

Handbook of Fungal Biotechnology

Second Edition, Revised and Expanded



edited by

Dilip K. Arora

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Handbook of Fungal Biotechnology

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Preface

The fungal kingdom comprises one of the most diverse groups of living organisms. They are numerous, ubiquitous, and undertake many roles, both independently and in association with other organisms. The fungal species range from those the size of a few micrometers up to larger fungi with fruiting bodies ranging from several centimeters to meters, and in extreme cases they can develop into a colonial organism that covers many hectares. This diversity of form is also mirrored by functional diversity as fungi can virtually occupy all ecological niches, from slow-growing endolithic communities in the polar regions to highly specialized plant and animal pathogens, and rapid degraders of organic materials in tropical environments. The span of functional diversity in fungi makes them the richest model system in cell biology. Recent developments in molecular biology techniques, including DNA amplification, cloning and expression systems, and modern genomic and proteomic analyses have yielded the discovery of new compounds, and also offered tools to investigate, characterize, and exploit both new and long established fungal activities. Fungi have played a significant role in several biotechnology-based industrial processes and the formulation of a variety of compounds. Fungi are also the target of many biotechnological applications, from the development and production of noteworthy pharmaceuticals and industrial products to their use as systems for homologous and heterologous gene overexpression.

The bulk of available literature covers all the major aspects of general mycology, much of which is themed into broad subject areas such as systematics, ecology, biochemistry, pathology, and molecular biology. However, there is a scarcity of compiled literature related strictly to the basic principles of applied mycology and fungal biotechnology. Their broader implications in published literature are fragmented over several specialized journals. In order to attempt to bring together such a diverse field, I, along with coeditors, ventured to edit the five-volume series *Handbook of Applied Mycology* in 1992. This series offered a comprehensive treatment of basic principles, methods, and applications of mycology as an integrated and multidisciplinary subject. These five volumes presented and collated the major aspects of applied mycology and served as the standard reference for students, teachers, and researchers. Since 1992, significant developments in both biological sciences and industry have broadened the conceptual basis of fungal ecology, physiology, and biochemical processes that are directly relevant to biotechnological usage and manipulation. As a result it seemed timely to revise the original Volume 4 (*Fungal Biotechnology*) and to review the current developments and highlight advances in rapidly expanding areas of molecular technologies in industry, commercial production technology, and medical biotechnology.

The revised second edition of the *Handbook of Fungal Biotechnology* is intended to provide a broad and detailed introduction to the different aspects of fungal biotechnology, with chapters covering molecular technologies, commercial fungal applications, medical mycology, culture collections, legal aspects, and biosafety. The contributions include

both reviews of existing fungal biotechnology applications and details of new processes that may become major applications in the future. For example, new chapters address topics ranging from cell biology of hyphae, protoplast fusion, metabolic regulation pathways, nuclei and chromosomes to genomics, gene clustering, gene cloning and sequencing, fungal mitochondrial genome, fungal genome and evolution, the role of GFP in fungal biotechnology, and DNA chips and microarrays. Coverage has been expanded on commercial applications of fungi, such as the application of genetic engineering for strain improvement, genetic importance of wine yeasts, fungi in brewing, alcohol production, fungal enzymes, role of chitin, polysaccharides, lipids in fungal biotechnology, production of citric acids, carotenoids, terpenoids, antibiotics, antifungal drugs, and antitumor and immunomodulatory compounds. Chapters that have direct or indirect significance in medical biotechnology have also been added.

The vast array of usage and properties is testimony to the countless ways in which mankind can harness the benefit of fungi; therefore, characterization techniques and methods of preservations of fungi, the recent development in biotechnology and intellectual property, access to genetic resources, and benefit sharing is essential. These challenging areas of fungal biotechnology are also covered in this book. Potential benefits and dangers of genetically modified foods and mycoherbicides are evaluated. Therefore, the aim of this handbook is to provide a snapshot in time as to the use of fungal biotechnology in different key areas, and to identify potential directions and possibilities for the future.

The subject areas related to agriculture, food, and environmental biotechnology are covered not in this volume but in the simultaneously published *Fungal Biotechnology in Agricultural, Food, and Environmental Applications* from the same editor.

In assembling this volume, I have collaborated with world-renowned scientists to illustrate many application areas of fungal biotechnology, from both industry and academia. The contributors are from a broad international background, and thus reflect the diverse activities occurring worldwide. I recognize serious difficulties in developing a comprehensive book on fungal biotechnology because of the range and complexity of the emerging knowledge. However, we have attempted to bring together pertinent information that may serve the needs of the reader, as a quick reference to a subject that might otherwise be difficult to locate, and by furnishing a starting point for further study. I hope that the comprehension of this material by readers will enhance their understanding and help them to gain new appreciation for many potential benefits of fungal biotechnology in a wide variety of fields. The book should be of great interest not only to students, teachers, and researchers but also to agricultural practitioners, mycologists, botanists, microbiologists, molecular biologists, food scientists, biochemists, ecologists, genetic engineers, environmental scientists, pharmacologists, and all those concerned with issues related to significant developments in the field of fungal biotechnology.

I am grateful to many colleagues for discussions and their advice during the preparation of this edition, and the academic niche of the Banaras Hindu University for the opportunity to complete this great task. I am grateful to many international authorities and specialists who have graciously consented to share their perspectives and expertise on the diverse applications of fungal biotechnology, and contributed chapters.

I am also indebted to Ms. Sandra Beberman, Vice President, Marcel Dekker, Inc., and Ms. Dana Bigelow, Production Editor, for their skill, patience, encouragement, guidance, and support.

Dilip K.Arora

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1

Cell Biology of Hyphae

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1 INTRODUCTION

Members of the fungal kingdom are present in almost every conceivable niche. Even though fungi are remarkably diverse, many fungi share common cellular characteristics that are instrumental to the success of fungal growth, development, proliferation, and survival. The purpose of this introductory chapter is to provide the reader with an overview of some of the fundamental aspects of one of the predominant forms of fungal structures—hyphae. The introductory chapter discusses the attributes that are common to many fungi as well as other organisms while also emphasizing some of the features that are unique to fungal species, as compared to other eukaryotes (some of the details will be discussed in depth in the following chapters of this book). Perhaps the primary recognizable difference between the hyphal “cell” and cells of other organisms is the predominantly coenocytic nature of the former. Characteristically, the hyphal cell harbors multiple nuclei that are evenly or unevenly distributed over relatively long distances of cytoplasmic continuity. Nonetheless, in this chapter, the term “cell” is used while discussing some of the fundamental as well as more unique attributes of hyphae. Identifying and understanding the nature of these attributes, and in particular, the regulatory mechanisms involved in orchestrating the growth of the fungal filament is an important step in the process of our intervention in fungal biology, be it curbing detriments or enhancing benefits these organisms are capable of exhibiting.

2 THE CELL WALL

The cell wall is a characteristic structure present in many organisms whose life style to grow in an environment with continuously changing water potential can be described as an absorptive one. Thus, the accumulation of large concentrations of osmotically active molecules requires the presence of a structure that will assist in maintaining the integrity of the cell membranes. In addition, the adventurous, and at times, invasive proliferation of the fungal filament in a variety of diverse environments requires the presence of effective mechanisms to defend the fungal cell from external perils. The cell wall is a prime example of an efficient mechanical exocellular defense system. Understanding the structure of the cell wall and the synthesis of its components is essential for elucidation of the processes involved in fungal growth and development. This understanding includes the fundamental aspects of filament elongation and branching as well as various aspects of differentiation, pathogenicity, absorption, and secretion.

2.1 Composition

The fungal cell wall accounts for approximately 25% of the mycelial dry weight. Approximately 80% of the cell wall dry weight is comprised of polysaccharides (Ruiz-Herrera 1992). The remaining 20% is comprised of proteins, lipids, and various inorganic salts. The predominant carbohydrate polymers found in different fungi are various forms of glucans and chitin. These sugars, synthesized and positioned in a nonuniform, yet highly regulated manner provide the external skeleton of the hyphal cell and are synthesized mainly at the apical region of the growing hyphae. Nonetheless, additional components (in particular—cell wall-associated proteins) are involved in determining the cell surface properties of the growing or nascent hypha. Cell wall associated proteins are involved in the restriction of cell permeability to detrimental compounds and/or proteins present in the environment. These cell wall proteins are also involved in recognition of other fungi and regulation of processes such as anastomosis, sexual and asexual partner recognition, and interactions (e.g., recognition and adherence) with various hosts (Lora et al. 1995; Saupe et al. 1996). The interactions between fungal cell wall proteins and potential hosts is predominantly based on protein-protein recognition, even though there are convincing records of carbohydrate-protein interactions with the carbohydrate supplied by either the fungus or the host (Cormack et al. 1999).

2.2 Synthesis

As the extension of the hyphal filament occurs mainly in the apical region, most of the biosynthetic machinery (and most likely some degradative machinery as well) involved in this process is trafficked to that region of the apical cell. As the main components of the hyphal cell are glucan and chitin, the glucan and chitin synthases must be positioned properly (in the vicinity of the extension or repair areas) and their activity regulated so as to produce the relevant polymer in a site-specific manner. The mechanism by which the polymers are extruded is yet unclear. The presence of several chitin synthases in filamentous fungi has been documented at the respective genomic databases (NCBI and other, specific, databases as mentioned in Section 7.3 of this chapter). Based on the available information, *Neurospora crassa* and *Aspergillus fumigatus* each have seven genes encoding for chitin synthases, whereas *Saccharomyces cerevisiae* has three. The differential expression and functional consequence of chitin synthase gene inactivation have demonstrated that different chitin synthases have different roles during fungal growth and development (for a detailed perspective see Chapter 30). Some chitin synthases are essential for maintaining proper hyphal rigidity and form and are required for hyphal elongation. The specific roles of other members of this gene family have yet to be elucidated. The cell wall biosynthetic (chitin and glucan synthases) as well as cell wall lytic (chitinases and glucanases) enzymes required for cell elongation and branching processes are conveyed to the required location, and at least in some cases, compartmentalized trafficking is carried out in membranous vesicles (Sietsma et al. 1996). The wall at the tip is plastic allowing the extension of the cell by insertion of new material. As extension progresses, the material at the former position of the apex is progressively rigidified as it becomes the lateral wall of the growing cell. This

rigidification is brought about by the covalent cross-linking of wall materials, especially chitin and $\beta(1\rightarrow 3)$ glucans, and the hydrogen bonding of adjacent polysaccharide chains, especially chitin, to give microfibrils.

Many of the cell wall-associated proteins are heavily glycosylated. Many of the proteins secreted to the external face of the plasma membrane are linked via a remnant of the glycosylphosphatidylinositol anchor to the polysaccharide cell wall component. *N*-Glycosylation of proteins in the fungal endoplasmic reticulum is most likely very similar to that observed in mammalian cells, yet the process occurring in the Golgi apparatus is different than that described in mammals (Dean 1999). *O*-Glycosylation also occurs in filamentous fungi and is mediated by a conserved family of protein-mannosyl-transferases (Strahl-Bolsinger et al. 1999).

2.3 Hydrophobins

Hydrophobins are among the unique protein components of the fungal cell wall. These small proteins, secreted during a variety of developmental processes are present in most filamentous fungi and have, so far, not been found in nonfungal species (Talbot 1999). These proteins play essential roles in fungal adherence, development of aerial structures, and infection of host plants by phytopathogens (Ebbole 1997). Fungal hydrophobins are hydrophobic in nature and can be defined by the presence of eight cysteine residues that are spaced in a particular manner within the amino acid sequence. They have been divided into two classes, based on solubility characteristics brought about by differences in cysteine residue spacing and distribution of hydrophobic and hydrophilic amino acids within the hydrophobin polypeptide sequences (Wösten et al. 1999). Beyond the involvement of fungal hydrophobins in natural processes of fungal growth and proliferation, the unique attributes of these proteins have been the basis of several suggested industrial applications. These include the use of hydrophobins as molecular anchor points for industrial proteins/enzymes, by attaching them to hydrophobic plastic surfaces. Other potential uses include the production of a natural protein coating over artificial organs or other transplants (Kershaw and Talbot 1998).

Though many structural components and organizational features are common to a wide range of fungi, differences in cell walls can be observed among different fungal species. Furthermore, the composition and structure of the cell wall can diverge immensely during the growth and development of the fungal colony.

3 THE PLASMA MEMBRANE

The presence of a cell wall provides the fungal cell with the ability to survive and grow without the need to equalize the cellular osmotic potential to that of its environment. In fact, the difference in osmotic potential contributes to the ability of the hyphal cell to elongate and branch by creating turgor pressure. The primary active barrier between the fungal cell and the environment is the plasma membrane. As seen in other organisms, the plasma membrane is a selective divide involved in flow of material and information between the cell and its environment.

The maintenance of a plasma membrane potential and appropriate ion gradients are the basis of the ability to transport solutes in and out of the fungal cell. This ability is achieved via a variety of proton pumps, carrier proteins, and ion channels. *N. crassa* has been a prime model for analysis of proton pumping and membrane potential (Davis 2000). The maintenance of a pH gradient and a high membrane potential (≈ 200 mV, inside negative) is mainly dependent on the efflux of protons, rather than Na^+ (the common mechanism in animal cells). Nonetheless, even though the plasma membrane ATPase (a key element in the maintenance of the pH gradient) is essential, it is most likely that other processes contribute significantly to the stabilization of cytoplasmic pH. These, most likely, include the modulation of the passive membrane permeability to protons, modulation of oxidative metabolism (a source of cytoplasmic protons), modulation of various proton-coupled transporters, and modulation of the flux between the cytosol and storage organelles (the vacuole in particular). A comprehensive overview of the transport processes operating in the fungal plasma membrane and the tonoplast has been compiled by Garrill (1994).

Apart from the maintenance-related functions mentioned previously, the plasma membrane (and plasma membrane-associated proteins) plays a key role in additional biological functions of the hyphal cell. This includes serving as the anchoring structure for a variety of receptors involved in environmental sensing, a key location for initiation of endocytosis, and the final step in secretion. Furthermore, all steps of hyphal fusion, be it self-fusion or fusion occurring with other strains during vegetative and/or mating processes involve recognition and changes in the plasma membrane.

4 SEPTATION

Compartmentalization of the fungal cell is dependent under most circumstances on the formation of septa. The initiation of septum formation has been associated with the recruitment of actin to a specific site along the hyphal cell. The events that follow include the deposition of cell wall material outside the plasma membrane. The septal wall provides a structural component for maintaining the hyphal architecture, yet is perforated in a way that provides the maintenance of cytoplasmic continuity and organelle movement (including, in some cases, nuclei) between compartments. Septation results in the cessation of growth and nuclear division in subapical cells. These subapical cells provide a reservoir of growth potential for the extending hypha. In most fungi, the nuclei residing within the subapical compartments ultimately give rise to new branches and various spore types. Thus, septation is most likely involved in determining both cell size and shape.

In ascomycetes the septa generally appear to be homogeneous with a simple perforation while in basidiomycetes some septa are trilaminar. The primary septal plate carries at its center a thickened ring (the septal swelling) that surrounds the septal pore. Either face of the septal swelling is enclosed by the concave surface of a dome-shaped structure which is composed of an irregularly perforated multilayer membrane. This complex septal structure, termed the “dolipore septum” is characteristic of most basidiomycetes (excluding, for example, the rusts and the smuts). Occlusions within the

pore and the parenthesomes on either side (in some fungi) delimit the pore domain (Moore 1985). The dolipore septum plays a role in nuclear migration, as the size of the pore and the parenthesome membrane provide a considerable barrier to nuclear movement. In fact, nuclear migration (as part of the dikaryon formation process) has been shown to be associated with disruption of the septum by lytic enzymes (Casselton and Economou 1985). Once the dikaryon is established, septal disruption no longer occurs. In contrast, it is important that septa remain intact for the maintenance of the binucleate stage. The cessation of septal disruption is probably one of the early postmating events regulated by the mating factors.

One of the major risks imposed by cytoplasmic continuity characteristic of many filamentous fungi is the catastrophic event of massive leakage. Such events may occur following mechanical or chemical damage to the cell wall, which in severe instances can result in impairment of the filament structure to the point where cell contents can no longer be retained. A key mechanism in the process of minimizing the damage following the formation of a rupture in the fungal cell is the induction of rapid cell wall repair activities (Ruiz-Herrera 1992) while at the same time activating a mechanism aimed at blocking the septal pores. One of the key factors involved in the latter process (in ascomycetes) is the Woronin body. Woronin bodies are proteinaceous granules that can reach a size of $\approx 0.5 \mu\text{m}$. They are of peroxisomal origin (Jedd and Chua 2000; Keller et al. 1991) and usually reside along the peripheral regions of the fungal cell and in association with the septum. The Woronin body is comprised of a crystalline subunit made of a protein with a peroxisome-targeting signal that most likely indicates that these bodies originate from the peroxisomal membrane. Even though the Woronin bodies do not move along with normal cytoplasmic streaming, the dramatic increase in cytoplasmic flow following cell rupture may provide sufficient force to pry the Woronin bodies from their plasma membrane site of attachment, and provide the vehicle for a rapid direction of the bodies to the septal pore.

5 THE CYTOSKELETON

The hyphal cytoskeleton plays a key role in cell shape determination, maintenance, and growth. A broad spectrum of proteins interacts with the basic components of the hyphal cytoskeleton that is comprised of microtubules and actin filaments. The dynamics of the hyphal cytoskeleton that involves constant change and adjustment in concert with cellular changes is characteristic of filamentous fungi. Furthermore, it is most likely that cytoskeletal elements interact directly with components of the cell wall. Thus, structure, sensing, and cellular response are closely associated with the hyphal cytoskeleton.

In contrast to many other cells types (including yeasts and higher eukaryotic cells) the distances that some cellular components must travel within the elongated hyphal cell are enormous. The continuous supply of vesicles (produced in the Golgi apparatus) to the apical cluster is critical for tip growth. This cluster of vesicles also termed the Spitzenkörper (apical body) appears as a dense spherical body with no discrete outline and is associated only with growing hyphae (apical cells) or immediately prior to the formation of new branches. The vesicles within the apical body range in diameter from

30 to 400 nm. The content of these vesicles, which most likely includes (among other proteins) enzymes involved in cell wall biosynthesis, has yet to be properly determined. Perhaps the most studied components of the apical body are a subpopulation of the smaller vesicles—the chitosomes. The pioneering work of Braker et al. (1976) established the linkage between these vesicles and chitin synthase activity. Following the isolation of genes encoding chitin synthases and the availability of antichitin synthase antibodies, localization of chitin synthases to chitosomes by immunohistochemistry was made possible (Sietsma et al. 1996). The capacity to transfer cargo along the cytoskeletal backbone of the hyphal cell is provided by molecular motors that are responsible for both forward and retrograde transport along the hyphal filament. The proteins that provide the ATP-driven motor function along actin and microtubules are myosin and kinesin/dynein, respectively (see later).

Recent observations by Seiler et al. (1999) suggest that significant retrograde transport, at times exceeding the forward traffic of vesicles occurs in the vicinity of the hyphal tip. Thus, transport of compounds absorbed from the environment, or recycling of cellular proteins and other components from the hyphal tip region are also key processes involved in hyphal elongation. The efficacy of vesicle transport is much dependent on the proper function of nascent and mobile cytoskeletal elements. Actin microfilaments form longitudinally oriented cables and fine filaments in the cytoplasm extending, in some cases, to the apical dome and they are also associated with the Spitzenkörper. Microtubules are longitudinally oriented and they do not usually extend into the apical dome. Interestingly, not every case of functional disruption of cytoskeletal elements (by genetic alteration or drug treatment) results in measurable impairment of vesicle traffic or hyphal tip growth (Yamashita and May 1998). Furthermore, it appears that the organization of microtubules, as are the cytoskeletal motor complexes may well be diverse in different filamentous fungal species (and, apart from the structural conservation of the core elements, are also functionally diverse from yeasts).

Microtubules are involved in the formation of the mitotic spindle, intracellular transport of secretory vesicles (Howard 1981), positioning of the Spitzenkörper (Riquel et al. 1998), and organelle movement (Steinberg and Schliwa 1993). The initiation of microtubule polymerization (which involves the structurally-related α and β tubulin proteins) is determined by spindle pole bodies (microtubule organization centers) (Heath 1994). In vegetative hyphae, another tubulin, designated γ -tubulin (which is slightly less structurally similar to α and β tubulin than they are to each other) is present only in the spindle pole bodies. These spindle pole bodies are a critical determinant in the establishment of microtubule polarity and the concomitant direction of cargo transport within the hyphal cell.

Kinesins: The presence of members of the kinesin superfamily (comprised of the “founder” family of conventional kinesins along with kinesin-like proteins) in fungi is well documented. Lee and Plamann (2001) have described the structural and functional attributes of fungal kinesins (and other cytoskeletal components). The role of conventional and kinesin-like proteins in vesicle transport towards the hyphal tip as well as mitochondrial and nuclear movement have been demonstrated, yet their function in different filamentous fungi may vary (Lee and Plamann 2001; Steinberg and Schliwa 1995; 1996).

Dynein: Dynein and its accompanying multisubunit component, known as dynactin have been implicated to be involved in vesicle trafficking and mitosis. The multisubunit dynein/dynactin motor is complex and all indications are that their structure is highly similar to those present in higher eukaryotes. The complex is involved in retrograde transport, vesicle movement associated with the endocytic pathway, formation of the endoplasmic reticulum network, organization of the Golgi apparatus, and formation of the mitotic spindle (Karki and Holzbaur 1999). Plamann et al. (1994) have analyzed the abnormalities in nuclear distribution associated with defects in cytoplasmic dynein and have proposed models for the possible mechanisms by which the complex is involved in nuclear migration.

6 REGULATION OF ELONGATION/ BRANCHING

Hyphal elongation and branching are fundamental processes that define the morphology of filamentous fungi. The efficacy and success of these processes are essential for the outreach and acquisition of nutrients from the environment and in many cases, are part of the proliferative and reproductive cycle. The formation of the common cylindrical cell extension/branches by the polarized synthesis of new membrane and cell wall material must be both temporally and spatially regulated. Though information concerning the regulation of hyphal growth is accumulating, this aspect of hyphal cell biology is still in its infancy and a comprehensive picture of the network of processes involved has yet to be obtained.

The use of genetics has been instrumental in identifying and analyzing factors involved in the regulation of polar growth in both yeasts and filamentous fungi. The significant morphological differences between yeast and filamentous fungi suggest that in addition to the common basic machinery involved in establishment, maintenance, and changes in cell polarity, filamentous fungi may have additional or alternate regulatory pathways and are most likely to have more downstream elements (as required by their more complex morphology) governed by the regulatory systems involved. A case illustrating a significant difference in a key regulatory pathway involved in polar growth of yeasts and filamentous fungi is described later.

Mutants that exhibit abnormal morphology of hyphal growth have served for identifying some of the genes involved in polar extension/branching of hyphae and the linkage between cell polarity and hyphal extension. Bruno et al. (1996) analyzed a temperature-sensitive *Neurospora* mutant (*mcb*) that exhibited apolar hyphal growth, which was not restricted to the apical cell of the hyphal filament. The mutant was shown to be defective in the gene encoding the regulatory subunit of cAMP-dependent protein kinase (PKA). The fact that the PKA pathway is involved in the regulation of polarized hyphal growth (the mutant is also defective in septum localization) in *N. crassa* is consistent with reports on the involvement of an activated PKA pathway in developmental switches that affect growth polarity in *Ustilago maydis* and *Magnaporthe grisea* (filamentous growth and proper appressorium formation, respectively). Later events in the penetration and infection processes of these fungi are stimulated by a mitogen-activated protein (MAP) kinase cascade, presumably through a MAP kinase module that

may respond to the cAMP signal (see Chapter 4).

