

Isolation and Characterization of a Novel Cytokinesis-Deficient Mutant in *Dictyostelium discoideum*

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Abstract Cytokinesis is a dramatic event in the life of any cell during which numerous mechanisms must coordinate the legitimate and complete mechanical separation into two daughter cells. We have used *Dictyostelium discoideum* as a model system to study this highly orchestrated event through genetic analysis. Transformants were generated using a method of insertional mutagenesis known as restriction enzyme-mediated integration (REMI) and subsequently screened for defects in cytokinesis. Mutants isolated in a similar screen suffered a disruption in the myosin II heavy chain gene, a protein known to be essential for cytokinesis and in a novel gene encoding a rho-like protein termed *racE* [Larochelle et al., 1996]. In the screen reported here we isolated a third type of mutant, called 10BH2, which also had a complete defect in cytokinesis. 10BH2 mutant cells are able to propagate on tissue culture plates by fragmenting into smaller cells by a process known as traction-mediated cytofission. However, when grown in suspension culture, 10BH2 cells fail to divide and become large and multinucleate. Phenotypic characterization of the mutant cells showed that other cytoskeletal functions are preserved. The distribution of myosin and actin is identical to wild type cells. The cells can chemotax, phagocytose, cap crosslinked receptors, and contract normally. However, the 10BH2 mutants are unable to complete the *Dictyostelium* developmental program beyond the finger stage. The mutant cells contain functional genes for myosin II heavy and light chains and the *racE* gene. Thus, based on these findings, we conclude that 10BH2 represents a novel cytokinesis-deficient mutant. © 1996 Wiley-Liss, Inc.

Key words: cell division, motility, contraction, myosin, actin, capping, development, mutagenesis

INTRODUCTION

Cell division is one of the most dramatic events in the life of a cell during which numerous complex mechanisms need to ensure that the complete genetic complement as well as the full battery of cytoplasmic organelles is correctly distributed to each daughter cell. This highly regulated event culminates with cytokinesis, which results in the mechanical separation of the two daughter cells via the formation of a circumferential contractile ring. The formation of the contractile ring is both temporally and spatially coordinated with the mitotic cycle. Strong evidence that the mitotic apparatus (and in particular the asters) controls the formation of the contractile ring came from elegant micro-manipulation experiments of Rappaport [1986]. Yet questions remain regarding the precise mo-

lecular and biochemical means by which the mitotic apparatus transmits signals to the cell cortex to initiate formation of the contractile ring at precisely the desired orientation and time.

Recent studies utilizing molecular genetic advances in mammalian cells, flies, yeast, fungi, and cellular molds have shed light onto some of the central questions challenging the field since its emergence earlier this century [Satterwhite and Pollard, 1992]. Certainly, the inventory of proteins involved at different stages of cell cleavage has expanded. In addition, a link between the cell cycle machinery and the contractile ring has been postulated by Satterwhite et al. [1992]. In this model, the p34^{cdc2} kinase (whose activity is high during prophase and metaphase) would inhibit the formation of the contractile ring by phosphorylating the regulatory myosin II light chain at an inhibitory site. At the onset of anaphase, when the p34^{cdc2} kinase activity plummets, myosin II would regain its function and would form the contractile ring. Furthermore, a

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direct interaction between the mitotic apparatus and the contractile ring was revealed by the translocation of the INCENP-B protein from the metaphase plate to the contractile ring [Earnshaw and Cooke, 1991]. Finally, calcium transients during the cell cycle may also play a part in controlling the formation of the contractile ring [Fluck et al., 1991]. However further studies are required to elucidate the regulatory mechanisms which govern the assembly, contraction, and dismantling of the contractile ring.

We have chosen to isolate novel genes essential for cytokinesis using *Dictyostelium discoideum* as a model system. This highly motile organism is an excellent candidate for molecular and biochemical studies of numerous processes, including cell migration, differentiation, and cell-cell interactions. It is especially well suited for the study of cell division as it undergoes cytokinesis much like higher eukaryotic cells [Fukui and Inoue, 1991]. Furthermore, several *Dictyostelium* mutants have been isolated with defects in cytokinesis [De Lozanne and Spudich, 1987; Liu et al., 1992; Pollenz et al., 1992; de Hostos et al., 1993; Chen et al., 1994; Haugwitz et al., 1994]. These mutants helped to define the essential proteins required for cytokinesis and also revealed that cytokinesis is not an essential process in *Dictyostelium*. When these mutants are placed on a solid substratum they can fragment into smaller cells by "pinching-off" portions of their cytoplasm in a process known as traction-mediated cytofission [Fukui et al., 1990]. Based on this phenotype, we screened *D. discoideum* cells mutagenized by restriction enzyme-mediated integration (REMI) [Kuspa and Loomis, 1992] to identify clones that are completely impaired in cytokinesis. Four such mutants have been isolated in a separate screen, which bore disruptions of either the myosin II heavy chain or the *racE* gene [Larochelle et al., 1996]. We have identified a new mutant, named 10BH2, which is also completely impaired in cytokinesis. The characterization of this mutant is discussed in this paper.

MATERIALS AND METHODS

Strains

Dictyostelium discoideum strain HL330 [Kuspa and Loomis, 1992] lacking the *pyr5-6* (UMP synthase) gene [Shi and Thornburg, 1993] was maintained at titers between 1×10^5 cells/ml and 4×10^6 cells/ml in HL5 medium supplemented with uracil at 40 μ g/ml. Bacterial strain

DH5- α was used to propagate plasmids containing cloned PCR fragments.

Restriction Enzyme-Mediated Integration (REMI)

Plasmid pDIV2 was a kind gift from Dr. Adam Kuspa [Kuspa and Loomis, 1992]. Prior to transformation, pDIV2 was linearized with BamHI (Boeringer Mannheim, Indianapolis, IN), purified by phenol extraction and ethanol precipitation and brought to a final concentration of 1 μ g/ μ l in TE (10 mM Tris, pH 8.0 and 1 mM EDTA).

REMI was carried out as described originally [Kuspa and Loomis, 1992]. Briefly, HL330 cells at titers of 1 to 2×10^6 cell/ml, were pelleted in 50 ml conical tubes at 4°C and 82 g for 5 min. The nutrient media was aspirated and the cells resuspended in ice-cold electroporation buffer (50 mM sucrose, 10 mM NaPhosphate, pH 6.1) to 1×10^7 cells/ml. Cells and cuvettes were kept on ice for the remaining time. Cells (8×10^6 per transformation) were mixed with 40 μ g of BamHI-cut pDIV2 and 150 units of Sau3AI (IBI, New Haven, CT) in a 0.4-cm-gap disposable electroporation cuvette (BioRad, Hercules, CA). This cocktail was then immediately electroporated at 0.9 kV, 3 μ F and 200 Ω in a Biorad Gene Pulser[™] and the cells allowed to recover on ice for 10 min. To obtain clonal transformants cells from one electroporation were gently mixed with 57 ml of FM minimal media lacking uracil [Franke and Kessin, 1977], and distributed into ten 96-well plates using a dodeca-pipette such that each well received 60 μ l of the cell suspension. The plates were maintained in FM minimal media at 21°C to select for uracil prototrophy. The FM media was changed every week until colonies of transformed cells appeared.

