of Ascomycetes, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Aspergillus nidulans*, having life cycles that make common biological phenomena accessible to genetic analysis. Techniques for genetic manipulation are particularly straightforward and highly developed for these species. Due to their long histories of genetic investigation, numerous genes have been located on their chromosomes. All can be grown in the haploid state, permitting direct observation of genotype. However, heterokaryons or diploids or both can be formed and maintained, facilitating analysis of genetic interactions. These organisms grow rapidly, complete their life cycles in short periods of time, ranging from one to a few weeks, have simple nutritional requirements, and lend themselves to a wide variety of genetic selection strategies. In addition, methods for DNA-mediated transformation have been devised for them (1-5). Procedures have been developed for replacing genes with novel alleles created *in vitro* by using recombinant DNA techniques (6-8) and for cloning genes by direct complementation of mutations (9-11). The genomes of these organisms can now be altered with a degree of precision that is not currently possible with higher organisms and that rivals the precision attainable with Gram-negative bacteria.

The ascomycetous fungi are also well suited for modern biochemical studies. Large quantities of cells can be grown readily and inexpensively and convenient and rapid procedures exist for the isolation of organelles, enzymes, enzyme systems, nucleic acids, and nucleic acid-protein complexes. Nucleic acids and proteins can be radiolabeled *in vivo* to useful specific activities simply by adding appropriate radioactive precursors to the growth medium. Fungal genomes are only a few times larger than that of *Escherichia coli*, and contain only limited amounts of repetitive DNA (12-15), facilitating the construction of chromosomal gene banks and the execution of "chromosome walks." The diversities of their mRNA populations are several times less than those typically found in higher organisms (13,16), and fungal protein-coding genes are generally simple in structure. These properties simplify many types of transcript analysis.

The utility of certain fungal species for genetic and biochemical studies of cellular processes, however, has not been extensively exploited for the investigation of the mechanisms controlling cell differentiation and development, although the fungi produce a variety of differentiated cell types which are frequently organized into higher order structures. A notable exception has been the analysis of the genetic regulatory mechanisms and biochemical processes that determine mating type in *S. cerevisiae* (yeast) (17-20). The rapid advances in our understanding of yeast mating type control serve to illustrate the effectiveness of the sophisticated experimental strategies available for analysis of complex biological phenomena in some fungi.

The processes of cell differentiation and development in fungi frequently lead to the formation of specialized propagules, spores, that can be responsible for the distribution of the organisms over large geographical areas, can permit them to increase their numbers very rapidly when conditions are favorable, or can serve to maintain them through periods of time when environmental factors preclude growth. Filamentous Ascomycetes, like *N. crassa* and *A. nidulans*, reproduce by forming both meiotically-derived (sexual) spores, called ascospores, and mitotically-derived (asexual) spores, called conidia. Ascospores are produced in sac-shaped cells, asci, that form within multicellular fruiting bodies, termed either perithecia (*Neurospora*) or cleistothecia (*Aspergillus*), and are adapted for long term survival under adverse conditions. Conidia, by contrast, are produced in large numbers on modified hyphal elements, called conidiophores, and are adapted for aerial dispersal. Conidiophores have morphologies characteristic of individual species. For example, the conidiophore of *N. crassa* is relatively simple in structure, consisting of a branched hypha that produces conidia (blastospores) by acropetal budding. On the other hand, the conidiophore of *A. nidulans* is complex in

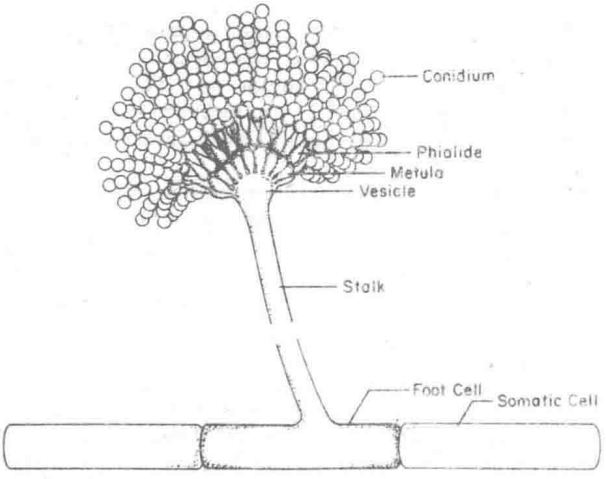

Figure 1. Artist's representation of an A. nidulans conidiophore. Reprinted by permission from Gwynne, D.I., Miller,B.L., Miller, K.Y. and Timberlake, W.E. (1984) J. Mol. Biol. 180, 91-109.

structure. It consists of a basal foot cell with thickened walls; an erect aerial stalk terminating in a globose, multiuncleate vesicle; a layer of uninucleate primary sterigmata, sometimes called metulae; and a layer of sporogenous secondary sterigmata, termed phialides, that successively form conidia (phialospores) from their open apexes (Figure 1; 21-24). As will be described in this chapter, formation of the complex, multicellular conidiophore of Aspergillus is accompanied by major alterations in gene activity.