In contrast, even though an increase in PKA activity in *S. cerevisiae* has morphological consequences, these do not really involve cell polarity. This difference may reflect the different nature of growth of filamentous fungi, in which septation/cell separation is not linked with polar elongation (yet involves “cross-talk” between mechanisms regulating cell size and cell cycle). The comprehensive mechanistic involvement of PKA in regulation of polar growth and other cellular processes has yet to be obtained; most likely it involves the complex regulation of PKA expression itself, the interaction between PKA and its substrates, and interactions between PKA and other regulatory pathways.

A variety of hyperbranching mutants have been obtained in different filamentous fungi. The genetic dissection of these mutants is at its infancy. An example of an immediate need to understand such a mutant was the appearance of a hyperbranched colonial mutant of *Fusarium graminearum* strain A3/5, used to produce Quorn[®] mycoprotein. The unexpected increase in the population of these colonial mutants in continuous flow cultures suggested a selective advantage over the sparsely branched wild type strain. The apparent advantage is an altered capability of glucose metabolism. Thus, the hyperbranching phenotype is due to a pleiotropic effect of the mutation in the colonial strain (Trinci et al. 1999). The influence of nutrient sources and the availability it has on colony morphology, as well as the effects paramorphogens have on fungal growth, and the means available to study the growth of the fungal colony have been recently reviewed by Olsson (2001).

The analysis of *Neurospora* colonial temperature sensitive (*cot*) mutants has yielded information concerning elongation and branching. Close examination of these mutants has clarified the fact that not all hyperbranching events are identical in rate of the elongation/branching frequencies or morphology of the branching pattern. Thus, it has become clear that diverse factors can result in considerably similar alterations in gross morphology while the mechanisms involved in imposing these changes can be very diverse, thus requiring much additional investigation.

At the permissive temperature, *cot-1* grows in a manner almost indistinguishable from that of the wild type, yet when shifted to the restrictive temperature hyphal elongation ceases concomitant with a massive induction of hyphal branching (Collinge et al. 1978; Yarden et al. 1992). The newly formed hyphal tips are unable to continue elongating at the restrictive-temperature, but returning the culture to the permissive-temperature results in the rapid restoration of normal hyphal growth. The *cot-1* gene has been cloned and, based on the deduced COT1 amino acid sequence it encodes a Ser/Thr-specific protein kinase that can be membrane associated (Gorovits et al. 1999; Yarden et al. 1992). Furthermore, evidence for a linkage between branching and fungal cytoskeleton assembly and function (Plamann et al. 1994) has set the stage for further analysis of the regulation and interaction of components governing the polarity of fungal filament growth.

Ser/Thr kinases that are highly similar to COT1 have been identified in other fungi (Buhr et al. 1996; Verde et al. 1998) and many higher eukaryotes, including *Candida elegans*, *Drosophila*, and mammals (Gorovits et al. 1999). The COT1 is highly similar to the product of the human *DM* kinase gene, which when mutated can cause myotonic dystrophy (Mahadevan et al. 1993). The common feature of most cells with alterations in their COT1-related kinase is abnormal morphology and observable defects in their

polarity.

In *N. crassa*, COT1 kinase is essential, as insertional inactivation of the *cot-1* gene is lethal (Yarden et al. 1992). Lauter et al. (1998) have demonstrated that *cot-1* transcription is photoregulated. Using co-immunoprecipitation with antiCOT1 antibodies, Gorovits et al. (1999) have suggested the feasibility of a physical interaction between COT1 kinase and the catalytic subunit of type 2B phosphatase (calcineurin). Interestingly, a reduction in calcineurin activity (via antisense expression of the *cna-1* gene) also results in reduced hyphal elongation rate accompanied by hyperbranching (Prokisch et al. 1997). It is not surprising that different kinases/phosphatases are involved in hyphal elongation/branching, as they are involved in almost every cellular process. If and how the various components of different signal transduction pathways interact in filamentous fungi is far from being fully understood. The regulation of hyphal elongation/branching is probably complex, and involves a variety of signal transduction pathways. In addition to these pathways, it is conceivable that master regulators of gene expression involved in hyphal growth are involved. One such example may be the B genes in *U. maydis* (Brachmann et al. 2001). Another potential regulator of hyphal branching is the *pahl* homeobox gene of *Podospira anserina* (Arnaise et al. 2001).

Overall, even with the abundance of colonial mutants isolated from a variety of filamentous fungi, the understanding of the genetic basis of these morphological abnormalities remains scarce (Turner and Harris 1999). Nonetheless, the availability of so many mutants may well prove an asset for expansion of the current attempts to functionally analyze genes involved in hyphal elongation and branching. Elucidation of this process is important not only for satisfying the basic need of understanding such a fundamental process, but also as a starting point for rationally intervening in this process. Curbing the proliferation of pathogens, or enhancing growth and morphogenesis in industrial strains, would both benefit once a clearer mechanistic picture of these events is obtained. Much hope has been put on the expected increase in secretion from hyperbranching mutants. The components involved in secretion in filamentous fungi are highly similar to those found in yeasts and higher eukaryotes. However, in contrast to other organisms, the hyphal cell with its polar apical extension creates a unique morphological arena for the process of secretion. Much focus has been put on understanding secretion at the apical zone of growing hyphae (Conesa et al. 2001). However, even though a link was proposed between growth and secretion at hyphal tips in filamentous fungi (Wessels 1993), the increase in hyphal tips per unit of biomass does not necessarily result in a parallel increase in secretion of extracellular enzymes (Conesa et al. 2001; Trinci et al. 1999).

7 STUDYING THE BIOLOGY OF THE HYPHAL CELL

7.1 Biochemical Genetics

A filamentous fungus was used to open the door to biochemical genetics (Beadle and Tatum 1941). Since that bridge between genes and their biochemical function was formed, the biochemical genetics approach has become part and parcel of almost every

analytical venture in biology. Thus, understanding biochemical pathways and the genetic basis of the structural and regulatory elements from which they are comprised is still a common and efficient strategy to advance our understanding of any cellular process. The haploid nature of many fungi (during most of their life cycle), the availability of mutants (or the means to produce them) and the development of methodology by which almost any fungal species can be made amenable to DNA-mediated manipulations provide a likely assurance that biochemical genetics approach, along with others, will continue to be in the mainstream of fungal biology research. The availability of complete fungal genomes has become a reality (see later) and the high-throughput technology encompassing the genomics and postgenomics era are certain to contribute immensely to the future advances in understanding fungal biology. Nonetheless, the power of classical genetics linked to biochemistry has not diminished and will most likely continue to serve as a solid foundation for resolving many fundamental questions in fungal biology.

7.2 Cell Imaging

Macroscopic and microscopic observation has always been a key element in the analysis of the fungal cell. Thus, as early as the third quarter of the 17th century Sir Robert Hooke, utilizing his optical magnification apparatus described the fungal cell (amongst those of other organisms he observed). Microscopy has accompanied the mycologist ever since and still provides an important means in studying fungal biology. The availability of reporter systems along with molecular transformation techniques and recent advances (as well as traditional technologies) in microscopy (light, fluorescent, confocal, and electron) allows the detailed probing of events at the cellular level. Measuring the relative intracellular ion concentrations in specific areas of hyphae (and other fungal structures) is possible by X-ray microanalysis procedures (Connolly et al. 1999). Immunocytochemical techniques (utilizing fluorescent or gold particle-labeled antibodies) are accessible and have been integrated into a growing number of study systems for localization studies, colorimetric visualization, and measurement of reporter gene expression in fixed or live tissues (e.g., β -glucuronidase or green fluorescence protein systems). Perhaps the most exciting current developments are the improvements in live fungal cell imaging. The use of classic dyes that are sensitive to changes within the living cell (e.g., pH, specific ions, reactive oxygen species, etc.) along with dyes that are membrane selective (e.g., FM4-64) and in combination with fluorescent and confocal microscopy will certainly open new avenues of research that will shed light on the cellular biology of the hyphal cell (Fischer-Parton et al. 2000; Heath 2000).

7.3 High-Throughput Analyses (The “X-omics” Age)

The first fungal genome to be sequenced in its entirety was that of *S. cerevisiae* in 1997. Since then, several fungal genomes have been sequenced (some more than once) by private ventures. Recently, the first complete sequence of a filamentous fungus, *N. crassa*, has been made available to the public (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>). The *Aspergillus nidulans* genome is also available, with restrictions (<http://www.fgsc.net/aspergenome.htm>). Others are likely

to follow soon, as public efforts for sequencing the genomes of several additional filamentous fungi are under way. These include *Cryptococcus neoformans* (<http://www-sequence.stanford.edu/group/C.neoformans/>), *M. grisea* (<http://www.riceblast.org/>), *A. fumigatus* (http://www.sanger.ac.uk/Projects/A_fumigatus/), *Candida albicans* (<http://sequence-www.stanford.edu/group/candida/index.html>), *Phanerochaete chrysosporium* (<http://www.jgi.doe.gov/programs/whiterot.htm>), and others. The sequencing of entire genomes will provide the required information for enhancing the ability to perform highthroughput analyses of gene structure and function (Sweigard and Ebbole 2001). The age of structural and functional genomics, transcriptomics, proteomics, metabolomics, and additional categories and subcategories has begun. The prospects for utilizing the new data and technological tools for understanding the molecular basis of cellular events in the hyphal cell are enormous (see Chapters 12 and 17). It will now be easier to combine reductionist approaches (e.g., analysis of single gene/protein function) with some of the more holistic approaches involving the concurrent study of several components affecting a specific trait.

8 CONCLUSIONS

Most fungi possess most of the common cellular features shared by other eukaryotes. However, the distinct shape and some of the metabolic features of the hyphal cell require that the activities of the fundamental, conserved, cellular machinery be amended with the unique structural and functional elements required to maintain the hyphal cell and support its growth. Thus, identifying the structural components (common and unique), determining their function and elucidating the regulatory mechanisms responsible for the concerted interactions between subcellular components on the one hand and the intact organism and its environment, on the other, are instrumental for our efforts to intervene with the activities of these fascinating and diverse organisms.

The advances made in dissection of individual components of the hyphal cell (e.g., cell wall, organelles, and septa) are significant. The combination of biochemistry, genetics (classical and molecular), and microscopy have yielded impressive results in the process of identifying many of the components involved, determining their significance in hyphal growth, and associating them with the topography of the hyphal cell. However, we are still challenged with a need to understand many of the mechanistic aspects of hyphal cell biology. At times, the difficulties involved in the analysis of these aspects are shared by those studying other eukaryotic cells (e.g., function of membrane-associated proteins, organization and function of cytoskeletal elements, and regulation of the cell cycle, etc.) and conceptual or technological breakthroughs in the field of cell biology are likely to be of significant value to fungal biologists. However, understanding the unique aspects linked to fungal morphology and growth, and intervening in these processes, depend on advances in mycological research in general and the in-depth analysis of fungal species of interest.

The outcome of the current effort invested by members of the fungal biology community in advancing the fields of fungal genomics and proteomics may well prove to be an invaluable stepping-stone in better understanding the hyphal cell. Comparative genomics (among filamentous fungi and between fungi and other organisms) will assist in identifying “missing links” in conserved pathways, while at the same time point to unique proteins in fungal species. Nonetheless, at the end of the day, classical conceptual approaches used in cell biology research during the past decades will be required in order to answer the fundamental questions relating to growth and development of the hyphal cell.

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2

Protoplast Isolation, Regeneration, and Fusion in Filamentous Fungi

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1 INTRODUCTION

In fungi, protoplasts can be defined as spherical cells whose chitinous cell walls have been removed by appropriate digestive enzymes (Bachmann and Bonner 1959). Based on the considerable literature that has accumulated over the past 40 years, protoplast studies in fungi can be divided in two main sections: (a) production of stable, complete protoplasts, and (b) use of protoplasts, for example, to obtain intact chromosomes, as partners in fusion studies, and as recipients of exogenously introduced DNA (a process termed as transformation). The successful applications of such protoplast manipulations has led to a better understanding of many biochemical, physiological, and genetic phenomena in fungi.

The technique of protoplast fusion in particular, continues to contribute positively to our understanding of fungal biology in topics ranging from secondary metabolism to vegetative incompatibility (discussed in Section 8). However, to the best of our knowledge, the topic of protoplast fusion has not been reviewed in recent times. Another technique involving protoplasts, that of DNA transformation, has also been very useful in fungal research, particularly in those fungi where sexual crosses are not feasible (imperfect fungi). For example, DNA-mediated transformations were instrumental in deciphering the molecular regulation of aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus* (Hornig et al. 1990; Payne and Woloshuk 1989; Trail et al. 1995; Woloshuk et al. 1989; Yu et al. 1995). Since the topic of fungal transformation has been reviewed in many current and comprehensive reviews (Hamer et al. 2001; Punt and van den Hondel 1992; Ruiz-Diez 2002; Mullins and Kang 2001; Shishido 1994), this chapter will not cover this topic, but will focus on protoplast fusion. The contents of this chapter include first a review of the salient features of protoplast isolation, followed by a discussion of fusion studies specifically in filamentous fungi in the context of fungal genetics. Where applicable, both the advantages and the limitations of the protoplast fusion technique will be highlighted. This article is targeted for those relatively “new” to the world of protoplasts, but should also serve as a source of literature to the more established fungal researchers.

2 HISTORICAL PERSPECTIVE

The earliest studies on the formation and development of fungal protoplasts were reported in *Neurospora crassa* (Bachman and Bonner 1959; Emerson and Emerson 1958) and *Fusarium culmorum* (Rodriguez-Aguirre et al. 1964). However, successful application of these protoplasts in biochemical or genetic studies would depend on the optimization of several parameters influencing their yield and stability including the effectiveness of the digestive enzymes and the ability of the protoplasts to regenerate (discussed later).

Initially, protoplasts were used mainly to isolate intact organelles and to prepare cell lysates for biochemical analyses [reviewed by Villanueva and Garcia (1971)]. Soon after, the first reports on spontaneous protoplast fusion appeared for *Geotrichum candidum* (Ferenczy et al. 1974) and *A. nidulans* (Ferenczy et al. 1975). Almost simultaneously, Anne and Peberdy (1975) reported their results on the induced protoplast fusion in *Penicillium chrysogenum* using polyethylene glycol (PEG) MW 6000 as a stimulant. Around the same time, the first report of DNA mediated transformation for a filamentous fungus appeared in *N. crassa* (Case et al. 1979). Since then, protoplasts have continued to serve as valuable tools in a wide range of basic and applied research projects.

3 PROTOPLAST ISOLATION

The basic approach for preparation of protoplasts from filamentous fungi has changed little over the years. Essentially, it involves the treatment of the hyphae with enzymes that will digest the cell wall contents. Since, the spherical protoplasts are osmotically fragile, organic or inorganic stabilizers must be added to the medium to help maintain their morphology. The major factors influencing the process of protoplast isolation are summarized below.

3.1 The Fungal Cell Wall and Digestive Enzymes

The fungal cell wall is a hardy structure that gives the cell its shape, protects it under the normally hypotonic environments, plays an important role in cell-cell interactions due to the presence of surface antigens, and acts as a site of extracellular enzymes with hydrolytic or metabolic activities (Farkas 1985).

In general, a fungal wall is composed of 70% or more polysaccharides (homo and hetero) and variable amounts of proteins, lipids, and melanins (Bainbridge et al. 1979; Bartnicki-garcia 1968; Rosenberger 1976). The polysaccharides are further classified into skeletal polysaccharides composed mainly of chitin and R-glucan, and wall-matrix polysaccharides composed mainly of protein-polysaccharides (Frakas 1985). Ultrastructure studies on *Pythium acanthicum* and *N. crassa* cell walls have shown that the skeletal polysaccharides are embedded in an amorphous matrix of protein-polysaccharide complexes such that the outer surface of the wall is usually smooth while the microfibrils of the skeletal polysaccharides are visible on the inner layer (Burnett

1979; Sietsma et al. 1975). The general cell wall composition, along with the functions of its major components are shown in Figure 1.

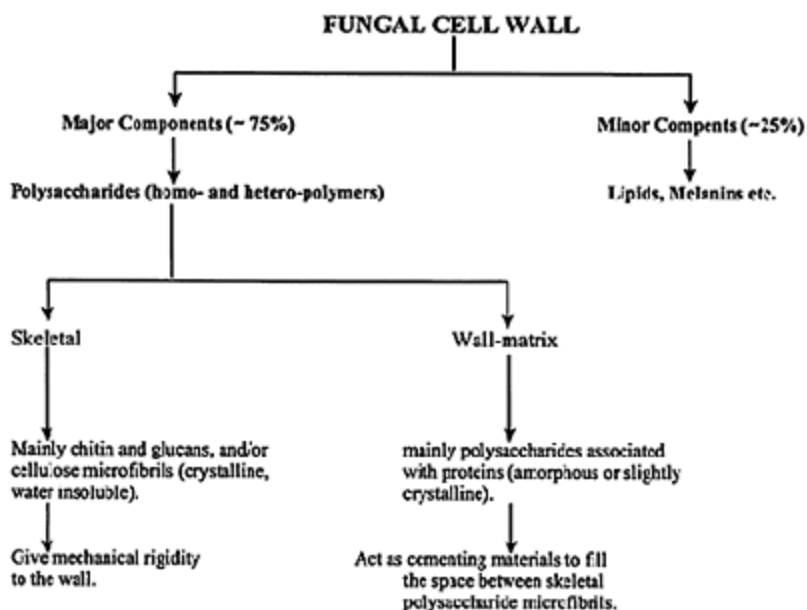


Figure 1 Schematic representation of the fungal cell wall [summarized from Farkas (1985)].

Since, protoplasts are able to form cell walls *de novo*, an understanding of the sequence of events that leads to this phenomenon is essential. Although advances in this area have been made (see Section 4), details of the assembly of the wall components and the genetic factors that are involved in the regulation of wall synthesis remain poorly understood.

With respect to cell wall lysis, for several years, the snail *Helix pomatia* digestive juice preparation was used to obtain protoplasts in *N. crassa* (Emerson and Emerson 1958), *A. nidulans* (Ferenczy et al. 1975) and *G. candidum* (Ferenczy et al. 1974). Mycolytic enzymes obtained from bacterial [*Arthrobacter*, *Bacillus*, and *Streptomyces* (Doi et al. 1971; Kitamura et al. 1974)] and fungal [*Trichoderma* (Benitez et al. 1975a,b; de Vries and Wessels 1973) and *Aspergillus* (Isaac and Gokhle 1982)] sources have also been used to produce fungal protoplasts. Regardless of the sources, these enzyme preparations have polysaccharases as a common activity, with differing amounts of individual subtypes like glucanase I and II, cellulase, mannanase, and chitinase [reviewed by Peberdy (1985)]. Often, the enzymes are not pure and are present as a mixture of polysaccharase, protease, and lipase activities (Scott and Schekman 1980; Villanueva and Garcia 1971). Although many laboratories preferred making their own lytic preparations, these preparations were laborious, varied in their lytic activities and gave variable yields of protoplasts (Villanueva and Garcia 1971). In 1981, findings of a comprehensive study that involved

the comparison of activities of several commercial polysaccharases (Novozym 234, Cereflo 200L, cellulase, helicase, chitinase, D-glucuronidase, and D-glucanase) for their ability to liberate fungal protoplasts was reported (Hamlyn et al. 1981). It was found that while enzymes individually, or in combination, varied in their ability to produce protoplasts depending on the type of fungus, in general, Novozym 234 (Novo), an enzyme mixture of chitinase, cellulase, and protease activities from *Trichoderma* species, was the most effective and produced sufficient numbers of protoplasts in 2–3 h (Hamlyn et al. 1981). Novozym 234 is also available from other commercial sources like Sigma and continues to be widely used for fungal protoplasting [for example in *P. chrysogenum* (Hong and Robbers 1985), *A. flavus* (Keller et al. 1992), *A. nidulans* (Bird and Bradshaw 1997), and *Lentinus lepideus* (Kim et al. 2000a)].

3.2 Age of the Mycelium and Pretreatment of the Organism

Although fungal protoplasts can be obtained from ungerminated fungal spores (Bos and Slakhorst 1981; Cheng and Belanger 2000; Moore and Peberdy 1976), most laboratories use mycelia as the starting material mainly because the spore wall is highly resistant to lysis (Peberdy 1979a). With respect to hyphal age, results vary from fungus to fungus but in general, 18–48 h old mycelia, corresponding to the early to mid-exponential growth phase result in the best protoplast yield (Peberdy 1979b). Occasionally, stationary-phase mycelia also yield protoplasts (as a second peak after the log-phase protoplasting) as was the case in *P. chrysogenum* (Anne et al. 1974), which suggested the possible involvement of an endogenous lytic activity. More recently, it has been reported that older hyphae not only resulted in release of fewer protoplasts, but the resulting protoplasts tended to be fragile and contained large vacuoles (Harman and Stasz 1991). Based on the early observations on protoplast formation in *Fusarium* species (Lopez-Belmonte et al. 1966), the hyphal walls swell at specific points before release of the protoplasts. Presumably these points are the initial sites of the enzyme attack and are most accessible when the hyphae are actively growing. In any case, the log-phase cultures also require lesser amounts of the lytic enzyme and shorter incubation times to obtain protoplasts so most studies report the use of 18–48 h old mycelia.

Certain media additives have been helpful in that, when the fungi are preincubated in these media, higher numbers of released protoplasts are obtained. These include reducing agents (e.g., dithiothreitol, 2-mercaptoethanol, and sodium thioglycolate), detergents (e.g., triton X-100 and deoxycholate), and chelating compounds (e.g., EDTA), [reviewed by Davis (1985)]. The thiol compounds may cleave the disulfide linkages in the cell wall (Peterson et al. 1976), thus facilitating the activities of the digestive enzymes, while the detergents may be involved in the removal of the lipid components of the cell wall (Davis 1985).

3.3 Osmotic Stabilizers, Temperature, and PH

Osmotic stabilizers are needed to maintain the protoplast morphology, which is otherwise maintained by only a cell membrane. In the past two and a half decades, a wide range of inorganic salts (KCl , CaCl_2 , $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , MgSO_4 , NaCl) and organic compounds (sugars and sugar alcohols) have been used in this capacity [reviewed by Davis (1985) and Villanueva and Garcia (1971)]. The experimental data on a range of systems has suggested that in general, inorganic salts (in 0.6 M range) work better for filamentous fungi while sugar and sugar alcohols are more suitable for yeasts (Peberdy 1979b). Further, depending on the enzyme preparation, some stabilizers may actually assist in the wall lysis. For example, a combination of CaCl_2 and KCl greatly enhanced the protoplast release from *A. niger* and *A. fumigatus* when chitinase was included as the lytic enzyme, but if CaCl_2 alone was used as a stabilizer, the protoplast yield actually decreased (Thomas 1981; Thomas and Davis 1980).

Other variables like pH and temperature also influence protoplast production based in part, on their influences on the activities of the lytic enzymes. As with other influencing factors, optimum pH and temperatures have to be determined experimentally for each fungus, but it appears that a range of 20–40°C work well with most filamentous fungi (Davis et al. 1977; Petrini 2001; Thomas 1981). Temperatures higher than 40°C may inhibit the activity of the lytic enzymes while lower temperatures may affect the protoplast membrane stability (Kovac and Subik 1970). With regards to the pH of the incubation medium, in general, acidic environments (pH below 4) tend to decrease the lytic activities of the digestive enzymes although optimum pH varies from one organism to another. Additional details on these factors and their possible side-effects on protoplast metabolism have been discussed by Davis (1985) and Peberdy (1979b).

