*Buffers and Stock Solutions*

Protease inhibitor stock: 1 m*M* benzamidine-HCl, 0.1 mg/ml phenanthroline, 1mg/ml each of aprotinun, leupeptin, and pepstatin A (this stock is used at dilutions of $\frac{1}{100}$ to $\frac{1}{1000}$, as noted)

C buffer: 50 m*M* HEPES-KOH, pH 7.6, 1 m*M* $MgCl_2$, 1 m*M* $Na_3EGTA$

CX buffer: C buffer supplemented with 10% glycerol, 25 m*M* KCl, 0.5 m*M* dithiothreitol (DTT), and protease inhibitor stock ($\frac{1}{1000}$)

BRB80 buffer (microtubule assembly buffer): 80 m*M* PIPES-KOH, pH 6.8, 1 m*M* $MgCl_2$, 1 m*M* $Na_3$ EGTA

F buffer (polymerizing conditions for actin filaments): 50 m*M* HEPES-KOH, pH 7.5, 0.1 *M* KCl, 0.2 m*M* $CaCl_2$, 0.2 m*M* ATP, 5 m*M* $MgCl_2$

G buffer (depolymerizing conditions for actin filaments): 5 m*M* HEPES-KOH, pH 7.5, 0.2 m*M* $CaCl_2$, 0.2 m*M* ATP

E buffer: 5 m*M* HEPES-KOH, pH 7.5, 0.05% Nonidet P-40, 0.5 m*M* $Na_3EDTA$, 0.5 m*M* $Na_3EGTA$, and protease inhibitor stock ($\frac{1}{100}$)

A buffer: 50 m*M* HEPES-KOH, pH 7.5, 50 m*M* KCl, 2 m*M* DTT, 0.5 m*M* $Na_3EDTA$, 0.5 m*M* $Na_3EGTA$, 0.05% Nonidet P-40, and protease inhibitor stock ($\frac{1}{1000}$)

Polyacrylamide gel sample buffer: 63 m*M* Tris-HCl, pH 6.8, 3% sodium dodecyl sulfate (SDS), 5% 2- mercaptoethanol, 10% glycerol

# [27] Molecular Genetic Tools for Study of the Cytoskeleton in *Dictyostelium*

*By* THOMAS T. EGELHOFF, MARGARET A. TITUS, DIETMAR J. MANSTEIN, KATHLEEN M. RUPPEL, and JAMES A. SPUDICH

*Dictyostelium discoideum* has a number of features that make it an attractive system for cell biological studies. The ability of *Dictyostelium* cells to perform active ameboid crawling and chemotaxis have made it a popular system for cell motility and signal transduction studies. Synchronous development of multicellular fruiting bodies occurs upon starvation, allowing basic developmental questions to be addressed as well. The absence of a cell wall, which is common in many other lower eukaryotes, allows much higher resolution microscopy than is possible in yeasts or filamentous fungi. Although wild-type isolates are generally cultivated with

bacteria as a food source, axenic lines have been generated that grow well in inexpensive nutritive media. The lack of a cell wall and ease of cultivation make *Dictyostelium* an excellent organism for biochemical approaches, allowing large quantities of material to be obtained and lysed without difficulty.

We have taken advantage of these attributes, together with recently developed molecular genetic tools, to begin structure–function studies on the cloned *Dictyostelium* myosin gene. Straightforward gene disruption protocols have been developed and used to construct myosin null lines of *Dictyostelium.* Studies of the resultant mutant cell lines have provided insights into the role of myosin in cell motility, cytokinesis, and development.[1,2] These techniques have also been successfully applied to the study of other genes in *Dictyostelium,* such as the α-actinin gene.[3,4] Transformation conditions and vectors have recently been established in our laboratory that allow null cells to be transformed to hygromycin resistance.[5] Combining the myosin null cells and the second drug selection system now available, we are expressing in *Dictyostelium* the cloned myosin gene and myosin subfragments that have been subjected to site-directed mutagenesis to study the effects of particular mutations on both the *in vivo* and *in vitro* properties of myosin.

In this chapter we describe molecular genetic tools that we are using for transformation, construction of null cell lines, and expression of the cloned myosin gene fragments. Brief coverage will be given to other tools and methods common in the field, but the emphasis will be on those approaches that we are currently using.

## Transformation Vectors

A well-established transformation system has been developed in *Dictyostelium* and is based on the selection for resistance to the synthetic neomycin analog G418.[6,7] Two G418-resistance cartridges have been developed, each containing a different actin promoter fused in phase to a

