WHY STUDY THE CYTOSKELETON IN YEAST?

DAVID BOTSTEIN

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

I. INTRODUCTION

THE internal architecture of eukaryotic cells, often referred to as the cytoskeleton, is organized around a number of filamentous structures called microtubules (made principally of tubulin), microfilaments (made principally of actin), and intermediate filaments (made of any of a number of related proteins). One or more of the cytoskeletal elements have been implicated in virtually all cellular functions, often by a combination of evidence including observation and experiments involving inhibitory agents. For example, in the electron microscope one can see that the mitotic spindle consists largely of microtubules, and the major specific inhibitors of mitosis like colchicine bind to tubulin.

Satisfactory as these attributions of function have sometimes been, they are surprisingly difficult to make in general, particularly so when details of function are sought. For example, the actin filaments found in virtually all cells have a function that is quite uncertain in nonmuscle cells. Similarly, major uncertainties remain concerning the nonmitotic functions of microtubules. Thus, there remain many issues left to be resolved regarding the functions of the cytoskeleton.

For some years my associates and I have been exploring the possibilities that genetic analysis might offer. The basic idea is to connect the cytoskeletal proteins with the functions they perform *in vivo* by studying the phenotypes of mutations in the genes that specify these proteins. Our hopes for this kind of analysis were based on the prior success of this approach in understanding phage morphogenesis and, less obviously, the analysis of bacterial as well as phage DNA replication. In those systems, it was

The Harvey Lectures, Series 82, pages 157–167

relatively easy to use mutations to associate gene with product, on the one hand, and gene with function, on the other.

The idea in general is illustrated in Figure 1. A connection between a protein and its function can be achieved by classic biochemistry, generally through the establishment of a specific assay *in vitro*. Cloning of the gene that specifies the protein provides a second route (the gene) for connecting a protein to its biologic function: assessment of the phenotype of mutations. This route has the special advantage that the determination of function is made *in vivo*. Logical connections between a protein and its function can be achieved by beginning with classic genetics—that is, by finding mutations defective in the function of interest. The genes thus identified can be cloned, and the clones can then be used to identify the proteins they specify. The peculiar power of the molecular biologic approach, especially since the advent of the recombinant DNA technology, is in the ability to join biochemical and genetic ways of looking at proteins and their functions in the cell.

Even with the elaboration of recombinant DNA methods, such a program of associating a gene with its product and its function cannot straightforwardly be applied directly to higher animal cell systems. This is unfortunate, because considerably more is known about the structure of the cytoskeleton of higher eukaryotes than of genetically more tractable lower eukaryotic systems. Nevertheless, I decided to study the cytoskeleton of the budding yeast *Saccharomyces cerevisiae*, reasoning that the excellent genetics, the low incidence of repeated genes, and the relatively advanced

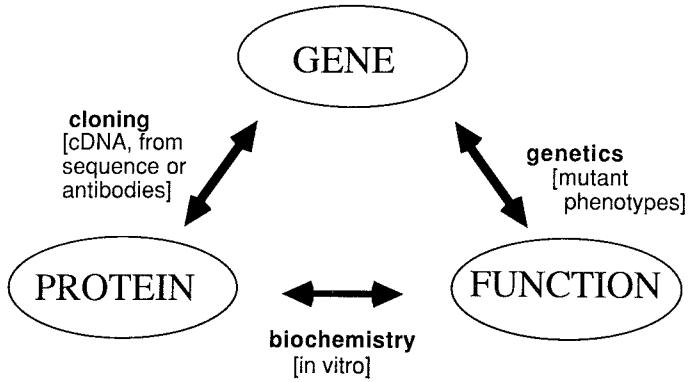

Figure 1

knowledge of the regulation of the cell division cycle would have to make up for the problems posed by our relative ignorance of even basic aspects of this yeast's cytoskeleton.

A major uncertainty concerned the degree to which any understanding gained by cytoskeletal studies using genetics in yeast might apply to higher cell cytoskeletons as well. Our initial hopes for this were greatly encouraged by the observation of extreme conservation in the amino acid sequences of cytoskeletal structural proteins. Yeast actin has 91% identity to the actin of higher animals (Ng and Abelson, 1980; Gallwitz and Sures, 1980); the yeast tubulins are more than 70% identical to their higher animal counterparts (Neff *et al.*, 1983; Schatz *et al.*, 1986). This picture has been supported by the observation that polyclonal antibodies made against higher animal actins and tubulins frequently cross-react strongly with their yeast counterparts and vice versa.