Conidiation in Aspergillus is useful for the study of developmental gene regulation in fungi for at least three reasons. First, it is convenient to control for experimentation. Cells may either be maintained in the vegetative state or induced to form conidiophores and conidia with considerable synchrony. Large quantities of hyphae or spores can be readily isolated in pure form. Second, the molecular processes controlling the formation, maintenance and function of the various differentiated cells comprising the conidiophore can be easily investigated by using biochemical techniques. Reliable procedures for isolating macromolecules from Aspergillus cultures at all stages of development are available. Finally, conidiation in Aspergillus is well suited to genetic analysis. Conidiation is a dispensable function in the laboratory, so developmentally incompetent mutant strains can be maintained as haploids or homozygous diploids as

desired. In addition, Aspergillus is homothallic. Thus, large numbers of genetically uniform ascospores can be obtained and used for the preparation of stocks and for inoculating cultures of mutants that are aconidial. In mutant searches, many colonies can be grown in a single Petri dish and screened quickly for developmental abnormalities.

In this chapter we will attempt to summarize the progress that has been made toward understanding the molecular and genetic mechanisms that control elaboration of the Aspergillus conidiophore. We will begin with descriptions of the Aspergillus genetic system, including the recent advances that have been made in DNA-mediated transformation, and the types of laboratory manipulations that are used to study development. We will then review results showing that extensive gene regulation occurs during conidiophore development, describe the physical isolation of many of these genes, and discuss their organization in the Aspergillus genome. We will place particular emphasis on a large cluster of developmentally regulated genes, called SpoC1. We will conclude by indicating what types of strategies are currently being used in attempts to recognize the genetic elements that control gene expression during conidiophore development and in efforts to determine the molecular mechanisms responsible for their activities. Our hope is that this chapter, which is not intended to be an exhaustive review, will serve to make the reader aware of the many types of experimental strategies that can be adopted with A. nidulans to study eukaryotic cell differentiation and development.

MOLECULAR AND GENETIC MANIPULATIONS OF A. NIDULANS

Procedures for handling Aspergillus in the laboratory have been described in detail (25-27). They are summarized here to provide an overview of the methods and procedures that are used to grow and store the organism, to subject it to genetic analysis, and to isolate macromolecules from it.

Growth and Storage of Cells

A. nidulans is cultured on a variety of simple or complex growth media, depending on the genotype of the strain being used and the purpose for which it is being grown. NO_3^- or NH_4^+ are most frequently used as sources of nitrogen, although A. nidulans can utilize numerous organic nitrogen sources, $SO_4^=$ is most commonly used as a source of sulfur and D-glucose or sucrose is most often used as a source of carbon. The wild-type strain has no requirements for vitamins, amino acids, or nucleic acid bases. Petri plate cultures are usually inoculated by transfer-

ring conidia to the surface of agar-solidified medium by using a microbiological loop or a glass inoculating needle. A finely drawn glass needle permits the transfer of spores from a single conidial head. Strains are purified by streaking or spreading for colonies arising from single, well isolated conidia, which are uninucleate. Some investigators prefer to suspend conidia in H_2O or dilute (0.001 to 0.01%) Tween 80 before streaking them, in order to reduce the possibility of obtaining a single colony from two or more spores. Detergents (deoxycholate or Triton X100, 0.01 to 0.08%) may be added to the medium to cause the formation of compact colonies.

Aconidial strains can be transferred by cutting out small blocks of agar from near the periphery of colonies, by using a sterile spatula, and transferring them to fresh medium. Alternatively, cleistothecia, which usually form spontaneously in cultures after 7 to 10 days of growth, can be dissected from colonies (see below), placed in a microfuge tube containing H_2O and crushed with a forceps or glass rod. The resultant ascospore suspension can then be used directly to inoculate plates. Aspergillus ascospores will germinate without a heat treatment. Although the ascospores are binucleate, they remain useful for strain purification, because the nuclei are genetically identical, being derived from a single post-meiotic mitosis. Cultures can be grown at 20 to 42°C, but 37°C is most frequently used.