[1] A. De Lozanne and J. A. Spudich, *Science* **236,** 1086 (1987).
[2] D. J. Manstein, M. A. Titus, A. De Lozanne, and J. A. Spudich, *EMBO J.* **8,** 923 (1989).
[3] W. Witke, W. Nellen, and A. Noegel, *EMBO J.* **6,** 4143 (1987).
[4] A. A. Noegel, B. Leiting, W. Witke, C. Gurniak, C. Harloff, H. Hartmann, E. Weismuller, and M. Schleicher, *Cell Motil. Cytoskeleton* **14,** 69 (1989).
[5] T. T. Egelhoff, S. S. Brown, D. J. Manstein, and J. A. Spudich, *Mol. Cell. Biol.* **9,** 1965 (1989).
[6] W. Nellen, C. Silan, and R. A. Firtel, *Mol. Cell. Biol.* **4,** 2890 (1984).
[7] W. Nellen, S. Datta, C. Reymond, A. Siversten, S. Mann, T. Crowley, and R. A. Firtel, *Methods Cell Biol.* **28,** 67 (1987).

neomycin resistance gene (neo). One of the available cassettes consists of the *Dictyostelium* actin 6 promoter fused to the Tn*5* neomycin resistance gene.[6] The second cassette carries the *Dictyostelium* actin 15 promoter fused to the Tn*903* neomycin resistance gene with the 3′ terminator region of actin 15 placed downstream.[8] These two neo-resistance cartridges have been used extensively by many researchers to date, and both are effective in conferring resistance. The initial transformation vectors developed for *Dictyostelium,* and the ones most commonly used, rely on the random integration of the construct into a *Dictyostelium* chromosome. These integrating vectors are often present in tandem arrays in transformed cell lines. A number of vectors employing the neo-resistance cartridges have been described and a sample of these is shown in Table I.

Several autonomously replicating extrachromosomal plasmids have been identified in wild-type *Dictyostelium* isolates.[9,10] Two of these, Ddp1 and Ddp2, have been exploited to construct transformation plasmids that carry the neo-resistance marker and can be introduced into *Dictyostelium* cells via G418 selection. Ddp1 has been reported to be present in 50–100 copies/cell,[11] and Ddp2 has been found to be present in about 300 copies/cell.[12] The advantage of these vectors is that they are maintained as extrachromosomal plasmids, without integrating into the genome, thus avoiding any potential positional effects that may influence expression of the introduced plasmids. In addition they offer the potential for consistently high copy number once transformed into *Dictyostelium.* These points become relevant when cloned genes that have been mutagenized or altered are reintroduced to study phenotypes or to obtain biochemical quantities of the mutated protein. Finally, the extrachromosomal vectors are efficiently and easily introduced into cells by electroporation, described below.

Additional selection systems have recently been developed, providing greater flexibility in the application of molecular genetics to *Dictyostelium.* The availability of a second selection system has been critical for the reintroduction of cloned, mutated myosin gene fragments into myosin null cells,[13] as the null cells were constructed by the introduction of the neo-resistance cartridge into the myosin locus.[2] For this purpose we constructed a

[8] D. A. Knecht, S. M. Cohen, W. F. Loomis, and H. F. Lodish, *Mol. Cell. Biol.* **6**, 3973 (1986).
[9] B. A. Metz, T. E. Ward, D. L. Welker, and K. L. Williams, *EMBO J.* **2**, 515 (1983).
[10] A. Noegel, D. Welker, B. A. Metz, and K. L. Williams, *J. Mol. Biol.* **185**, 447 (1985).
[11] R. A. Firtel, C. Silan, T. E. Ward, P. Howard, B. A. Metz, W. Nellen, and A. Jacobson, *Mol. Cell. Biol.* **5**, 3241 (1985).
[12] B. Leiting and A. Noegel, *Plasmid* **20**, 241 (1989).
[13] T. T. Egelhoff, S. S. Brown, and J. A. Spudich, *J. Cell Biol.* **109**, 85a (1989).

TABLE I
*Dictyostelium* TRANSFORMATION VECTORS AND PROPERTIES

| Vector | Selection | Size (kb) | Comments | Single sites |
|---|---|---|---|---|
| B10[a] | G418 | 5.7 | Integrating | *Bam*HI, *Eco*RI, *Sal*I |
| B10SX[a,b] | G418 | 4.8 | Integrating | *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I |
| B10TP1[c] | G418 | 7.0 | Integrating. Good for site-directed mutagenesis and directed deletions | *Bam*HI, *Bgl*II, *Hin*dIII, *Kpn*I, *Nsi*I, *Sal*I, *Xba*I |
| B10TP2[c] | G418 | 6.2 | Integrating. Derived from B10TP1 | Same as B10TP1, also *Eco*RI |
| pA15TX[d] | G418 | 4.5 | Integrating | *Bam*HI, *Bgl*II, *Eco*RI, *Sal*I, *Xba*I |
| pA6NPTII[e] | G418 | 6.7 | Integrating. Has been used for antisense RNA expression | *Bam*HI |
| pBMWN1[f] | G418 | 19.0 | Extrachromosomal. Contains a Ddp1 origin of replication | *Bam*HI, *Spe*I, *Xba*I |
| pnDSal[g] | G418 | 10.7 | Extrachromosomal. Contains a Ddp2 origin of replication | *Aat*II, *Bam*HI, *Dra*II, *Kpn*I, *Nar*I, *Sph*I, *Sst*I |
| pnDeI[g] | G418 | 9.8 | Extrachromosomal. Contains a Ddp2 origin of replication | *Aat*II, *Bam*HI, *Dra*II, *Kpn*I, *Nar*I, *Sal*I, *Sst*I |
| pDE109[h] | Hygromycin | 10.8 | Extrachromosomal. Contains a Ddp2 origin of replication | *Kpn*I, *Sac*I, *Sma*I, *Sph*I |
| pTS1[i] | Thymidine | 10.1 | Integrating. Transformation must be performed using thymidine-requiring strain HPS400 | |

