The \textit{S. cerevisiae} \textit{CLU1} and \textit{D. discoideum} \textit{cluA} genes are functional homologues that influence mitochondrial morphology and distribution

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\section*{SUMMARY}

The \textit{cluA} gene, encoding a novel 150 kDa protein, was recently characterized in \textit{Dictyostelium discoideum}; disruption of \textit{cluA} impaired cytokinesis and caused mitochondria to cluster at the cell center. The genome of \textit{Saccharomyces cerevisiae} contains an open reading frame (\textit{CLU1}) that encodes a protein that is 27\% identical, 50\% similar, to this \textit{Dictyostelium} protein. Deletion of \textit{CLU1} from \textit{S. cerevisiae} did not affect cell viability, growth properties, sporulation efficiency, or frequency of occurrence of cells lacking functional mitochondria. However, in \textit{CLU1} cells the mitochondrial reticulum, which is normally highly branched, was condensed to one side of the cell. Transformation of \textit{CLU1} \textit{Dictyostelium} mutants with the yeast \textit{CLU1} gene yielded amoebae that divided normally and had dispersed mitochondria. The mitochondria in \textit{CLU1} \textit{Dictyostelium} cells complemented with \textit{CLU1} were not as widely scattered as in \textit{CLU1} \textit{Dictyostelium} cells, but formed loose clusters throughout the cytoplasm. These results indicate that the products of the \textit{CLU1} and \textit{cluA} genes, in spite of their limited homology, are functional homologues.

Key words: \textit{S. cerevisiae}, \textit{D. discoideum}, Mitochondrion

\section*{INTRODUCTION}

Mitochondria supply most of the cellular ATP in eukaryotic cells. The accumulation of mitochondria near sites of high ATP consumption (i.e. near flagellar basal bodies and between myofibrils) suggests that mitochondrial positioning is important for efficient cellular metabolism. Furthermore, even cells that can survive anaerobically still require mitochondria (Gbelska et al., 1983; Yaffe and Schatz, 1984), because these compartments play an essential role in biosynthesis of pyrimidines, phospholipids, nucleotides, folate enzymes, sterols, and some amino acids (Shimizu et al., 1973; Zelikson and Luzzati, 1977; Goewert et al., 1981). Mitochondrial membranes cannot arise de novo, so proper transport, division, and partitioning of mitochondria to daughter cells is essential. The mechanisms by which mitochondria are replicated, transported, and partitioned are not well understood and may vary among different cell types.

The three major cytoskeletal elements, microtubules, microfilaments and intermediate filaments, act to varying degrees as the scaffolding along which mitochondria are transported (see Yaffe, 1996, for an extensive review of mitochondrial movement). Mitochondrial distribution has been linked most consistently to microtubules, with numerous studies demonstrating that mitochondrial position correlates with and/or depends upon the microtubular array (Heggeness et al., 1978; Ball and Singer, 1982; Couchman and Rees, 1982; Summerhayes et al., 1983; Yaffe et al., 1996). In axons, anterograde transport of mitochondria takes place along microtubules, suggesting that kinesin is the motor protein (Morris and Hollenbeck, 1995). Several microtubule-based, kinesin-like motor proteins specific for mitochondrial transport have been identified, including KIF1B in frog neurons (Nangaku et al., 1994; Hirokawa, 1996) and KLP67A in mitotic cells of \textit{Drosophila} (Pereira et al., 1997).

Intermediate filaments and microfilaments have also been implicated in mitochondrial distribution. Mitochondria co-distribute with intermediate filaments by immunofluorescence (Mose-Larsen et al., 1982; Summerhayes et al., 1983), and crossbridges between the outer mitochondrial membrane and intermediate filaments have been observed by electron microscopy (David-Ferreira and David-Ferreira, 1980). The lack of direct evidence for organelle movement along intermediate filaments may indicate that these filaments serve as scaffolding for other cytoskeletal elements or as anchorage points for mitochondria, rather than participating directly in mitochondrial transport.