Mutant Screening

Following selection for uracil prototrophy in FM medium, the number of colonies per 96-well plate were counted. To increase the probability of working with clonal transformants as predicted by the Poisson distribution, only those plates with less than 35 colonies per plate were kept. For the primary screen, each colony was manually transferred in duplicate to 24-well plates in 0.5 ml per well of FM medium. One of the 24-well plates was kept stationary while the other was shaken at 240 rpm for 1 week. The duplicate plates were then examined for clones that failed to grow in suspension (visibly clear culture), but that grew in the stationary plate (turbid culture). As a secondary screen, such

clones were picked from the stationary plate and subjected to the same procedure as above. Clones that maintained the "no-growth-in-suspension" phenotype were passed up to six-well plates and subjected to two more rounds of screening in a total volume of 10 ml of media in 50 ml flasks. The cells were counted and also stained with DAPI to check for multiple nuclei (see below). After these four rounds of screening, clones that grew on plates but that failed to grow in suspension and became multinucleated, were treated as positive mutants. They were then grown in larger quantities on plates to prepare genomic DNA for molecular analysis.

Southern Analysis

Typically, 20 µg per lane of genomic DNA was digested with several different restriction enzymes, electrophoresed into a 1% agarose gel, blotted onto Hybond-N (Amersham, Arlington Heights, IL) and probed with ³²P-labeled pDIV2. The bands were visualized on a Fujix Bas 1000 phosphor-imager or by autoradiography using Kodak (X-OMAT AR) film.

Genomic Cloning

Recovery of the plasmid inserted into the 10BH2 mutant, together with the flanking genomic regions was attempted by digesting 20 µg of genomic DNA from the mutant cells with a variety of enzymes. The digested DNA was ligated in a volume of 10 µl to favor intramolecular ligation and 1 µl of the ligation cocktail was electroporated into SURE™ cells from Stratagene (La Jolla, CA). When these attempts were unsuccessful, PCR methods were adopted. PCR conditions were optimized for the experiment to be done. Typically the melting temperature depended on the melting point of the primers being used and a minute of extension time was allowed per kilobase of expected product. Also, regular AmpliTaq® DNA Polymerase (Perkin Elmer, Norwalk, CT) was supplemented with Taq-extender™ PCR additive (Stratagene, La Jolla, CA) and used according to the manufacturer's suggestions in order to improve the quality of the products. 1 µl of the ligation cocktail was used as template for nested PCR while 2 to 3 µl from the first round of PCR was used as template for the second set of primers. The 1.3 kb 5' region was obtained by digesting genomic DNA with ClaI and HindIII, blunting and ligating the ends, and amplifying using primers **AO-101R** and **SP6** for the first amplification and primers

AO-104 and **SP6** used for the following nested PCR amplification. The 1.7kb 3' flanking region was obtained by digesting genomic DNA with EcoRI, ligating the ends, and amplifying using primers **AO-107** and **DIV2R2** followed by **AO-106** and **DIV2-R1**. The primers used were: **AO-101R**-ACC TAC AGC GTG AGC TAT GAG AAA G, **AO-104**-CTC CCT CGT GCG CTC TCC TG, **SP6**-CAC ATA CGA TTT AGG TGA CAC, **AO-107**-CTT GGA GCG AAC GAC CTA CAC C, **DIV2R2**-CTT CTG AAT GTC CAT TTG ATT G, **AO-106**-ACC TAC AGC GTG AGC TAT GAG AAA G, **DIV2R1**-GTA AAA ATA ACT AAT CGA CTC TAG AGG ATC. PCR products were cloned into the pT7Blue-T vector from Novagen and transformed into *E. coli* DH5α cells.

Staining for DNA, Myosin, and Actin

Mutant cells maintained on plates were prepared for agar-overlay immunofluorescence [Fukui et al., 1987] in order to determine both the number of nuclei and myosin II localization. They were harvested and allowed to attach to glass coverslips for 15 min after which the nutrient media was exchanged for Sorenson's Buffer (SB, 15 mM KH₂PO₄, 2 mM NA₂PO₄, pH 6.5). The amoebae were overlaid with thin sheets (0.2 mm thick) of 1% agarose M (Pharmacia Biotech, Piscataway, NJ) and excess buffer carefully wicked away. The agar-overlaid coverslips were fixed in a 1% formaldehyde-methanol solution for 5 min at -20°C, washed in TBS (25 mM Tris, 135 mM NaCl, 2.7 mM KCl, pH 7.4), blocked with a 5% BSA solution for 30 min at 37°C, and incubated with a polyclonal antibody against the myosin II heavy chain for 1 h at 37°C. After washing again in TBS the cells were incubated for 1 h at 37°C in a preadsorbed FITC-conjugated goat-anti-rabbit antibody (Molecular Probes, Eugene, OR). The coverslips were washed, stained with a 1 µg/ml solution of DAPI in SB for 10 min, and rinsed with water before mounting onto slides for visualization by immunofluorescence microscopy (Zeiss, Thornwood, NY).

To visualize actin distribution, cells were harvested as above and allowed to attach to glass coverslips for 15 min and fixed with a 3.7% solution of formaldehyde in 150mM KCl, 5mM MgCl₂, 20mM K-phosphate, 10mM EGTA, pH 6.1 for 10 min at RT (room temperature). Cells were washed in TBS and incubated at RT for 10 min with a 1:100 dilution of Rhodamine-Phalloidin (Molecular Probes, Eugene, OR) in a 0.05% NP-40-PBS buffer. The coverslips were rinsed and mounted for visualization.

ConA Capping Assay

Cells were harvested from Petri plates, attached to glass coverslips for 15 min and washed free of media with SB. A fresh 1 mg/ml solution of FITC-ConA (Sigma, St. Louis, MO) in SB was clarified by centrifugation for 10 min at 16,000g in an Eppendorf Micro Centrifuge 5415C. 100 μ l of this solution was then added to the cells for exactly 2 min at room temperature (RT), quickly washed off with SB, and the cells incubated for 5, 10, and 15 min at RT. The cells were fixed in 1% formaldehyde-methanol at -20°C for 5 min, rinsed with water, and the coverslips mounted.