[a] W. Nellen, C. Silan, and R. A. Firtel, *Mol. Cell. Biol.* **4,** 2890 (1984).
[b] W. Nellen and R. A. Firtel, *Gene* **39,** 155 (1985).
[c] A. E. Early and J. G. Williams, *Gene* **59,** 99 (1987).
[d] S. M. Cohen, D. Knecht, H. Lodish, and W. Loomis, *EMBO J.* **5,** 3361 (1986).
[e] D. Knecht and W. F. Loomis, *Science* **236,** 1081 (1987).
[f] D. Knecht, unpublished.
[g] B. Leiting and A. Noegel, *Plasmid* **20,** 241 (1989).
[h] T. T. Egelhoff, S. S. Brown, D. J. Manstein, and J. A. Spudich, *Mol. Cell. Biol.* **9,** 1965 (1989).
[i] A. C. M. Chang, K. L. Williams, J. G. Williams, and A. Ceccarelli, *Nucleic Acids Res.* **17,** 3655 (1989).

gene cartridge that confers resistance to the antibiotic hygromycin B in *Dictyostelium*.[5] This cassette contains the actin 15 promoter fused in phase to a hygromycin-resistance gene (hph) with 265 base pairs (bp) of the 3′ end of the actin 15 gene downstream which serves as a terminator. This resistance cartridge was placed into a vector that also contains the high-copy-number extrachromosomal sequence Ddp2. The resulting plasmid, pDE109, is now routinely used in our laboratory for transformation of the G418-resistant myosin null cells. For reasons that have not been fully resolved, the hygromycin-resistance cartridge functions much more poorly when it is introduced as an integrating plasmid. Stable transformants can be isolated,[14] but the frequency of transformation is low and variable. The cloned myosin gene, as well as several subfragments, have been successfully introduced into wild type and null cells using these hygromycin vectors.[13,14]

Other recently reported transformation technologies promise to strengthen further the available tools for *Dictyostelium*. The first of these is the demonstration that a thymidine-requiring auxotroph of *Dictyostelium* can be complemented with the mouse thymidylate synthase gene, offering an additional selectable marker.[15] This thymidine-requiring mutant has also been complemented with a *Dictyostelium* gene library, providing an endogenous *Dictyostelium* selectable marker.[16] The isolation of this gene also provides a demonstration that *Dictyostelium* genes can be isolated from libraries by direct selection for complementation, which should prove to be a powerful tool in the future. An additional selection system recently described relies upon the *Dictyostelium* UMP-synthase gene. With technology similar to that currently used with the yeast *ura3* gene, it appears that both positive selection for transformants and negative selection for loss of the UMP-synthase gene will be possible.[17]

## Transformation Protocols

The standard and most widely used method for introducing DNA into *Dictyostelium* is to incubate cells with calcium phosphate–DNA precipitates.[6,7] Many minor variations of this method have been described, with differences in growth medium buffers, glycerol shock, and the subsequent

[14] D. J. Manstein, K. M. Ruppel, and J. A. Spudich, *Science* **246**, 656 (1989).
[15] A. C. M. Chang, K. L. Williams, J. G. Williams, and A. Ceccarelli, *Nucleic Acids Res.* **17**, 3655 (1989).
[16] J. L. Dynes and R. A. Firtel, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7966 (1989).
[17] D. Kalpaxis, H. Werner, E. Boy-Marcotte, M. Jacquet, and T. Dingerman, *Dev. Genet.* in press (1990).

selection step. Recently Howard *et al.*[18] established conditions for introduction of DNA via electroporation. Electroporation is useful for introducing vectors that contain extrachromosomal origins of replication, but does not seem to work as efficiently for integrating vectors. With extrachromosomal plasmids we find electroporation to be consistent and somewhat more efficient than introduction via calcium phosphate precipitates. An additional benefit of electroporation is that it is simpler to perform than calcium phosphate-mediated transformation. Described below are the details of how we perform these manipulations in our laboratory.

Ax2, Ax3, and Ax4 cell lines are all widely used for DNA-mediated transformation. Culture maintenance will be covered briefly here. Consult Sussman[19] for detailed procedures for culturing, storing, and cloning *Dictyostelium* cell lines. It is worth noting that the brand of peptone is important.[19] Stock cultures are commonly kept as suspension cultures in HL5, which are passed into fresh media every 3–4 days. Cultures should be passed once they reach a density of $2-3 \times 10^6$ cells/ml. *Dictyostelium* cell lines have a strong tendency to change with prolonged passage, resulting in altered growth or development properties. It is therefore advisable to restart stock cultures from spores every 4 weeks. The best way to do this is to store many aliquots of spores from a culture that has been tested for proper growth and development. Aliquots can then be germinated once a month and used to start new stock cultures.