Microfilaments have been shown to play an active role in mitochondrial transport in some cell types. In axons, mitochondria are transported slowly along microfilaments in a net retrograde direction, contrasting with the fast microtubule-based anterograde movement (Morris and Hollenbeck, 1995). In \textit{S. cerevisiae}, mitochondria localize to actin cables in vivo and move along actin filaments in vitro (Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1997). Disruption of actin cables leads to defects in
mitochondrial motility and delays transfer of mitochondria to bud cells (Simon et al., 1997; Hermann et al., 1997). The presence of an actin-based, ATP-dependent motor activity on the mitochondrial surface (Lazzarino et al., 1994) and the finding that mutations in the myosin-binding site of the ACT1 gene of S. cerevisiae result in abnormally organized mitochondria (Simon et al., 1995), both suggest that a myosin-like protein acts as a motor for the transport of this organelle. However, mutations in each of the yeast myosin genes (or pairs of those genes) produce no significant effect on mitochondrial movement or arrangement (Simon et al., 1995), so the identity of this presumptive actin-based motor is uncertain.

Although motor and linker proteins involved specifically in yeast mitochondrial movement have not been characterized, a number of studies in S. cerevisiae have identified mutants that are deficient in mitochondrial inheritance, movement, and morphology (reviewed by Yaffe, 1996). The yeast gene MMM1 encodes a protein that localizes to the outer mitochondrial membrane and is involved in maintaining proper mitochondrial morphology and inheritance. Burgess et al. (1994) have proposed that Mmm1p attaches the mitochondrion to the cytoskeleton, thereby helping to maintain the shape and perhaps aiding in organizing and moving the mitochondrion. Lack of the protein encoded by MDM10 results in giant spherical mitochondria as well as a defect in mitochondrial inheritance; Mdm10p is found in the outer mitochondrial membrane (Sogo and Yaffe, 1994). Another mitochondrial membrane protein, the product of the MDM12 gene, is also required for proper mitochondrial distribution and inheritance (Berger et al., 1997). Disruption of MDM2, a gene involved in the synthesis of unsaturated fatty acids, also impairs mitochondrial transmission to buds (McConnell et al., 1990); this defect can be complemented by addition of oleic acid to mdm2 mutants (Stewart and Yaffe, 1991). The gene Mgm1p encodes a protein with homology to dynamin; this gene is required for the propagation of functional mitochondria in yeast, apparently through an effect on the replication or partitioning of mitochondrial DNA (Jones and Fangman, 1992; Guan et al., 1993). In the absence of Mgm1p, the distribution of mitochondria is also abnormal. The gene MDM1 has multiple functions in organellar inheritance. This gene encodes an intermediate filament-like protein that localizes to punctate arrays throughout the cytoplasm of yeast cells (McConnell and Yaffe, 1992). Disruption of MDM1 results in failure to transfer both mitochondria and nuclei to buds (McConnell et al., 1990); these two functions have been separated in different mutant alleles (Fisk and Yaffe, 1997). Overall, these data indicate that both cytoskeletal elements and components of mitochondrial membranes contribute to mitochondrial morphology and inheritance.

In the present report, we describe a new yeast gene, CLU1, whose product affects mitochondrial morphology. The predicted sequence of Clu1p is 27% identical (50% similar) to the product of the cluA gene of Dictyostelium discoideum, whose disruption results in clustering of mitochondria near the cell center (Zhu et al., 1997). We show here that the CLU1 gene of S. cerevisiae is a functional homologue of Dictyostelium cluA and that the lack of Clu1p affects the morphology of the mitochondrial network in yeast cells.

Materials and methods

Strains, culture media and genetic methods

S. cerevisiae strains used in this study are listed in Table 1. Yeast culture media were prepared, and standard genetic manipulations were carried out, as described by Rose et al. (1990). Media with non-fermentable carbon sources included YPG (3% glycerol), YPE (3% ethanol) and YPL (2.5% lactic acid).YPD contained 2% dextrose. D. discoideum strains were grown in HL5 medium. Growth conditions, transformation by electroporation, and selection in G418-containing medium were as previously described (Liu and Clarke, 1996).