Cortical Contraction Assay

Cells were allowed to grow overnight at 21°C either in six well plates or on coverslips. Media in the plate was then exchanged for media plus 2 mM azide and the cells observed at $20\times$ for up to 10 or 15 min. The coverslips were placed in a continuous-flow chamber, and media containing 2 mM azide was diffused into the chamber while monitoring the cellular response at $40\times$.

Development

To prepare bacterial lawns SM/5 agar plates (0.2% glucose, 0.2% peptone, 0.2% yeast extract, 2% agar, 0.8 mM MgSO_4 , 14 mM KH_2PO_4 , 5.7 mM K_2HPO_4 , pH 6.4) were inoculated with *E. coli* B/r and incubated at 37°C overnight. Plates were cooled down to RT before seeding the bacterial lawn with a 250 μ l drop of cells containing 5×10^6 cells. The plates were incubated at 21°C for 7 to 10 days to allow the cells to clear the bacteria and undergo their developmental cycle. The plates were observed daily and the final stages of development were then photographed under a dissecting microscope.

RESULTS

Screen for Cytokinesis-Deficient Mutants

The goal of this study was to identify novel genes essential for cytokinesis using a method of random insertional mutagenesis. Restriction enzyme-mediated integration (REMI) was used to generate a panel of 4,500 *D. discoideum* transformants which were subsequently screened for their inability to divide in suspension culture. The screen was designed to isolate transformants which emulated cells lacking myosin II, a protein known to be essential for cytokinesis [De Lozanne and Spudich, 1987; Knecht and Loomis, 1987]. These cells can propagate on a sub-

stratum by an illegitimate means known as traction mediated cytofission [Fukui et al., 1990]. In suspension however, they cannot divide and since mitosis is normal, they become strikingly large and multinucleate. Accordingly, in our screen we looked for those transformants that could not grow in suspension culture, but that could grow attached to plates. Four cytokinesis mutants were isolated in a separate screen [Larochelle et al., 1995]. Two of those mutants suffered mutations at the myosin II heavy chain locus demonstrating that our screen could positively identify cytokinesis mutants. Two other mutants harbored disruptions of a novel member of the *rho* subfamily of small GTP-binding proteins named *racE* [Larochelle et al., 1996]. In the screen described here we isolated a fifth mutant (10BH2) which also had a complete defect in cytokinesis.

Cytokinesis Is Impaired in 10BH2 Cells

In accordance with the design of our screen, 10BH2 cells, like myosin II null cells cannot divide in suspension culture, although karyokinesis proceeds normally. When grown in suspension, wild type cells grow exponentially and saturate the culture within 4 days, however myosin null mutants and the 10BH2 mutant cells fail to propagate (Fig. 1A). This failure is not due to an overall growth deficiency since 10BH2 mutant cells multiply at almost the same rate as the parental wild-type strain when grown on plates (Fig. 1B). Under these conditions, the 10BH2 mutant cells have a similar size and morphology as wild-type cells. Staining with the nuclear stain DAPI showed that wild-type cells contained 1 or 2 nuclei when grown attached to plates (Fig. 2A). In suspension cultures, most wild-type cells contained 1 or 2 nuclei and very few cells contained more than 2 nuclei (Fig. 2B). However, 10BH2 mutants were uninucleate only when grown on plates (Fig. 2C). In suspension cultures the mutant cells became very large and multinucleate (Fig. 2D).

10BH2 Cells Contain a Mutation Distinct from Myosin II and *racE* Mutants

In attempting to identify the gene affected in the 10BH2 mutant we first determined whether it had suffered a mutation in the myosin II heavy chain and light chain or the *racE* gene, proteins essential for cytokinesis. Southern blot and Western blot analyses, indicated that these genes were intact and functional in the 10BH2

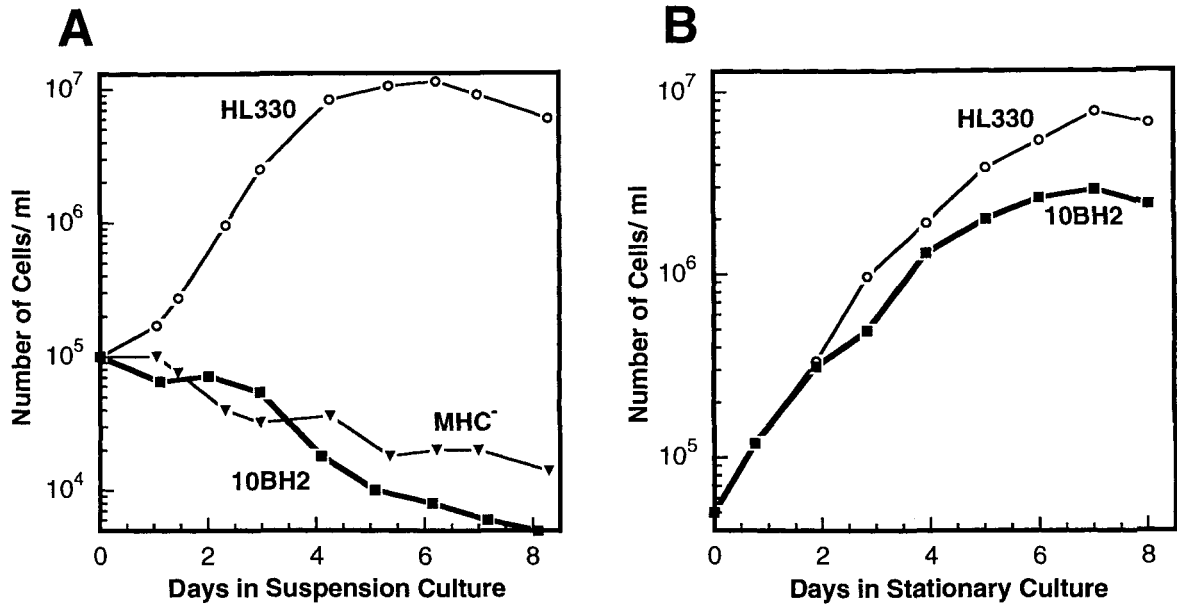


Fig. 1. Growth curves of wild type and 10BH2 mutant cells in suspension and stationary cultures. **A:** Cells were harvested from stationary plates, seeded at 1×10^5 cells/ml, shaken at 240 rpm and counted every day. Wild type cells grow exponentially and saturate the culture within 4 days, while 10BH2 mutant cells as well as myosin II null cells become large and

multinucleate and eventually lyse leading to a loss in cell number. **B:** Cells were harvested from stationary plates, diluted to 5×10^4 cells/ml, and 1.5 ml plated onto six-well plastic dishes. Every day cells from a well were resuspended and counted. Wild type and 10BH2 mutants grow at similar rates.