### *Calcium Phosphate-Mediated Transformation*

#### *Materials*

HL5 (standard growth medium for *Dictyostelium;* slightly modified from Sussman[19]): 10 g/liter proteose peptone (Oxoid, Columbia, MD), 5 g/liter yeast extract (Oxoid), 10 g/liter glucose, 1.2 g/liter $KH_2PO_4$, 0.35 g/liter $Na_2HPO_4$, pH 6.5 (autoclave 20–25 min; overautoclaving causes medium to caramelize)

HL5–Bis-Tris (medium used for calcium phosphate-mediated transformation): 10 g/liter proteose peptone (Oxoid), 5 g/liter yeast extract (Oxoid), 10 g/liter glucose, 4.18 g/liter Bis-Tris, pH to 7.10 with HCl (autoclave)

HBS (2×): 4 g NaCl, 0.18 g KCl, 0.05 g $NaH_2PO_4$, 2.5 g HEPES, 0.5 g dextrose, pH to 7.05 with NaOH and bring to 250 ml. Filter sterilize and store frozen in 50- to 100-ml aliquots.

[18] P. K. Howard, K. G. Ahern, and R. A. Firtel, *Nucleic Acids Res.* **16**, 2613 (1988).
[19] M. Sussman, *Methods Cell Biol.* **28**, 9 (1987).

$CaCl_2$, 2 *M* (filter sterilize; store frozen)
Glycerol, 60% (autoclave)
Penicillin–streptomycin stock, 100× (filter sterilize; store frozen): 10,000 U/ml penicillin, 10 mg/ml streptomycin
G418 (also called Geneticin; GIBCO, Grand Island, NY): 10 mg/ml in 10 m*M* HEPES, pH 7.5 (filter sterilize; store frozen)
Hygromycin B (Calbiochem, San Diego, CA): 10 mg/ml in 10 m*M* HEPES, pH 7.5 (filter sterilize; store frozen)

*Procedure*

1. Plate $5 \times 10^6$ cells in a 10-cm plastic Petri dish in 10 ml HL5 and allow to attach for 30 min to several hours. It is advisable to include 1× penicillin–streptomycin (PenStrep) in HL5 and HL5-Bis-Tris from this point on to avoid any bacterial contamination. Aspirate medium off from one corner and gently add 10 ml of HL5-Bis-Tris. It is useful to place a mark at one spot on the edge of the Petri dish and perform all media removals and additions at that spot. Leave HL5-Bis-Tris on cells for 30 min to 1 hr.

2. Place 10 μg DNA in 0.6 ml 1× HBS in a sterile 5-ml glass tube, and add 38 μl of 2 *M* $CaCl_2$ with continuous vortexing. Allow 25–30 min at room temperature for the DNA precipitate to form. Remove HL5–Bis-Tris from the attached cell layer and gently apply the calcium phosphate–DNA mixture to the cell layer. We do this using a pipetman P1000, slowly expelling the solution while moving the tip of the pipetman back and forth just above the cell layer. Leave the precipitate on the cells for 30 min with occasional gentle rocking, then add 10 ml HL5–Bis-Tris.

3. At 4–8 hr remove the medium and add 3 ml of 15% glycerol in 1× HBS for 3–5 min. This should be gently spread across the cell layer with the pipette tip as it is being applied. This step should be done gently to avoid dislodging cells. Aspirate to remove the glycerol and add 10 ml HL5–Bis-Tris. Leave plates overnight (8–20 hr).

4. Remove medium and add 10 ml regular HL5 containing appropriate antibiotic selection. We generally use G418 at 10 μg/ml. With this level of selection, cells appear fairly normal for 1–2 days when observed with an inverted microscope at low power. By 2–3 days they begin to appear rounded and become increasingly refractile. By 4–5 days the majority of the cells have usually detached from the Petri plate. We find that the appropriate hygromycin selection level varies much more between cell lines than does the correct G418 concentration. With our isolate of Ax4 (obtained from D. Knecht, University of Connecticut), selection levels of 30–35 μg/ml have generally worked best, while with our isolate of Ax2

(obtained from G. Gerisch, Max Planck Institut für Biochemie), 20–25 μg/ml works best. It is advisable to test a series of hygromycin concentrations when first using it with a new cell line. Selection levels that give a rate of killing similar to that described above for G418 generally work best.

5. A number of alternative methods have been described for outgrowth of the transformation mixture and isolation of transformed cells. Described below are two methods that are commonly employed in our laboratory.

A. Add HL5 containing appropriate antibiotic selection to the Petri dish 8–20 hr after the glycerol shock, as described above. Change medium at 3-day intervals, keeping the entire sample in the original Petri dish. The bulk of the untransformed cells detach and are removed during the first two media changes. Depending on exact conditions, colonies usually become visible by eye at 5–8 days. With 10 μg of an integrating $G418^R$ vector, we generally obtain between 20 and 200 colonies. Once colonies are clearly visible, gently aspirate them off the surface with a P20 Pipetman and transfer them to another 10-cm Petri dish containing selective HL5. Colonies appear on the second plate within several days. Harvest individual colonies as above. Transfer these to 24-well microtiter dishes containing selective HL5 for propagation.