The best evidence for meaningful conservation is functional interchangeability. Indeed, a variety of experiments both *in vitro* and *in vivo* indicate conservation of function and compatibility of cytoskeletal proteins over the entire evolutionary scale. Two notable instances are worth special mention. First is the ability of chimeric proteins consisting of part yeast and part human β-tubulin to polymerize and function in animal cells (Bond *et al.*, 1986). Second is the ability of filaments of purified yeast actin to move *in vitro* on sheets of purified rabbit muscle myosin (S. Kron, D. Drubin, J. Spudich, and D. Botstein, unpublished, 1986).

This theme of functional interchangeability is not limited to structural proteins. The yeast RAS homologues, when both mutant, result in lethality; but the human gene can, when expressed in yeast, suppress this lethality, indicating conservation of function *in vivo*. Another more recent example is the complementation of a *Schizosaccharomyces pombe cdc2* mutation by a hitherto undescribed but homologous human gene (Lee and Nurse, 1987).

Thus there are real reasons to believe that what might be learned through the application of the entire panoply of molecular genetic methods about the yeast cytoskeleton will have direct application in understanding the cytoskeleton of higher eukaryotic cells. The analysis of the yeast cytoskeleton began with the cloning of the yeast actin gene in 1980, by methods relying on the sequence homology with the actin genes of higher eukaryotes (Ng and Abelson, 1980; Gallwitz and Sures, 1980). Within a few years, the β-tubulin gene and then the α-tubulin genes had been cloned (Neff *et al.*, 1983; Schatz *et al.*, 1986).

II. Phenotypes of Actin and Tubulin Mutants

The finding that the actin and β-tubulin genes were present in only a single copy per haploid genome immediately presented an opportunity to make mutations in these genes and then to observe, for the first time, the phenotypes associated with lesions in these central cytoskeletal proteins. The peculiar ability of yeast cells to integrate DNA by homologous recombination was used to perform integrative gene disruption experiments that showed that the null phenotype of mutations in the actin (*ACT1*) and β-tubulin (*TUB2*) genes is recessive lethality (Shortle *et al.*, 1982; Neff *et al.*, 1983).

A variation on the integrative gene disruption theme (illustrated in Fig. 2) was devised (Shortle *et al.*, 1984). This allows the production of mutations *in vitro* on an incomplete copy of the gene to be altered, followed by its directed integration into the genome so that the mutation is left in the intact copy of the gene while the other copy is disrupted. This method permits the recognition of recessive conditional-lethal mutations directly after transformation of yeast with the mutagenized plasmid. One can then derive strains with only the mutation (no vector) simply by selecting for loss of the *URA3*

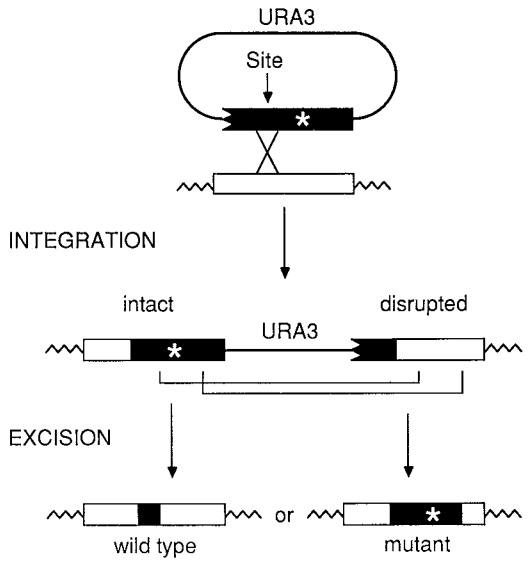

Figure 2

gene on the vector followed by screening for those derivatives that retain the conditional-lethal phenotype.

In this way we were able to construct several heat-sensitive *act1* mutations (Shortle *et al.*, 1984) and a number of cold-sensitive *tub2* mutations (T. Huffaker and D. Botstein, unpublished). These mutants were then studied in detail in the expectation that one might learn something about the role of actin and tubulin in the cell.

