Perspectives

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Chlamydomonas: The Cell and Its Genomes

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GREAT strides in understanding fundamental processes in eukaryotic cells have come from genetic studies on budding and fission yeasts and other fungi. The exquisite experimental tools developed for use with these systems have made them the undisputed first choice for studying a variety of metabolic pathways and other cellular processes, such as cell cycle control, cell signaling, and the regulation of gene expression. However, these systems represent only a small fraction of the immense diversity of form and function found in eukaryotic systems (see Margulis and Schwartz 1982). To understand this diversity at the molecular level, experimentalists must tap the vast array of unicellular eukaryotes, many of which provide insights into biological processes that cannot be studied in the usual fungal systems. Recent advances in the genetic analysis of protistans, including Dictyostelium (*e.g.*, Kuspa and Loomis 1996); Physarum (reviewed by Burland *et al.* 1993); and the ciliates Tetrahymena (*e.g.*, Brickner *et al.* 1996) and Paramecium (reviewed by Preer 1997), have provided the tools for studying fascinating cellular processes exhibited by these systems. In this article we will focus on the unicellular eukaryote *Chlamydomonas reinhardtii* by looking back at recent technical advances and ahead to the prospect of functional genomics being applied to this powerful model system.

WHY CHLAMYDOMONAS?

Chlamydomonas occupies an important niche in the world of eukaryotic cell biology. It is a unicellular eukaryote with well-understood haploid genetics, like yeast, but unlike yeast it has both flagella and a chloroplast. Thus, it is possible to use Chlamydomonas as an experimental system to understand flagellar motility and basal body function, as well as chloroplast assembly and function, using the powerful techniques of microbial genetics.

The genetic analysis of the flagellar apparatus of Chlamydomonas, including the flagella, basal bodies, and connecting structures, has been developing for a number of years. The flagella are not necessary for cell growth, but they are required for motility, providing a powerful screen for mutations that affect these organelles specifically. To date, more than 80 different mutant loci have been shown to affect the assembly and function of the flagellar apparatus. Biochemical analyses of wild-type and mutant flagella have demonstrated that the flagellar axoneme alone is composed of more than 200 proteins, most of which appear to be unique to the axoneme (reviewed by Dutcher 1995). The basal bodies and their connecting structures are at least as complex as the flagella, structurally and biochemically.

Early investigators studying photosynthesis also took advantage of the fact that this process is not essential in Chlamydomonas. Probably the single most important experimental opportunity offered by Chlamydomonas in the analysis of photosynthesis is its ability to grow either photosynthetically or on a carbon source. Because Chlamydomonas is a facultative heterotroph, mutations affecting genes required for photosynthesis are not lethal but conditional, requiring acetate for growth. Hundreds of such "ac" mutants have been isolated, and at least 50 have been placed on the genetic map (Harris 1989). Many of these genes probably identify proteins important for chloroplast assembly or function in higher plants as well as in Chlamydomonas.

We shall return at the end of this essay to look more deeply at the biological issues presented by the flagella, basal bodies, and chloroplasts of Chlamydomonas. First, it is informative to summarize the past, present, and possible future of the genetics of this protist.

Tetrad analysis, which has been so useful in the development of yeast and fungal genetics, was first described in Chlamydomonas (Pascher 1918). The first genetic evidence that chloroplasts maintained a separate genome and that recombination could occur between

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chloroplast genomes during mating was obtained in Chlamydomonas (reviewed by Sager 1960). The first transformation of a chloroplast genome was obtained in Chlamydomonas (Boynton *et al.* 1988), as was the first transformation of the mitochondrial genome of a photosynthetic eukaryote (Randolph-Anderson *et al.* 1993). In fact, Chlamydomonas is the only organism in which transformation techniques have been developed for all three genomes: nuclear, chloroplast, and mitochondrial.

CLONING GENES IDENTIFIED BY MUTANT PHENOTYPES

To take advantage of the power of Chlamydomonas genetics, a number of techniques have recently been developed to clone the genes in which mutations produce interesting phenotypes. The most widely used method requires that the new mutations be generated by the insertion of a known sequence, either a plasmid or a transposable element, which can then be used as a hybridization probe to clone the gene of interest (Tam and Lefebvre 1993; Davies *et al.* 1996). A second method for cloning genes involves complementation of mutant phenotypes by transformation with libraries of wild-type DNA to rescue mutant phenotypes. In the near future, the molecular mapping of the entire genome should make positional cloning the method of choice for the molecular identification of most genes of interest. These three cloning strategies will be discussed in turn.