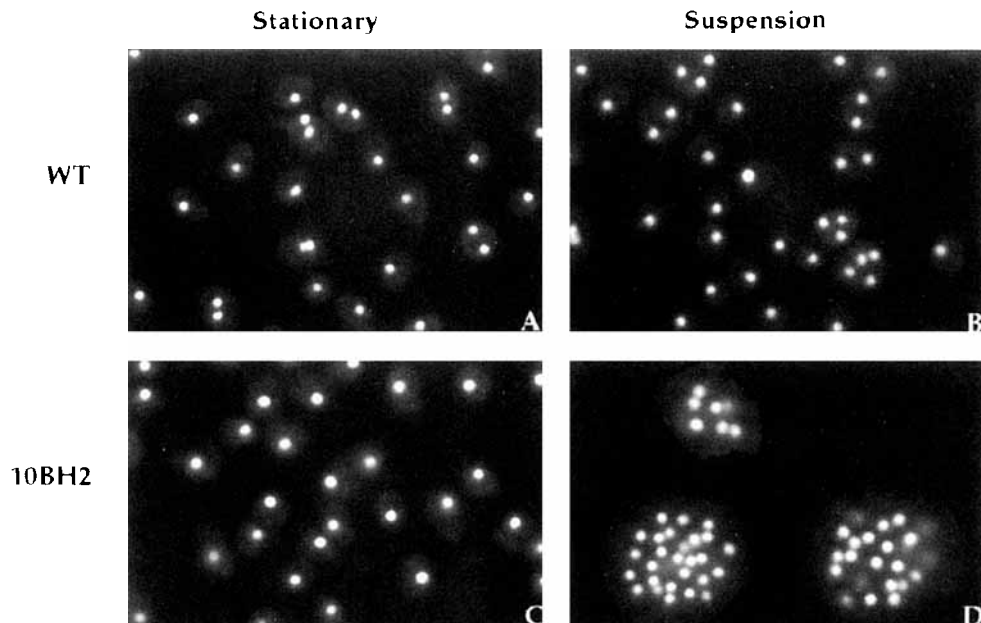


Fig. 2. 10BH2 cells fail to grow in suspension and become large and multinucleate in suspension culture. Parental wild type and 10BH2 mutant cells were grown either on stationary plates or in suspension culture for seven days. Their nuclei were

stained with DAPI. **A:** Wild type cells grown on stationary plates; **B:** wild type cells grown in suspension culture; **C:** 10BH2 mutant cells grown on stationary plates; **D:** 10BH2 mutant cells grown in suspension culture.

cells (data not shown). Furthermore, the phenotype of the 10BH2 mutant cells described below, is distinct from the phenotype of the myosin II or *racE* mutants. Thus, the mutation suffered by the 10BH2 cells defines a novel locus that is essential for cytokinesis.

Cloning of the 10BH2 Disruption Locus

One of the major advantages of REMI mutagenesis is that the gene of interest is tagged with a plasmid that facilitates its cloning. In order to map convenient sites flanking the insertion site, we prepared genomic DNA from 10BH2 cells for Southern blot analysis and probed it with the integrating plasmid pDIV2. This analysis indicated that only one copy of pDIV2 had integrated into 10BH2 (Fig. 3, pGEM probe). This analysis also revealed that the plasmid suffered some rearrangement that led to the formation of a small 1.2 kb EcoRI fragment that hybridizes with the plasmid probe (Fig. 3, pGEM probe, EcoRI lane). Through very detailed Southern analysis of the 10BH2 genomic DNA and from sequence analysis of a PCR product obtained from within the inserted plasmid we discovered that a rearrangement had indeed occurred during plasmid insertion. This rearrangement resulted in an inversion of 455 bp at one end of pDIV2 in such a way that the plasmid origin of replication (*ori*) was cut in half and rendered dysfunctional (Fig. 4A, arrows). Thus, we were not able to retrieve the plasmid and its flanking sequences by conventional methods since it could no longer replicate in bacteria.

With this knowledge, however, we designed an inverted PCR scheme to isolate the genomic regions surrounding the inserted plasmid. Genomic DNA from 10BH2 was digested with appropriately chosen enzymes, self-ligated, and used as template for nested inverted PCR. The PCR reactions yielded bands of the expected size which were then subcloned into pT7Blue and sequenced. Figure 4A shows that in the 5' direction we isolated a 1.3-kb fragment that extended into a neighboring *Cla*I site. In the 3' direction a 1.8-kb fragment extending to an *Eco*RI site was isolated.

Sequence Analysis of the 10BH2 Disrupted Locus

Surprisingly, when we compared the sequences from our PCR fragments we discovered that the region immediately adjacent to the 5' end of the plasmid constituted a *Dictyostelium*

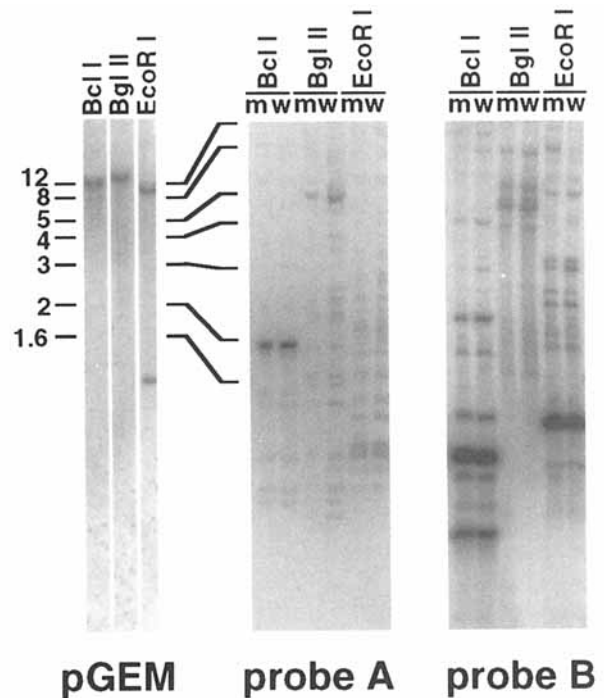


Fig. 3. Southern analysis of wild type and 10BH2 cells. DNA from the 10BH2 mutant cells was digested with *Bcl*I, *Bgl*II, and *Eco*RI, blotted and probed with a pGEM-3 probe to show that only one plasmid inserted into 10BH2 (*pGEM* panel). The *Eco*RI lane in this panel shows the 1.2 kb band produced by the plasmid rearrangement illustrated in Figure 4A. The same three digests were carried out on both mutant (*m*) and wild type (*w*) DNA and probed either with the 5' flanking (*Probe A* panel) or with the 3' flanking (*Probe B* panel) fragments indicated in Figure 4A. Both these probes bind to multiple bands, indicating that the plasmid in 10BH2 did in fact insert into a stretch of repetitive elements.