Indeed we found some consistent phenotypes among the actin mutants (Novick and Botstein, 1985). At their nonpermissive temperature, *act1* mutants showed aberrant or absent actin filament structure, especially a complete loss of the normal asymmetry in the distribution of actin filaments through the cell; they showed unusual sensitivity to high osmotic pressure in the medium; they had a clear, but incomplete, defect in secretion of invertase; and the distribution of chitin on the surface of the cells was aberrant. Although they failed to show a standard defect in the progress of the cell division cycle, they showed a clearly aberrant distribution of cell types, with more than 70% of the cells accumulating as single cells with no bud, suggesting a difficulty in bud emergence. All this is consistent with a primary function of actin in controlling biosynthesis and morphogenesis of the cell surface. The phenotypes of the mutants suggested strongly that actin has no direct role in other functions, specifically mating, nuclear movements, or mitosis.

The phenotypes of the cold-sensitive *tub2* mutants were simpler to interpret. They showed a clear cell-division-cycle phenotype after incubation at their nonpermissive temperature (Thomas *et al.*, 1985; T. Huffaker and D. Botstein, unpublished, 1987). Many showed more than 85% of the cells arrested with a large bud and an undivided nucleus. There was no defect in bud emergence, DNA replication, secretion of invertase, or chitin deposition. On the other hand, meiosis was defective, as might be expected. All the mutants display defects in chromosome segregation. The actin cytoskeleton appeared relatively undisturbed when viewed by immunofluorescence, but the different mutants showed different degrees of loss of the microtubule cytoskeleton: some appear to lose all their staining microtubules, others lose only the extranuclear microtubules, and others retain a substantial but clearly functionally defective spindle. The gross phenotypes of the tubulin mutants suggested that microtubules are involved primarily in mitosis and meiosis, when chromosomes are moved.

A more detailed analysis of phenotypes of the β-tubulin mutants resulted in the observation of a correlation between the presence in some mutants of extranuclear microtubules and the ability to carry out nuclear movements. Those *tub2* mutants with extranuclear microtubules arrested at the nonpermissive temperature with the nucleus in the mother at the neck between mother and bud, whereas the remaining mutants arrested with the nucleus at apparently random positions in the mother cell. Even more impressively, the mutants without extranuclear microtubules failed to carry out nuclear fusion after mating (i.e., they had a defect in karyogamy) whereas those with extranuclear microtubules showed nearly normal karyogamy at the nonpermissive temperature (Thomas, 1984; T. Huffaker and D. Botstein, unpublished, 1987).

Thus the ability to make conditional-lethal mutations in the major structural cytoskeletal proteins actin and β-tubulin has allowed us to define the range of function of the actin filaments and microtubules in the cell, in some cases to quite fine detail.

III. Genetic Methods for Studying Proteins Associated With Cytoskeletal Filaments

The major protein components of the cytoskeleton are unlikely to be the only functional components. Proteins essential to both assembly and function of both actin filaments and microtubules have been described. A major goal of any genetic analysis of the cytoskeleton must include methods for finding and analyzing genes that specify the associated proteins.

A. Pseudoreversion

A number of genetic techniques for identifying genes of associated proteins have been devised in our laboratory. The most classic of these is pseudoreversion (Jarvick and Botstein, 1975; Moir *et al.*, 1982). Phenotypic revertants of mutations in one gene (let us say Ts^+ revertants of *act1-ts*) that simultaneously have acquired a second phenotype (let us say cold sensitivity) are sought. These are called Sup/Cs mutants to denote their double properties: suppression of the ts-actin mutation and the new cold sensitivity. The Sup/Ts are commonly extragenic suppressors that define a new gene. The virtue of this way of defining the new genes is that they can

be studied in their own right directly through the new (Cs, in this example) phenotype.

There are serious potential problems with the pseudoreversion scheme as outlined thus far: the suppressor mutations may in fact be quite nonspecific, not actually changing an interacting protein but changing instead the intracellular environment in a more general way. For example, a tubulin mutant might be suppressed by a change in the intracellular availability of Ca^{2+} ions. Another example might be that a nonspecific lowering of the overall growth rate might allow a marginally defective spindle to act in time to successfully separate the chromosomes.