4 PROTOPLAST REGENERATION

This process refers to the ability of the protoplasts to revert back to the normal filamentous form by first synthesizing the cell wall. While producing protoplasts is a crucial first step, the success of any experiment involving protoplasts largely depends on whether they can regenerate through cytokinesis and form visible colonial growth on osmotically balanced solid media. Over the years, most studies on the dynamics of cell wall regeneration have been conducted in yeast, in part because of the ability of some to undergo the yeast/ mycelial (Y/M) shift, making comparative studies possible. Such studies (discussed later) have aimed at understanding the specific events of wall polymer synthesis in regenerating protoplasts and the regulatory factors that control their sequence. Data from some early studies indicated that the first structure to be assembled on the surface of the regenerating protoplasts is the microfibrillar network of chitin and glucan synthesized probably by enzymes associated with the plasma membrane (Van der Valk and Wessels 1976; 1977), and this event is usually followed by the assembly of the amorphous matrix (Necas 1971; Peberdy 1979a). In another study on *Candida albicans*, the zymogenic stage (when protoplasts are getting formed) was associated with incorporation of higher amounts of chitin synthetase in the cell membrane (Elorza et al.

1983). The protein synthesis was initiated within the first 40min and after a few hours, chitin was the most abundant polymer in the aberrant wall of the regenerating protoplasts (Elorza et al. 1983). More recently, quantitative measurements of the fluorescence emitted by individual *C. albicans* protoplasts indicated that chitin and mannoproteins are among the first components to be laid down on the regenerating protoplasts followed by the glucans (Rico et al. 1997). In a related study (Nishiyama et al. 1995), similar results were obtained based on electron microscopic observations using a fluorescent dye (Fungiflora Y) that specifically binds to beta-linked polysaccharides. In this study, fluorescence was detected on multiple sites of the fresh protoplasts within 15 min of incubation; by 1 h the entire structure was fluorescing, and after 24 h of incubation, the reverted cells were capable of proliferating (Nishiyama et al. 1995). On a molecular level, in the fission yeast, experiments involving two novel protein kinase C homologs *pck1+* and *pck2+* have shown that the *pck2+* gene is essential for protoplast regeneration, but *pck1* is not (Kobori et al. 1994). In another recent study, mRNA expression levels of 38 stress response and cell wall protein genes from *Saccharomyces cerevisiae* were investigated during spheroplast regeneration by Braley and Chaffin (1999); a spheroplast being defined as a cell which may have portions of the cell wall still attached (Davis 1985). It was observed that although the expression of some of these genes did increase between 5- and 10-fold during attempted regeneration, their transcription was not sufficient to initiate the replacement of the cell wall (Braley and Chaffin 1999). From these recent examples, it is evident that the genetic basis of protoplast regeneration is a series of complex interactions of several factors and a better understanding of this process will have to await further research.

5 PROTOPLAST FUSION

The process by which the protoplast membranes fuse (plasmogamy) to have a common, multinucleate cytoplasm is called protoplast fusion. As mentioned earlier, protoplast fusion can occur naturally but the frequency is low and so is the yield of the fusants. With respect to induced fusion, perhaps the most important development was the demonstration by Anne and Peberdy (1976) that PEG, a known fusagen of plant protoplasts (Kao and Michaylink 1974) can successfully be adapted for that purpose in fungi as well. The PEG is thought to be involved in forming the hydrogen bonds (by itself or in the presence of Ca^{+2} ions) between the cell membranes (Peberdy 1979b). To determine the optimal conditions for induced protoplast fusion, a comprehensive study was undertaken by Anne and Peberdy (1975). Using complementing auxotrophs of *P. chrysogenum*, they reported that best results were obtained when the PEG (MW 6000) solution was at least 20% (w/v) and was supplemented with Ca^{+2} (in form of 10 mM CaCl_2) under alkaline conditions. The significance of this report is reflected in the fact that even today, almost every laboratory utilizes these conditions with occasional modifications for successful protoplast fusions (Brauer and Robbers 1987; Couteaudier et al. 1996; Martinkova et al. 1990; Ogawa et al. 2000).

It should be noted that protoplast fusion can also be accomplished using electric currents in the form of DC pulses (Fukuda et al. 1995; Zimmerman 1982) but this

technique has not been very widely used.

In experiments involving PEG, the fusion events are random and if two parents are used, fusants can be formed which are like (partner protoplasts are from the same parent) or mixed [partner protoplasts are from two different parents (Peberdy 1987)]. Further, since the numbers and the types of nuclei vary in the protoplast aggregates, the proportion of the two parental types of nuclei is thought to most influence the stability and development of the first generation of fusion products, the heterokaryons (Peberdy 1987). Once the fusion products are obtained, they are recovered and analyzed by using several selective techniques as discussed in the later sections.

6 THE PARASEXUAL CYCLE AND PROTOPLAST FUSION CONNECTION

Parasexual cycle is one of the ways by which fungi can exchange genetic information. In fact, in the imperfect fungi which lack a sexual phase, it is the only known means for genetic recombination.

The parasexual cycle was first discovered in *A. nidulans* (Roper 1952) and shortly after, was shown to occur in *P. chrysogenum* (Pontecorvo and Sermonti 1953) and the pathogenic fungus *Fusarium oxysporium* (Buxton 1956). It consists of three basic stages: heterokaryosis, diploidization, and recombination through mitotic crossing over or haploidization. Heterokaryosis is the anastomosis or fusion of the hyphae. The schematic representation of formation of protoplast fusants and their analysis is shown in Figure 2. In a laboratory setting, this process can be observed using two compatible haploid strains differing in one or more characteristics that serve as genetic markers. So, for example, if the marker is that of a nutritional requirement (different one in each parent), neither parent can grow on a minimal medium (MM). However, if a heterokaryon forms due to hyphal fusion, it can grow on the MM. This is because it contains the nuclei of both parents which complement each other's defect. In this instance, the MM serves as the selective medium. Often, the auxotrophic crossing parents also carry different spore color markers. Although not essential, spore colors are useful markers, because if the two parents produce spores of contrasting colors, the heterokaryon is seen as a mixture of both spore colors (Bennett 1979). Similarly, among the auxotrophs some are easier to isolate and hence more often used. For example, mutants defective in the genes of nitrate assimilation pathway like the nitrate reductase (*niaD*) mutants can be easily isolated, based simply on their resistance to chlorate without the need of any mutagen (Cove 1976). Auxotrophic nutritional markers, however, can be "leaky" (a small amount of the nutrient is still made by the fungus under certain growth conditions; Bennett 1979; Hsieh et al. 1976) and sometimes, can negatively influence the growth or secondary metabolite production of the fungus (Ferenczy et al. 1987; Kikuchi 1980). In response, other genetic markers have been used, including those for resistance to antibiotics and fungicides (Tudzynski et al. 1982) which aid in the identification of the heterokaryon.

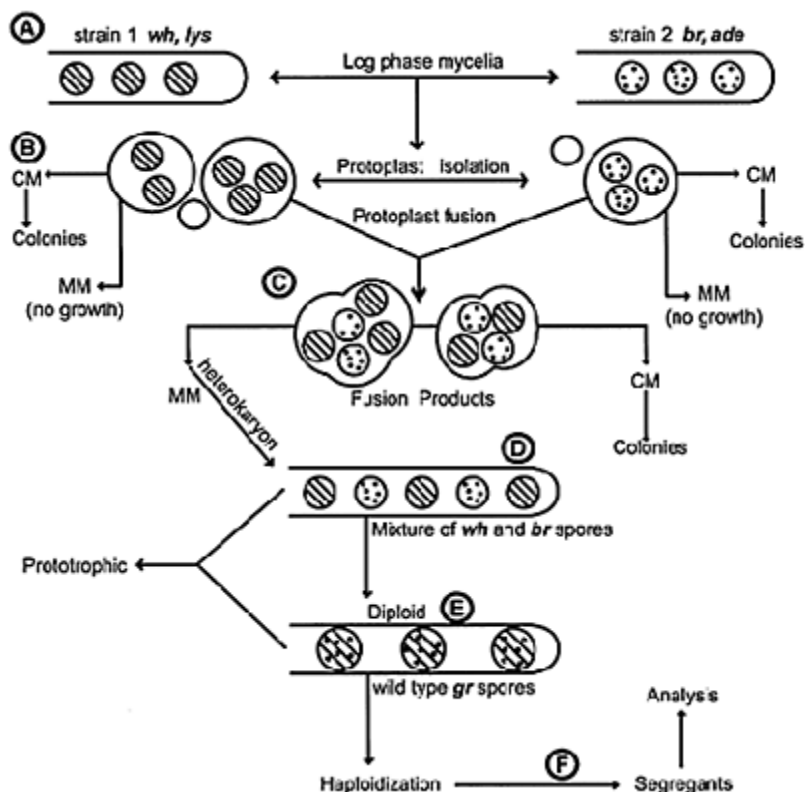


Figure 2 Schematic representation of fungal protoplast fusion and general scheme of fusion progeny analysis. A: Strain 1 is auxotrophic for lysine (*lys*) and has white (*wh*) spores. Strain 2 is auxotrophic for adenine (*ade*) and has brown (*br*) spores. Both strains are derivatives of wild type (WT) prototrophic cultures that have green (*gr*) spores. B: Heterogeneous protoplasts of varying sizes are obtained from logphase mycelia of strains 1 and 2. Neither type of unfused protoplasts grow on agarbased MM because of their respective auxotrophic requirements, but growth is obtained on agarbased nutritionally CM. C: Protoplast fusion products are formed as aggregates containing varying numbers of parental nuclei. Growth occurs when these fusants are plated on CM and MM. Fusion frequency is defined as a ratio of number of colonies on MM/number of colonies on CM. D: A heterokaryon carrying the two types of parental nuclei. On MM, the heterokaryon will grow as a prototrophic mixture of *wh* and *br* spores, with the two types of parental nuclei complementing each others' nutritional deficiencies. E: Nuclear fusion in the heterokaryon gives rise to prototrophic diploids that have the wild type (*gr*) spore color. F: Haploidization of the diploids gives rise to segregants in form of sectors, which can be purified and

further analyzed. [Based on Anne and Peberdy (1976), Bennett (1979), Peberdy (1979b), and Scott et al. (1979). Additional details are given in the text].

In the second step of the parasexual cycle, rare nuclear fusion in the heterokaryon produces the heterozygous diploid which is identified as a nonsectoring stable prototroph on MM with the wild type mycelial and spore color. During vegetative multiplication of the nuclei in the diploid, mitotic crossing-over events can lead to diploid segregants, or nondisjunction phenomena can give rise to haploid recombinant segregants. In this way, even in the absence of a sexual cycle, genetic exchange can occur (Papa 1978; Roper 1966; Scott et al. 1979).

The events that occur in most protoplast fusions parallel those in the parasexual cycle with one advantage. Fusion of the protoplasts can bypass the incompatibility barriers that exist at the hyphal level which, in the parasexual cycle prevent the formation of a heterokaryon (Dales and Croft 1977, and discussed later). Like in parasexual analysis, as long as there are appropriate genetic markers and tests in place to distinguish the heterokaryons vs. the diploids vs. the haploid segregants, protoplast fusion can serve as a powerful tool for a wide range of genetic studies. For instance, once the products of the parasexual cycle (diploids, haploids, etc.) are obtained, they can be analyzed for linkage studies, secondary metabolite production, virulence, and other characteristics under study (Bennett 1985; and as discussed in Section 8).

7 MOLECULAR MARKERS

For the past several years, molecular markers such as isozyme analysis, restriction fragment length polymorphism (RFLP), and rapid amplified polymorphic DNA have been employed to more accurately identify the recombinant progenies obtained from the postfusion events. Isoenzyme analysis is based on the ability of an enzyme to exist in various forms, which (depending on the difference in their electrophoretic mobilities) can be visualized as distinguishable bands when separated according to their charge distribution at a specific pH (Anne 1977; Stasz et al. 1988). Thus, once isoenzyme profiles for a particular strain are established, they can serve as efficient molecular markers to demonstrate the formation of a heterokaryon and/or to identify recombinant haploids (Anne 1977; Stasz et al. 1988).

Restriction fragment length polymorphism is based on the fact that relatively common differences in the DNA sequences exist in all organisms including filamentous fungi. Such DNA polymorphisms can be visualized by cutting the genomic DNA with restriction enzymes, separating the resulting DNA fragments by agarose gel electrophoresis and then performing Southern blot using the cloned gene or gene-fragment of interest as a probe (Hartl and Jones 2002). Using this technique, any process (e.g., mutation, recombination, retrotransposition, etc.) which yields different-sized DNA fragments can be visualized as a novel RFLP pattern. Among its many applications, this technique can be used to analyze DNA similarities of the samples. For example, recombinant DNA clones carrying sequences from *A. nidulans* and *N. crassa* were used

to identify diagnostic RFLPs for members of the *A. flavus* groups (*A. flavus*, *A. parasiticus*, and *A. nomius*), and the fungi were resolved in three different categories based on the RFLP profiles (Moody and Tyler 1990). More recently, RFLP markers were isolated and tested for *A. fumigatus*, and the RFLP analysis revealed clear polymorphism in these otherwise closely related fungi, thus establishing their status as two distinct species (Parenicova et al. 2001). In the context of protoplast fusions, RFLPs can determine whether the genetic makeup of the fusion progeny is parental (RFLP pattern identical to one of the parents) or recombinant (RFLP pattern different from either parents). Specific applications are discussed in the following section.

Random amplified polymorphic DNA (RAPD) is a relatively new assay (Williams et al. 1990) which also detects DNA polymorphisms. However, instead of cloning a gene and conducting laborious Southern analysis for RFLP, it is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequence. The polymorphisms of the primer DNA fragments (RAPD profiles) are then visualized simply by gel electrophoresis and ethidium bromide staining of the DNA (Durand et al. 1993; Williams et al. 1990).

8 PROTOPLAST FUSION APPLICATIONS IN BASIC AND APPLIED GENETICS

Often, a genetically well-understood organism is also the more commonly used in advanced studies. In the filamentous fungi, this has been true for *N. crassa* and *A. nidulans*; and the latter in particular, has been extensively employed for protoplast studies, some of which are discussed below.

8.1 Vegetative Incompatibility and Related Phenomena

Also referred to as somatic or heterokaryon incompatibility, this phenomenon can be defined as the inability of fungal mycelia to fuse, to form heterokaryons, and to exchange genetic information. Its occurrence is well documented in several *Aspergillus* species and is thought to be a mechanism for isolation within a species (Croft 1987). As a case study, in *A. nidulans*, wild isolates were classified into heterokaryon compatibility (h-c) groups and the compatibility differences shown to be under nuclear control (now called the *het* genes) as early as 1963 (Grindle 1963). Based on this classification, members within any one h-c group can readily form heterokaryons with each other, while members of different h-c groups do not form heterokaryons. Since, the *het* genes do not interfere with sexual reproduction, early studies based on their segregation patterns in the progeny of sexual crosses between members of differing h-c groups resulted in the demonstration of their heterogenic nature (Esser and Blach 1973). However, such work was rather laborious, and the problem was overcome by the advent of protoplast fusion. By fusing protoplasts of vegetatively incompatible strains, heterokaryons were obtained which were then subjected to standard parasexual analysis in order to understand vegetative incompatibility (Dales and Croft 1977). Using the “master Glasgow strains” [which have eight scorable markers, one on each linkage group (McCully and Forbes 1965)] and

members in other h-c groups like h-c B, h-c A, h-c R, and h-c Q as crossing parents, the following general scheme was employed by Dales and Croft (1983; 1990); Dales et al. (1983):

(a) Protoplasts of the two parental strains (having differing spore color and auxotrophic markers) were prepared, induced to fuse, and plated on osmotically stabilized MM. Appropriate controls were also set up to ensure that the results seen were indeed due to the fusion of the protoplasts and not due to back mutation or other phenomena. (b) Detailed observations were made regarding the phenotype of the presumptive heterokaryons especially with respect to growth patterns, amount of sporulation, and the color of the spores. Heterokaryon formation was confirmed by subculturing colonies on complete media (CM) and recovering parental phenotypes. (c) Presumptive diploids were isolated based on their wild type spore color (or sometimes a shade lighter than wild type spore color), the spore diameter (whether or not it was significantly larger than that of haploid conidia), and mycelial color. (d) Representative diploids were analyzed by haploidization analysis using the inducing agent benlate (Hastie 1970). (e) Analysis was done to determine the linkage group on which the *het* difference(s) resided. For example, pairs of haploid progeny identical for all nuclear markers except on the linkage group under question (say group 1) were subjected to heterokaryon compatibility tests. If the results were positive for heterokaryon formation for all (or almost all pairs), it was deduced that the original crossing parents did not have any *het* difference(s) on linkage group 1.

Using this approach, extensive data was obtained from all five compatibility test groups (h-c GL, h-c A, h-c B, h-c R, and h-c Q). A compilation and comparison of these results indicated that a minimum of eight and a maximum of 18 loci spread over five linkage groups were necessary to explain the compatibility relationships among the five h-c groups (Anwar et al. 1993).

Based on this work and related studies in other fungi like *Gibberella* (Adams et al. 1987) and *Cochliobolus* (Leach and Yoder 1983), it is quite evident that the vegetative incompatibility phenomenon is heterogenic, and having dissimilar alleles at any one of the several loci can make the strains incompatible. Further, based on the degree of such difference(s), "levels" of incompatibility may exist. In general, greater the relatedness between the two fungal partners, greater is the possibility that they are vegetatively compatible. Another generality—although protoplast fusion can force two incompatible strains to form a heterokaryon, frequency of diploid formation is not always high, and recovery of the haploid progeny may be slow. Finally, protoplast fusion cannot solve the postfusion vegetative incompatibility that may be due to other influences such as those of mitochondria and other cytoplasmic elements (Harman and Stasz 1991; Peberdy 1987).

Certain aspects of our work on strain degeneration in filamentous fungi with respect to secondary metabolite production (Kale and Bennett 1992; Kale et al. 1994) using aflatoxin producing *A. parasiticus* (an imperfect fungus) as a model system appear to be related to the phenomenon of heterokaryon incompatibility as follows: A collection of degenerate strains (designated *sec*–) which no longer produce any aflatoxins or its intermediates have been generated from the toxigenic parental strains simply by transfer of mycelial macerates (Kale et al. 1994). The *sec*– strains were found to be pleiotropic in nature in that, apart from loss of secondary metabolite production, they also displayed

altered morphology and reduced sporulation compared to their *sec*⁺ parents (Kale et al. 1994). Since then, we have subjected the isogenic *sec*⁺/*sec*[−] pairs to exhaustive stability testing, biochemical, and molecular studies and have demonstrated that the *sec*[−] forms are highly stable (none have reverted back to the *sec*⁺ form), that they have intact aflatoxin pathway structural genes, and that there is no (or very little) expression of any of these genes including the *afl* R regulatory gene (Chang et al. 1993; Payne et al. 1993) in these *sec*[−] variants (Kale et al. 1996). Along with the ongoing molecular work, we have also conducted comprehensive parasexual analysis of the *sec*[−] strains (Kale et al. unpublished study). Results of parasexual crosses show that when *A. parasiticus sec*⁺ and *sec*[−] strains of contrasting spore color and auxotrophic markers are used in crossing, *sec*⁺/*sec*⁺ crosses yield heterokaryons and diploids with ease, *sec*⁺/*sec*[−] crosses generate heterokaryons and diploids with an intermediate level of success, and *sec*[−]/*sec*[−] crosses yield no viable heterokaryons and consequently no diploids even after 10 attempts and compensating for reduced spore counts of the *sec*[−] forms. The cytoplasmic inheritance test (Arlett et al. 1962) on the *sec*⁺/*sec*[−] heterokaryons suggests that the *sec*[−] phenotype is not under cytoplasmic control. Combined, these data suggest that in the process of loss of aflatoxin production, the *sec*[−] forms may have developed some form of vegetative incompatibility, even though they all belong to the same species. To explore this hypothesis, we are in the process of conducting protoplast fusions between representative *sec*[−]/*sec*[−] strains. If protoplast fusions in these crosses result in formation of viable heterokaryons and/or diploids, clearly, the barrier in the parasexual cycle experiments exists at the anastomosis (hyphal) level, bypassed by protoplast fusions.

More recent work on the cloning and analysis of the mating type locus in *N. crassa* (alleles A and a, Glass et al. 1988; Metzenberg and Glass 1990) and that in *Podospora anserina* (alleles S and s, Turcq et al. 1990) has offered a better insight into the molecular basis of vegetative incompatibility. In *N. crassa* for example, the A/a mating locus is involved in both vegetative incompatibility and in sexual reproduction (while in *A. nidulans*, the *het* genes do not influence sexual reproduction) and the sequence required for the expression of incompatibility has been localized to a single open reading frame with the putative polypeptide having similarity to the MAT alpha 1 polypeptide of *S. cerevisiae* (Glass et al. 1990). More recent work has further identified a cluster of genes surrounding the mating locus that may be involved in the heterokaryon incompatibility (Randall and Metzenberg 1998). Additional research is certain to result in characterization of these genes and will perhaps assist in discovering their corresponding homologs in other fungal systems.

8.2 Intra or Interspecies Protoplast Fusion to Study Phenomena Other than Vegetative Compatibility

In filamentous fungi, protoplast fusion experiments at the interspecies level (Anne et al. 1976) were reported around the same time as those at the intraspecies level (Anne and Peberdy 1976). Both shared similar techniques for protoplast fusions and for data analysis, using the principles of parasexual cycle. Such studies in representative fungi of academic, economic, or commercial significance are discussed below.

8.2.1 *Penicillium*

Over the span of about 15 years, several auxotrophically marked species of *Penicillium* including *P. chrysogenum*, *P. notatum*, *P. cyaneofulvum*, *P. citrinum*, *P. roqueforti*, and *P. stoloniferum* were employed in interspecies hybridizations using protoplast fusion techniques [Anne 1977; 1982a,b; Anne and Peberdy 1976; Anne et al. 1976; reviewed by Anne and Peberdy (1985)]. Since, *Penicillium* is a well-known producer of β lactam antibiotics, the main goals of these studies were two-fold: (a) to conduct basic genetic analysis, and (b) to possibly create a stable recombinant (hybrid) with improved antibiotic production. Detailed analysis of results revealed that in general, crosses involving related species like *P. chrysogenum* X *P. notatum* (Anne 1977; Anne and Peberdy 1976), and *P. cyaneofulvum* X *P. citrinum* (Anne and Eysen 1978) formed healthy heterokaryons and upon longer incubation, gave rise to prototrophic colonies on MM that segregated under nonselective conditions. Such colonies were presumed to be diploids, but since it was not possible to say with certainty whether or not all the chromosomes from both parents were present in the potential diploid, the term “hybrid” was used (Anne and Peberdy 1985). Alternative terms like “allopolyploids” or “allodiploids” have also been used to describe such fusion products (Kevei and Peberdy 1985). On the other hand, if the crossing parents were from relatively less related species like *P. chrysogenum* X *P. roqueforti* (Anne et al. 1976) or *P. chrysogenum* X *P. stoloniferum* (Anne 1982a), the heterokaryons grew poorly, did not sporulate on MM, showed more aberrant morphology and under nonselective conditions, produced spores of only one of the parents, suggesting imbalanced heterokaryons and incompatibility at the chromosomal level. While it was possible to force the poorly growing heterokaryons to produce prototrophic hybrids, they often showed variable stabilities (Anne 1982a). In fact, natural or induced haploid segregants stable enough to withstand further genetic or biochemical analysis could mostly be obtained only from hybrids of related species (Anne and Peberdy 1985). Analysis showed that in general, as long as one of the parents was an antibiotic producer, the penicillin production in the hybrids and/or the segregants ranged between the two parents, providing evidence for genetic recombination (Anne 1983; Anne and Peberdy 1985). Similar tests for isoenzyme analysis (e.g., catalase) in the hybrids and their segregants showed profiles of either one parent, both parents, or novel bands, the latter indicating genetic recombination (Anne and Peberdy 1981). Based on such analyses, Anne and Peberdy (1985) discussed the potential use of *Penicillium* interspecies protoplast fusions in obtaining novel secondary metabolites, provided the limitations of the screening process and the instabilities of certain fusion products could be overcome. Since the mid-80s, there have been few reports on creation of novel penicillin producing strains. However, one relatively recent study used a slightly different approach—that of overcoming the use of auxotrophic markers (which can have a decreased effect on penicillin production, Ferenczy et al. 1987; Kikuchi 1980) by obtaining nitrate nonutilizing and acetate nonutilizing mutants of high penicillin producing strains of *P. chrysogenum* and fusing their protoplasts (Tahoun 1993). Since, the cross was at the intraspecies level, diploids were readily obtained and induced haploidization allowed recovery of stable segregants, one of which reached a penicillin G

titer of 290–390% over that of the parental strains (Tahoun 1993). The antibiotic production by this novel segregant at a scale-up batch or continuous-level and its long-term stability was not discussed in this study. These are critical issues for any industrial strain, because it has been shown that in the absence of selection pressure, high penicillin producing industrial strains (usually ploidy mutants) segregate to lower producing strains by chromosomal losses (Kunkel et al. 1992). This may be one of the reasons why protoplast fusions have not always been the best choice for industrial strain improvement purposes.