transposable element. The sequence shown in Figure 4B was obtained from the 1.3-kb 5' flanking fragment in which nucleotides 1047 to 1340 (where position 1340 is immediately adjacent to the pDIV2 plasmid) are identical to the known sequence from transposon Tdd-3 (*Transposon Dictyostelium discoideum*) [Hofmann et al., 1991]. In addition, the sequence from 184 to 1340 constitutes an open reading frame (ORF) which has many residues identical to the ORF of DDPONA2, a gene flanking the *Dictyostelium* ponticulin gene [Hitt et al., 1994]. Through this analysis we found that DDPONA2 also represents another transposable element described previously as RED (Repetitive Element *Dictyostelium*) [Marschalek et al., 1992]. The small portion of known sequence from RED is identical to a portion of DDPONA2 (data not shown). Figure 3 (Probe A) shows that this probe hybridizes to numerous bands for various digests indi-

cating that the 5' flanking sequence represents a multigene family of repetitive elements. Our results also suggest that our sequence represents a copy of Tdd-3 and that the repetitive elements Tdd-3 and RED are closely related since they share significant homology in their ORFs. It is also clear that our sequence contains only a truncated form of Tdd-3. It has been shown that the repetitive elements in *Dictyostelium* are often associated with tRNA genes. Not surprisingly, we found that nucleotides 4 to 91 (Fig. 4B) are identical to a tRNA-Ile gene, and nucleotides 108 to 124 represent an ex-B motif which is always present at the 3' end of *Dictyostelium* tRNA genes [Hofmann et al., 1991].

Similarly, in the sequence obtained from the 3' flanking region (Fig. 4C) we found that residues 1 to 45 are identical to tRNA-Phe, residues 60 to 76 constitute an ex-B motif, and residues 91 to 215 are identical to sequences flanking a tRNA-Val. However, the rest of this sequence did not share similarities with any gene in the GenBank database. To determine if this novel sequence represents a single copy gene, we used a fragment from bp 410 to 1819 as a probe in a Southern analysis of *Dictyostelium* genomic DNA. Figure 3 (Probe B) shows that this probe hybridizes very strongly to multiple bands for various digests. From the intensity of these bands compared to similar blots probed with single-copy genes, we concluded that our novel sequence also represents repetitive elements.

Taken together, our results indicate that the plasmid pDIV2 inserted into a genomic region rich in tRNA genes and repetitive elements. It may be possible that a gene essential for cytokinesis is surrounded by repetitive elements and was deleted in the 10BH2 mutant. To address this possibility we attempted to recapitulate the same mutation in the wild type parental cells using the flanking regions that we cloned. Unfortunately, we were not able to construct a transformation vector that contained both the 5' and 3' flanking regions. Conceivably, the combination of these repetitive sequences may be particularly unstable in *E. coli*.

Phenotypic Characterization of the 10BH2 Mutant

In order to determine if the mutation suffered by the 10BH2 cells had other pleiotropic effects on the cytoskeleton, we characterized several aspects of cell motility. Myosin II mutant cells for example, have defects not only in cytokine-

sis, but also in capping of concanavalin A (conA) receptors, cell motility, and development.

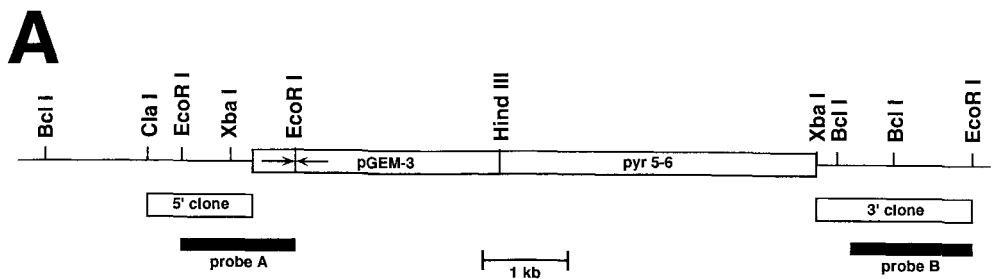
We first explored whether the 10BH2 mutation had any effects on the distribution of actin and myosin II cytoskeleton. Mutant cells stained with rhodamine-phalloidin displayed actin-rich structures identical to those of wild type cells (Fig. 5A and D). Similarly, myosin II localized by indirect immunofluorescence, was found to be primarily in the cortex of both wild type and mutant cells with a small amount also found in their cytoplasm (Fig. 5B and E).

Although actin and myosin localization was found to be normal in 10BH2 cells, it was equally important to confirm that other functional parameters of the cytoskeleton were also conserved. The contractile tension generated by myosin II drives not only the capping of crosslinked cell surface receptors, but also other changes in the cell shape [Pasternak et al., 1989]. In order to test the ability of 10BH2 cells to carry out these functions, the mutant cells were first treated with the tetravalent lectin concanavalin A (conA) to crosslink their membrane proteins. The mutant cells cap their crosslinked surface receptors as readily as do wild type cells (Fig. 5C and F). Similarly, treatment of cells with azide depletes cellular ATP and elicits a 'rigor' contraction, which in turn causes cells to stiffen and become spherical. When 10BH2 cells are treated with azide they contract in a manner identical to wild type cells (data not shown). These cytoskeletal functions are therefore well preserved in 10BH2 cells.

Moreover, 10BH2 cells are able to phagocytose at the same rate as the wild type parental cells (data not shown). When plated on a solid agar substratum coated with nutrient bacteria 10BH2 cells feed on the bacteria and move across the plate as efficiently as the parental HL330 cells. Hence the mutant cells appear to have an intact cytoskeleton as demonstrated by these assays.