Disruption of CLU1

The S. cerevisiae CLU1 gene was deleted in the diploid strains MCY387 and MDD1 by one-step gene disruption, using the technique of Baudin et al. (1993). The shuttle vector pRS304, which contains the TRP1 gene (Sikorski and Hieter, 1989), was used as template for polymerase chain reaction (PCR). The forward primer was identical to bases 25,801-25,853 of YM8270.16 (Saccharomyces Genome Database) with the addition at the 3’-end of the bases 5’-CAGACGAGATTGACTGAGATCC-3’, which are homologous to the pRS304 region upstream of TRP1. The reverse primer was an oligonucleotide complementary to bases 29,615-29,670 of YM8270.16, plus an additional 3’ sequence (5’-GCATCGTGCCGATTTCACCC-3’) homologous to the pRS304 region downstream of TRP1. The MYC387 and MDD1 cells (both trpA) were transformed by the lithium acetate method with the PCR disruption cassette, and transformants were selected as tryptophan prototrophs. Several transformants from each strain were sporulated and tetrads dissected. The resulting haploid segregants were screened by PCR to verify disruption of CLU1, as described in Results. The segregants were subjected to standard genetic analyses (Sherman, 1991).

Complementation methods

For complementation studies, the CLU1 gene was synthesized by PCR with Deep Vent polymerase (New England Biolabs) using as template the phage clone lPM-6114 (ATCC #70677), which contains the CLU1 gene. Primers were positioned 300 bp upstream of the 5’-end and 100 bp downstream of the 3’-end of the gene. The forward primer was 5’-GGATCCCTGAGAAACTTGTGCA TGA TTTG-3’ and the reverse primer was 5’-GGTACCTGGGGATTATGCATGTG-3’. The PCR product was then cloned in-frame into the expression vector pDXA-HC (Manstein et al., 1995). Dictyostelium product was then cloned in-frame into the expression vector pDXA-HC (Manstein et al., 1995). Dictyostelium

Database) with the addition at the 3’-end of the bases 5’-CAGACGAGATTGACTGAGATCC-3’, which are homologous to the pRS304 region upstream of TRP1. The reverse primer was an oligonucleotide complementary to bases 29,615-29,670 of YM8270.16, plus an additional 3’ sequence (5’-GCATCGTGCCGATTTCACCC-3’) homologous to the pRS304 region downstream of TRP1. The MYC387 and MDD1 cells (both trpA) were transformed by the lithium acetate method with the PCR disruption cassette, and transformants were selected as tryptophan prototrophs. Several transformants from each strain were sporulated and tetrads dissected. The resulting haploid segregants were screened by PCR to verify disruption of CLU1, as described in Results. The segregants were subjected to standard genetic analyses (Sherman, 1991).
**RESULTS**

**CLU1 is not required for growth in *S. cerevisiae***

A search of the GenEMBL database detected a single open reading frame in the *S. cerevisiae* genome with significant homology to the cluA gene of *D. discoideum*. This open reading frame, now designated *CLU1*, is located on chromosome XIII, locus YMS270.16 (*Saccharomyces* Genome Database ORF YMR012w and GenBank accession number Z48613). Comparison of the predicted amino acid sequences of the *D. discoideum* and *S. cerevisiae* proteins showed 27% identity between these two proteins, with only scattered short stretches of highly conserved sequence (Fig. 1). As had been found for the *Dictyostelium* protein, the only homology between Clu1p and known proteins in the database was to a series of 42-residue imperfect repeats present in the kinesin light chain; these repeats are thought to function in protein-protein interaction (Gindhart and Goldstein, 1996).