8.2.2 *Aspergillus*

Results obtained from interspecies hybridizations in *Aspergillus* differ to a certain degree from those in *Penicillium* in that, even crosses of closely related species such as *A. nidulans*, *A. rugulosus*, *A. quadrilineatus*, *A. nidulans* var *echinulatus* and *A. violaceus* did not easily yield healthy heterokaryons and allodiploids (Kevei and Peberdy 1984). When protoplasts of taxonomically unrelated species like *A. nidulans* and *A. fumigatus* were crossed, fusion events were rare, the fusion products were highly unstable and when subcultured on CM, conidia from the fusion segregants produced only *A. nidulans* colonies (Ferenczy et al. 1977). Analysis of a small percentage of such prototrophic segregants showed that part of the *A. fumigatus* genome was responsible for overcoming the nutritional auxotrophy (Ferenczy et al. 1977), hence the term “partial aneuploid” was coined (Ferenczy 1981) to describe them. On the basis of this data, it therefore appears that vegetative incompatibility and postfusion incompatibility is more prevalent in *Aspergillus* than in *Penicillium* species. Kevei and Peberdy (1985) suggested that the use of other techniques for genetic transfers (like isolated nuclei and mitochondria) could allow scientists to induce genetic interactions at a wider level. Indeed a few years later, an efficient method for isolation of *Aspergillus* nuclei from protoplasts was reported (Vagvolgyi and Ferenczy 1991), and purified nuclei from a haploid auxotrophic *A. nidulans* strain were transferred into the protoplasts of a complementary haploid auxotroph (Vagvolgyi and Ferenczy 1992). This process, also called karyoduction, resulted in both heterokaryotic and diploid colonies with a low but reproducible frequency of nuclear uptake (Vagvolgyi and Ferenczy 1992). Production of heterokaryotic nuclear hybrids have helped overcome the barriers of incompatibility in other fungi as well as shown in a recent study where nuclei from an auxotrophic strain of *Lentinula edodes* (a pharmacologically active basidiomycete) were transferred into complementing auxotrophic protoplasts of *Coriolus versicolor* (an incompatible partner) and nuclear hybrids with mixed nonparental isozyme patterns were obtained (Kim et al. 2000b).

In another recent study, isolated nuclei of the diploid fungus *Phytophthora parasitica* were fused with protoplasts of another strain of the same species (Gu and Ko 1998; 2001). The resulting regenerated nuclear hybrids expressed increased vigor in that, they grew faster than the parental isolates. These hybrids also expressed increased fungicide resistance. Since, such a phenomenon did not occur in hybrids regenerated from mitochondrion-protoplast or protoplast-protoplast fusion products between the two strains, it indicated that the hybrid vigor was the result of the interaction between the two

different kinds of nuclei, but not between the two types of mitochondria. Such increased vigor was also observed in the nuclear hybrids within two different species of *Phytophthora*, suggesting potential application of this fusion technique in creation of superior fungal strains for usage in agriculture and industry (Gu and Ko 2001).

The first studies on protoplast fusion in another industrially important organism *A. niger* (the koji fungus and well-known producer of citric acid) were reported by Kirimura et al. (1986; 1988), where they succeeded in producing fusion strains with higher productivity in solid culture methods compared to the parental strains. In another study, using protoplast fusion, a recombinant strain producing slightly higher (2.5%) beta-glucosidase production was obtained by Hoh et al. (1992). Other koji molds like *A. oryzae* and *A. sojae* have also been used in protoplast fusion experiments with some success in generating stable recombinants with desirable traits of both parents [e.g., increased protease, glutamine, or ethanol production; reviewed by Hara et al. (1992)].

8.2.3 *Claviceps*

Claviceps purpurea is another commercially important deuteromycete that is used in the production of a broad spectrum ergot alkaloids with therapeutic uses (Spalla and Marnati 1978). Both intra and interspecific level protoplast fusions have been carried out in this organism with some success in obtaining hybrids capable of either producing greater amounts of alkaloids or a combination of alkaloids [compared to the parental strains, Robbers (1984); Spalla and Marnati (1982)]. More recently, protoplast fusions between strains of *C. purpurea* carrying auxotrophic and fungicide resistance markers were conducted (Didek-Brumec et al. 1991). In this study, one parent actively produced two alkaloids and the second parent was inactive in producing a third type of alkaloid. Protoplast fusion experiments succeeded in generating prototrophic strains that produced all three types of alkaloids demonstrating recombination and activation of distinct alkaloid biosynthesis in the inactive partner (Didek-Brumec et al. 1991).

8.2.4 *Gibberella*

A group in Germany first optimized the conditions for protoplast formation in *Gibberella fujikuroi*, producing the horticulturally important plant growth hormones, gibberellins (Bruckner et al. 1990). Next, they isolated several auxotrophic, drug resistant, nitrate nonutilizing, and albino mutants of this species (Bermudez and Torres et al. 1992). Using contrastingly marked mutants; they then set up different combinations of crosses using the protoplast fusion technique, and made the following observations. Stable heterokaryon and diploids (induced by camphor treatment, Kirimura et al. 1988) were obtained from every cross except where both parents had drug resistance markers, and while most recombinants showed lower gibberellin titers than the parental strains, some diploids had greater productivities (Bermudez and Torres et al. 1992). Based on the poor understanding of the mechanisms of gibberellin biosynthesis especially at the regulatory level, the authors attributed their success of obtaining a higher producing diploid to a chance phenomenon, not unlike mutagenesis used in industrial strain improvement programs (Bermudez and Torres et al. 1992).

8.2.5 *Trichoderma*

Some filamentous fungi (e.g., *Trichoderma* species) have been developed as biological agents to control plant pathogens, weeds, and insects by using mutagenesis, screening of wild isolates, etc. (Stasz 1990). In *Trichoderma*, protoplast fusion has successfully been used to obtain improved biocontrol strains, but the genetic basis for the results could not be documented based on the usual parasexual cycle events (Harman et al. 1989; Ogawa et al. 1986). This is because unexpected data was obtained from studies at the intrastrain, intraspecies, or interspecies level. For example, even at the intrastrain level, while balanced heterokaryons were obtained, heterozygous diploids were not detected despite using inducing agents (Ogawa et al. 1986). Further, when the crossing parents were at the intraspecific or interspecific level, somatic hybrid colonies grew poorly even on nutritionally CM and upon subculturing on MM or CM, continued to undergo phenotypic changes (Stasz et al. 1988). When isozyme analysis was used, no evidence was found for heterokaryotic or heterozygous diploid intermediates, aneuploids or recombinants. It was therefore concluded that parasexual cycle could not explain the postfusion events and the authors raised the question of postfusion incompatibility occurring in this species (Harman and Stasz 1991; Stasz 1990).

Interestingly, a recent study reported the isolation of a recombinant between *Trichoderma reesei* and *A. niger* (Ai et al. 1997), demonstrating genomic compatibility and interactions between the two taxonomically diverse organisms. In addition, not only was this intergeneric recombinant stable, but its growth rate and cellulase biosynthetic activity was significantly dominant over the two parents (Ai et al. 1997). Also interestingly, other recent studies in *Trichoderma* have reported the evidence for parasexual cycle events (Furlaneto and Pizzirani-Kleiner 1992; Ogawa et al. 2000). In the latter study, protoplasts of two *T. harzianum* auxotrophic mutants (one having fungicide resistance, another, better growth pattern) were fused to obtain heterokaryons and subsequently a diploid that had both parents' desirable traits (Ogawa et al. 2000). In contrast with an earlier report (Ogawa et al. 1986), in this instance, the d-camphor treatment succeeded in inducing formation of the diploid, the ploidy of which was confirmed by various analyses. Finally, the biocontrol properties of this diploid were determined to be against *Fusarium* and *Pyricularia* (phytopathogenic fungi) species (Ogawa et al. 2000). From this recent work, it appears that some *Trichoderma* species probably participate in parasexual crossing, but depending on the compatibility issues and the nature of the genetic interaction, the products may vary in their stability and, hence may be rather difficult to study.

8.2.6 *Beauveria*

In the deuteromycete *Beauveria* (a biocontrol fungus), several protoplast fusions and parasexual recombinations have been reported (Kawamoto and Arzawa 1986; Paccola-Meirelles and Azevedo 1991; 1994; Paris 1977). More recently, an interspecies crossing in *Beauveria* resulted in obtaining a superior biocontrol strain, as follows: An entomopathogenic strain of *B. bassina* active against two insects was crossed with a

strain of *B. sulfurescens* capable of producing an insecticidal toxin (Couteaudier et al. 1996). Protoplast fusions between the diauxotrophic mutants of these strains resulted in the recovery of some stable products, two of which showed increased virulence against the susceptible insects (possibly because of the combining of both parents' characteristics). Molecular RFLP analysis suggested that these were recombinants or partial diploids with portions of the genome being heterozygous. A molecular mitochondrial marker indicated homoplasmy of these hybrids, with inheritance of the mitochondria from either of the two parents. The pathogenicities and the ploidies of the hybrids remained stable after passage through the host insect, suggesting that somatic hybridization through protoplast fusion may serve as a method for genetic improvement of biocontrol mechanisms in the entomopathogenic fungi (Couteaudier et al. 1996; Viaud et al. 1998).

9 CYTOPLASMIC INFLUENCES IN PROTOPLAST FUSIONS

Since, fungi contain nucleic acid bearing organelles like mitochondria and also carry plasmids and mycoviruses, the influence of these cytoplasmic elements in the postfusion events cannot be excluded. There have been some reports on protoplast fusions to examine the transfers of mycoviruses (van Diepeningen et al. 1998; Varga et al. 1994), but most studies in this regard have focused on mitochondria, the sites of cellular respiration and energy production. In fungi, as in other eukaryotes, mitochondrial DNA (mtDNA) is generally maternally inherited and does not normally undergo recombination during sexual reproduction (Mason and Turner 1975; Mitchell and Mitchell 1952). Specific reasons for this mitochondrial behavior from the evolutionary point are not well understood but in a recent report, Kevei et al. (1997) offer possible explanations including active degradation of mitochondria of one of the parents, and selective silencing (gradual disappearance) of mitochondria of one of the parents upon zygote formation.

During asexual reproduction, however, mtDNA recombination does occur, and can be easily followed using appropriate nuclear (auxotrophy and spore color) and mitochondrial (drug resistance) markers (Rowlands and Turner 1974; 1977). According to Ferenczy (1983), nonselective transfers involve transmittance of mitochondria along with the donor nucleus via protoplast fusion with the separation of the two types of genomes occurring at a later stage. Conversely, in a selective transfer, the mitochondria are first separated and then transfused selectively into the recipient protoplasts (Ferenczy 1981). Early experiments of such nature were carried out in *S. cerevisiae* and *Schizosaccharomyces pombe* (Ferenczy and Maraz 1977; Gunge and Tamaru 1978; Luckemann et al. 1979; Maraz and Subik 1981) which have been reviewed by Ferenczy (1985). Results from these studies which included comparative analyses of normal sexual crossing vs. protoplast fusion using several combinations of mitochondrial markers, nuclear markers, and mating types demonstrated that the transmission and recombination frequencies of mitochondrial genes were very similar in both processes (fusion vs. sexual crossing) regardless of the mating type (Ferenczy 1985). The basic experiments in yeast were soon extended to filamentous fungi demonstrating that using protoplast fusion, mitochondria

can successfully be transmitted at the intra or interspecific level (Croft and Dales 1983; Croft et al. 1980).

Most recent studies have used mtDNA RFLP comparisons in addition to the nuclear and mitochondrial genetic markers to better understand the nature of mitochondrial recombination. For example, in the edible mushroom *Lentinula edodes*, mitochondrial inheritance was compared in sexual crosses vs. those induced by protoplast fusions (Fukuda et al. 1995). Since, mtDNA had been implicated earlier in influencing mycelial growth of another mushroom *Agaricus bitorquis* (Hintz et al. 1988), it was of interest to determine if the same may be true for *L. edodes*. By comparing the RFLP patterns, it was observed that all newly established dikaryons formed by sexual crossing retained the mitochondrial RFLP of one of the monokaryons, suggesting the uniparental mode of the mitochondrial inheritance (Fukuda et al. 1995). This was not an expected result, since earlier, cytological experiments have clearly shown the movement of mitochondria from one parent to another through the opening formed by hyphal anastomosis during sexual crossing (Nakai 1986). Fukuda et al. (1995) suggested that perhaps dikaryotization and cell division in *L. edodes* may occur shortly after hyphal fusion not allowing sufficient time for mitochondrial recombination to occur. In the same study, genetically recombined mitochondria were obtained using protoplast fusion, possibly, (according to the authors) because it took at least 3 days for the protoplasts to regenerate, which allowed for the genetic exchange to occur (Fukuda et al. 1995).

Other mitochondrial studies have been performed in *Aspergillus* species predominantly to study mitochondrial inheritance and to characterize the recombinations patterns in the context of strain compatibility as model systems (Coenen et al. 1996; Hamari et al. 1997; Hamer et al. 2001; Kevei et al. 1997; 2001; Kirimura et al. 1992; Toth et al. 1998). For example, using protoplast fusions, mitochondria were transferred at the intra and interspecific level among incompatible *A. niger* strains and based on the RFLP patterns, three main types of mtDNA profiles were established (Kevei et al. 1997). Since, the vegetative incompatibility in the *A. niger* strains is so strong that even protoplast fusion cannot force heterokaryon formation and interaction of the two parents' nuclei, the mitochondrial experiments were successful only under selection pressure for the oligomycin-resistant donor mtDNA and the recipient nuclear phenotype (Kevei et al. 1997). Using this directed transfer strategy, mitochondrial transfers among three mtDNA types (and their subgroups) belonging to *A. niger*, *A. tubingensis*, and some Brazilian wild isolates were successful. However, such crosses among the more distantly related black *Aspergilli*-like *A. niger* and *A. japonicus* were unsuccessful (Kevei et al. 1997), possibly, according to the authors, due to the wide variety of naturally occurring polymorphic mtDNAs among the black *Aspergilli* (Varga et al. 1994). More recently, using similar strategy, oligomycin-resistant mitochondria were transferred to oligomycin sensitive *A. japonicus* recipients carrying appropriate nuclear markers like nutritional requirement and spore color (Kevei et al. 2001). In this study, based on the RFLP patterns, the progeny selected for oligomycin resistance with recipient nuclear phenotype had either unchanged donor mtDNA (substituted progeny) or recombinant mtDNA (recombinant progeny). The latter were found to harbor mobile elements characteristic of the recipient parents, while the former were either stable (due to compatible co-operation between the donor mitochondria and the recipient nuclei), or aconidial and unstable (due

to less compatible donor mitochondrial and recipient nuclear communication, Kevei et al. 2001). Finally, the aconidial substituted progeny was found to undergo a segregation process resulting in stable, wild-type strains with a new type of mtDNA similar to that of the recipient parent (Kevei et al. 2001). In a related study, detailed analysis of a protoplast-fusion generated *A. japonicus* strain harboring a recombinant mtDNA suggested that both mobile intron losses and acquisitions played a role in formation of the rearranged mtDNA (Hamari et al. 2001). In addition, with certain parental combinations, a particular intron was lost very frequently and since intron loss is an important phenomenon in eukaryotic gene expression, this process is currently under investigation (Hamari et al. 2001).

10 CONCLUDING REMARKS

Protoplast fusions continue to play a major role in fungal research. Even DNA transformation, where protoplasts serve as recipients of exogenous vectors, is thought to be a protoplast fusion-mediated event (Vagvolgyi and Ferenczy 1992). Further, protoplasts have also been transformed by total RNA extracts, an event termed as retrotransformation (Zucchi et al. 1989). Such RNA-mediated transformations have been shown to recover gene functions lost by point mutations or deletions (Zucchi 1996). It is quite possible that retrotransformation also occurs as a result of the fusion of the recipient protoplasts.

One important feature that serves as a common thread in all protoplast fusion experiments is the genetic compatibility of the two participating parents. As seen from many of the studies discussed in the earlier sections, the greatest advantage of protoplast fusion is to overcome the vegetative incompatibility that exists at the hyphal anastomosis level and to force the formation of a heterokaryon. Once this is accomplished, formation of diploids, recovery of recombinant segregants, etc., will depend on other factors such as interactions between the two types of nuclei, influence of cytoplasmic elements, and the level of compatibility/ tolerance among all the heterogenous DNA components existing in the fusion product. However, protoplast fusions cannot always force the formation of a stable, balanced heterokaryon because the incompatibility may be so extreme that it is not possible for the two types of nuclei to coexist. In such cases, nuclear DNA of only one of the parents (sometimes with portions of the other parents' genome) is likely to remain in the fusion product, determining the outcome of the parasexual analysis. In fact, compatibility issues are known to affect production of industrially important secondary metabolites (Anne 1983). Thus, due to the issues of strain stability and the complexity of genetic regulatory processes of secondary metabolic pathways, increased use of protoplast fusions for generation of novel industrial strains is not very probable. However, intra and interspecies protoplast fusions will very likely be widely used in relation to taxonomic (e.g., vegetative incompatibility), evolutionary (e.g., mitochondrial inheritance), and agricultural (e.g., generating superior biocontrol strains) processes.

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Metabolic Pathway Regulation

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1 INTRODUCTION

Research efforts in the past three decades have revealed the complex mechanisms employed by fungi to control gene activity. In their natural habitat, fungi are capable of using a variety of compounds as nitrogen and/or carbon sources. However, not all nitrogen and/or carbon sources support growth and development equally. This chapter will focus primarily on the regulatory mechanisms governing nitrogen and carbon metabolism, particularly on utilization of alternative nitrogen and carbon sources, in the model filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. Recent progress in the characterization of global factors affecting nitrogen and carbon metabolism, and pathway-specific metabolic gene regulation will be emphasized. Global regulators act on a wide range of unrelated activities involved in utilization of an array of different compounds, whereas pathway-specific regulators usually act specifically within a single or several related pathways to mediate coordinated expression of activities required for utilization of related compounds. A number of authoritative reviews of early aspects of regulation of nitrogen and carbon metabolism have been published (Caddick et al. 1994; Ebbole 1998; Kelly 1994; Felenbok and Kelly 1996; Marzluf 1997; Ruijter and Visser 1997; Scazzocchio et al. 1995).

2 REGULATION OF NITROGEN METABOLISM

In order to use a nitrogenous compound as a nitrogen source, the compound has to be converted to glutamate and glutamine, which serve as nitrogen donors for the synthesis of all other nitrogen-containing compounds in the cell. Both glutamate and glutamine can be synthesized using ammonium as the amino group donor. Therefore, ammonium, glutamate, and glutamine are the preferred nitrogen sources. In the absence of these favored compounds, secondary nitrogen sources such as nitrate, amides, purines, amino acids, and proteins are used. The phenomenon of preferential utilization of favored nitrogen sources is known as nitrogen metabolite repression (NMR). This mechanism enables fungi to efficiently manage the available nitrogen resources, and only under conditions of nitrogen limitation will alternative pathways responsible for the utilization of secondary nitrogen compounds be activated. Selection of the best nitrogen source must

include a sensing mechanism. Glutamine is a key effector of nitrogen metabolite repression but the intracellular concentration of glutamine appears not to be the sole determinant (Morozov et al. 2001). Ammonium, in combination with alpha-ketoglutarate, can be converted to glutamate through the action of NADPdependent glutamate dehydrogenase or the combined action of glutamine synthetase and glutamate synthase (Macheda et al. 1999). Glutamate and ammonium also act to signal nitrogen sufficiency independently from the glutamine signal (Margelis et al. 2001).

2.1 Global Positive-Acting Nitrogen Pathway Regulators

The utilization of secondary nitrogen sources requires the relief of nitrogen metabolite repression to allow *de novo* synthesis of specific permeases and catabolic enzymes. Relief of repression (derepression) is mediated by global positiveacting regulatory proteins. Such regulators are encoded by *areA* of *A. nidulans* (Kudla et al. 1990), *A. oryzae* (Christensen et al. 1998), *A. niger* (MacCabe et al. 1998) *A. parasiticus* (Chang et al. 2000), and *Gibberella fujikuroi* (Tudzynski et al. 1999), *nit-2* of *N. crassa* (Fu and Marzluf 1990a,b), *nre* of *Penicillium chrysogenum* (Haas et al. 1995; Haas and Marzluf 1995) and *NUT1* of *Magnaporthe grisea* (Froeliger and Carpenter 1996). All these regulators are members of the Cys-X₂-Cys-X₁₇-Cys-X₂-Cys zinc finger GATA-binding transcription factor family, and some have been shown to bind DNA with the consensus sequence HGATAR (Fu and Marzluf 1990b; Ravagnani et al. 1997). Various mutations that affect AreA activity have been extensively characterized (Arst and Cove 1973; Caddick et al. 1994; Langdon et al. 1995; Stankovich et al. 1993; Wilson and Arst 1998). Loss-of-function alleles confer inability to utilize nitrogen sources other than ammonium and are commonly associated with changes in the DNA-binding domain. The *areA102* mutation results in the substitution of the highly conserved leucine within the zinc finger for a valine, and causes a complex phenotype, including increased *amdS* and reduced *uapA* expression, due to altered GATA-site specificity (Hynes 1975; Kudla et al. 1990; Ravagnani et al. 1997).

In *A. nidulans*, AreA activity is modulated to facilitate expression of genes subject to nitrogen metabolite repression only under conditions of nitrogen limitation (Platt et al. 1996a). This modulation is achieved through control of *areA* transcription, altered stability of the *areA* mRNA, and interactions of AreA with other proteins (Langdon et al. 1995; Platt et al. 1996a,b; Small et al. 1999). The *areA* transcript exists at extremely low levels in nitrogen repressing conditions but is highly expressed in derepressing conditions. Transcripts of three sizes, approximately 3.9, 3.6, and 3.2 kb, are produced and appear functionally redundant. Synthesis of the 3.2-kb transcript is subject to autogenous regulation (Langdon et al. 1995). The turnover rate of the *areA* transcript depends on nitrogen status. The wild-type *areA* transcript has a half-life of 40 min in derepressing conditions compared to 7 min in repressing conditions (Platt et al. 1996a). Deletion of a 218 bp region in the 3' untranslated region (UTR) of *areA* results in partial derepression of *areA*-regulated activities (Platt et al. 1996a). This 218 bp sequence is sufficient to confer nitrogen-regulated transcript degradation on a reporter gene (Morozov et al. 2000). Deletion of a 58 nucleotide region within this sequence confers a half-life of about 25 min for *areA* mRNA in repressing conditions and a parallel derepressed

phenotype (Platt et al. 1996a). This sequence is required for accelerated shortening of the poly-A tail, which in turn leads to the rapid degradation of the *areA* transcript under repressing conditions (Morozov et al. 2000; Morozov et al. 2001). Intriguingly, the *N. crassa nit-2* mRNA and NIT2 protein are easily detectable regardless of the nitrogen source, and the *nit2* transcript is quite stable (Fu and Marzluf 1987). Nonetheless, the levels of the *nit-2* transcript and the NIT2 protein increase three to four-fold when a secondary nitrogen compound is the sole nitrogen source (Tao and Marzluf 1999).