10BH2 Cells Have a Developmental Defect

When deprived of nutrients, wild type *D. discoideum* amoebae cease growth and aggregate into a multicellular mound containing about 10^5 cells. Cell differentiation leads to the formation of tips at the apex of each mound which elongate to produce a migrating slug and finally culminate into mature fruiting bodies consisting of stalk and spore cells (Fig. 6A). The 10BH2 cells can proceed through the early stages of develop-



B

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ATCGAGCTCGATTAGCTCACTCGGCAGAGCGTGGTGCATAATAACGCCACGGTCGCGAGTTCCGATCCCTGCATCGAGCAACGATAATTTTAAATATTGT 100
GAAATAGATTACAGGTTCAATTTCCCACTTCTGAAAAGGGAAATAAAGATCCTGGAAATTAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAACAAAAATGC 200
TAGTGGATTCAAGTTATTAATTAATGAGAACCAAAAGCAAGATACCCACTAAAAAGTAGGGGGCTGGAACATTTGGAATATAGAAATGAGACAACTG 300
S G F Q V I N L M R T K R A R Y P L K V G G W N I W N I E L R Q L
GGCGAGAACTTTGGATATAAACCACTCATCTAGCACTAGAAACCAAAACAACACTCATCTCACAAGAGTGGGAATATCAGATCCCAAAAA 400
A Q K L W L I N Q F I L A L E T K Q T N S S H H K S W E Y Q I Q K
ACAAATTCACATCCGATACCTAAAAGAAAACCTAGACGAATGGAACAAAATAAGAAATAAAAAAGCAATTTTCAACAATAATCTCACATCCATAAAAA 500
N K F F T S R Y T K E N L D E W N K I R I K K A T I F N N N L T S I K T
GAAATGGCAAGCACTTCTACTGGCAAGTGGTATACCAAACTCAAGACCAAAATCCAAACATCCTTAAACGAGAAATCCCAATCATCTCTAAACTTA 600
K M D N H F W Q N G I P Q F K T K I Q P S G K R E F Q S S L N Z
AGAGCTACAGCTATAATCAACCTTCAACCAACATACGAAAGATAAAGACCGTAAACAAAGAGACAGAAATGTTAGATTCGATGGCAGGATGCCTCCAA 700
R G Y S Y N Q L P N N I L K I K D E K T R D T H F E E H A S G L P
TAAACCTCTCAACAACAACAATGCCCACTCTGCAAAAGAGATATGAGCAAGACGATACGGTCCCTCTCTCATGCAAGAAACAACAACAAT 800
I N Y L H N K Q C P L C K E D M S K D P Y G H L F F S C K K T K Q E
CATAAAAATAAATAAATCAAAATCTCATCTATATACACAGCAAGGTAATAAACTGGCACCATCAAGAATTAGGCAAAATAACAAATTCATTAAT 900
I K I N K L K N L I Y I T T G K G K N W H H T R I R Q N N N F I N
AGAATAACTCCAAAATATCACCGATAGCCAAAAGATCAAGAAAGTGAATGCAGATACGCCAACCAAGATTCACAAAGAATACTTGAATGGAACT 1000
R I T P K L S P I A K K D Q E V N A D Y A N H R F H K E Y F E W N
ATAAAGCAATAGACTATGATCTAACCAGATCATTCGCATATAGAAAACCTAATGCCCCTATCTCCCAACATTTGATTTGGATTTGCAATCAAAATA 1100
Y K A I D Y D L T R S F A V R N L M A L I L H N I W I W I C N Q I Y
CAGTGAAGATCCCTTAAACNAATGAATCACTATCATACAGCTCCCTCTAAAAGAAATGGCACAAATGGCCACTCTAGAATACATCAAAAAGCAAAAGAT 1200
S E D P L T N E S L S Y S S L L K K W H K L A T L E Y I K K A K D
CTAAAAAATTTATCAGCAAAAGACCAATAAATCTCAAAAGATCCCAAGATCCCTCTCACTCAAGATCAAGTAAAGAAAACAACAGCAATATCACTAT 1300
L K N L S A K D H N N F K D P K I L L T S T I K L R K K T T A N V Y
GTATCCAGAAATCATCTCTCCCAAAATATATATCTTTG 1340
C I F E S S L P N I I S F
    
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C

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GATCTTAAGGTCGCTGGTTCGATCCGGCCCTGAGGCAAAATTTTTTAATGTGGTATGATCGCCGGTTCGATCTATATGTTGATACCCGAAAACAAN 100
GNAATAAACGATCAATTTATACATATAAATAAATAAATCACTGTTATATCTTCACCGCCACATTCATTCCTTAATTTTCCCTTTTTCCTCAATAAAA 200
CAAGGCTATCGCTTATATACTTTTCAAAATGGCTGATGATCACTCACTGAAATACAGTTAAATCATACAGATAACAACAACACCTACAATAATAC 300
CTCCACAACCTCAACAACAACAACAAGTCAAAAATCGACACACCTAAAAACAACCCCAACAACAACCTCTCACTAAACAAGGACTTGATAAT 400
CAATTAGATATCATTTGCACCTCCATCAATCACAAGACCACTCTCTCTTAGAAAAAACAATAACACCATCAACATTAAGTGTACTTTAATCAACCAC 500
ACATCCGTGAAGAAATTAACCTCAAAAGTACACAAGAATCCAAACAAATTCACAAAACATTCACAACCTCTCGAAGATATCTGTCCCATCAGCATT 600
TAAAAAATTTGGTGAACCTGCCCTCGTAAATGCAACGTTGACAAATTAACCTCAATAATCAACCTCAAAACCAACTGCACTAAATATCGCAAAAAC 700
AACTATAGATTCGATATATCATCAATGATTTATGCAATCTTAAACATTCATTAACAGTACTCACCCAAACATGCAAAACGAGGCAATTAACAACAAC 800
TCACTAAATTCACCAAGTGTACATCAATGAAATCTTACTGTACATCTCATATGCTGTGGCTTAAAAAGTTACATTAACATATCATCTAATTTGA 900
TCACACACACAACATTTGATCAAAAGATCGAACCAACCTCTGGTATCACAAAGATATATCAAAAGTGCACAACGATAGGTCACTAAACAACAACAT 1000
ATGCAAAAATGAGCATATGGCAACAACATAAAAAATGCCATCACAAACCAACATGAACAACAACAATCAACCCAAAAACAACATTAACACCCC 1100
ACCAATCAACGAAATGCAAAATGCCAATAAATACTCACTCAAAATAGTGGTTCGAAAGCTATACATAAATGAAATCACTGATTAACAATCAA 1200
TCCCAAAAACAATAATGAAATTTCAACAAGAATGATCTCCAAATTAATAAAGGAAAAAACAATCATATCAATGAGCAATCAACCGATCCAAACAAT 1300
TACCACAAAAACCTCTACAATCTCAACCAACAACAACAAGTGCACCAACCTCCAATAACAATAAATAACAAGAAAGCAGCAATGCCCTGACTACACAAC 1400
AGTCAATCTGGTGTCTCCAAACCTAGTAAAGTATGAGCTCAAAAATCAACTTTATAACATGGAATTTGAGAGGATTCAGTAAAACCCCAACATCAA 1500
CACAATTCCTACCAAAAAGCTGTTGACACACTACAACACTTATATCAAAAACCTCCACCTCGTCCAAACAATCTCAATCTCACTGAATCAATTCATCA 1600
CCATTAACAATTAACAAGATTTTCCCGACACATCATCTCTGCTCACTAAACGGGGAAATGGTGAATCATCATTAACCAACAACAACAACATCAAC 1700
TCAAAACATATCAACAATTTGATGGTATGAGCTATTTAGCAGACGTTATTTCCAAATCAATTAACAATTTGGTATTTCTGGAATTTAGCCACCGACATC 1800
AAATAATACCATCAAAAAGAAATTC 1824
    