Insertional mutagenesis using plasmids: Despite a great deal of effort in many laboratories, reliable transformation of the nuclear genome of Chlamydomonas was not achieved until selectable marker genes from Chlamydomonas itself were cloned. When such intraspecific selectors did become available, taking the place of generic selections, such as neomycin resistance, transformation was quickly achieved by several different laboratories using different selectable markers (Debuchy *et al.* 1989; Fernandez *et al.* 1989; Kindle *et al.* 1989; Mayfield and Kindle 1990). Taking advantage of an earlier report from yeast, Kindle (1990) developed a simple and highly efficient method of transformation by simply vortexing cells with glass beads. It was clear from the first analyses that transformation almost never involves the homologous recombination of transforming DNA into the nuclear genome, even if a direct selection is applied for the inactivation of a target gene (Nelson and Lefebvre 1995). Chlamydomonas is similar to other photosynthetic eukaryotes in the extreme difficulty encountered in efforts to demonstrate gene disruption by homologous recombination during transformation. By contrast, in other unicellular protists such as Dictyostelium, Tetrahymena, and Physarum, insertion of transforming DNA by homologous recombination can be achieved, making it possible to disrupt and alter genes *in vivo* (*e.g.*, DeLozanne and Spudich 1987; Burland and Pallotta 1995; Hai and Gorovsky 1997). The absence of homologous gene disruption during transformation of the nuclear genome of Chlamydomonas is somewhat ironic; integration of transforming DNA into the chloroplast genome occurs almost exclusively by homologous recombination. Thus, the genes in the chloroplast genome can be mutated in preplanned ways by transformation with appropriately designed plasmids (Newman *et al.* 1991).

As stated above, the random insertions accompanying transformation of the nuclear genome have made it possible to clone genes of interest by insertional mutagenesis. In the first insertional mutagenesis experiments, we were surprised by the very large fraction of random transformants that exhibited defects in flagellar function. Of 3000 randomly picked transformant strains, 80 had defective flagellar motility (Tam and Lefebvre 1993). Among these strains were mutants affected in flagellar assembly (flagella-less or long flagella), in flagellar function (paralyzed), and in flagellar number. Even though several hundred gene products are involved in the assembly and function of the flagella and basal bodies, finding 80 motility mutants in 3000 transformants was surprising. When some of the affected genes were cloned and characterized, it became clear that the insertion of plasmid DNA is frequently accompanied by the deletion of up to tens of kilobases of genomic DNA at the site of insertion (*e.g.*, Smith and Lefebvre 1996). These large deletions may account for the high frequency of flagellar mutants obtained among the transformants; many regions of 10 to 20 kb must contain at least one gene required for flagellar motility, but no genes required for cell survival. Owing to these large deletions, the identification of the affected gene in insertional mutagenesis experiments requires that one identify by repeated subcloning and transformation the smallest possible fragment of genomic DNA capable of rescuing the mutant phenotype.

Insertional mutagenesis has become the method of choice for identifying and cloning genes of interest to investigators studying metabolism, photosynthesis, flagellar motility, and the many other questions addressed by Chlamydomonas researchers. At best, however, it is a blunt instrument for genetic analysis. Because most mutant alleles generated during transformation involve both the insertion of a large plasmid and the deletion of genomic DNA at the site of insertion, only null mutations can be expected. Such mutations are of no use for essential genes in this haploid system, and in many cases they provide limited information about the function of the affected gene product. Stable diploids can be produced as a result of failed maturation of zygotes, and because they are capable of mating it is possible to use tetraploid genetic crosses to study essential genes (Harris 1989).