The activity of the AreA protein, in addition to *areA* transcript levels, is modulated. The 12 amino acid residues at the carboxyl terminus, EWEWLTMSL, are necessary for modulation of AreA function. Noticeably, 9 out of the 12 amino acid residues at the carboxyl termini of AreA homologs from *Aspergillus* species (*nidulans*, *niger*, *oryzae*, and *parasiticus*), *P. chrysogenum*, *N. crassa*, and *G. fujikuroi* are identical (*M. grisea* has a conserved amino acid substitution, EWDWLTMSL), strongly suggesting functional conservation of this region. Deletions within the carboxyl terminus of AreA result in derepression to levels similar to those caused by 3' UTR deletions in *areA* (Platt et al. 1996b). As the effects of mutations in these two regions are additive, the carboxyl terminus and 3'UTR define independent control mechanisms for regulation of *areA* activity.

2.2 Repressors Involved in Nitrogen Metabolite Repression

The nitrogen control circuit for the utilization of secondary nitrogenous compounds involves negative-acting regulatory proteins. Their function is to antagonize the action of the corresponding activators. The *N. crassa* negative regulatory protein NMR, encoded by the *nmr* gene (for *nitrogen metabolite repression*) (Fu et al. 1988), modulates the activity of NIT2 and thereby alters expression of many nitrogen metabolic genes (Marzluf 1997). The *nmr* gene is constitutively expressed at a very low level. Up to 16% of the carboxyl terminus of NMR is not necessary for function (Young et al. 1990). The NMR repressor is able to interact with two distinct regions of the NIT2 activator: a short alpha-helical motif within the NIT2 DNA-binding domain and an alpha-helical motif at the carboxyl terminus (Xiao et al. 1995). These interactions probably block the functional domains of NIT2, thus either interfering with binding of NIT2 to GATA sites, shielding the nuclear localization sequence (NLS) located in the basic region, or making regions of NIT2 inaccessible to coactivators. Various mutations in *nmr* resulted in derepression of many activities in the presence of ammonium or glutamine suggesting that the overall conformation of NMR is critical for its activity (Tomsett et al. 1981). Disruption of *A. nidulans nmrA*, the homolog of *nmr*, resulted in a partially derepressed phenotype similar to those of *areA* mutants with alterations in the DNA binding domain and the carboxyl terminus (Andrianopoulos et al. 1998). The predicted NmrA protein contains five regions highly conserved with *N. crassa* NMR. It is likely that protein-protein interactions occur between NmrA and the AreA DNA-binding domain and carboxy-terminal 12 amino acid region. Most recently, the crystal structure of NmrA has been resolved (Nichols et al. 2001) and, unexpectedly, NmrA displays structural similarity to the short-chain dehydrogenase/reductase family (Stammers et al. 2001).

2.3 Other Regulatory Genes Related to Nitrogen Metabolism

2.3.1 *Aspergillus nidulans* TamA is a Coactivator of AreA

Mutations in *A. nidulans tamA* result in simultaneous resistance to toxic analogues such as thiourea, aspartate hydroxamate and methylammonium (Kinghorn and Pateman 1975). These analogues are substrates of enzymes or permeases, which are regulated by nitrogen metabolite repression. The *tamA* mutants were referred to as “partially repressed” in terms of nitrogen metabolite repression, showing reduced growth on ammonium and reduced levels of a variety of nitrogen metabolic enzymes (Kinghorn 1999). The *tamA* gene has been cloned by complementation (Davis et al. 1996). The predicted TamA protein contains a Zn(II)₂Cys₆ zinc binuclear cluster DNA-binding motif, similar to other known pathway-specific regulatory proteins found in fungi (see Section 4.1). However, the zinc cluster motif is not required for function (Davis et al. 1996). Domainswap experiments show that residues 153–651 of TamA are sufficient for function, and TamA apparently acts as a transcriptional coactivator (Small et al. 1999). Random amino acid sequence changes and internal deletions abolish the function of TamA, which suggest that overall conformation is critical for its activity (Small et al. 2001). TamA interacts with the carboxy-terminal region of AreA (Small et al. 1999). Interestingly, this is the same region in AreA predicted to interact with the negative-acting NmrA protein of *A. nidulans* (Platt et al. 1996b). Thus, TamA may function to displace the negative-acting NmrA protein under derepressing conditions (Small et al. 2001). Consistent with its role as a transcriptional coactivator, TamA contains sequences showing similarity to putative NLSs (Small et al. 2001). Furthermore, a GFP-TamA fusion protein was found predominantly in the nucleus regardless of the presence or absence of ammonium, and *areA* was not required for this distribution (Small et al. 2001). TamA is highly conserved, with homologs in *A. oryzae* (Small et al. 2001) and in the *N. crassa* genome sequence (R.B.T., unpublished observations), suggesting that TamA homologs function similarly in other fungi.

2.3.2 Negative-Acting DNA-Binding Factors: *Penicillium chrysogenum* NreB and *Aspergillus nidulans* AreB

The *nreB* gene from *P. chrysogenum* encodes a GATA zinc finger DNA-binding factor with similarity to Dal80p/Uga43p and Gzf3p/Nillp, both repressors of nitrogen metabolism in *Saccharomyces cerevisiae* (Haas et al. 1997). NreB also contains a putative basic leucine zipper domain. Overexpression of *nreB* leads to repression of nitrogen catabolic genes. Two *nreB* transcripts, 1.5 and 1.8 kb in length, are produced, and the steady state level of these transcripts is subject to nitrogen metabolite repression (Haas et al. 1997). Antisense *nre* (the *areA* homolog) simultaneously represses the expression of *nre* and *nreB*, which further suggests that *nreB* is under *nre* regulation (Zadra et al. 2000).

The *A. nidulans nreB* homolog, named *areB*, was identified by screening for suppressors of *areA* loss-of-function mutations (Conlon et al. 2001). The *areB* gene is

predicted to encode at least three distinct proteins, which arise from different promoters, differential splicing and AUG and non-AUG translation start codons. All the putative products include a GATA zinc finger domain and a leucine zipper motif similar to the one identified in NreB (Conlon et al. 2001). Three classes of mutations in *areB* have been identified; (a) loss-of-function mutations resulting from termination of translation within or before the GATA domain, (b) mutations resulting in proteins retaining the GATA domain but truncated either at or upstream of the leucine zipper, and (c) gain-of-function rearrangements fusing novel gene sequences to *areB* to produce chimeric polypeptides. The novel fusion genes observed in the gain-of-function mutants convert the putative negative-acting transcription factor into an activator that can partially replace *areA*.

2.3.3 The *meaB* Gene Of *Aspergillus nidulans*

Methylammonium, a toxic substance, can induce nitrogen metabolite repression in wild type *A. nidulans*. Methyl-ammonium toxicity can be reversed by ammonium and by mutations that increase the conversion of this substance to ammonium. Mutations at the *meaB* locus result in derepression of a number of activities subject to nitrogen metabolite repression (Arst and Bailey 1980). The *meaB* gene has been isolated (Polley and Caddick 1996). The expression of *meaB* is independent of nitrogen metabolite repression. In addition, *meaB* mutants showed no changes in the level of the transcript produced, which suggests that *meaB* is not autoregulated. Pfam (protein families database of alignments and hidden Markov models) common domain search reveals weak support for MeaB as a member of the basic leucine zipper (bZIP) transcription factor family. However, like bZIP proteins, MeaB contains a putative basic DNA-binding domain, an invariant asparagine, and a 6-amino acid connector (McKnight 1991) positioned N-terminal to a putative leucine zipper. The predicted MeaB leucine zipperlike region is Leu-X6-Asn-X6-Leu-X6-Ala-X6-Leu-X6-Leu compared to the consensus sequence Leu-X6-Leu-X6-Leu-X6-Leu-X6-Leu. Asparagine and alanine are found at leucine positions 2 and 4, respectively, but are not predicted to interrupt the extensive alpha-helical structure. Substitutions of a leucine residue(s) for other residues in various positions that do not disrupt function have been reported for bZIP proteins (McKnight 1991; Peters et al. 2001). The Leu to Ala substitution also is present in the putative leucine zipper of AreB and NreB. Leucine zippers are commonly involved in protein dimerization. Whether MeaB engages in specific protein-protein interactions with the global nitrogen regulatory protein, AreA, thereby eliciting important regulatory responses remains to be studied.

3 REGULATION OF CARBON METABOLISM

Fungi encounter a wide range of potential carbon sources in the environment and have evolved a large number of metabolic pathways to allow utilization of many carbon compounds. Some carbon sources are more complex, and consequently less energetically efficient to utilize, than others. Metabolic pathways for carbon source utilization are highly regulated to allow the preferential utilization of energetically favorable compounds such as glucose.

3.1 Mutations Affecting Carbon Catabolite Repression (CCR) In *Aspergillus nidulans*

Carbon catabolite repression (CCR) is the repression in the presence of a preferred carbon source (e.g., glucose) of genes required for utilization of nonpreferred carbon sources (e.g., ethanol, starch, acetate, and quinate). CCR acts on many metabolic pathways that are required for utilization of carbon sources. Genetic screens for mutations affecting CCR in *A. nidulans* exploited the regulation of utilization of compounds that serve as both carbon and nitrogen sources, such as proline or acetamide [reviewed in Felenbok and Kelly (1996); Scazzocchio et al. (1995)]. Relief of either NMR or CCR is sufficient for expression of the genes required for utilization of these compounds. *areA* loss-of-function mutations confer inability to grow on compounds that serve as both carbon and nitrogen sources in the presence of glucose. Mutations affecting CCR were selected as extragenic suppressors of *areA* loss-of-function mutations on such compounds in the presence of glucose and mapped to three genes: *creA*, *creB*, and *creC* (Arst and Cove 1973; Kelly 1980; Hynes and Kelly 1977). *creA* mutants have also been isolated using other selection strategies (Bailey and Arst 1975; Shroff et al. 1997). Mutations in *creA* cause derepression of a wide range of activities, such as those required for utilization of ethanol, acetate, and starch, which would normally be repressed in the presence of repressing carbon sources.

3.2 Global Regulators of Carbon Metabolism

3.2.1 CreA, a DNA-Binding Transcriptional Repressor

The *A. nidulans creA* gene encodes a 416 residue protein containing two Cys2His2 zinc finger DNA-binding motifs (residues 66–116) and an alanine-rich region (residues 131–132) (Dowzer and Kelly 1989; Arst et al. 1990; Dowzer and Kelly 1991). CreA also contains a putative ubiquitination consensus sequence (residues 258–267, HEDESYASH) (Strauss et al. 1999). CreA shows similarity through the zinc finger region to Mig1p, the major repressor of carbon catabolite repressed genes in *S. cerevisiae* (Nehlin and Ronne 1990) (see Section 3.2.2). CreA contains neither obvious NLS nor sequences with strong similarity to the NLS of Mig1p (De Vit et al. 1997). However, the predicted size of CreA is approximately 45 kDa and thus CreA may be sufficiently small to diffuse through the nuclear pore into the nucleus without active transport.

A number of studies have shown that a CreA fusion protein binds to specific DNA binding sites upstream of many genes that are subject to CCR with the consensus sequence 5'-[G/ C][C/T]GG[G/A]G-3' (Cubero and Scazzocchio 1994; Dzikowska et al. 1999; Espeso and Penalva 1994; Ho et al. 1995; Kulmburg et al. 1993; Mathieu and Felenbok 1994; Orejas et al. 1999). *In vivo* studies have demonstrated that at least some *in vitro* CreA DNA-binding sites and sequences conforming to the consensus binding site are functional (Cubero and Scazzocchio 1994; Mathieu et al. 2000; Orejas et al. 1999; Panozzo et al. 1998).

A comprehensive molecular analysis of *creA* mutant alleles showed that seven alleles

carried missense mutations, and 12 alleles contained nonsense or frameshift mutations resulting in truncated CreA proteins (Shroff et al. 1996; Shroff et al. 1997). All of the missense mutations, including the extreme *creA306* allele, mapped to the DNA-binding domain, suggesting that they affect CreA DNA-binding activity. Truncation of the C-terminal 80 residues results in derepression of several activities subject to CCR, suggesting that the CreA C-terminus is required for mediating repression (Shroff et al. 1997). The *creA303* mutation truncates the CreA protein within the N-terminal zinc finger domain at residue 68, and, consistent with the extreme *creA303* phenotype, is therefore expected to be a null mutant (Shroff et al. 1997). Thus, *creA* null alleles are viable (Shroff et al. 1997). In contrast, initial deletion experiments only obtained gene replacement of *creA* in a diploid or a heterokaryon and deletion of *creA* appeared lethal (Dowzer and Kelly 1991). As haploid *creA* deletion conidia germinated but then arrested in growth, the *creA* deletion phenotype was termed “leaky-lethal” (Dowzer and Kelly 1991). Subsequently, precise gene replacement of *creA* demonstrated that a *creA* deletion mutant is viable and the leaky-lethal phenotype was due to deletion of sequences 3' to the *creA* gene (Shroff et al. 1997).

Northern analysis revealed that *creA* is expressed in both carbon repressing and derepressing conditions, with expression levels varying according to the carbon source (Strauss et al. 1999; Shroff et al. 1996). *creA* mRNA levels were higher in derepressing than repressing conditions (Strauss et al. 1999; Shroff et al. 1996). Furthermore, in loss-of-function *creA* mutants the *creA* transcript levels in repressing conditions were increased compared with wild type to levels similar to those found in derepressing conditions suggesting that *creA* may be autoregulated (Shroff et al. 1996). As the 5' DNA sequence of *creA* contains consensus CreA DNA-binding sites (Dowzer and Kelly 1991; Shroff et al. 1996), Shroff et al. (1996) suggested that *creA* autoregulation may be direct. *In vitro* mutation of two of the closely spaced CreA consensus DNA-binding sites in the *creA* 5' regulatory region resulted in derepression of *creA* under repressing conditions when introduced into *A. nidulans* (Strauss et al. 1999). The CreA fusion protein binds oligonucleotides containing these two DNA-binding sites *in vitro* and mutation of these DNA-binding sites virtually abolished DNA-binding (Strauss et al. 1999). The levels of *in vitro* binding to these oligonucleotides by a protein that may correspond to CreA in *A. nidulans* cell-free extracts decreased in derepressing compared with repressing conditions (Strauss et al. 1999). Thus in derepressing conditions, although *creA* mRNA levels are increased, CreA protein may be subject to post-transcriptional modification resulting in degradation or modulation of DNA-binding activity.

3.2.2 Phosphorylation and Nuclear Localization Regulate Repression Function of CreA Homologs

Homologs of *creA* have been isolated from a number of filamentous fungi including *A. niger* (Drysdale et al. 1993), *G. fujikuroi* (Tudzynski et al. 2000), *Botrytis cinerea* (Tudzynski et al. 2000), *Trichoderma reesei* (Ilmen et al. 1996; Strauss et al. 1995), *Trichoderma harzianum* (Ilmen et al. 1996), *N. crassa* (de la Serna et al. 1999), *Metarhizium anisopliae* (Screen et al. 1997) and *Sclerotinia sclerotiorum* (Vautard et al. 1999). Protein sequence alignments of CreA homologs reveal strong conservation

through the DNA-binding domain and several additional regions of weak conservation (Drysdale et al. 1993; Ilmen et al. 1996). One region shows similarity to RGR1, which is involved in CCR in *S. cerevisiae* (Drysdale et al. 1993; Sakai et al. 1988). However, domain-swap experiments showed that RGR1 cannot substitute for this region in *A. nidulans* CreA (Shroff et al. 1996). These *creA* homologs appear to function similarly in CCR to repress transcription of regulated genes in the presence of a repressing carbon source. Some differences are apparent amongst these CreA homologs. *A. nidulans creA*, *T. harzianum cre1* and *S. sclerotiorum cre1* mRNA levels are regulated according to carbon source. However, unlike *A. nidulans creA* and *T. harzianum cre1*, where transcript levels are higher in derepressing than repressing conditions, *S. sclerotiorum cre1* transcript levels are higher in repressing than derepressing conditions (Vautard et al. 1999). The *S. sclerotiorum* Cre1 protein is observed under derepressing conditions, though at reduced levels compared with repressing conditions (Vautard-Mey et al. 1999).

Subcellular fractionation studies indicated that *S. sclerotiorum* Cre1 was both nuclear and cytosolic under repressing conditions but largely excluded from the nucleus under carbon derepressing conditions, implicating regulated subcellular localization as a mechanism for modulating Cre1 activity (Vautard-Mey et al. 1999). Furthermore, in the same study, Cre1 showed regulated nuclear localization dependent on carbon source when expressed as a GFP fusion in *A. nidulans*. Thus, the machinery for regulation of Cre1 subcellular localization is conserved in *A. nidulans*. The repression activity, but not subcellular localization, of *S. sclerotiorum* Cre1 is modulated by phosphorylation when expressed in *A. nidulans* (Vautard-Mey and Fevre 2000). It is unknown if *A. nidulans* CreA is regulated in response to carbon source by phosphorylation or subcellular localization.

In *S. cerevisiae*, CCR is effected predominantly by the DNA-binding transcriptional repressor Mig1p, a Cys2His2 DNA-binding protein that shows similarity through the DNA-binding domain with CreA (Nehlin and Ronne 1990). Mig2p and Mig3p are paralogs of Mig1p and appear to be at least partially redundant in function with Mig1p (Lutfiyya and Johnston 1996; Lutfiyya et al. 1998). The function of Mig1p has been well characterized (Carlson 1998; Carlson 1999; Gancedo 1998; Johnston 1999). Mig1p acts by binding to a specific sequence similar to that bound by CreA in the upstream regulatory regions of regulated genes. Mig1p mediates glucose repression by recruitment of the Tup1p pleiotropic transcriptional repressor to glucose repressed promoters (Treitel and Carlson 1995). Tup1p is a seven WD40 repeat protein, which acts as a complex with the tetratricopeptide (TPR) protein Ssn6p (Komachi et al. 1994; Treitel and Carlson 1995; Williams and Trumbly 1990). Tup1p confers repression by interaction with RNA polymerase II holoenzyme components and, alteration of the chromatin structure at regulated promoters through interactions with histone deacetylases (HDAC) and histones H3 and H4 (Edmondson et al. 1996; Wu et al. 2001; Zaman et al. 2001). Mig1p activity is regulated by Snf1p kinase-dependent phosphorylation (De Vit et al. 1997; Ostling and Ronne 1998; Smith et al. 1999; Treitel et al. 1998) and subcellular localization (De Vit et al. 1997; De Vit and Johnston 1999). Under carbon limiting conditions Mig1p is phosphorylated and found in the cytoplasm whereas in the presence of glucose Mig1p is not phosphorylated and accumulates in the nucleus.

3.2.3 *Aspergillus nidulans* CreB and CreC Are Involved in Deubiquitination and CCR

The *creB* and *creC* genes were identified in the same manner as *creA* in screens for suppressors of *areA* loss-of-function mutations on compounds that serve as both sole nitrogen and sole carbon sources in the presence of glucose (Hynes and Kelly 1977). *creB* was previously identified in a screen for molybdate resistance as *molB* (Arst et al. 1970). The *creB* and *creC* mutants share the same range of pleiotropic phenotypes, including derepression of a subset of *creA*-repressed activities in the presence of glucose, poorer growth on a range of sole carbon sources (e.g., 0.5% quinate and 50 mM proline) and elevated levels of some enzymes, such as extracellular proteases, in both repressing and derepressing conditions (Arst 1981; Hynes and Kelly 1977; Kelly and Hynes 1977). Furthermore, the *creB* and *creC* phenotypes are not additive, suggesting that *creB* and *creC* act in the same complex or pathway and that each of these gene products is required for the activity of the other and that is its only function (Arst 1981; Lockington and Kelly 2001).

The *creB* gene was cloned by complementation and sequence analysis revealed an encoded protein of 767 amino acids containing ubiquitin carboxy-terminal hydrolase family 1 and 2 signature sequences and showing similarity with the UBP family of deubiquitinating enzymes (Lockington and Kelly 2001). CreB also contains four PEST sequences (Lockington and Kelly 2002). The determination of sequence changes in *creB* mutants revealed two alleles with missense mutations in residues absolutely conserved among deubiquitinating enzymes and two alleles containing mutations leading to a truncated CreB protein (Lockington and Kelly 2001). One of these truncation mutations results in expression of only the first 12 residues of CreB and is therefore essentially a null allele. CreB was shown to have deubiquitinating activity when expressed in a bacterial system (Lockington and Kelly 2001).

The *creC* gene was cloned by complementation and sequence analysis showed that *creC* encodes a protein of 630 amino acids containing an N-terminal proline-rich region (residues 43–123), a putative NLS (residues 168–174), and five WD40 repeat units (residues 286–595) (Todd et al. 2000). CreC shows weak sequence similarity with the *S. cerevisiae* glucose transcriptional repressor Tup1p (see Section 3.2.3). Determination of the molecular nature of mutations in three *creC* alleles and deletion analysis revealed that the C-terminal 32 residues are dispensable and residues 588–598 within the most C-terminal WD40 repeat are required for CreC function (Todd et al. 2000). Gene replacement of the *creC* gene resulted in a phenotype identical to that of the *creC* truncation mutants (Lockington and Kelly 2001). CreB and CreC are both highly conserved, each with homologs in higher organisms, but neither protein has close sequence homologs in *S. cerevisiae* (Lockington and Kelly 2001; Todd et al. 2000).

The identical range of phenotypic effects and non-additivity of mutations in *creB* and *creC* has led to the suggestion that CreB and CreC act together in a complex to deubiquitinate target proteins with both proteins required for activity (Lockington and Kelly 2001). This hypothesis is supported by co-immunoprecipitation studies, which show physical interaction of CreB and CreC in *A. nidulans* protein extracts (Lockington

and Kelly 2002). Overexpression analysis showed that when overexpressed *creB* can function in a *creC* deletion mutant whereas overexpressed *creC* does not function in a *creB*⁻ mutant (Lockington and Kelly 2002). Therefore it appears that CreB acts downstream of CreC and encodes the active component of the CreB–CreC complex whereas CreC acts to stabilize the CreB deubiquitinating enzyme. Furthermore, the deubiquitinating activity of CreB implicates a deubiquitination mechanism in CCR in *A. nidulans*. In *S. cerevisiae*, Skp1p–Grr1p mediated ubiquitination has been implicated in CCR as introduction of a heterologous *SKP1* homolog leads to destabilization of the F-box protein Grr1p and inactivation of Mig1p (Li and Johnston 1997; Schouten et al. 2000).

The CreA protein contains a ubiquitination motif (Strauss et al. 1999) and therefore is likely to be ubiquitinated and a target for CreB-dependent deubiquitination. A genetic association has been established between *creA* and *creB*—the *creB9730* mutation was selected as a suppressor of the most extreme *creA306* allele (Lockington and Kelly 2001). Lockington and Kelly (2002) have proposed a scheme in which CreB functions as both a free entity and in a complex with CreC. Free CreB acts under carbon catabolite repressing conditions to deubiquitinate CreA thereby preventing targeted proteasomal degradation of CreA. Deubiquitinated CreA would bind to carbon catabolite repressible promoters and repress transcription. Under carbon catabolite derepressing conditions free CreB would be degraded and CreA would be ubiquitinated and targeted for degradation via the proteasome, allowing derepression of transcription. The CreC-bound CreB is required for expression of some pathway-specific genes under both repressing and derepressing conditions.