B. An alternative method for isolating transformed cells involves dilution into 96-well microtiter plates. On day 2 (8–20 hr after glycerol shock) resuspend cells in medium containing antibiotic selection, and dilute a portion with more selective HL5 for transfer to microtiter wells. The amount of dilution depends on how many colonies are expected, but one-fifth to one-tenth is generally appropriate. We resuspend the original transformation plate in 10 ml of selective medium, and transfer 1 ml of this suspension to 7 ml additional selective medium. Transfer this dilution to a 96-well microtiter plate using a 12-channel pipette, applying 60 μl/well. Leave 9 ml of the initial resuspension in the original transformation plate. Keep both this original plate and the microtiter plate and monitor for colonies. Change medium in the original Petri plate at 3-day intervals. The first medium change on the microtiter plate is usually performed at 5 days. The next change is done after an additional 7–8 days. By this time colonies are usually clearly apparent and can be transferred to larger plates for maintenance. With 10% well occupancy 95% of the colonies will be derived from single cells.[20]

Method A is generally the easier one for obtaining transformed cells, and for most purposes works fine. In some circumstances method B can be

[20] H. A. Coller and B. S. Coller, this series, Vol. 121, p. 412.

of value. For example, with some *Dictyostelium* isolates spontaneous hygromycin-resistant colonies appear during transformation at frequencies approaching the transformation frequency, and may outgrow the true transformants. In this situation dilution into microtiter plates prevents contamination of true transformants with spontaneously resistant cells.

In most cases we maintain transformed cell lines on Petri dishes in HL5 containing selection for the transformation marker. Once confluent, these dishes contain approximately $10^7$ cells. Pass the cells by drawing the medium into a 10-ml pipette and blowing it out onto the cell lawn to detach the cells. Once they are resuspended, make a one-twentieth to one-fiftieth dilution into a fresh plate.

### *Electroporation*

Electroporation offers a method for introducing DNA into *Dictyostelium* that is simplier than the more widely used calcium phosphate method. This application of electroporation for *Dictyostelium* was first reported by Howard *et al.*[18] We use a minor modification of this method as described below. We generally use electroporation for the introduction of all plasmids that contain the extrachromosomal sequences Ddp1 or Ddp2. Although these extrachromosomal plasmids can be introduced with similar efficiency via calcium phosphate precipitates, electroporation is the method of choice, being simpler and of slightly higher efficiency. As reported by Howard *et al.*, we have found that electroporation does not work as efficiently with transformation vectors that integrate after transformation into *Dictyostelium*. Therefore with integrating vectors we generally use the calcium phosphate method described above.

*Procedure.* Harvest cells for transformation from HL5 medium at densities of $2-3 \times 10^6$ cells/ml or less by centrifugation at 1000 *g* for 5 min, washing twice with cold 10 m*M* sodium phosphate, pH 7.0, 50 m*M* sucrose. Resuspend cells in the same buffer at $10^7$ cells/ml. We use a Bio-Rad (Richmond, CA) Gene Pulser unit for electroporation, with the 0.8-ml cuvettes. Mix 0.8 ml of the cell suspension with 5 $\mu$g of the transformation plasmid and place in a 0.8-ml electroporation cuvette. Place the cuvette on ice for 5–10 min to ensure complete chilling before the electroporation step. Flick the cuvette briefly to resuspend settled cells, and immediately subject to one pulse at 1200-V and 3-$\mu$F settings. This should generate a time constant of 0.6–1.0 sec. Return the sample immediately to ice, and after 5 min transfer the cells to a 10-cm Petri dish containing 10 ml of HL5 (with PenStrep). It is critical with these conditions that the cells be well chilled when the charge is delivered. Considerable lysis occurs if cells are subjected to these electroporation conditions at room temperature. Cells

can be checked with an inverted microscope 10–20 min after transfer to HL5. At this stage they should appear healthy and the majority of them should be attached to the dish. No detectable lysis should have occurred. Allow cells to recover 8–20 hr, and then apply selection for transformants by one of the methods described above for calcium phosphate-mediated transformation. Although we have used only the Bio-Rad electroporator, several other manufacturers have similar machines on the market. The data of Howard *et al.*[18] should be consulted for information regarding exact conditions with different instruments.