Insertional mutagenesis using transposable elements:

A number of active transposons have been identified in the Chlamydomonas genome, although transposon tagging has not yet been used extensively to clone genes in this organism. The first transposon, a retrotransposon known as *TOC1* (for transposon of Chlamydomonas), was identified in the oxygen-evolving enhancer 1 (*OEE1*) gene (Day *et al.* 1988). The element first used for cloning a gene by transposon tagging was *Gulliver*, first identified by Ferris (1989) during an extensive chromosome walk through the mating type locus (Ferris and Goodenough 1994). The *Gulliver* transposon was used as a hybridization tag to clone the *NIT2* gene, a regulatory gene for the nitrate assimilation pathway, from a spontaneous mutant that was caused by the transposition of a copy of *Gulliver* into the *NIT2* coding region (Schnell and Lefebvre 1993). The use of *Gulliver* to clone this gene benefited from the observation that a field isolate of *Chlamydomonas reinhardtii* had no *Gulliver* elements in its genome. It has become clear that a substantial number of active transposable elements exist in Chlamydomonas. For example, among 14 spontaneous *nit2* mutations, 8 were caused by the insertion of known transposons (*TOC1*, *Gulliver*, *tcr1*, *tcr2*, *tcr3*), and 6 others were caused by the insertion of previously unknown transposable elements (R. Schnell, unpublished observations). Many of the mutations caused by the insertion of the putative transposons were unstable, reverting at a high frequency, indicating that the elements can excise readily after insertion. The ingredients are clearly present in the Chlamydomonas genome to develop procedures for efficient transposon tagging.

Cloning by complementation: For genes identified by mutations that create a counterselectable phenotype, cloning by complementation has become feasible. Indexed cosmid libraries and yeast artificial chromosome (YAC) libraries have been produced and used to clone genes by rescuing mutant phenotypes upon transformation with pools of clones (Purton and Rochaix 1994; Zhang *et al.* 1994; Vashishtha *et al.* 1996). Improved methods for high-efficiency transformation using electroporation have recently been introduced (Shimogawara *et al.* 1998), and these should make cloning by complementation an important tool in the gene isolation tool kit for Chlamydomonas.

Positional cloning: Given the hundreds of mutants already placed on the Chlamydomonas genetic map (Harris 1989), the simplest way to determine the function of a cloned gene of interest is to place the cloned gene on the genetic map and look for potentially interesting mutations with the expected phenotype in the genetic neighborhood. The first molecular map of the Chlamydomonas genome, constructed from restriction fragment length polymorphism (RFLP) markers, was published in Genetics 10 years ago (Ranum *et al.* 1988). To generate this map, multiply marked *C. reinhardtii* strains were crossed with the wild-type *C. smithii* strain, and the segregation of RFLP markers among the progeny was scored by genomic Southern analysis. This early molecular map facilitated the cloning of a number of genes of interest, in particular the nitrate reductase structural gene, NIT1, which then became widely used as an intraspecies selectable marker for transformation experiments (Fernandez *et al.* 1989). This early mapping effort was hampered by the scarcity of RFLPs for many cloned probes between the *C. reinhardtii* and *C. smithii* genomes. Many clones of interest could not be mapped for this reason, even after screening many different restriction endonucleases. The solution to this problem became available in the same year when an undergraduate student in our laboratories, Christian Gross, discovered a field isolate of Chlamydomonas (strain S1-D2) that is completely interfertile with the laboratory strains, but that shows an exceptionally high frequency of RFLPs when compared with standard laboratory strains. Using the S1-D2 strain, we have expanded the molecular map of the genome to include now more than 200 markers, identifying each of the 17 linkage groups in Chlamydomonas. This map is being expanded further by using as molecular markers the abundant simple sequence repeats (usually GT repeats) found in the Chlamydomonas genome (Kang and Fawley 1997).

Given that Chlamydomonas has a densely populated genetic and molecular map and a genome of moderate size (100 Mbp), a major effort to cover the genetic map with overlapping clones is under way. We have recently constructed an indexed bacterial artificial chromosome (BAC) library containing more than 15,000 clones with an average insert size of approximately 70 kb, giving 10- to 12-fold coverage of the Chlamydomonas genome. Work is under way to isolate contigs of overlapping BAC clones anchored to the 200 molecular markers we have placed on the genetic map. Given an approximate size for each contig of 150 kb, these 200 contigs should cover 30–40% of the genome. For many investigators, these initial contigs will provide the starting material for positional cloning of genes identified by mutations that map near the anchor loci. As new molecular markers are mapped, they will in turn be used to anchor new contigs. Dedicated walking from the ends of contigs and screening of new large-insert BAC and YAC libraries will be used to complete the map and cover the entire genome with ordered clones.