3.2.4 Filamentous Fungal Homologs of Tup1p Play Little or no Role in CCR

In *S. cerevisiae*, CCR is mediated by Tup1p, a pleiotropic transcriptional repressor (see Section 3.2.2). Sequence homologs of *S. cerevisiae* Tup1p have been identified in filamentous fungi. The *A. nidulans rcoA* gene was cloned by homology to *TUP1* (Hicks et al. 2001). Analysis of the *rcoA* deletion phenotype indicated that *rcoA* is involved in regulation of morphogenesis and sterigmatocytin biosynthesis but plays little or no role in CCR (Hicks et al. 2001; RB Todd, unpublished data). Similarly, the *Penicillium marneffei* Tup1p homolog TupA is involved in regulation of developmental programs and plays only a minor role in CCR (Todd et al. 2003; RB Todd, MJ Hynes, and A Andrianopoulos, submitted). In *N. crassa* the Tup1p homolog RCO1 also is involved in regulation of development and repression of several genes under carbon limiting but not carbon repressing conditions (Yamashiro et al. 1996; Ebbole 1998; Lee and Ebbole 1998). In *A. nidulans*, *P. marneffei*, and *N. crassa* it appears that RcoA, TupA, and RCO1, respectively, regulate development, but it is possible that these Tup1p homologs regulate development in response to carbon status.

3.2.5 How Does CreA Effect Repression?

It is clear that RcoA does not play a major role in mediating CreA-dependent repression of carbon catabolic genes. Moreover, CreB and CreC appear to function in repression in a

deubiquitination mediated process distinct from the Tup1p repression mechanism. Thus the mechanisms of CCR are not conserved between the filamentous fungi and *S. cerevisiae*. A key question remaining is how CreA effects repression at promoters of carbon catabolite repressed genes. A protein equivalent in function to Tup1p, which mediates repression by interaction with the transcriptional machinery and chromatin reorganization, may exist in *A. nidulans*. Alternatively, CreA itself may actively repress transcription by direct interaction with the transcriptional apparatus or directly influencing chromatin structure.

3.2.6 Other Global Regulators of CCR

The *creD* gene was identified as *cre-34*, a suppressor of the toxic effects in a *creC27* mutant of fluoroacetamide in the presence of glucose (Kelly and Hynes 1977). The *creD* mutation suppresses some but not all of the pleiotropic phenotypes of both the *creB15* and *creC27* mutants. The role of *creD* in CCR remains to be elucidated. The signals for CCR are not understood in filamentous fungi. In *N. crassa rco-3* encodes a low affinity glucose transporter which may function as a glucose sensor to signal extracellular glucose concentration (Madi et al. 1997). Thus, RCO3 may be a key element in triggering CCR. In *S. cerevisiae* hexokinases may be involved in triggering CCR [reviewed in Ruijter and Visser (1997)]. The *A. nidulans fra* gene, which encodes a hexokinase, does not appear to be involved in CCR of a number of enzymes analyzed (Ruijter et al. 1996). However, the *creA30* mutation was selected as a suppressor of *frA1* (Arst et al. 1990). Furthermore, the *xprF* gene has been recently shown to encode a hexokinase-like protein involved in the regulation of extracellular proteases (Katz et al. 2000). Katz et al. (2000) also have identified *xprG* as a suppressor of *xprF*. The role of these genes in CCR remains to be determined.

4 PATHWAY-SPECIFIC REGULATORS OF NITROGEN AND CARBON METABOLISM

In some cases, the global regulators are the only transcription factors that control gene expression, with no additional pathway-specific regulatory factors required for expression [e.g., *fmdS* (Fraser et al. 2001)]. At many promoters the globally acting protein(s) do not function alone, but require a pathway-specific regulatory protein to mediate induction by a substrate or an intermediate of the pathway. Dissection of the *amdS* and *prnB-D* promoter regulatory regions has established that the pathway-specific activators act independently of global regulators, as induction and global controls are separable (Davis et al. 1993; Scazzocchio 1992).

4.1 Domain Structure of Pathway-Specific Regulators

Pathway-specific regulators generally comprise a DNA-binding domain for interaction with specific DNA-binding sites, an effector domain for response to environmental cues, and a transcriptional activation domain that mediates transcription. In fungi the Zn(II)

2Cys6 zinc binuclear cluster has been extensively used as the DNA-binding domain in pathway-specific regulators (Todd and Andrianopoulos 1997), although other DNA-binding domains, such as the Cys2His2 zinc finger, are known [e.g., AmdA, AmdX (Andrianopoulos et al. 1997; Murphy et al. 1997)]. The structure of the DNA-binding domains, recognition sites, and DNA-binding specificity of various Zn(II)2Cys6 pathwayspecific regulatory proteins have been well defined [e.g., NirA, NIT4, UaY, FacB (Fu et al. 1995; Punt et al. 1995; Suarez et al. 1995; Todd et al. 1998)] and extensively reviewed (Todd and Andrianopoulos 1997). The domain structure of Zn(II)2Cys6 regulators has been established by experiments swapping DNA-binding and transcriptional activation domains between members of this class, e.g., AmdR and FacB (Parsons et al. 1992; Todd et al. 1997). Most Zn(II)2Cys6 proteins appear to bind DNA as a dimer, e.g., NirA, NIT4 (Fu et al. 1995; Strauss et al. 1998), although AlcR binds as a monomer (Nikolaev et al. 1999). Dimerization is mediated by a coiled-coil motif C-terminal to the DNA-binding domain and these domains are spaced by a linker region. Activation domains are not conserved, indicating that there may be numerous mechanisms for recruitment of the general transcriptional machinery.

4.2 Action of Pathway-Specific Regulators

There appears to be no common mechanism by which pathway-specific activators respond to signals to modulate their activity and confer changes in gene expression. Some pathway-specific regulators require inducer for DNA-binding [e.g., UaY, NirA, PrnA (Gomez et al. 2002; Oestreicher et al. 1997; Narendja et al. 2002)] whereas others may bind to promoters in the absence of added inducer [e.g., AmdR (van Heeswijck and Hynes 1991)]. Some regulators [e.g., PrnA (Gomez et al. 2002)] are constitutively localized to the nucleus whereas others show regulated nuclear localization [e.g., FacB (A. Andrianopoulos, pers. comm.)]. The activity of the QutA activator appears to be modulated by binding of the QutR repressor (Levett et al. 2000).

Many regulatory genes are expressed constitutively, e.g., *nit-4*, *nirA*, *prnA* (Fu et al. 1989; Cazelle et al. 1998; Burger et al. 1991). In contrast, expression of others is repressed by CreA and induced by an autoregulatory mechanism [e.g., *alcR*, *qutA*, *qa-1F* (Patel and Giles 1985; Levesley et al. 1996; Lockington et al. 1987)]. In the well-studied case of *alcR*, a “double-lock mechanism” occurs, where both the regulatory gene and the structural genes are directly controlled by CreA (Felenbok et al. 2001; Kulmburg et al. 1993). Furthermore, AlcR and CreA binding sites are overlapping in some co-regulated genes and CreA also acts by competition with AlcR for DNA-binding (Mathieu and Felenbok 1994; Mathieu et al. 2000). Competition for the same DNA-binding sites in the *amdS* promoter may occur between CreA and the Cys2His2 zinc finger pathway-specific activators AmdA and AmdX (Andrianopoulos et al. 1997; Murphy et al. 1997). Competition has also been proposed between CreA and a positive-acting factor in the *prnB-D* intergenic region (Gonzalez et al. 1997).

5 THE PROMOTER, GENE CLUSTERING, AND CHROMATIN REMODELING

The promoter of a metabolic structural gene provides a platform for modulating gene expression. Promoters comprise a regulatory region, which contains DNA-binding sites for the global and pathway-specific regulatory factors, and core elements for interaction of the general transcription machinery. Mutational and molecular studies have defined the arrangement of regulatory DNA-binding sites in the *amdS*, *prnB-C*, and *niiA-niaD* promoters (Davis et al. 1993; Punt et al. 1995; Scazzocchio 1992). Transcription factors may exert their effects directly on the general transcriptional machinery or indirectly by altering chromatin structure. Many metabolic pathway genes are clustered in fungi (Cary et al. 2001). Gene clustering allows coordination of changes in chromatin structure for co-expressed genes.

Regulation of the expression of genes encoding the nitrate utilizing enzymes in *N. crassa* and *A. nidulans* are welldocumented examples (Caddick et al. 1994; Marzluf 1997). Nitrate is taken up by permeases, and reduced to nitrite by nitrate reductase. Nitrite is further reduced to ammonium by nitrite reductase. Genes encoding these proteins are organized differently in several fungi (Cary et al. 2001). For example, the nitrate permease (*crnA*=*nrtA*), nitrate reductase (*niaD*), and nitrite reductase (*niiA*) genes in *A. nidulans* are clustered (Johnstone et al. 1990) whereas the homologs of *niaD* and *niiA* in *N. crassa*, *nit-3*, and *nit-6*, respectively, are located on separate chromosomes.

Two NIT4 DNA-binding sites of significantly different affinities have been identified in the 1.3-kb promoter region of the *nit-3* gene (Fu et al. 1995). The stronger binding site contains the palindromic sequence, TCCGCGGA, whereas the weaker site has a related sequence (Fu et al. 1995). NIT2 binds strongly to regions in the promoter *ofnit-3* containing at least two GTAT elements, which are 30 bp apart from each other (Chiang and Marzluf 1994). NIT2 DNA-binding site II, which contains only a single GATA element, also plays a major role in *nit-3* gene expression (Tao and Marzluf 1998). Thus, all the NIT4 DNA-binding sites and GATA elements in the *nit-3* promoter serve a regulatory function for nitrate utilization. Interestingly, a direct interaction between NIT2 and NIT4 is essential for optimal *nit-3* expression (Feng and Marzluf 1998), but the NIT2 and NIT4 binding sites can be separated without affecting either the expression level or the precise regulation of the *nit-3* gene (Tao and Marzluf 1998).

In the *A. nidulans niaD-niiA* intergenic promoter region, all four NirA DNA-binding sites, 5'-CTCCGHGG-3' (H=A, C or T), have a regulatory function. It appears that site 1 is necessary for the inducibility of *niiA* alone, while sites 2, 3, and 4 act bidirectionally (Punt et al. 1995). The binding of NirA to the promoter is dependent on nitrate induction (Narendja et al. 2002). Of the ten AreA DNA-binding sites in the *niaD-niiA* intergenic region, the central clustered sites 5–8, which neighbor NirA DNA-binding site 2, are responsible for more than 80% of the transcriptional activity of both genes (Muro-Pastor et al. 1999). It appears that AreA is essential for chromatin remodeling (nucleosome repositioning) of the *niaD-niiA* intergenic region, and this process is independent from NirA-mediated transcription activation (Muro-Pastor et al. 1999). However, AreA is required for DNA-binding by NirA (Narendja et al. 2002). Thus, one function of AreA is

to open the chromatin structure in the promoter region to allow access for NirA and general transcription factors to initiate gene expression. The chromatin arrangement also appears important at other promoters. In the *A. nidulans amdS* and *fmdS* promoters, the AnCF CCAAT-binding complex and CCAAT sequence [see Brakhage et al. (1999) for a review] are necessary for formation of nucleosome-free DNase I-hypersensitive sites and determine basal transcription levels (Fraser et al. 2001; Narendja et al. 1999).

6 CONCLUSIONS

Great progress has been made in the past decade in our understanding of the combined action of global and pathway-specific regulatory circuits, which mediate the preferential utilization of nitrogen and carbon sources, especially in the model fungi *A. nidulans* and *N. crassa*. A major challenge of future research is to understand how binding of a global regulator to a promoter modulates gene expression and how pathway-specific regulators recruit co-activators to a promoter to initiate gene transcription. Other challenges include elucidation of the sensors of nutritional limitation or sufficiency and the signal transduction systems and inducer molecules that relay these signals to the key effectors of the transcriptional response. The powerful combination of genetics, biochemistry, molecular and cell biology approaches and the recent characterization of novel regulatory genes and mechanisms have provided the framework to overcome the missing links in nitrogen and carbon metabolic gene regulation.

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Fungal Nuclei and Chromosomes

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1 INTRODUCTION

Since George Beadle and Edward Tatum (1941) experimented on genetic controls of biochemical processes using the fungus *Neurospora crassa* as a model system, fungi have taken the center stage for research in genetics, molecular and cellular biology, and more recently molecular cytogenetics. The unicellular yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have gained wide acceptance by many to be the organism of choice besides *Neurospora* and *Aspergillus*. In retrospect, progress appears to have followed the innovation of new technologies. For example, the creation of temperature-sensitive mutations has made it possible to analyze genes and their functions, gene manipulations can create truncated genes whereby specific domain and its function may be analyzed, and the yeast two-hybrid system provides a powerful tool to study protein-protein interactions. More recently, fusion proteins, created with green fluorescent protein (GFP), yellow fluorescent protein (YFP), and the like, have allowed live subcellular localization of specific proteins in question under confocal and/or fluorescence microscopy. For more accurate subcellular localization under electron microscopy, Myc- or Protein-A conjugates, or Glutathion-S-transferase (GST) conjugates have been used in conjunction with indirect immunogold localization. In addition, fluorescence *in situ* hybridization (FISH) has allowed localization of specific DNA or RNA species, such as mRNA transport or localization of telomere clusters (see references in this chapter). In the last six or so years, there has been an enormous information explosion in the area of fungal nuclei and chromosomes, especially the nucleolus, the spindle pole body (SPB), the nuclear pore complex (NPC), the centromere and the telomere. It is most gratifying to have the privilege to provide a brief review on these findings, but it is also with a humbling sense of inadequacy that many works may not be included for the lack of time and space. For those authors that I missed, my sincere apologies.

*I wish to dedicate this chapter to Ms Iris Hseung who showed me, during my freshman year at National Taiwan University, that Biology is a beautiful area to explore.

2 A REFLECTION OF TIMES—A PERSONAL ANECDOTE

When I started my graduate studies, my professor H.J.Brodie asked me what I would like to do for research. At that time I was fascinated by studies of meiosis of *N. crassa*

(McClintock 1945; Singleton 1953) and *Sodaria fimicola* (Carr and Olive 1958), and there were no clear pictures of chromosomes of the Basidiomycetes. What attracted my attention the most was a review by Lindsey Olive (1953) that, without exception, every species of the Basidiomycetes ever studied had four chromosomes. Since Brodie's main interest involved the genus *Cyathus*, I offered to study the chromosomes of the Bird's Nest fungus. He told me to forget the idea because he said, "I had two internationally renowned cytologists look at it once and they could not see anything" instead, he wanted me to study the effect of light on fruiting and I did (Lu 1965). The rebellion inside me, however, wanted to try anyway. I first developed a staining technique using *Neurospora* (Lu 1962) as a test case. As I got *Cyathus stercoreus* to fruit, I prepared a slide, and was surprised to see some chromosome figures but the biggest prize of all was that I found, on a second look at the slide the following day, one nucleus showing 12 clearly separated chromosomes of *C. stercoreus* (Figure 1B) and we wrote a letter to Nature (Lu and Brodie 1962). When I cut out

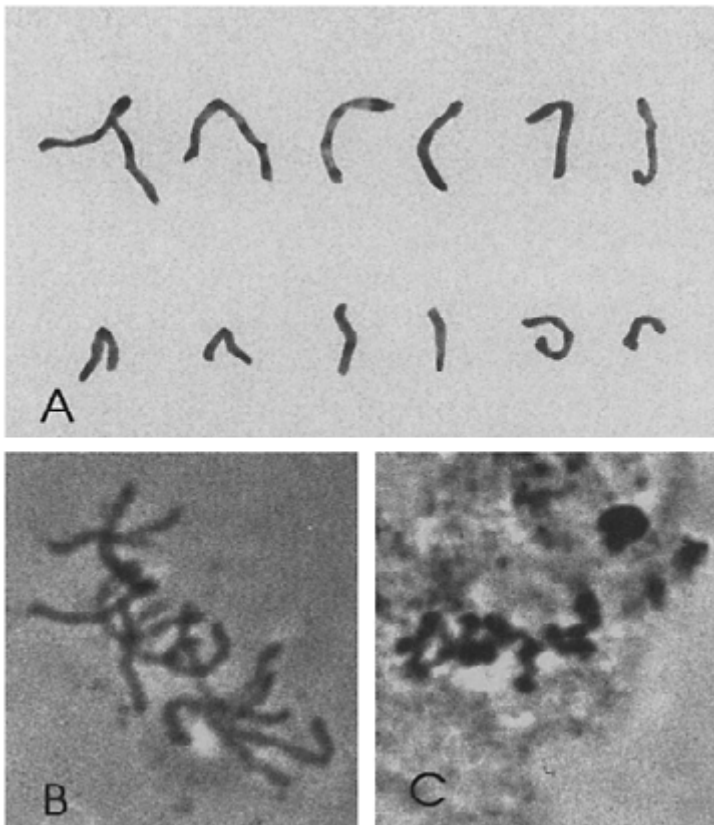


Figure 1 Chromosomes of *C. stercoreus*: A, 12 cut out chromosomes from Figure 1B and C, diakinesis showing quadrivalents for 1+2, 3+4, 5+6, 7+8, and 9+10. Reproduced from Lu (1964).

all 12 chromosomes and aligned them according to sizes (Figure 1A), I was intrigued by the similarity between chromosomes 1 and 2, 3 and 4, 5 and 6, etc. I decided to look for an answer. I discovered that chromosomes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10 formed quadrivalents (Figure 1C) and I realized that I was dealing with a tetraploid Basidiomycete (Lu 1964).

In the early sixties, it was commonly thought that filamentous fungi have no mitosis and that chromatin simply separated into two tear-drop-like lumps (Robinow and Bakerspigel 1965). However, I found prophase chromosomes with associated nucleolus and the SPB in the mycelium (Figure 3B). I also found metaphaselike figures with two SPBs as well as anaphase-telophase figures. I presented my findings in a conference. After my presentation, a professor of cytology glared down at me and said, “Don’t make any more chromosomes of fungi!” Times have changed and advances made and research in fungi is leading the way on many fronts in genetics, molecular and cellular biology, and recently in molecular cytogenetics. This is especially worth noting in analyses of different components of the nucleus and the chromosome. Fungal chromosomes, mitosis and meiosis have been reviewed (Lu 1996) and will not be repeated here. In this chapter, emphases will be given to some of the recent advances in nuclear organelles.

3 THE NUCLEOLUS

The nucleolus is the largest and most conspicuous body in the nucleus. It can be easily visualized under a light microscope by the help of a simple basic stain, such as acetocarmine or propiono-hematoxylin (Lu 1967a; Raju 1978). All fungi appear to have only one nucleolar organizer (NOR) located in a specific chromosome, which is known as the nucleolar chromosome. For example, it is chromosome 2 for *N. crassa*, (McClintock 1945), chromosome 10 for *Coprinus cinereus* (Pukkila and Lu 1985) and chromosome 12 for the yeast *S. cerevisiae*. A translocation that occurs at or near the NOR in *N. crassa*, such as T(IL-VL)OY321 and T(V-IV)AR33, could organize two nucleoli (Perkins et al. 1980; 1984). In the fission yeast *S. pombe*, where mitosis is intranuclear, the nucleolus persists throughout mitosis and eventually divided into the daughter cells (McCully and Robinow 1971). Under the squashed preparation, the NOR is often seen as a highly condensed body associated with the nucleolus. By FISH technology, the rDNA of *S. cerevisiae* exhibits changes according to the cell cycle, i.e., decondensed and dispersed in G1 and highly condensed as a distinct looped or linelike structure in M-phase (Lavoie et al. 2002).

3.1 Nucleolar Structure

With the help of electron microscopy, the nucleolus is seen to have substructures—the fibrillar component, the granular component (GC), the DNA containing component, and sometimes nucleolar vacuole. Actually, the subcompartmentalization was described in animal cells much earlier by Estable and associates in the early 30s and 50s using acid fixation and silver impregnation techniques. These authors described the “stranded network in a generally amorphous background” and they named it nucleolonema and pars

amorphous. Their finding was not well received initially and the skeptics criticized it as an artifact because of the “nasty” treatments that were used. At the International Symposium on Nucleolus held in Montevideo, Uruguay, Estable (1966) demonstrated the same network in stained preparations and in live specimens under phase contrast microscopy. In fact, the clearest demonstration of nucleolonema was that of corn pollen mother cells provided by Swift and Stevens (1966). In light of recent studies with *Locusta* oocytes by conventional electron microscopy as compared with the Miller spreads (Scheer et al. 1997), the nucleolonema in the intact nucleus really represent the “Christmas trees” showing rRNA transcription in progress so elegantly demonstrated by Miller and Beatty (1969).

3.1.1 Nucleolar Subcompartmentalization

The presence of nucleolonema has recently been confirmed by using cryofixation and cryosubstitution techniques in yeast, *S. cerevisiae* and *S. pombe* (Leger-Silvestre et al. 1997; 1999), an example of which is shown in Figure 2A. These authors demonstrated three major compartments of the nucleolus: the dense fibrillar component (DFC), the fibrillar center (FC) and GC. Moreover, they demonstrated that the FC contains rDNA by *in situ* hybridization (after RNase treatment) using the cloned 35S rDNA as a probe that was labeled with digoxigenin-11-UTP and the probe was detected by antidigoxigenin antibody-gold conjugate. They found that the gold particles are concentrated around the FC (Figure 2B). In the same token, they demonstrated that the nascent rRNA transcripts are mainly located in the DFC and never in the FC regions. This is proven by colocalization of three key rRNA transcription and processing proteins in the DFC regions: RNA polymerase I, which transcribes the pre-rRNAs, Gar1p, which is required for pseudouridylation of yeast rRNAs, and Nop1p, which is a component of the box C/D and plays a role in pre-rRNA methylation. The granular particles are preribosomes that eventually are transported to the cytoplasm through the nuclear pores. The same results were obtained in mouse cells using the 5' external transcribed spacer (5'ETS) as a probe for *in situ* hybridization, which showed that nascent rRNA transcripts are restricted to the DFC regions (Puvion-Dutilleul et al. 1997).

It is interesting that yeast mutants, deleted of the rDNA repeats (but carrying many copies of a plasmid that includes a single rDNA sequence), showed no FC and no nucleolonema (Trumtel et al. 2000). Obviously, the rDNA sequences carried by the plasmids are not silenced and do not organize the FC zones or the nucleolonema, probably because they are not in tandem repeats. It is now clear that the chromosomal localization of the NOR containing tandem repeat of 100–200 copies of rDNA sequence is not essential for the transcription and processing of rRNAs or for recruiting ribosomal proteins to the nuclear site (Nierras et al. 1997; Trumtel et al. 2000). However, the transcription by RNA pol I appears to be one of the main requirements for nucleologenesis (Trumtel et al. 2000).

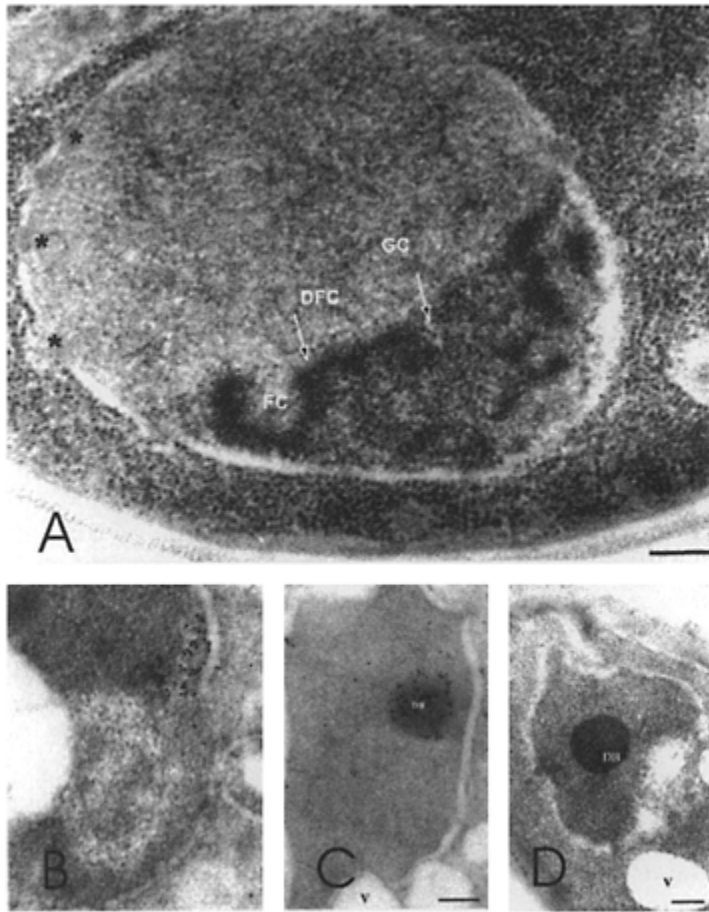


Figure 2 Electron micrographs of yeast nucleus showing nucleolar subcompartmentalization: A–B—from *S. cerevisiae* showing FC, GC, and DFC (A) and rDNA localization only in the FC (B); C–D—the nucleolus of *S. pombe* showing NDB from *gar-1* mutant cells, the truncated Gar2p is accumulated in the DB (C). The bar=1 μ m. Reproduced from Chromosoma 1997, 105:542–552 and Chromosoma 1999, 108:103–113, by copyright permission of Springer-Verlag.