Liquid cultures are inoculated with suspensions of conidia or ascospores to a concentration of $5x10^4$ to $5x10^6$ spores/ml. Certain strains may be self-sterile and aconidial. With these, liquid cultures are inoculated with hyphal macerates. Mycelia are peeled from the surface of agar medium by using a spatula and forceps, placed in a sterile, stainless steel blender cup containing physiological saline, and blended for about 5 min. If only a small amount of inoculum is needed, the mycelia can be suspended in H_2O or physiological saline and forced through a hypodermic needle. Cultures are grown by agitating them in a rotary shaker at 200 to 300 rpm. Pre-treatment of culture vessels with a siliconizing agent helps to prevent hyphae from adhering to the walls of the vessels.

Suspensions of conidia are prepared by lightly streaking or spreading plates and growing them for 3 to 7 days, until the surface of the culture is covered with conidia. In our experience, minimal medium, containing NO_3^- as nitrogen source and appropriate nutritional supplements, encourages vigorous conidiation. Strains containing multiple genetic lesions, especially in amino acid biosynthetic pathways (28,29), may conidiate poorly. The yield of conidia from these strains can often be increased by incorporating high levels of supplements into the growth medium and by increasing the osmotic pressure of the medium through the addition of 1.2 M D-sorbitol or 0.7 M KCl. Conidia are suspended in a sterile solution of Tween 80 by scraping with a spatula or a glass rod. Suspensions are filtered through sterile cotton or

Miracloth and washed 2 to 4 times with H_2O by centrifugation at about 800 x g.

Suspensions of ascospores are prepared by inoculating Petri dishes at single points near their centers. The cultures are incubated for 3 to 4 days and then the plates are sealed with Parafilm or tape. Cleistothecia usually form after an additional 3 to 10 days of incubation. Cleistothecia are spherical structures, 0.2 to 0.5 mm in diameter, surrounded by specialized cells, called Hülle cells, and occur on the surface of the mycelium, often in clumps. They are dissected from the culture by using a fine glass or metal needle, transferred to a Petri dish containing 3% H_2O-agar, and rolled along the surface to remove adherent Hülle cells, conidia, and hyphae. They are then placed in a microfuge tube and crushed, as described above. Disruption of the cleistothecia is obvious, because the ascospores are red and will form a pink suspension. A cruder procedure is suitable when large amounts of suspension are needed. Cleistothecia are simply scraped off the plate *en masse* with a spatula, transferred to a Dounce homogenizer with a loose fitting pestle, and homogenized in H_2O or physiological saline. Suspensions of conidia or ascospores can be stored at 4°C for one to several months. Spore densities may be determined by counting appropriate dilutions in a hemacytometer, whereas viable spore counts are made by plating serial dilutions.

Stock cultures are generally maintained on silica gel granules as described for *Neurospora* (30). In brief, conidia or ascospores are suspended in sterile, reconstituted dry skim milk. Small, screw-cap vials are half filled with silica gel granules and sterilized in an oven at high temperature. Samples of the spore suspension are transferred to the vials, cooled with ice, and the vials are sealed. Silica gel stocks can be stored at room temperature indefinitely. They are reactivated by sprinkling granules onto the surface of agar-solidified growth medium. For shorter term storage, Petri dishes or slants can be sealed with tape or Parafilm and stored at 4°C.

Genetic Analysis

The parasexual cycle. *A. nidulans* readily undergoes heterokaryosis, and diploid nuclei are formed in heterokaryons at easily detectable frequencies, permitting use of the parasexual cycle for genetic analysis (25). Most laboratory strains have been derived from the Glasgow wild-type isolate and are therefore in the same heterokaryon incompatibility group. Thus heterokaryons can be formed at will. Balanced heterokaryons may be produced in a variety of ways. We prefer to inoculate conidia from two strains onto the surface of liquid complete medium in a test tube by using a microbiological loop. The spores are

allowed to germinate and grow at 37°C overnight, without agitation. The floating mycelial mat that forms is removed with a forceps, rinsed several times with sterile H_2O to remove medium, and placed onto the surface of agar-solidified medium having a composition that selects against growth of either homokaryotic strain but permits growth of the heterokaryon. Small pieces of mycelium are dissected away from the mat and spaced evenly on the plate. After one to several days of incubation, heterokaryotic mycelia can be seen growing away from the areas of inoculation. If different conidial color mutations are incorporated into the two homokaryotic strains, the nature of the heterokaryon can be determined by observing the mixture of differently colored conidial heads. Wild-type conidia are green. The most frequently used spore color mutations are white (wA⁻) and yellow (yA⁻); wA and yA mutations are recessive, and wA mutations are epistatic to yA mutations. The heterokaryon is transferred by cutting out small blocks of agar from near the periphery of a sector and placing them on fresh, selective medium. A balanced heterokaryon will have a mixture of differently colored conidial heads if spore color markers are incorporated into the strains being used.