In order to minimize the effect of these difficulties, we have restricted our attention to suppressors that pass a number of tests. First, the same suppressor locus should be found more than once among independent pseudorevertants. Second, the secondary phenotype (i.e., the Cs of a Sup/Cs) should resemble in some way the phenotype of the original mutation that was suppressed (i.e., the original actin mutation), even in the absence of the original mutation (i.e., in an $ACT1^+$ background). Third, if we anticipate genes that specify proteins that truly interact, we restrict our attention to suppressors that show allele specificity (i.e., we study suppressors that suppress one or more, but not all, actin mutations).

We have searched for pseudorevertants of the actin mutations, with the result that six new genes (called *SAC* for *S*uppressor of *ac*tin) have been identified (P. Novick, B. Osmond, and D. Botstein, unpublished). Each has the Sup/Cs phenotypes, and all are unlinked to the *ACT1* locus. Likewise, we searched for pseudorevertants of a *tub2-cs* mutation, with the result that we found more than 16 apparent Sup/Ts loci unlinked to the *TUB2* locus, a number rather larger than one might reasonably expect (J. Thomas and D. Botstein, unpublished). Indeed, only three of these loci were represented twice or more among some 31 pseudorevertants, and only one of these has a clear tubulin-like phenotype in a TUB^+ background at its nonpermissive temperature.

The *SAC* mutant phenotypes closely resemble the original actin mutant phenotypes, including the loss of filamentous structure and loss of the asymmetry of the actin cable structure, the defects in chitin deposition, and, in the case of *sac2* closely resemble the original actin mutant phenotypes, including the loss of filamentous structure and loss of the asymmetry of the actin cable structure, the defects in chitin deposition, and, in the case of *sac2*, the partial secretion defect. Particularly striking is the similarity in

appearance of *act1* mutants and *sac2* mutants when the actin structures are visualized by immunofluorescence with antiactin antibodies: Both show similar, characteristic thick bars of actin staining (P. Novick and D. Botstein, unpublished, 1986).

B. Unlinked Noncomplementation

If one screens for mutations that fail to complement a mutation already in hand, one expects to find only additional alleles of the same gene or, sometimes, mutations in regulatory sequences that act in *cis* to prevent expression of the same gene's products. Sometimes, however, one finds mutations that fail to complement that are certainly not novel alleles or *cis*-acting elements because they are unlinked to the original mutation. Specifically, Raff and Fuller (1984) found a number of unlinked mutations that fail to complement mutations in one of the β-tubulin genes of *Drosophila melanogaster*; one of the loci at which these unlinked noncomplementing mutations arise seems to encode an α-tubulin.

Following the lead of Raff and Fuller (1984), we sought and found mutations that fail to complement *tub2-cs* (cold-sensitive β-tubulin) mutations that lie in the unlinked *TUB1* (α-tubulin) gene (T. Stearns and D. Botstein, unpublished). These mutations were found to be cold sensitive in their own right and display all the conditional-lethal phenotypes typical of tub1 mutations obtained by direct mutagenesis of the gene *in vitro*. Searches for unlinked mutations that fail to complement *tub1-cs* mutations yielded mutations in the *TUB3* (minor α-tubulin) gene as well additional *TUB2* alleles.

Further genetic investigations with unlinked noncomplementing mutations suggest that many of the criteria used to separate the interesting from the trivial pseudorevertants should be applied here as well. As one might expect, when the mutations are in related genes and have phenotypes of their own, those phenotypes strongly resemble the phenotype of the original mutations, as was the case in related genes found by pseudoreversion. The case of tubulin genes, where *TUB1* and *TUB2* specify the two components of a heterodimer, virtually requires such a similarity in phenotype. We have also found that noncomplementing mutations in unlinked genes often show a marked allele specificity with respect to their failure to complement.

C. Synthetic Lethality

During the analysis of the allele-specificity of some suppressors of actin mutants, we found that an allele that might suppress one actin mutation would be lethal at all temperatures in combination with another (P. Novick, B. Osmond, and D. Botstein, unpublished, 1986). Likewise, we found mutations that show high rates of chromosome loss that were inviable in combination with *tub2* mutations (A. Hoyt, T. Stearns, and D. Botstein, unpublished, 1987). This phenomenon we call "synthetic lethality," following the precedent of Dobzhansky and Sturtevant, each of whom described examples in *Drosophila*.