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**Table 1. Genotypes of yeast strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>MCY387</td>
<td>MATα/α CLU1/CLU1 his4::HIS3/HIS4 ade2-101/ADE2</td>
<td>Conrad et al. (1997)</td>
</tr>
<tr>
<td>SFY200B</td>
<td>MATα clu1 his leu2 lys2 trp1 ura3-52</td>
<td>Segregant from MCY387</td>
</tr>
<tr>
<td>SFY225A</td>
<td>MATα clu1::TRP1 his leu2 lys2 trp1 Δura3-52</td>
<td>Segregant from MCY387</td>
</tr>
<tr>
<td>SFY225B</td>
<td>MATα clu1::TRP1 his leu2 lys2 trp1 Δura3-52</td>
<td>Segregant from MCY387</td>
</tr>
<tr>
<td>SFY290</td>
<td>MATα/α clu1::ADE2/ade2 ade1-101 his/his leu2/leu2 trp1/Δura3-52</td>
<td>SFY225A × SFY225B</td>
</tr>
<tr>
<td>MDD1</td>
<td>MATα/α CLU1/CLU1 ADE2/ade2 ADE5/ad5 his5·1-his2-7 leu-c/leu4-d</td>
<td>Dresser et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>lys2-2/fys2-2 met13-c/met13-d trp1-63/trp1-64 ura3-13/trp1-63 ura3-13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tyr1-2/trp1-1</td>
<td></td>
</tr>
<tr>
<td>MDY433</td>
<td>MATα CLU1 ade5 his7 leu1-12 lys2-2 met13-d trp1-63 tyr1-1 ura3-13</td>
<td>Dresser et al. (1997); used to construct MDD1</td>
</tr>
<tr>
<td>SFY562B</td>
<td>MATα CLU1 his7 leu2 lys2 met13 trp1-63/trp1-64 tyr1-1 ura3-13</td>
<td>Segregant from MDD1 transformant</td>
</tr>
<tr>
<td>SFY561A</td>
<td>MATα clu1::TRP1 his7 leu2 met13 trp1-65 tyr1 ura3-13</td>
<td>Segregant from MDD1 transformant</td>
</tr>
<tr>
<td>SFY563B</td>
<td>MATα clu1::TRP1 ade5 his7 leu2 met13 trp1-63 tyr1 ura3-13</td>
<td>Segregant from MDD1 transformant</td>
</tr>
<tr>
<td>SFY565D</td>
<td>MATα clu1::TRP1 ade5 his7 leu2 met13 trp1-63 tyr1 ura3-13</td>
<td>Segregant from MDD1 transformant</td>
</tr>
</tbody>
</table>
Using standard methods (see Materials and Methods) we disrupted the \textit{CLU1} gene (\textit{clu1::TRP1}) in two different diploid strains. Homologous recombination between the disruption cassette and the chromosomal \textit{CLU1} gene was expected to result in deletion of all but 7 bases at the 5'-end and 66 bases at the 3'-end of \textit{CLU1}, with insertion of the \textit{TRP1} gene at the site of the deletion. Transformants were screened by PCR using the strategy shown in Fig. 2 to verify that this homologous recombination event had occurred, and a number of \textit{clu1A} transformants were identified. Diploid transformants containing a disrupted copy of \textit{CLU1} were sporulated. The tetrads produced four viable spores, 2 Trp+ and 2 Trp-. PCR analysis (Fig. 2) confirmed that the Trp+ spores were \textit{clu1D}. All other relevant markers displayed normal segregation as well (data not shown). A homoyzogous diploid \textit{clu1A} strain, SFY290, was generated by mating of appropriate haploid segregants, and the sporulation efficiency of this strain was determined. 95% of the spores from SFY290 were viable. The diploid parental strain, MCY387, displayed similar spore viability.

The growth properties of four haploid \textit{clu1D} segregants (SFY225B, SFY561A, SFY563B, and SFY565D) and two \textit{CLU1} segregants (SFY562B and SFY200B) were examined (Table 2). Growth of \textit{clu1D} segregants resembled that of wild-type cells under all conditions tested. The growth rates of normal and mutant cells were similar using non-fermentable carbon sources (glycerol, lactate, or ethanol) as well as glucose. Even under extreme temperature conditions, the growth rate on different carbon sources remained unaffected by deletion of the \textit{CLU1} gene. Deletion of \textit{CLU1} also did not impair growth under conditions of high osmolarity, even at restrictive temperatures. Finally, viability after heat shock was similar for wild-type and mutant cells. Thus, within the limits of these assays, \textit{clu1D} had no effects on normal growth.

Possible defects in the ability to synthesize the cell wall were tested by exposure of cells to calcofluor white (Ram et al., 1994). Neither mutant nor wild-type strains displayed sensitivity to concentrations of 20 \textmu g/ml calcofluor in agar plates. Both mutant and wild-type strains also exhibited the same degree of susceptibility to the fluorescent lipophilic dye DiOC6, which is accumulated by mitochondrial membranes.