LESSONS IN BIOLOGY FROM CHLAMYDOMONAS

What biological questions will be addressed with the molecular and genetic tools accumulating in the Chlamydomonas toolbox? Among the many research areas being productively studied in this system, two stand out for their fundamental biological importance and for the particular advantages of Chlamydomonas for research in these areas.

Chloroplast biogenesis and function: The ease of transformation of both the nuclear and chloroplast genomes and the ease with which genes in the chloroplast genome can be altered by homologous recombination make Chlamydomonas an ideal system for studying the chloroplast. The chloroplast and nuclear genomes carry on a constant conversation that regulates the synthesis, transport, and assembly of chloroplast proteins. It has been shown recently that part of the language used in this conversation involves a signal from the chloroplast to the nucleus generated by the accumulation of certain precursors in the chlorophyll biosynthetic pathway (Kropat *et al.* 1997). Large, multiprotein complexes must be assembled in the stromal and thylakoid membrane compartments of the chloroplast, and many of the proteins of these complexes must be maintained in precise relative stoichiometries. Chloroplast assembly requires the synthesis and processing of proteins in both the cytoplasm and the chloroplast, along with the transport, further processing, and assembly of these proteins in the proper compartments. Each of these steps is subject to precise regulation, and this regulation must respond to environmental signals such as light intensity (for a review, see articles in Rochaix *et al.* 1998).

As pointed out at the beginning of this essay, photosynthetic mutants in Chlamydomonas are viable when provided with acetate as a carbon source. Thus, screens for acetate-requiring auxotrophic mutants have been used to identify hundreds of mutants with defects in chloroplast function (*e.g.*, Levine 1969). More recently, elegant genetic screens have been designed to isolate mutants with defects in particular aspects of chloroplast function, such as protein transport across the chloroplast membrane (*e.g.*, Smith and Kohorn 1994) and the protection of chloroplasts from damage by high-intensity light (Niyogi *et al.* 1997). Traditional suppressor screens have proven to be very useful in uncovering the role of the products of nuclear genes in the expression of genes in the chloroplast (*e.g.*, Chen *et al.* 1997; Hong and Spreitzer 1998). Until recently, it has not been possible to clone the nuclear genes affected by mutation unless an insertional mutant allele was available. Given the recent advances in cloning by complementation and in positional cloning, it should be possible to obtain the genes affected in most mutant strains of interest, regardless of the mutagen used. With these tools, it will be possible to address major outstanding questions of chloroplast development and function. How many nuclear gene products are involved in the expression of genetic information in the chloroplast, and what do they do? How are proteins targeted for assembly into the different compartments of the chloroplast, and how are these proteins transported to the proper site of assembly? How are the proteins in the chloroplast maintained in the appropriate stoichiometry? What environmental signals are transmitted to the chloroplast, and how does the chloroplast respond to these signals?

The assembly and function of basal bodies and flagella: Probably the least understood organelle in the cell is the basal body (and its somewhat better known cousin, the centriole). There is still not a single protein that can be identified unequivocally as a "basal body protein," although proteins such as the tubulins and tektins have been shown to be components of basal bodies and other organelles. There are two fundamental reasons for our lack of understanding of even the protein composition of basal bodies: they are difficult to isolate in pure form, and it is difficult to isolate mutants of Chlamydomonas that lack basal bodies. Presumably a mutation causing the loss of basal bodies in Chlamydomonas would be lethal, because the mutant cells would also lack the centrioles needed for mitosis. Some mutants with ultrastructural defects in the basal bodies have been described. The most extreme of these, *bld2*, has no more than a ring of singlet microtubules as a remnant of the basal bodies (Johnson and Porter 1968). *Bld2* mutants exhibit severe displacement of the basket of cytoskeletal microtubules and defects in establishing the plane of cell division (Ehler *et al.* 1995). Uni mutants with a single flagellum have been shown to have ultrastructural defects in the flagellar transition zone, marking the intersection between each basal body and its attached flagellum (Huang *et al.* 1982). In the *uni3* mutant, the ultrastructural defect has been shown to be the loss of the outer C tubule of the triplet microtubules of the basal body. The *uni3* gene product is a new member of the tubulin superfamily, δ-tubulin, which has degrees of similarity at the amino acid level approximately equal to those of α-, β-, and γ-tubulins (Dutcher and Trabuco 1998).