Diploid nuclei form spontaneously at low frequencies in heterokaryons. Diploid strains may be selected as follows. A dilution series is prepared from a conidial suspension from a balanced heterokaryon. Dilutions are plated individually by adding them to 25 ml of selective medium, containing 1.5% melted agar, maintained at 42 to 45°C and pouring the medium into empty Petri dishes. The medium is allowed to solidify and the plates are then incubated for 2 to 4 days at 37°C, after which time diploid colonies are usually evident. Diploids can be most readily recognized by the color of their spores, if different spore color mutations are incorporated into the haploid strains. For example, a diploid derived from yA⁻;wA⁺ and yA⁺;wA⁻ strains will produce green spores. Diploids also produce morphologically distinct conidiophores that can be recognized after a little practice by the irregular arrangement of the conidial chains. In addition, diploid conidia are larger than haploid conidia. It should be noted that the selection of diploid mycelia in Aspergillus is an artificial process and that diploids cannot be induced to undergo meiosis by nutrient depletion, as is the case for S. cerevisiae and related yeasts. Pre-meiotic diploid nuclei are formed naturally in ascus mother cells in the fruiting bodies and exist only transiently. Diploids are most conveniently "haploidized" by plating them on complete or supplemented minimal medium containing 0.5 to 2 µg/ml of the antitubulin fungicide benomyl (31). Diploid mycelia are more sensitive to benomyl than are haploid mycelia, growing and conidiating poorly in the presence of low concentrations of the fungicide. Haploid segregants are readily identified as more rapidly growing sectors that

conidiate vigorously. Segregation of recessive spore color markers may also be used to identify haploid sectors. New mutations are assigned to linkage groups by constructing diploids with mapping strains that are marked on all eight linkage groups (32) and then examining segregation of the new mutation and the markers in haploids. As mitotic recombination is a rare event, the new mutation will be completely linked to one of the markers. Only a limited number of haploid segregants from a diploid need to be examined to make an unambiguous assignment of a new mutation to a linkage group. Thus, the chromosomal locations of DNA fragments that have been introduced into the genome by transformation and do not cause a visible or easily scored phenotype can be determined by blot analysis of DNA isolated from the haploid segregants.

Mitotic crossing over may be used to determine the relative positions of genes along chromosome arms. Diploids are constructed with strains carrying multiple markers in coupling, including a centromere-distal marker that can be selected for homozygosity (for example, recessive spore color or drug resistance markers). Conidia are plated and diploids that are homozygous for the distal marker are selected, purified, and examined for the relevant phenotypes. Mitotic crossing over, followed by appropriate strand segregations, results in homozygosity for all of the markers distal to the crossover point.

Meiotic recombination. Although A. nidulans is homothallic, meiotic mapping is possible because cleistothecia arising in mixed cultures contain only self-fertilized or cross-fertilized asci. Crosses are initiated by inoculating agar-solidified medium that selects against both parental strains with approximately equal numbers of spores from each strain. We do this by cutting a small well in the middle of the plate, filling it with about 0.1 ml of liquid complete medium, to allow spore germination and a limited amount of mycelial growth, and placing a loopful of spores from each parent into the well. A previously formed, balanced heterokaryon may also be used to initiate crosses. The plates are incubated for 3 to 4 days, sealed with tape or Parafilm, and incubated until mature cleistothecia are observed, usually in less than a week. Individual cleistothecia are dissected from the plate, crushed, and samples of ascospores are streaked onto appropriate medium. It is most convenient to incorporate unlinked spore color mutations into the parental strains so that hybrid cleistothecia can be identified directly by the appearance of approximately equal numbers of differently colored colonies. Once a crossed cleistothecium has been identified, ascospores are plated by spreading, and the resultant colonies are scored for the relevant phenotypes. A single cleistothecium can produce enough ascospores to allow detection of even intragenic recombination. Replica plating may be done by