```

Figure 4.

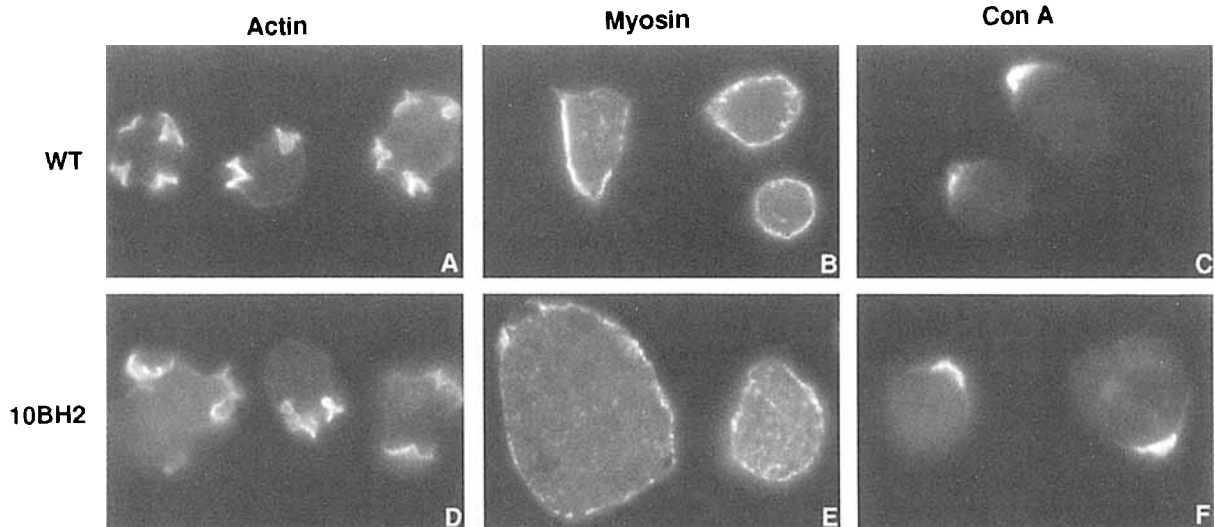


Fig. 5. Actin and myosin II distribution and concanavalin A capping in wild type and mutant cells. Wild type cells (A, B, C) and 10BH2 mutant cells (D, E, F) from stationary cultures were stained with rhodamine phalloidin (A, D) to determine the distribution of actin in these cells. Similarly, the distribution of

myosin II in these cells was visualized by immunostaining with a polyclonal antibody against myosin II heavy chain (B, E). Both cell populations were also challenged with FITC-concanavalin A for 2 min and fixed after 5 min of incubation (C, F) to test their ability to cap cell surface receptors.

ment, forming apparently normal streams and mounds. However, the mutant aggregates form only abnormal finger structures and fail to develop further (Fig. 6B). This is different from the myosin II mutant which arrests in development at the mound stage [De Lozanne and Spudich, 1987].

DISCUSSION

Dictyostelium discoideum is a very useful organism for the genetic analysis of cytokinesis because it offers several advantages. Most importantly among them is that *Dictyostelium* cells carry out cytokinesis in a manner very similar to

higher eukaryotic cells [Fukui and Inoue, 1991]. Furthermore a vast array of *Dictyostelium* cytoskeletal and regulatory proteins have been isolated and cloned and, in some cases, mutagenized by gene disruption or antisense mutagenesis. These studies have revealed that several of these proteins are involved in cytokinesis such as myosin II [De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Pollenz et al., 1992; Chen et al., 1994], calmodulin [Liu et al., 1992], profilin [Haugwitz et al., 1994], and coronin [de Hostos et al., 1993]. In this study we set out to isolate novel genes involved in cytokinesis based on the cytokinesis-deficient pheno-

Fig. 4. Map of the plasmid insertion in the 10BH2 mutant and sequence of the cloned flanking regions. **A:** The single lines indicate the genomic regions flanking the plasmid pDIV-2 (*open bars*) inserted into the 10BH2 mutant. pDIV-2 consists of the *Dictyostelium* pyr-5-6 gene cloned into the plasmid pGEM-3. The plasmid portion suffered an inversion of 455 bp which disrupted the origin of replication (*arrows*). Inverted PCR was used to clone the 5' flanking region up to a Cla I site and the 3' flanking region up to an EcoR I site as indicated. The regions indicated as *probe A* and *probe B* were used as probes in the Southern blots shown in Figure 3. **B:** Sequence of the 5' flanking clone indicated in A. The sequence starts at the 5' flanking Cla I site and ends at the junction with the plasmid pDIV-2. The nucleotide sequences identical to the tRNA-Ile are indicated as white letters on black background. The Ex-B motif found at the 3' of all *Dictyostelium* tRNA genes is indicated by the underlined nucleotides. A long open reading frame extends from nucleo-

tides 184 to 1340 and is displayed with the single letter code underneath the nucleotide sequence. This open reading frame shares many identical residues with the open reading frame of transposon RED (white letters on black background). The boxed nucleotide residues are identical to the sequenced portion of the *Dictyostelium* transposon TDD-3. This sequence is available from GenBank under accession number U46204. **C:** Sequence of the 3' flanking clone indicated in A. The sequence starts at the junction with the plasmid pDIV-2 and ends at the 3' flanking EcoR I site. The residues identical to the tRNA-Phe are indicated as white letters on black background. The Ex-B motif found at the 3' of all *Dictyostelium* tRNA genes is indicated by the underlined nucleotides. The boxed residues are identical to those found at the 3' flanking region of the *Dictyostelium* tRNA-Val. This sequence is available from GenBank under accession number U46205.

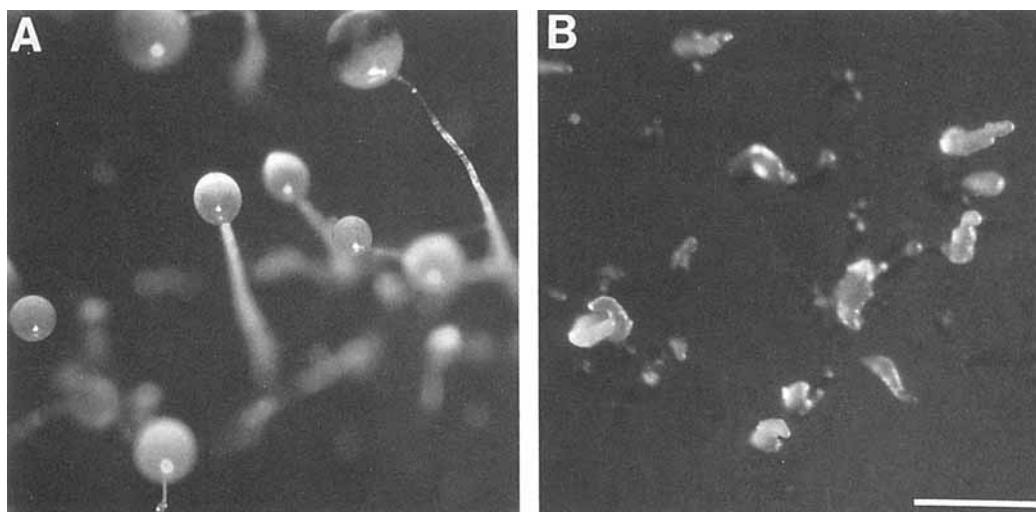


Fig. 6. 10BH2 mutants fail to complete the developmental program. Wild type (A) and 10BH2 mutant (B) cells were plated onto a bacterial lawn and allowed to grow and develop. Under

these conditions wild-type cells develop into mature fruiting bodies. In contrast, the mutant cells form only abnormal finger structures and fail to complete development. Bar = 0.5 mm.

type of *Dictyostelium* myosin II-null cells. Following insertional mutagenesis by restriction enzyme-mediated integration [Kuspa and Loomis, 1992] five such mutant cell lines have been isolated in two independent screens. Two of these mutants bore disruptions of the myosin II heavy chain gene and two others contained disruptions of the *racE* gene [Larochelle et al., 1996]. The fifth mutant, called 10BH2, is also completely impaired in cell division and represents yet another novel locus that is essential for cytokinesis.

Although REMI has been used successfully to tag and subsequently clone numerous genes, there are many unreported instances where the inserted plasmid cannot be cloned with the flanking sequences. We found that the plasmid inserted in the 10BH2 mutant could not be cloned by conventional methods due to a small inversion event that destroyed the plasmid's origin of replication. It is possible that this inversion represents a type of recombination process that may occur concomitantly with the plasmid insertion by REMI. Sequence rearrangements that affect the origin of replication or the ampicillin operon would destroy the ability of the plasmid to be recovered in bacteria. This may explain many of the unrecoverable plasmids from some REMI mutants.