We have devised a screening procedure for mutants that show absolute or conditional lethality only in the presence of a mutation in another gene (A. Hoyt and D. Botstein, unpublished, 1987). Such a screen constitutes a third way (the first two are pseudoreversion and unlinked noncomplementation) to identify mutations in genes that interact with genes in which one already has mutations. Our system begins with a haploid *ura3*$^-$ strain that carries mutation in a gene of interest; if the mutation is a conditional-lethal, the procedure is carried out at the permissive temperature. The strain also carries a plasmid with a wild-type copy of the gene of interest plus the intact *URA3* gene. The strain is mutagenized and screened by replica plating: one looks for mutants that fail to grow only when the *URA3* gene is selected against by the presence of 5-fluoro-orotic acid.

By application of these methods, we have been able to identify a very large number of mutations in genes that appear to interact with actin or tubulin and/or each other. In a number of cases, we have been able to show by phenotypic analysis that the mutations indeed identify genes of phenotype similar to the actin or tubulin-defective phenotypes; in other cases, we have managed to find mutations in genes already known to interact (e.g., *TUB1* and *TUB2*).

IV. Closing the Circle: Finding Cytoskeleton-Associated Proteins Biochemically

Biochemical methods can, of course also be used to study the composition of filamentous structure in yeast. We have concentrated mainly on actin-containing structure. Chromatography of yeast extracts on columns of filaments of purified yeast actin (stabilized with phalloidin and coupled to

sepharose) allowed us to identify and purify three major proteins having molecular masses of about 195 kd, 85 kd, and 67 kd (D. Drubin, K. Miller, D. Botstein, and B. Alberts, unpublished, 1986). The 195 kd appears to be a myosin-like molecule, based on its ability to react with antibodies directed against the myosins of other species.

Affinity-purified antibodies directed against the 85-kd and 67-kd proteins appear to colocalize with actin antibodies (D. Drubin and D. Botstein, unpublished, 1987). The genes specifying 85-kd and 67-kd species have been cloned, and genetic analysis (disruptions and generation of point mutations) is in progress.

The ultimate aim, of course, is to unify the genetic and the biochemical approaches. Several important preliminary indications suggest that we may be very close to closing the circle: the 67-kd actin-binding protein is overproduced in strains carrying a *sac2* (suppressor of actin) mutation. Overproduction of the 85-kd protein results in a conditional-lethal phenotype featuring disruption of the actin cables as visualized in immunofluorescence microscopy.

V. Conclusion

I believe that yeast is rapidly proving itself to be a very favorable organism with which to try to understand the function of the eukaryotic cytoskeleton. The combination of biochemistry, genetics, and DNA manipulation methods is particularly powerful in yeast, and the cell biology is beginning to catch up. When viewed in the context of the unexpectedly high degree of conservation of the sequences and function of the cytoskeletal proteins, it seems likely that many of the basic proteins and their functions in the cell cycle will soon be understood.

References

Bond, J.F., Fridovich-Keil, J.L., Pillus, L., Mulligan, R.C., and Solomon, F. (1986). *Cell* **44,** 461–68.

Gallwitz, D., and Sures, I. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2546–2550.

Jarvik, J., and Botstein, D. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 2738–2742.

Lee, M.G., and Nurse, P. (1987). *Nature* **327,** 31–34.

Moir, D., Stewart, S.E., Osmond, B.C., and Botstein, D. (1982). *Genetics* **100,** 547–552.

Neff, N.F., Thomas, J.H., Grisafi, P., and Botstein, D. (1983). *Cell* **33,** 211–219.

Ng, R., and Abelson, J. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 3912–3916.

Novick, P., and Botstein, D. (1985). *Cell* **40,** 405–416.

Raff, E.C., and Fuller, M. (1984). *In* "Molecular Biology of the Cytoskeleton" (G.G. Borisy, D.W. Cleveland, and D.B. Murphy, eds.), pp. 293–304. Cold Spring Harbor Press, New York.

Schatz, P.J., Pillus, L., Grisafi, P., Solomon, F., and Botstein, D. (1986). *Mol. Cell. Biol.* **6,** 3711–3721.

Shortle, D., Haber, J.E., and Botstein, D. (1982). *Science* **217,** 371–373.

Shortle, D., Novick, P., and Botstein, D. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4889–4893.

Thomas, J.H. (1984). "Genes Controlling the Mitotic Spindle and Chromosome Segregation in Yeast." Ph.D. Thesis, Massachusetts Institute of Technology.

Thomas, J.H., Neff, N.F., and Botstein, D. (1985). *Genetics* **112,** 715–734.