\textbf{\textit{CLU1} is not required for inheritance of functional mitochondria}

The frequency of defects in mitochondrial inheritance was determined using the tetrazolium overlay method (Ogur et al., 1957; Newlon et al., 1979). Haploid wild-type and \textit{clu1D} cells were spread on YPD plates with 5% glucose and incubated for 3 days at 30°C. Colonies were then overlaid with agar containing 0.1% 2,3,5-triphenyl tetrazolium chloride (TTC). In this colony-sectoring assay, cells with normal mitochondrial activity reduce the TTC to formazan. Normal cells were clearly distinguished from \textit{clu1D} cells by their blue-purple coloration. Clones were then marked with \textit{BglI} to remove plasmid DNA. In this manner, the percentage of colonies with defective mitochondria was determined for wild-type and \textit{clu1D} strains. The results showed that the frequency of defective mitochondria in \textit{clu1D} was significantly lower than in wild-type cells, indicating that \textit{CLU1} is not required for inheritance of functional mitochondria.

\textbf{Fig. 1. Comparison of deduced amino acid sequences of proteins encoded by S. cerevisiae \textit{CLU1} and Dictyostelium \textit{cluA}.}

The figure was generated with the GAP program of the University of Wisconsin Genetics Computer Group package. The upper line shows the yeast sequence (Sc) and the lower line the \textit{Dictyostelium} sequence (Dd). Vertical bars connecting the sequences mark identical residues, and dots indicate similar ones. Gaps introduced during the alignment are shown by dots in the sequence line. The default parameters of the GAP program calculated 27% identity and 50% similarity for the two proteins.
the TTC to a crimson product, while those cells lacking mitochondria or mitochondrial function (‘petite’ cells) do not show a color change. Petites were detected at a similar frequency in wild-type and mutant strains. Half-sectored colonies comprised less than 1% of the population in four wild-type strains and in three of the four clu1Δ haploid segregants that were tested. In the fourth mutant, SFY225B, nearly 4% of the colonies were half-sectored, presumably owing to the presence of some other marker carried by this segregant. Observation of mitochondria by fluorescence microscopy, as described in the next section, also indicated that mitochondria were present in buds.

**CLU1 affects mitochondrial morphology**

The most obvious difference between clu1Δ and wild-type cells was the morphology of their mitochondria, which was visualized by fluorescence microscopy using an antibody against the mitochondrial porin protein. Wild-type cells taken from log phase growth on YPG (density ~2×10^6 cells/ml) had a reticulated mitochondrial network that was dispersed around the cell periphery (Fig. 3A). A portion of this network was visible in all focal planes, but its branching structure was most clearly seen in the focal planes corresponding to the top and bottom of the cells. In contrast, clu1Δ cells possessed a more condensed mitochondrial mass, usually found at one side of the cell (Fig. 3B, most cells), and occasionally appearing as a ring (Fig. 3B, cell at far left). Fluorescence microscopy revealed that buds contained mitochondrial protein (Fig. 3B, arrows), indicating that the aggregated state of the mitochondrial network did not significantly impair its transmission to daughter cells. This finding is consistent with the tetracism overlay results and with the similar growth rates of mutant and wild-type cells on non-fermentable carbon sources.

Mitochondria were also visualized by staining living yeast cells with the mitochondrion-specific dye, DiOC6. Fig. 4 shows higher magnification views of representative wild-type (A,B) and mutant (C,D) cells stained with this vital dye. The mitochondrion of a typical mutant cell, with staining restricted to one side of the cell, is shown in C, while the less common ring-type staining, which probably represents an en face view, is shown in D. The ring-type staining of mutant cells was restricted to a single focal plane and was tightly compacted, distinguishing it from the open cortical reticulum of wild-type cells, which was visible at both the upper and lower cell surfaces. Observation of late log phase (5×10^7 cells/ml) and stationary phase clu1Δ cells indicated that the aggregated mitochondria became fragmented and dispersed under these growth conditions, as did the mitochondria of wild-type cells (not shown).

Transformation of clu1Δ yeast strains with a centromeric plasmid carrying the **CLU1** gene resulted in restoration of the normal mitochondrial network in log phase cells (Fig. 4E and F), while cells transformed with the empty vector remained mutant in appearance (not shown). Mitochondrial fragmentation during stationary phase also occurred normally in the complemented cells.