To surmount the rearrangement in the 10BH2 mutant we used inverted PCR to isolate the genomic regions flanking the inserted plasmid. In this manner we cloned 1.3 kb of 5' and 1.8 kb

of 3' flanking sequences. Interestingly, the sequence of these fragments revealed that the plasmid was inserted in a genomic region rich in *Dictyostelium* transposable elements and tRNA genes. We identified a portion of the 5'-flanking sequence as a truncated copy of the transposable element Tdd-3. This transposon, which had not been completely sequenced before, appears to contain an open reading frame (ORF) similarly to other *Dictyostelium* transposable elements [Hofmann et al., 1991]. The ORF of Tdd-3 shares significant homology with the ORF of the transposable element RED [Hofmann et al., 1991] but very little homology with the ORF of transposable element DRE [Marschalek et al., 1992]. Thus, it seems that Tdd-3 and RED are closely related transposons. It has been previously shown that greater than 50% of tRNA genes in *Dictyostelium* are associated the RED, DRE, or Tdd-3 transposable elements [Hofmann et al., 1991]. Hence, it was not surprising to find several tRNA genes both at the 5' and 3' flanking regions of the plasmid inserted in the 10BH2 mutant. Intriguingly, most of the sequence at the 3' flanking region did not have any significant homology to any sequence in the Genbank database. However, we showed by Southern blot analysis that this region is also highly repetitive in the *Dictyostelium* genome. It is possible that this sequence represents other unsequenced portions of transposon Tdd-3 or that it represents a novel uncharacterized repetitive element.

How can the cytokinesis-defective phenotype of the 10BH2 mutant be reconciled with the insertion of a plasmid in repetitive elements? A trivial explanation is that the 10BH2 mutant contains a mutation at another locus which is unrelated to the site of plasmid insertion. An alternative possibility is that a gene essential for cytokinesis is nearby or surrounded by repetitive elements in wild-type cells and was deleted during the plasmid insertion in the 10BH2 mutant. For example, analysis of other REMI mutants in our laboratory has shown that genomic deletions (as large as 2 kb) can often be associated with the plasmid insertion (A.D., unpublished results). Theoretically, a way to distinguish among these possibilities is to reproduce the same 10BH2 plasmid insertion into a wild-type cell and determine if it is associated with the loss of cytokinesis. Unfortunately, every attempt at placing the two cloned flanking sequences into a single plasmid for transformation into *Dictyostelium* cells has not been successful.

Nonetheless, the 10BH2 mutant represents a novel locus that is essential for cytokinesis. We found by Southern and Western analysis that the myosin II heavy and light chains and *racE*, proteins known to be essential for cytokinesis, are still present in 10BH2 cells. Moreover, phenotypic characterization of the 10BH2 cells revealed that they are not similar to the other existing cytokinesis mutants with defects in calmodulin, profilin, and coronin. The calmodulin antisense mutants can form a cleavage furrow but cannot break the cytoplasmic bridge that connects the two daughter cells [Liu et al., 1992]. However, in suspension cultures, the bridge is broken by shear forces and the cells are able to multiply. This is in contrast with the 10BH2 mutants which cannot multiply in suspension cultures and never form cleavage furrows. Similarly, profilin-minus mutants cannot grow in suspension cultures due to a strong cytokinesis defect, however, it is necessary to knock out the two *Dictyostelium* profilin genes in order to get a profilin-minus mutant [Haugwitz et al., 1994]. It is very unlikely that the two profilin genes would have been affected in the 10BH2 mutant. Furthermore, the profilin-minus cells arrest in development at the early mound stage, whereas the 10BH2 mutant arrests at the finger stage. Finally, the coronin mutants have only a mild cytokinesis defect and are able to complete the *Dictyostelium* develop-

mental program. In addition, the coronin mutants can divide normally in the presence of bacteria [de Hostos et al., 1993]. In contrast, the 10BH2 mutants cannot divide even in the presence of bacteria. Thus, we conclude that the 10BH2 cells have suffered a mutation in a novel gene essential for cytokinesis. Our future efforts are directed at trying to identify this gene by rescue of the 10BH2 mutants with a wild-type cDNA expression library. Furthermore, this mutant cell line may be very useful to dissect the molecular signaling events that emanate from the mitotic spindle and determine, possibly through *racE*, the formation of the contractile ring.

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