3.1.2 The Nucleolar Dense Body (NDB)

The NDB or simply nucleolar body (NB) or dense body (DB) is another prominent fibrillar compartment that has not received due recognition. It was initially noted only in meiotic nuclei, in the wild-type *C. cinereus* (Lu 1984; 1996) and in yeast mutants (Horesh et al. 1979). It was never observed in mitotic nuclei. However, in a fission yeast

mutant with a truncated *GAR2*⁻ nucleolar protein gene, the nucleolus is reorganized to display a prominent NDB in mitotic cells and the defective gar2 protein is localized only in this NDB (Figure 2C and D); no rRNA is located in this compartment (Léger-Silvestre et al. 1997). In a separate case, budding yeast cells overexpressing the box C/D snoRNAs from multicopy vector also exhibit the additional NB structure in the nucleolus and the snoRNAs are seen to localize in this body as shown by electron microscopy and by *in situ* hybridization (Verheggen et al. 2001). These authors also showed similarities between NB of yeast and the Cajal or coiled body (CB), which exist in normal vertebrates (Gall 2000). These observations raise the question of whether or not the organization of this NDB compartment is related to some specific function(s), be it in meiotic or in mitotic nucleolus. For example, the central components of the synaptonemal complex were observed to assemble in the NDB in meiotic nucleolus (Haskins et al. 1971; Lu 1984).

3.2 Nucleolar Functions

The nucleolus is known to be the site of rRNA transcription and processing, i.e., the 35S rRNA was first transcribed from rDNA and then processed into 18S, 25S, and 5.8S rRNAs. These are combined with 5S rRNA and ribosomal proteins to form preribosomal particles for export to the cytoplasm (reviewed in Shaw and Jordan 1995). A nucleolar protein Rlp7p of the budding yeast is required for processing 27S rRNA into 5.8S and 25S rRNAs for the large subunit; defect in Rlp7p results in accumulation of 27S RNA in the nucleolus (Dunbar et al. 2000). Another essential and highly conserved protein, Zpr1p, has been identified in yeast. It is a cytoplasmic Zinc finger protein that accumulates in the nucleolus of proliferating cells and it may be involved in the regulation of 35S rRNA transcription (Galcheva-Gargova et al. 1998). It is possible that the nucleolus has other important functions that are not commonly known.

3.2.1 The Nucleolus May Be a Depot for mRNA Exports from Nucleus to Cytoplasm

The earliest hint that the nucleolus has functions other than ribosome biogenesis came from pioneering studies in the RNA transcription and transport from the nucleus to the cytoplasm in embryonic chick erythrocytes as well as in HeLa cells (Sidebottom and Harris 1969). These authors found that the RNA transport occurs only after the embryonic chick erythrocytes develop the nucleolus, suggesting that the nucleolus may play a role in the RNA transport. To confirm this hypothesis, they irradiated the nucleolus of chick erythrocytes as well as that of the HeLa cells with a microbeam of UV light and found that the RNA transport is impaired while the control cells (unirradiated, extranucleolar area of the nucleus irradiated, or even one of the two nucleoli irradiated) show normal or near normal RNA transport. The effect of the nucleolus on RNA transport is not limited to rRNAs, mRNA transport is also affected by UV irradiation of the nucleolus (Deak et al. 1972). These authors concluded that “some function located at or near the nucleolus is essential for the full expression of structural genes.” After nearly three decades of silence, molecular biology finally brought evidence to support their claims (Schneiter et al. 1995).

To study the role of the nucleolus in mRNA export from the nucleus to the cytoplasm, genetics and molecular cytology are the most powerful tools, especially valuable are the temperature sensitive mutations, and the yeasts are the organisms of choice. A large number of temperature sensitive mutants have been isolated that are defective in mRNA transport at the nonpermissive temperature. The genes are named *MTR* for Messenger RNA TRansport genes (Kadowaki et al. 1994). In addition to its known function for rRNA transcription, processing and export, the studies that follow have demonstrated that the nucleolus is decisively involved in the mRNA export.

The indication that the nucleolus plays a role in the mRNA export came from temperature sensitive (ts) *MTR* mutants that are defective in mRNA transport at 37°C. In *S. cerevisiae*, *MTR3* codes for a 28-KDa protein that appears to be a part of the mRNA transport machinery and *mtr3-1* is a ts mutant. At 37°C, *mtr3-1* failed to transport mRNAs to the cytoplasm resulting in an accumulation of polyA⁺ RNAs in the nucleus. By *in situ* hybridization using oligo-(dT)₅₀ as a probe, accumulation of polyA⁺ RNAs is restricted only to the nucleolus (Kadowaki et al. 1995). Since the wild-type cells show poor presence of polyA⁺ RNA in the nucleoplasm and in the nucleolus, the transport pathway is uncertain. However, a clearer picture is shown in the fission yeast, *S. pombe*, by electron microscopic *in situ* hybridization with an oligo (dT)₅₀ probe. Poly(A)⁺ RNAs are uniformly distributed in the chromatin-rich region, in the nucleolar region, as well as in the cytoplasm in control cells at 30°C, whereas poly(A)⁺ RNAs are accumulated in the fragmented condensed nucleolar regions in heat-shocked cells, suggesting that exports of mRNA normally pass through the nucleolus (Tani et al. 1996). Under severe heat-shock conditions, that impair RNA transport, accumulation will occur at the location where the last stop of the transport pathway is, i.e., the nucleolus. In *S. pombe*, *pim1*, which has a defect in mRNA transport at 37°C, also accumulates poly(A)⁺ RNA in the nucleolar region (Tani et al. 1996).

Further evidence came from the cellular localization of Mtr3p. A gene encoding Mtr3p with the HA epitope at its carboxy terminus was constructed in YTK306 strain (with tagged Mtr3p). By subcellular fractionation and by immunofluorescence microscopy, HA-epitope-tagged Mtr3p cofractionates with the nucleolar-protein Noplp, which is required for rRNA processing, and colocalizes with Rpa190p, which is the largest subunit of RNA polymerase I confirming that Mtr3p is localized in the nucleolus (Kadowaki et al. 1995). Since Mtr3p is involved in the mRNA export and it is located in the nucleolus, it seems reasonable to conclude that the nucleolus is a depot for mRNA exports.

3.2.2 The Nucleolus is the Site of Signal Recognition Particle (SRP) Assembly and Export

The secretory and membrane proteins are targeted to the endoplasmic reticulum (ER) membrane vesicles by the SRP because the nascent polypeptides of these proteins all carry a signal sequence. All SRP proteins, except one, are concentrated in the nucleolus as shown by fluorescence microscopy (Grosshans et al. 2001). Other RNA factors for RNA processing such as snRNA, snoRNA, and box C/D proteins are also known to use CB/NB and/or nucleolus as transitory station before reaching their target sites (Verheggen et al. 2001).

3.2.3 The Nucleolus May Hold Keys to Cell Cycle and Check Point Controls

In eukaryotes, the maintenance of genome integrity is achieved through the operation of cell-cycle checkpoints that ensure the completion of one cellular event before the initiation of another, and the checkpoint controls operate in mitotic as well as in meiotic cells (Hartwell and Weinert 1989; Murakami and Nurse 2000). In meiotic prophase, the main event is the synapsis of homologous chromosomes and genetic recombination between nonsister chromatids. Failure to complete one or both events, such as in *dmc1*, *zip1*, or *hop2* mutant of the yeast, *S. cerevisiae*, will lead to slowing down of meiotic progression and eventual arrest at pachytene; this control mechanism has been named pachytene checkpoint (Roeder and Bailis 2000). Genes that participate in the checkpoint controls can be identified because their mutations cause a bypass of pachytene arrest. A number of checkpoint proteins and their genes have been identified (Roeder and Bailis 2000). Here, only three proteins will be discussed, as they are closely associated with the nucleolus, and they are Dot1p (also known as Pch1p), Pch2p, and Sir2p; all three are silencing factors and all three are nucleolar proteins. In mitotic cells, using the help of the green protein, Dot1p-GFP, the signal is located in the nuclear region not stained by D API, i.e., the nucleolus. This nucleolus accumulation is predominant in 70% of the cell population. The nucleolar localization is confirmed by colocalization with the nucleolar protein Nsr1p (San-Segundo and Roeder 2000). In meiotic cells, Dot1p is uniformly distributed in all chromatin regions. In contrast, the meiosis-specific protein Pch2p and chromatin silencing factor Sir2p are localized predominantly in the nucleolus (San-Segundo and Roeder 2000). It is most interesting that the nucleolar localization of Pch2p is essential for the checkpoint to be operative (e.g., in *zip1* cells), when Pch2p is delocalized from the nucleolus (e.g., in *sir2* mutant cells), the checkpoint is inactive (Roeder and Bailis 2000). It appears that Dot1p is required for the nucleolar localization of Sir2p, which in turn is required for the nucleolar localization of Pch2p. This is shown in the *zip1 dot1* double mutant that bypassed the pachytene arrest and in which Pch2 proteins are mislocalized throughout the chromatin regions (San-Segundo and Roeder 2000).

The nucleolus may also have a role in the mitotic cell cycle controls. There are three transitions in mitotic cell cycles: G1 to S-phase, metaphase to telophase, and the exit from mitosis. The key players are genes for the cyclin-dependent kinases (CDKs) and their associated stage specific cyclins, namely, G1 cyclins (*CLN1*, *CLN2*, and *CZN3*), S phase cyclins (*CLB5* and *CLB6*), and mitosis cyclins (*CLB1* through *CLB4*), and the key kinase is p34^{cdc2/cdc28}, which is also known as maturation promoting factor (MPF): *Cdc2p* for the fission yeast and *Cdc28p* for the budding yeast (King et al. 1996; Visintin et al. 1997). Activation of this kinase system promotes the entry to mitosis and its inactivation leads to exit from mitosis (Visintin et al. 1999). The point of interest where the nucleolus plays a part is the exit from mitosis which, in the budding yeast, is controlled by two redundant mechanisms to inactivate Cdc28—by proteolysis of the mitotic cyclin Clb2p, and by accumulation of Cdc28p/Clbp inhibitor Sic1p that binds and inhibits the kinase activity (Visintin et al. 1997; 1999). Both processes are controlled by the anaphase promoting complex (APC) and its substrate-specific activator Cdc20p and

Cdh1p/Hct1p. Cdc20p controls the metaphase-anaphase transition and Cdh1p/Hct1p controls the exit from mitosis by degrading mitotic cyclin B (Visintin et al. 1997). In addition to APC, another set of genes is essential and these genes are collectively referred to as the mitotic exit network (MEN). They include *TEM1*, *LTE1*, *CDC15*, *DBF2/DBF20*, *CDC5*, *MOB1*, and *CDC14* (Morgan 1999). Inactivation of any one of these genes leads to cell-cycle arrest at late anaphase-telophase (Visintin et al. 1998). Of the MEN, Cdc14p is a dual specificity protein phosphatase and it may be the key player because over expression of Cdc14p causes degradation of Clb2p and accumulation of Sic1p (Visintin et al. 1998). Cdc14p is sequestered to the nucleolus by a nucleolar protein, Cfi1p (for Cdc14 inhibitor; Visintin et al. 1999), which is also known as Net1p (for nucleolar silencing establishing factor and telophase regulator; Shou et al. 1999). Net1p (Cfi1p) is an inhibitor and a negative regulator of Cdc14p (Visintin et al. 1999; Shou et al. 1999). Net1p (Cfi1p) forms a complex with both Cdc14p and Sir2p and the Cdc14p-Net1p-Sir2p complex is referred to as "RENT" for regulator of nucleolar silencing and telophase (Shou et al. 1999). Net1p is responsible for anchoring Cdc14p to the nucleolus, where its phosphatase activity is suppressed during G1, S, and M phase of the cell cycle (Shou et al. 1999; Traverso et al. 2001). To facilitate exit from mitosis, Cdc14p is released from the nucleolus and is activated during late anaphase/telophase, when the spindle is elongated, but Net1p remains in the nucleolus. Here the nucleolus functions as a sequestration center, by the help of Net1p, to prevent premature exit from mitosis (Bachant and Elledge 1999; Shou et al. 1999; Visintin et al. 1999).

What controls the release of Cdc14p from RENT? When the budding yeast was synchronized and the localization of Cdc14p was monitored at 10 min intervals, a clearer picture emerged. Cdc14p is transiently released from the nucleolus, not only in the wild type cells, but also in the majority of the MEN mutants, except in *cdc5-1* cells. By using this assay, a number of mutants were identified that are defective in the MEN-independent transient release of Cdc14p and these are *esp1*, *slk19*, and *spo12* as well as *cdc5-1*. Thus, the transient release of Cdc14p from the nucleolus at early anaphase requires *ESP1*, *CDC5*, *SLK19*, and *SPO12* that collectively are named the fourteen early anaphase release (Cdc FEAR) network of which *ESP1* may be the major player (Stegmeier et al. 2002). The FEAR network is not essential because mutation in any one of its components leads only to a delay in exit from mitosis. However, it is absolutely essential for release of Cdc14p and exit from mitosis in cells lacking *LTE1*, for *esp1-1 lte1Δ*, *lte1Δ slk19Δ*, and *spo12Δ lte1Δ* double mutants are inviable (Stegmeier et al. 2002). Taken all together, the sequence of events may be summarized as follow: (a) Cdc20p of APC controls the metaphase-anaphase transition, (b) the FEAR network initiates the transient release of Cdc14p from the nucleolus during early anaphase and activates its phosphatase activity that dephosphorylates Cdc15p and activates the MEN network, (c) MEN maintains Cdc14p in the permanent released state at late anaphase-telophase, (d) the active Cdc14p phosphatase dephosphorylates Cdh1p/Hct1p, Swi5p, and Sic1p which in turn inactivate mitotic cyclin, Clb2p, and protein kinase, Cdk1p, to allow exit from mitosis, and (e) after mitosis, Cdc14p is returned to the nucleolus.

3.2.4 Nucleolar Silencing and Its Control of Aging

The main DNA component of the nucleolus is the nucleolus organizer, which contains 100–200 tandem repeats of rDNA whose transcription by RNA polymerase I is essential for the structural integrity of this organelle (Trumtel et al. 2000). In general, there is no recombination activity in these ribosomal repeats as exemplified in *C. cinereus* (Cassidy et al. 1984). The understanding of this unusual phenomenon has come to light in recent years through genetics and molecular cytological research especially in *S. cerevisiae*. Yeast cells divide asymmetrically (the mother is larger than the daughter) and there is a finite number of daughters each mother can produce before senescence sets in and this is controlled by silencing factors. A number of genes have been identified that control silencing in the telomere, in mating type loci (HML and HMR), and in the nucleolus. They are the silence information regulators (SIR) 2–4. Furthermore, silencing in the nucleolus appears to be related to aging (reviewed in Guarente 2000; Johnson et al. 1998; Shore 2000). By careful experimentation, Kaeberlein et al. (1999) provided evidence that although all *SIR* genes are involved in silencing, *SIR2* appears to play a central role in yeast aging and it does so by preventing recombination in rDNA repeats. Recombination is the main cause of aging because it creates extrachromosomal rDNA circles (ERC), which are preferentially retained by the mother, as shown by elevated rate of ERC formation in *sir2* mutants, which have a reduced life span by 50% (Kaeberlein et al. 1999). Moreover, the Sir2p appears to be the limiting factor, because a *SIR2/sir2* heterozygous diploid has a shorter life span than the *SIR2/SIR2* homozygote, and an integration of a second copy of *SIR2* into the wild-type strain gave rise to an ~30% extension of life span (Kaeberlein et al. 1999).

Sir2p is immuno-localized to the nucleolus of yeast cells using affinity-purified antibodies against Sir2p. A strong Sir2p signal in the nucleolar domain is confirmed by its unambiguous colocalization with the nucleolus specific protein Nop1p (Gotta et al. 1997). A weaker punctate pattern of Sir2p signals is also found at or near the nuclear periphery. These signal foci are confirmed to be on the telomeres by virtue of their colocalization with the telomere binding protein, Rap1p (also known as repression activator protein). Thus, Sir2p is located mainly in the nucleolus. In a somewhat lesser way, it is also located in the telomeres. The association of Sir2p with the telomeres is lost when Sir3p, Sir4p, or Sir2p are mutated (Gotta et al. 1997). Sir3/4p are not DNA binding proteins, they are recruited to the telomeres by Rap1p (Moretti and Shore 2001). In the old cells, the Sir3p proteins are redirected to the nucleolus and the proper relocation to the nucleolus requires Sir2p as well as yet another protein Uth4p (Gotta et al. 1997; Sinclair et al. 1997). Kaeberlein et al. (1999) suggested that the telomeres may be holding a regulatory balancing act and that the relocation of the Sir proteins from the telomeres to the nucleolus in the old cells is to counter aging (Gotta et al. 1997; Johnson et al. 1998; Kaeberlein et al. 1999). Indeed, the Sir3p redirection to the nucleolus was shown to delay aging process (Sinclair et al. 1997).

4 THE SPINDLE POLE BODY

Before the electron microscope (EM), the SPB was variously called centrioles, centrosomes, and, after EM, kinetochore equivalent (KCE) or microtubules organizing center (MTOC). At the first international mycological congress, the term SPB was proposed. It is equivalent to the centrosomes of the animal cells in the functional sense. In fungi, it is an organelle closely associated with the nucleus (Figure 3 A and B) but located outside of the nuclear envelope (NE) (Girbardt 1971; Raju and Lu 1973). During mitosis and meiosis, the SPB divides at prophase and moves to the opposite poles at metaphase to help organize the spindle. In Basidiomycetes, the SPB is globular in shape before division, and is dumbbell-shaped during division and its morphology does not change in mitosis or in meiosis (Raju and Lu 1973). In the Ascomycetes, such as *Neurospora*, *Gelasinospora*, and *Podospora*, etc. the SPB changes from a small rod in meiosis I to a large plaque in division II and III in the ascus (Lu 1967a; Raju 1978; Raju 1980; Zickler 1971). In addition to spindle organization, the SPB also help delimit the spore formation at the end of division III in the ascus (Lu 1967b; Raju 1980).

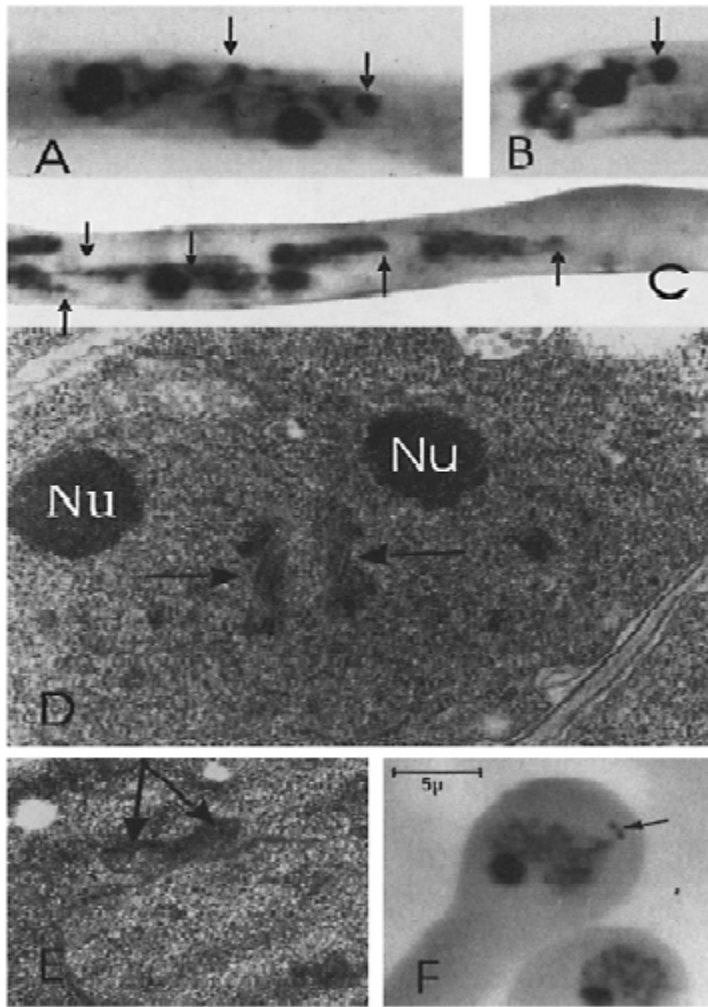


Figure 3 A–B—prophase nuclei in the mycelium of *N. crassa* each contains a nucleolus and an SPB (arrowed); C—*N. crassa* showing “horse tail” nuclei traveling to and from the hyphal tip with the SPB at the leading edge, traveling right to the tip (up arrows), and to the left on return flow (down arrows); D—electron micrograph (EM) of a basidium of *C. cinereus* during karyogamy showing two fusing haploid nuclei at the point of contact between two respective SPBs (arrowed); note the darkly stained chromatin (centromere clusters?) from each nucleus are situated symmetrically opposite each other; Nu, nucleolus; E—EM image of a diploid nucleus at diplotene showing divided SPBs; F—diplotene nucleus showing divided SPBs, reproduced from Raju and Lu (1973).

4.1 The SPB is At the Leading Edge of Nuclear Migration

When *N. crassa* was grown on a thin layer of agar medium on a microscope slide and examined live under a phase contrast microscope, rapid protoplasmic streaming to and from the hyphal tips was observed. When this slide culture was fixed and processed, the SPB was seen located at the tip of what is recently described as a “horse tail” nucleus suggesting that the SPB is leading the way with the nucleolus trailing behind (Figure 3A–C). Recent studies using fluorescence staining demonstrated that Kms1p, a component of the SPB of *S. pombe*, is located at the leading edge of the two traveling haploid nuclei to be fused at karyogamy (Niwa et al. 2000; Chikashige et al. 1994). Moreover, nuclear migration in the budding yeast is controlled by the core SPB component Nud1p as nuclear migration is defective in *nud1–2* mutant cells (Gruneberg et al. 2000). Thus, the SPB does lead the movement during nuclear migration.

Meiosis in the basidia is initiated by the fusion of two haploid nuclei. It is possible that the SPBs lead the movement to consummate karyogamy in *C. cinereus*; Figure 3D depicts the two SPBs from two haploid nuclei positioned face to face just before nuclear fusion. The condensed chromatin symmetrically placed near the SPBs may be the centromere clusters (Figure 3D). The diploid nucleus should have two SPBs and this is observed in *C. cinereus* at diplotene by electron and light microscopy (Figure 3E and F; Raju and Lu 1973).

4.2 The SPB is Always Associated with the Chromosomes

The SPB is not just a part of the mitotic or meiotic spindle mechanism, it is intimately associated with the chromosomes even during interphase and prophase. Using a whole mount electron microscopy, a rare image was captured where chromosomes at prophase III of *N. crassa* were found to be intimately associated with the divided SPBs (Figure 4). In mitotic cells, the association is between the SPB and the centromere. However, during meiosis, nuclear reorganization occurs and the centromere association is replaced by the telomere clusters (Trelles-Sticken et al. 2000).