## Gene Targeting

Gene targeting, the recombination of DNA sequences residing in the chromosome with newly introduced homologous sequences, has become an important tool in eukaryotic cell and developmental biology. With this technique one can direct alterations to any gene for which molecular probes are available, introducing point mutations, gene truncations, or completely removing a gene from the genome. *Dictyostelium* has several features that make it appealing for gene targeting experiments. It resembles yeast in having a relatively small genome size (50,000 kb),[21] while in its appearance and motile behavior it resembles more closely mammalian cells. *Dictyostelium* is generally worked with in its haploid state, a feature that considerably simplifies gene targeting experiments. Most importantly, homologous recombination occurs at high efficiency relative to random integration events, unlike mammalian cells.[1,4]

### *General Considerations*

Gene targeting has been reported for five genes in *Dictyostelium* to date.[1,3,22–24] In our experiments with the myosin *(mhcA)* locus, we have performed two types of gene targeting experiments. These can be classified as gene disruption and gene replacement methods. The first type of targeting, gene disruption, involves a single recombination event and results in integration of the introduced circular plasmid into the myosin locus. This type of event is represented in Fig. 1A. In this representation, only a single integrated plasmid is shown, but in most cases there are probably multiple plasmid copies present as a tandem array. The initial demonstration of homologous recombination in *Dictyostelium* involved this type of event.[1]

[21] R. A. Firtel and J. T. Bonner, *J. Mol. Biol.* **66**, 339 (1972).
[22] G. Jung and J. A. Hammer III, *J. Cell Biol.* **110**, 1955 (1990).
[23] M. Maniak, U. Saur, and W. Nellen, *Anal. Biochem.* **176**, 78 (1989).
[24] C. Harloff, G. Gerisch, and A. A. Noegel, *Genes Dev.* **3**, 2011 (1989).

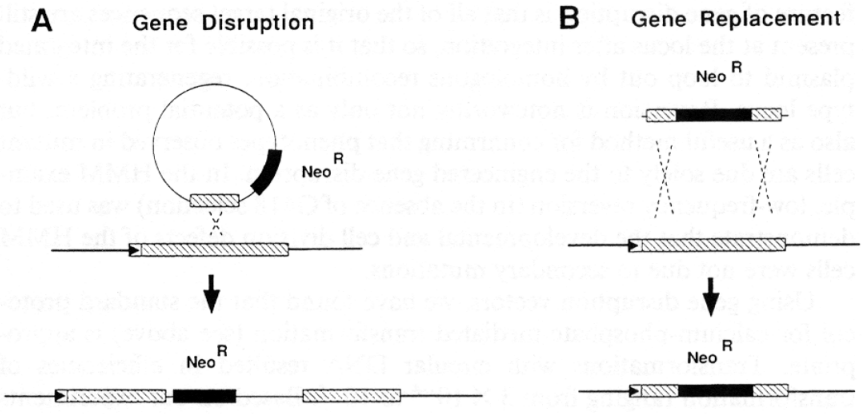


FIG. 1. Schematic diagram of (A) gene disruption and (B) gene replacement. Circular plasmid (A) or linear gene replacement fragment (B) is shown at top, aligned with native genomic copy of the homologous gene. Products of the recombination events are shown at the bottom. Thin line, plasmid DNA; thick line, flanking DNA in genome; black box, neo-resistance gene cartridge; hatched boxes, native gene and cloned segments of homology; boxed arrowhead, native promoter.

In that experiment a myosin coding region fragment corresponding to the heavy meromyosin (HMM) portion of the myosin was introduced into cells and recombined at the myosin locus. The result was a cell line that contained the HMM portion of the myosin gene fused to the native promoter (indicated by boxed arrowheads in Fig. 1) residing upstream of the myosin gene. In the integrated configuration, plasmid sequences lie downstream of this HMM segment, followed by the rest of the native portion of the myosin gene. This arrangement resulted in expression of HMM protein from the myosin promoter. The downstream portion of the myosin gene failed to be expressed because there was no promoter positioned to drive it. Several noteworthy features of gene disruption are apparent in this example. First, it is clear that this approach allows one to disrupt expression of the normal gene product at a given locus. The resulting phenotypes can provide insights into the cellular roles of the gene. In the HMM example, the phenotypes of the resulting cells proved to be very similar to that of myosin null cells that were created later. In most cases production of severely truncated proteins can be expected to give phenotypes similar to total removal of the protein. The ability to produce truncated proteins such as HMM is the second noteworthy feature of gene disruption. This feature can be taken advantage of to engineer the expression of specifically tailored gene products. This application allows a variety of altered proteins to be produced in place of the wild-type protein. A final

feature of gene disruption is that all of the original target sequences are still present at the locus after integration, so that it is possible for the integrated plasmid to loop out by homologous recombination, regenerating a wild-type locus. Reversion is noteworthy not only as a potential problem, but also as a useful method for confirming that phenotypes observed in mutant cells are due solely to the engineered gene disruption. In the HMM example, low-frequency reversion (in the absence of G418 selection) was used to demonstrate that the developmental and cell division defects of the HMM cells were not due to secondary mutations.

Using gene disruption vectors, we have found that the standard protocol for calcium-phosphate-mediated transformation (see above) is appropriate. Transformations with circular DNA resulted in efficiencies of transformation ranging from $3 \times 10^{-6}$ to $10^{-5}$. Based on our experiments with the myosin locus,[1] and other studies with the $\alpha$-actinin locus,[4] it appears that introduction of homologous sequences in the range of 0.7 to 5 kb results in gene disruption in 2–30% of the transformed cells. Southern blot analysis is the standard method for identifying transformed cell line clones bearing recombination events. It is critical that a genomic map of the gene be known for several restriction enzymes, and that predicted Southern blot hybridization patterns for a homologous integration versus a random integration be distinct from one another. It is also essential that transformed cell lines used for Southern analysis be clonal rather than being mixed populations of transformants.