Future research in Chlamydomonas will be critical to identifying the protein components of the basal body and defining their roles in the function of these organelles in nucleating flagellar assembly and in organizing the centrosome. The likelihood that most mutants with major defects in the basal body will be lethal in Chlamydomonas requires that conditional mutants be isolated and characterized. Such mutants may be identified, for example, among conditional-lethal strains with defects in flagellar assembly at the permissive temperature. The genes affected by mutations in these strains could then be cloned by the positional cloning procedures being developed. In addition, research on nonconditional mutants with more subtle phenotypes, such as the Uni mutants, should continue to be fruitful in understanding basal body function.

The basal bodies nucleate the assembly of equally complex but better understood organelles, the flagella. Decades of research on the biochemistry of Chlamydomonas flagella have produced an impressive body of knowledge about flagellar proteins and their role in motility. Efficient fractionation and reactivation proce-

dures have made it possible to dissect the flagellar axoneme into its component parts and reconstitute flagellar motility *in vitro*. The genetic dissection of flagellar function has been fruitful for many years, because mutants with defective flagellar motility are very easy to isolate in mutant screens (reviewed by Luck 1984). A great deal more information needs to be gathered, however, before an understanding of flagellar motility and its regulation can be brought to the molecular level. How, for example, is the dynein-mediated sliding of adjacent doublet microtubules translated into complex wave forms? How does the cell switch its flagellar motility from the asymmetric (ciliary) stroke, used for forward motility, to the symmetric (flagellar) stroke used in backward swimming after a photoshock? How is the perception of light by the eyespot, located micrometers away from the flagellum, translated into a precise steering of the flagellum to achieve phototaxis?

Recently a new form of motility has been described for the Chlamydomonas flagellum. Particles can move rapidly (up to 2 μm/sec) up and down the flagellum just beneath the flagellar membrane (Kozminski *et al.* 1993). The anterograde movement of particles away from the cell into the flagellum requires the activity of a kinesin-like protein (FLA10; Kozminski *et al.* 1995; Piperno and Mead 1997), whereas the retrograde movement of particles from the flagellum toward the cell body may involve a dynein motor (Pazour *et al.* 1998). This latter possibility is raised by the analysis of a mutant (*fla14*) that blocks the retrograde motility and causes flagella to be shortened and thickened as they accumulate a series of protein aggregates called "rafts." The deduced *fla14* gene product is very similar in amino acid sequence to a dynein light chain. This new form of motility may be of general importance in the assembly of the modified cilia present in sensory neurons, such as the chemosensory neurons of the nematode *C. elegans*. The sequence of several proteins isolated from Chlamydomonas rafts is similar to proteins of *C. elegans* that have been shown by mutant analysis to be required for the assembly of the modified cilia of the chemosensory neurons (Cole *et al.* 1998). Thus, the mechanisms used to move particles within Chlamydomonas flagella may be of general importance for the assembly of cilia and the function of ciliated organelles in a wide variety of cell types.

AN INVITATION

There was a time in the last decade when it appeared that the exponential increase in the number of yeast molecular biologists meant that by the early part of the next millennium every person on the planet would be a yeast molecular biologist. Recently a small reduction in this rate of growth has made the doomsday scenario seem less likely. The Chlamydomonas community, by contrast, has adhered to a more sustainable growth rate and increased with less alarming kinetics. Starting 20 years ago with a small roomful of enthusiasts at the Chlamydomonas/Euglena session, held annually for part of 1 day at the American Society for Cell Biology Meetings, the Chlamydomonas meetings have grown to attract more than 200 participants every 2 years. There is, however, ample room for new investigators to find a sparsely populated research niche. The hundreds of genes required for basal body function, for example, are being studied genetically by fewer than 10 laboratories. Given the particular biological questions that can be productively addressed in Chlamydomonas, it is clear that there is room for more researchers to employ this useful little organism.

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