4.3 The SPB Plays a Role in MEN Regulation

Recent advances in molecular cytology, using GFP (or YFP) fluorescence microscopy, or using *in situ* immunofluorescence microscopy, has made it possible to localize proteins in the SPB during mitosis. The results are most revealing. In *S. cerevisiae*, many of MEN components are localized to the SPB at anaphase/telophase and the SPB localization of Tem1p, Lte1p, Cdc15p and Dbf2p depends on the SPB core protein Nud1p. The recruitment appears to be orderly; Tem1p is recruited by Nud1p, Cdc15p by Tem1p, and Dbf2p by Cdc15p (Visintin and Amon 2001; Xu et al. 2000). Bub2p-Bfa1p/Byr4p complex localizes to SPBs constitutively by directly interacting with Nul1p (Xu et al. 2000). The SPB is a multilayered structure. It consists of an outer plaque, a central plaque and an inner plaque. The outer plaque is responsible for organizing cytoplasmic (astral) microtubules while the inner plaque is for nuclear microtubules. The central plaque is inserted in the NE [reviewed in Pereira and Schiebel (2001)]. It is now clear that the SPB

has a role in MEN regulation and in the control of exit from mitosis.

It is interesting to note that, for its proper function, the SPB requires small GTPase Tem1p, its GDP/GTP exchange factor Lte1p and the GTPase activating protein (GAP) complex Bub2p-Bfa1p/Byr4p together with protein kinases Cdc15p and Dbf2p (Gruneberg et al. 2000; Visintin and Amon 2001). Taken together, the SPB controls of nuclear migration and of exit from mitosis may be energy requiring processes.

5 THE NUCLEAR PORE COMPLEX (NPC)

Like all eukaryotic cells, the fungal nucleus is enclosed by a double membrane system, called the NE, which differs from other double membrane systems in having many NPCs embedded in it. The NPC is a very specialized organelle, which is the gateway for all nucleocytoplasmic transports. NPCs of the budding yeast have been isolated and examined by transmission and by scanning electron microscopy (Rout and Blobel 1993). The NPC has an overall diameter of 97 nm, which is composed of eight interconnected spokes that span perpendicularly between the outer and inner nuclear membranes and arranged in an 8-fold symmetry forming the central ring. The center of this ring is the gate channel (or transporter). The radial length of each spoke is ~26 nm. Sitting over the gate channel is a plug of ~35 nm in diameter that may have an aperture. The height of the NPC is ~30 nm, and the molecular mass is ~66 MDa. On the inner nuclear membrane is embedded a nucleoplasmic coaxial ring, attached to its periphery are eight long fibers of ~40 nm in length. These extend into the nucleoplasm to form a “nuclear basket.” On the outer nuclear membrane, at the cytoplasmic face, is embedded another coaxial ring from which eight short fibers protrude into the cytoplasm [Rout and Blobel 1993; reviewed by Fabre and Hurt (1997)]. From purified NPCs, about 30 nuclear pore proteins have been identified, which are collectively named nucleoporins or nups, only three of these are pore membrane (Pom) proteins (Rout et al. 2000). A specific subset of these contains Phe-Gly repeats (FG nups) and appears to constitute most of the filamentous structures emanating from the NPC [cited by Rout et al. (2000)]. These nups have been found by immunolocalization to be symmetrically located between the nuclear and cytoplasmic faces; a few are biased toward nucleoplasm (Nup145N) or cytoplasm (Nup100p and Nup116p). Two FG nups (Nup1p and Nup60p) are exclusively nucleoplasmic that are significantly outward from the NPC mid-plane. These two proteins may constitute the nuclear basket. On the other hand, Nup159p, Nup42p, and Nup82p are exclusively cytoplasmic and they may be the cytoplasmic filaments. It is possible that these FG-nups provide docking sites for nucleocytoplasmic trafficking. The architecture of the NPC is shown in Figure 5 (Rout et al. 2000).

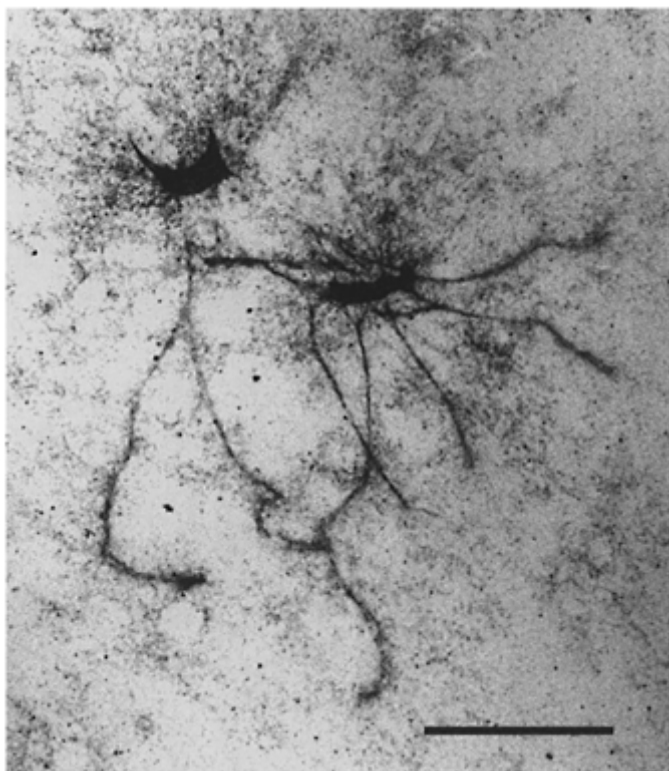


Figure 4 Whole mount electron microscopy of a prophase III nucleus from the ascus of *N. crassa*, showing divided SPBs and associated 7 chromosomes. The bar=10 μ m. (B.C.Lu, unpublished photo.)

Nucleocytoplasmic exchange of macromolecules takes place through the NPCs and this transport is an energy dependent process. Many proteins are involved and these include Ran-GTP, some soluble factors for mRNA export (e.g., Mex67p, Mtr2p, Yrb2p), import/export receptors (e.g., Xpo1p), and proteins that shuttle their cargoes from the nucleus to the cytoplasm through the NE (e.g., the DEAD-box protein, Rip1p, etc). The DEAD-box protein Dbp5p (Rat8p) of the budding yeast is a highly conserved family of proteins and it is essential for mRNA export (Hodge et al. 1999). ProtA-Dbp5p is cytoplasmic and associated with the cytoplasmic fibrils (Strahm et al. 1999). In contrast, ProtA-Rip1p was detected in the nucleoplasm as well as on both sides of the NPC, in association with the nuclear basket and the cytoplasmic fibrils. It is interesting to note that its localization is not changed under the heat shock stress at 42°C (Strahm

microtubule per chromosome of the budding yeast (Peterson and Ris 1976), the same may be true for other fungi (Kubai 1975). The number of centromeres per haploid cell for any given species is highly controlled and addition of extra cloned copies is toxic to haploid yeast (Futcher and Carbon 1986; Runge et al. 1991).

6.1 Centromere Organization

The centromere sequences have been analyzed in the budding yeast (Bloom et al. 1983; Clarke and Carbon 1995). The centromere sequence of *S. cerevisiae* is the simplest among fungi. It contains a 220–250-bp sequence, which is divided into three functionally distinct sequences, namely CDEI, CDEII, and CDEIII. The CDEII is an A+T rich central region of 78–86 bp. A deletion of all or part of it leads to increased nondisjunction of chromosomes in mitosis and premature separation of sister chromatids in meiosis (Gaudet and Fitzgerald-Hayes 1989). CDEIII is the most critical one. A point mutation of the central C to T in the inverted repeat will abolish the centromere function and structure (Kenna et al. 1988). The centromere DNA sequence of *S. cerevisiae* is very small compared to that of the fission yeast *S. pombe* (Clarke and Baum 1990; Murakami et al. 1991), and *N. crassa* (Centola and Carbon 1994), the organization of which may be closer to those of the higher eukaryotes. It contains repeated sequences reminiscent of the pericentric heterochromatin of higher eukaryote [see Lu (1996) for diagrams].

6.2 The Kinetochore is a Complex Organelle

Although the centromere is simple, the kinetochore is complex with a large number of centromere binding proteins and associated proteins. It is made up of three distinctive compartments: the inner, the central, and the outer kinetochore [see review by Cheeseman et al. (2002)]. The centromere DNA of *S. cerevisiae* is organized into centromere specific nucleosomes that contain specialized histone H3-like proteins, Cse4p, which is a homolog of CENP-A. In the inner kinetochore, the most important one of the centromere binding proteins is the CBF3 complex (Ndc10p, Cep3p, Ctf13p, and Skp1p), which binds to CDEIII; without it, the kinetochore function is abolished. Other inner kinetochore proteins are Cbf1p/CENP-B, and Mif2p/CENP-C [see review in Cheeseman et al. (2002)]. There are many more proteins associated with the inner kinetochore proteins and these form the central kinetochore: the Ctf19 complex (Ctf19p, Mcm21p, and Okp1p), the Ctf3 complex (Ctf3p, Mcm16p, and Mcm22p) and the Ndc80 complex [Ndc80p, Spc24p, Spc25p, and Nuf2p; reviewed in Cheeseman et al. (2002)]. The most important one is the Ndc80 complex, without it the kinetochore function is defective.

6.3 Kinetochore Involvement in Spindle Organization

The function of the kinetochore is the organization of spindle microtubules during nuclear division and this is the function of the outer kinetochore where microtubules are attached by the help of microtubule-associated proteins (MAPs). The first kinetochore-associated MAP to be identified is Dam1p that is localized to the periphery of the SPB

and clustered on the mitotic spindle as shown by immuno-electron microscopy suggesting that Dam1p is a component of the kinetochore. This is indeed the case as Dam1p is colocalized with the inner kinetochore component Ndc10p to the kinetochore on chromosome spreads by immunofluorescence microscopy (Jones et al. 2001). Dam1p plays a role in kinetochore attachment to the metaphase spindle and in anaphase spindle elongation (Jones et al. 2001). A large number of proteins are associated with Dam1p and this multiprotein complex is known as the Dam1p complex of the outer kinetochore (Dam1p, Duo1p, Dad1p, Spc19p, Spc34p, Dad2p, Ask1p, Dad3p, and Dad4p; Cheeseman et al. 2001; Janke et al. 2002; Cheeseman et al. 2002). It is also known as DASH (Dam1p, Duo1p, Ask1p, Spc34p, Spc19p, and Hsk1p) complex (Li et al. 2002). This complex is localized to kinetochores in an Ndc10p- and Ndc80p-dependent manner [Enquist-Newman et al. 2001; Jones et al. 2001; see review in Cheeseman et al. (2002)]. Ask1p has been analyzed in detail and it was found to play an essential role in mitosis (Li et al. 2002). Unlike Dam1p, Ask1p is localized along the spindle as well as in kinetochores. It exhibits a single dotlike GFP signal in G1 cells and two dot signals in division stage near the spindle pole bodies when the spindle is elongated. The dotlike localization depends on the presence of the inner kinetochore CBF3 subunit Ndc10p, because in *ndc10-1* mutants, the dotlike signals are mislocalized into a diffuse nuclear staining although the staining of Ask1p along the spindle is still present (Li et al. 2002). Further, Ask1p is unique among kinetochore proteins. It specifically associates with centromeres and its loading to kinetochores is dependent upon an intact spindle, because centromere binding of Ask1p is inhibited by nocodazole and this inhibition is reversible. It appears that the spindle microtubules are responsible for loading Ask1p to newly replicated kinetochores (Li et al. 2002). It is most interesting to note that *ask1-3* mutants undergo massive nondisjunction without sister chromatid separation suggesting a defect in the spindle-kinetochore interaction that leads to failure in setting up a bipolar attachment (Li et al. 2002). Two more proteins were recently found to promote chromosome bi-orientation in mitosis (Tanaka et al. 2002). Ip11p is the only Aurora kinase in *S. cerevisiae* and it forms a complex with Sli15p, a yeast ortholog of INCENP protein of animal cells. Both *ip11* and *sli15* mutants shows high degree of chromosome missegregation with a strong bias of paired sister kinetochores connected to the old SPB in the bud of *S. cerevisiae* (Biggins et al. 1999; He et al. 2001; Tanaka et al. 2002). It is possible that Ip11pSli15p complex is required to disrupt the spindle connection between the kinetochores and the old SPB to allow bi-orientation when new kinetochores are replicated (Tanaka et al. 2002). This hypothesis is consistent with the observations that Ip11p-Sli15p complex is colocalized with CBF3 subunit Ndc10p for much of the cell cycle except for the metaphase-anaphase transition when Ip11-GFP signals appear to move away from the kinetochores (Tanaka et al. 2002).

7 THE TELOMERES

Eukaryotic chromosomes are capped with a very specialized chromatin structure known as telomere. The telomere has two important functions: (a) to protect the integrity of the chromosome end, and (b) to ensure complete replication of the chromosomal DNA

[reviewed in Blackburn and Szostak (1984), Zakian 1996, and Blackburn (2001)]. The telomere sequence was first identified in *Tetrahymena* as $(3'\text{CCCCAA}5')^n/(5'\text{TTGGGG}3')^n$ (Blackburn and Szostak 1984). Similar but not identical hexanucleotide repeats are found in other eukaryotes. In fungi, *S. pombe* has $5'\text{-TTACAGG-}3'$, *N. crassa* has a hexanucleotide repeat of $5'\text{TTAGGG}3'$, a sequence it shares with human and other animals (Schechtman 1990). The yeast *S. cerevisiae* is different because its telomere sequence is degenerate; its sequence may be defined as $\text{G}_2\text{-}_3(\text{TG})_{1-6}$. Besides the hexanucleotide telomere sequence, there are subtelomeric (X and Y') sequences that are also important for the maintenance of telomere integrity (Zakian 1996).

7.1 Telomere Capping

Telomeres are capped and capping requires ~80–100bp of genuine TG repeat sequence (Grossi et al. 2001). Uncapping of telomeres by incorporation of mutant DNA sequences leads to telomere deregulation and detrimental cellular consequences, like defects in cell division, in the yeast *Kluyveromyces lactis*. However, recapping can be achieved by addition of only a few wild-type telomeric repeats to their ends (Smith and Blackburn 1999). The proper telomeric TG repeat sequences are required for the binding of the repressor activator protein 1 (Rap1p), an essential telomere binding protein, to complete the cap formation where Rap1p binds specifically to the duplex telomeric repeats. The Rap1p binding is possible even when the repeat sequences are in the wrong orientation so long as the end is capped with the correct sequence orientation. It is interesting that the wrong orientation can be corrected by the Rad52-dependent recombination pathway (Grossi et al. 2001). It appears that Rap1p binding requires a minimum of two consecutive telomeric repeats having a 16bp center-to-center spacing (Wahlin and Cohn 2002). Unlike human and fission yeast, where Rap1p is recruited by TRF proteins and Taz1p respectively, Rap1p of *S. cerevisiae* binds directly to the telomere repeats (Chikashige and Hiraoka 2001). The telomere sequence is further capped by other telomere binding proteins, namely, Ku proteins, Cdc13p, Stn1p, Ten1p, for the budding yeast [Grandin et al. 2001; Lin et al. 2001; Pennock et al. 2001; Wang et al. 2000; reviewed in Blackburn (2001)], and Pot1p and Ku proteins for the fission yeast (Baumann and Cech 2000; Baumann and Cech 2001).

7.2 Telomere Maintenance

Because of the biochemical nature of the DNA polymerases, the leading strand replication needs to be primed by RNA primer. Therefore, a few nucleotides will be lost in each round of replication. This is overcome by a specialized RNAenzyme called telomerase (or telomere terminal transferase). This enzyme carries its own RNA template for the G-rich strand extension by a reverse transcription process. This enzyme is encoded by genes *EST1-3* (for the polypeptide component) and *TLC1* (for the RNA template). Mutations or deletion of these genes will cause ever-shorter-telomere phenotype and eventual senescence, known as replicative senescence. It was found that yeast cells lose ~200nt (in *tlc-Δ*), and ~180nt (in *yku10Δ*) in 60–80 generations, or three successive restreaks of a colony (Förstemann et al. 2000). However, in the absence of

telomerase, two parallel bypass pathways involving *SGS1* and *RAD50/51* genes could generate survivors (Johnson et al. 2001).

Cdc13p is a single-strand and sequence-specific telomere binding protein of *S. cerevisiae* (Lin et al. 2001; Pennock et al. 2001; Qi and Zakian 2000) while Pot1p is for *S. pombe* (Baumann and Cech 2001). Cdc13p is an essential protein as *cdc13*-null mutants are lethal (Pennock et al. 2001). Cdc13p interacts with Est1p of telomerase, with DNA polymerase α , Pollp (Qi and Zakian 2000), and with telomere binding protein Stn1p, which is a primary effector of chromosome end protection (Pennock et al. 2001; Wang et al. 2000). Another telomere binding protein, Ten1p, was found to associate physically with Stn1p and it was able to suppress the mutant phenotype of *stn1*. Ten1p is also an essential protein as *ten1*- Δ is lethal. Like *stn1* mutants, some *ten1* mutants (*ten1*-16,-31) cause accumulation of single stranded DNA in telomeric regions of the chromosomes and they trigger DNA damage checkpoint and G2/M arrest, while others (*ten1*-3,-6,-13) cause telomere elongation, the expression of which is telomerase dependent (Grandin et al. 2001). Taken all together, it appears that the function of Cdc13p is to help recruit protein complexes, like Cdc13p-Stn1p-Ten1p, essential for both telomere length regulation and end protection (Grandin et al. 2001; Pennock et al. 2001).

7.3 Telomere Position Effect (TPE)

In addition to telomere length regulation and end protection of eukaryotic chromosomes, the telomere has yet another property, reminiscent of heterochromatin, genes adjacent to telomeres are transcriptionally repressed. This silencing effect is known as TPE, which is mediated by the SIR proteins, Sir2p, Sir3p, Sir4p (Bruin 2000; Laroche et al. 2000; Moretti and Shore 2001). The Sir2/3/4 protein complex is recruited to the chromosome sites by the telomere binding protein Rap1p that binds directly to the duplex telomere repeats. Experimental data showed that both Sir3p and Sir4p interact directly and independently with partially overlapping regions of the carboxyl terminus of Rap1p (Moretti and Shore 2001) and that the Sir protein complex interacts with the N-terminal tails of histones H3 and H4 in the subtelomeric nucleosomes (Hecht et al. 1995) where telomeric chromatin is hypoacetylated at each acetyltable histone H3/H4 lysine residue and this hypoacetylation is SIR dependent (Bruin 2000). Sir2p is shown to be a NAD-dependent histone deacetylase (Guarente 2000; Shore 2000) and hypoacetylation is directly correlated with transcriptional silencing (Bruin 2000). These authors also provided evidence that heterochromatin occludes the promoter, preventing its access to transcription factors. Cytologically, by double immunolocalization and FISH, Sir2/3/4p and subtelomeric sequences are found to colocalize in 6–10 perinuclear foci suggesting that these are sites of Sir-mediated silencing (Laroche et al. 2000). It is interesting to note that the telomere repeats are only ~300–400 bp from the chromosome end and yet Rap1p and Sir2/3/4 proteins were found at distances as far as 2–4kb from a telomere end as shown by chromatin immunoprecipitation studies (Moretti and Shore 2001). This can be explained on the basis of telomere folding back or more likely on the basis of protein-protein cooperative bindings (Moretti and Shore 2001). Thus, the subtelomeric regions are heterochromatic and the heterochromatin can spread its influence on the adjacent genes, reminiscent of the classical variegated position effect found in *Drosophila*.

7.4 Telomeres Play a Role in Homologous Chromosome Pairing in Meiosis

Telomeres play an important role in homologous chromosome pairing during meiosis. Earlier, I speculated that telomeres attached to the nuclear envelope like casters rolling on a two dimensional surface in search for homologous contacts (Lu 1996). Now clear evidence has been documented (Niwa et al. 2000; Rockmill and Roeder 1998; Trelles-Sticken et al. 2000) and the best is provided by Trelles-Sticken et al. (2000) who used double immunolabeling of SPB components with fluorescein and of meiotic telomeres with antibodies against HA-tagged Ndj1p with rhodamine in synchronously induced meiosis in *S. cerevisiae* where Ndj1p has previously been identified as a meiosis specific telomeric protein (Chua and Roeder 1997). At early meiosis, telomeres were seen in peripheral rimlike distribution. This is followed by the so-called bouquet stage with telomeres clustering around the SPB, and finally at a later stage in pachytene showing dispersed telomere signals separate from the SPB. This clustering of telomeres is Ndj1p dependent as the bouquet formation is impaired in *ndj1Δ* mutants. This is the first telomeric protein identified to be required for meiosis specific bouquet formation (Trelles-Sticken et al. 2000). Here Ndj1p (also known as Tam1p) may be that caster I suggested previously. Telomere-mediated chromosome movements in meiotic prophase have also been suggested to facilitate homologous chromosome pairing by Yamamoto and Hiraoka (2001).

The bouquet formation has also been documented in *S. pombe* (Chikashige et al. 1997; Niwa et al. 2000), and it takes two steps to complete. First, in premeiotic haploid nuclei, centromeres are intimately associated with the SPB while telomeres are located at the opposite end of the nucleus. During the mating reaction, by the influence of the mating pheromone, telomeres become associated with the SPB. Second, centromeres detach from the SPB when conjugation and nuclear fusion are completed (Chikashige et al. 1997). Here it is evident that the interaction between the telomere and the SPB plays a key role in homologous pairing, and the telomere binding protein Taz1p of the fission yeast and SPB component Kms1p are required for telomere clustering and bouquet formation (Cooper et al. 1998; Niwa et al. 2000). The bouquet stage has been observed by cytologists in plants and animals for a long time, and it is only now that its significance has become known. By careful stage-by-stage analysis of telomere clustering in corn, Bass et al. (1997) showed that telomere clustering persisted through zygotene and early pachytene with the nucleolus located adjacent to the cluster. This could be the synizetic knot cytologists observed and never understood (Lu 1996).

8 CONCLUSIONS

Recent advances in FISH, fluorescence microscopy using fusion proteins (e.g., GFP) and indirect immunolabeling of epitope-tagged proteins in electron microscopy have made it possible to examine the cellular localization of DNA, RNA, and proteins of interest where localization may reveal structural organization or site(s) of action. Genetically, creation of temperature sensitive mutants by DNA manipulation and performing complementation with two hybrid system analysis have allowed in depth analyses of gene

interactions as well as protein-to-protein interactions. Here the combination of molecular, cellular and genetic technologies has afforded a super powerful tool for life sciences research, some of which are high lighted in this chapter.

The biggest surprise is the finding that the nucleolus has varied functions not known before. Besides its known function for ribosome biosynthesis and its subcompartmentalization, it may be a transitory depot for mRNA export, it may hold keys to cell cycle and checkpoint control for mitosis and meiosis, and very importantly, it controls nucleolar silencing and aging.

The SPB is not just a nucleating site for spindle organization, it is intimately involved with mitosis exit network (MEN) as many of MEN components are localized to the SPB, and its position in the bud determines the time of exit from mitosis. The NPC is the gateway for nucleocytoplasmic trafficking. It is a very complex organelle. It includes—30 nucleoporins (or nups) that are intimately associated with the protein imports and ribonuclear protein particle (RNP) exports. The cytoplasmic filaments and the nuclear basket are the docking sites for importin α and exportin β and their cargoes.

The centromere of the budding yeast is simple as compared to that of the fission yeast, but the kinetochore is a very complex organelle. It is responsible for connecting the spindle microtubules to both the old and the new SPB to ensure bi-orientation of sister chromatids at metaphase. The telomere may be considered an organelle. Telomere capping, silencing and end maintenance are vital to the life of the cell. Most importantly, telomeres play a key role in homologous chromosome pairing in meiosis.

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