The second type of targeting experiment we have performed is gene replacement. In this procedure two recombination events occur, resulting in replacement of a native gene segment with introduced DNA. This is illustrated schematically in Fig. 1B. The transforming plasmid in this case contains the neo-resistance gene situated between 5′ and 3′ blocks of homology to the native gene. A recombination event in each of these homologous segments results in loss of the intervening portion of the native gene, and its replacement with the neo-resistance gene. 5′ and 3′ DNA segments can consist of coding or flanking sequences, so that it is possible to obtain cell lines that are virtually or entirely devoid of coding DNA for the gene of interest. The minimum sequence length that has given successful gene replacement to date is about 700 bp. The primary advantage of the gene replacement approach is that there is no possibility for a later recombination event causing reversion. Myosin null cell lines made by this method are extremely stable and show no reversion in their developmental or cell division defects. The absence of reversion is critical if one wishes to later use a mutant cell line as a recipient for modified versions of the original gene.

In studies with the myosin locus, gene replacement apparently occurred less frequently than gene disruption. For this reason several modifications were introduced in an attempt to improve the efficiency of the procedure. The first of these is that the transforming plasmid DNA was linearized adjacent to the replacement sequences. The creation of free DNA ends adjacent to the segments of homology may be expected to enhance recombination frequencies.[25,26] It has been observed, however, that linearized plasmid DNA transformed into *Dictyostelium* religates with very high efficiency.[27] For this reason an additional step was introduced to remove the complementary restriction site overhangs from the free ends of the DNA, rendering them less likely to religate. This was done by restricting the gene replacement plasmid on each side of the replacement cartridge with a restriction enzyme that leaves a 3′ overhang, and treating the resulting ends with T4 DNA polymerase in the presence of a single deoxynucleotide. Adjacent polylinker restriction sites were taken advantage of for this step. The 3′–5′ exonuclease activity of the polymerase removes all complementary bases at the termini of the DNA fragments, and the presence of the single deoxynucleotide ensures that only limited exonuclease digestion occurs.

Gene replacement with linear restricted DNA has the potential to create single-copy integration events. A potential concern in this situation is that a single copy of the drug resistance gene would be insufficient to confer resistance to standard selection conditions during the initial isolation of the mutants. For selection with G418 we were able to overcome this problem simply by lowering the concentration of the drug to 6 μg/ml. For selection with hygromycin suitable conditions have yet to be found. The procedure given below provides the conditions we have used to prepare the vector DNA, and indicates steps in the transformation procedure that differ from the standard protocol presented above. These modifications were introduced with the goal of increasing the number of recombinant cells obtained from the procedure.

*Protocol*

1. Restrict 10–20 μg of gene replacement vector with appropriate restriction enzymes to liberate the gene replacement fragment. At least one of the ends should be cleaved with an enzyme that creates a 3′ overhang.

[25] T. L. Orr-Weaver, J. W. Szostak, and R. J. Rothstein, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6534 (1981).
[26] T. L. Orr-Weaver, J. W. Szostak, and R. J. Rothstein, this series, Vol. 101, p. 228.
[27] K. S. Katz and D. I. Ratner, *Mol. Cell. Biol.* **8**, 2779 (1988).

Following the restriction enzyme digests incubate the DNA for 5–8 min with T4 DNA polymerase (1 unit/20 μg DNA) in the presence of a single deoxynucleotide triphosphate. Ethanol precipitate the DNA before proceeding to the transformation step. It is not necessary to remove the vector DNA that is still present in the sample with the gene replacement DNA.

2. Grow Ax2 or Ax4 cells to a density of $2-4 \times 10^6$/ml. Approximately 3 hr before transformation, transfer $2 \times 10^7$ cells to a 10-cm plastic Petri dish and allow them to attach for 10 min. Remove the medium and replace with HL5–Bis-Tris. Prepare calcium phosphate–DNA precipitates and perform the transformation procedure as described above.

3. Following the glycerol shock, allow cells to recover for 18–24 hr. On day 2 replace the medium with HL5 containing G418 at 6 μg/ml. Change the medium every 24 hr for 3 days, maintaining the G418 at 6 μg/ml. From day 5 on, increase the G418 concentration to 10 μg/ml with medium changes only every third day. Following the appearance of colonies, around the sixth day, remove cells from these colonies with a pipette and analyze and reclone as described in the section, Transformation Protocols.

## Expression of Recombinant Myosin Subfragments

The tools described in this chapter are currently being employed for two classes of studies in our laboratory. The first involves expression of head subfragments of the myosin protein in *Dictyostelium,* both as wild-type fragments and as fragments bearing site-directed mutations. Biochemical purification of these fragments allows specific questions regarding active domains to be addressed. The second class of experiments involves expression of wild-type and altered forms of the entire myosin heavy chain. These studies are designed to address the *in vivo* roles and activity of domains of the protein.

In the first class of experiments, recombinant myosin head fragments (HMM or S1 equivalents) are generally transformed into cell lines that contain the wild-type myosin gene, such as Ax2 cells, rather than into myosin null cells. This choice of recipient results in a later requirement to purify the recombinant fragment away from the wild-type myosin. This disadvantage is compensated for by the ability of the wild-type cells to grow in suspension. Myosin null lines, by contrast, grow only as surface-attached cells. Obtaining 150–200 g of cells for biochemical purification requires 30–40 liters of *Dictyostelium* culture to be grown. As suspension culture this is not a difficult task, but as surface-attached cultures it is very labor intensive.

An additional benefit of transformation into wild-type cells is that a wide array of G418-resistance vectors are available for transforming these

cell lines. Myosin head fragments have been expressed using a variety of the *Dictyostelium* vectors described above. Both integrating and extrachromosomal vectors have been utilized with similar results. A limited number of strong, vegetatively expressed *Dictyostelium* promoters are available to date, including the actin 6,[6] actin 15,[8] and heat shock promoters.[28] Although the myosin promoter is a good candidate for expression studies, it initially proved difficult to clone due to instability in *Escherichia coli*. For this reason expression constructs made in our laboratory over the past several years have employed the well-characterized promoter from the *Dictyostelium* actin 15 gene. Recombinant myosin gene fragments corresponding to both HMM and S1 portions of the protein have been introduced into *Dictyostelium* driven by the actin 15 promoter. When genes driven by this promoter are introduced into plasmids containing the actin 15–neomycin selection cartridge described above, we generally orient them such that they converge upon the actin 15 termination element located at the 3′ end of the neo-resistance gene. This terminator has previously been shown to function in both orientations.[1] The convergent orientation allows the expressed gene fragments to utilize the same terminator segment as the neo-resistance gene. It has also been suggested that this orientation may prevent homology-based loopout of segments lying between the two copies of the actin 15 promoter. Expression levels of the introduced recombinant proteins have typically been within a few fold of the levels of endogenous myosin. Expression from the actin 15 promoter increases in level early in development.[8] We have found that expression levels of several engineered proteins driven by this promoter can be enhanced by developing the cells for several hours prior to harvesting cells for protein purification. We are currently employing this system to express and purify milligram quantities of wild-type and mutant myosin head subfragments.[14,29]

In the second class of studies (*in vivo* analysis) it is desirable to introduce the altered myosin gene into myosin null cells, allowing *in vivo* effects of specific alterations to be studied in the absence of the wild-type protein. For these studies myosin null cells are transformed using vectors that confer resistance to hygromycin. As described above, we have fused these myosin gene constructs to actin 15 promoters. Hygromycin resistance is most reproducible as a selectable marker when present on high-copy-number extrachromosomal vectors, so the plasmid pDE109 is generally used. When this arrangement is used with the intact myosin gene, we obtain expression levels in the range of approximately 20–70% relative to wild-

[28] S. M. Cohen, J. Capello, and H. F. Lodish, *Mol. Cell. Biol.* **4**, 2332 (1984).
[29] K. M. Ruppel, T. T. Egelhoff, and J. A. Spudich, *Ann. N.Y. Acad. Sci.* **582**, 147 (1990).

type cells. An important point is that occasionally during a transformation spontaneous hygromycin-resistant colonies arise at frequencies (up to $10^{-6}$) approaching that of the real transformation frequency. It is essential that a negative control transformation be performed, using a DNA sample that does not confer hygromycin resistance. If this control produces no colonies, then colonies on the experimental samples can be assumed to be true transformants. Often we see one or two colonies on the negative control while obtaining 50–100 colonies with pDE109. In parallel samples of pDE109 containing a myosin gene expression construct, typically 20–30 colonies are obtained, and these tend to appear more slowly than colonies transformed with pDE109 alone. In this situation it is useful to have the transformation plated in 96-well microtiter plates. This ensures that any faster growing spontaneously resistant colonies that might be present will not overgrow the true transformants. With recipient cell lines that show no tendency to form spontaneous colonies, this precaution can be omitted.

### Concluding Remarks

Recent advances in the application of molecular genetics to the simple eukaryotic organism *Dictyostelium* have greatly increased our understanding of the role of cytoskeletal proteins *in vivo*. The ability to perform homologous recombination as well as introduce mutated or altered genes of interest and have them overexpressed greatly expand the range of options available in carrying out cell biological studies. The principles and techniques described here illustrate how one can undertake a multifaceted approach to study a protein or phenomenon of interest in *Dictyostelium*.

## [28] Screening for *Dictyostelium* Mutants Defective in Cytoskeletal Proteins by Colony Immunoblotting

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### Introduction

There are two ways of studying the activities of cytoskeletal proteins. One is to reconstitute systems *in vitro* that are composed of defined, purified components.[1,2] By adding more and more proteins to such systems one can simulate under defined conditions cellular functions of increasing