**CLU1 can complement cluA− Dictyostelium cells**

The cluA− Dictyostelium mutant (Zhu et al., 1997) was transformed with an expression vector, pDXA (Manstein et al., 1995), that carried either S. cerevisiae CLU1 or Dictyostelium cluA. Total cell lysates from the two types of transformants were examined by polyacrylamide gel electrophoresis (Fig. 5). The pDXA-CLUD transformant (lane a) contained a new polypeptide of ~145 kDa, in agreement with the predicted size of Clu1p (predicted molecular mass, 145,164), while the pDXA-cluA transformant (lane c) contained a slightly larger polypeptide, confirmed by antibody staining (not shown) to correspond to the ~150 kDa product of the **Dictyostelium cluA** gene (predicted molecular mass, 148,916). In both cases, the polypeptides expressed in the pDXA transformants were more abundant than the endogenous polypeptide of wild-type cells, which was not detectable by Coomassie staining (lane b). Because an antibody to yeast Clu1p is not yet available, we verified the identity of the presumptive Clu1p polypeptide by staining an immunoblot with an antibody that recognizes the (His)6 tag added to Clu1p upon cloning **CLU1** into pDXA (lane d).

As previously shown (Zhu et al., 1997), cluA− Dictyostelium cells are commonly large and multinucleated,

![Table 2. Comparison of the growth properties of clu1Δ and wild-type cells](image)

<table>
<thead>
<tr>
<th>Growth parameter</th>
<th>Result</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon source</td>
<td>No difference in utilization of acetate, glycerol, and glucose</td>
<td>Tested by replica-plating</td>
</tr>
<tr>
<td>Growth rate</td>
<td>YPD: wt = 1.2 hours; mt = 1.1 hours</td>
<td>Doubling times not significantly different</td>
</tr>
<tr>
<td></td>
<td>YPG: wt = 3.5 hours; mt = 3.6 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YPL: wt = 2.6 hours; mt = 2.7 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YPG: wt = 2.4 hours; mt = 2.6 hours</td>
<td></td>
</tr>
<tr>
<td>Temperature sensitivity (17°C, 30°C, 37°C)</td>
<td>YPE: wt = 3.4 hours; mt = 3.6 hours</td>
<td>Tested by replica-plating</td>
</tr>
<tr>
<td>DiOC6 sensitivity</td>
<td>Wild type and mutant sensitive to &gt;0.2 μg/ml in YPD. Not sensitive to 10 μg/ml in YPD</td>
<td>Tested in liquid YPG or replica-plated on YPD</td>
</tr>
<tr>
<td>Calcofluor sensitivity</td>
<td>wt and mt strains not sensitive to 20 μg/ml</td>
<td>Tested by replica-plating</td>
</tr>
<tr>
<td>High osmolality</td>
<td>wt and mt strains not sensitive to 0.9 M NaCl or 1.5 M sorbitol</td>
<td>Tested by replica-plating</td>
</tr>
<tr>
<td>Spore viability</td>
<td>95% viability for SFY290 and MCY387</td>
<td>SFY290 is a homozygous clu1Δ diploid</td>
</tr>
<tr>
<td>Heat shock viability</td>
<td>wt: 42°C-55%, 50°C&lt;1%</td>
<td>Percentages based on cell viability at 30°C</td>
</tr>
<tr>
<td></td>
<td>mt: 42°C-58%, 50°C&lt;1%</td>
<td></td>
</tr>
<tr>
<td>Petite formation (% half-sectored colonies)</td>
<td>wt (4 strains): &lt;1% (n=4,332)</td>
<td>Tetrazolium overlay</td>
</tr>
<tr>
<td></td>
<td>mt (3 strains): &lt;1% (n=1,730)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mt (1 strain): 3.6% (n=2,058)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: wt; wild type; mt, mutant; YPD, rich medium with dextrose; YPE, rich medium with ethanol; YPG, rich medium with glycerol; YPL, rich medium with lactic acid.
and their mitochondria are found in a single cluster. Transformation of cluA− cells with a plasmid expressing either Dictyostelium cluA or yeast CLU1, yielded transformants that were small and mostly mononucleated (Fig. 6), as are wild-type Dictyostelium cells. Thus, the Dictyostelium and yeast gene products were equally effective in correcting the cytokinesis defect of cluA− cells. However, the degree of mitochondrial dispersal in the complemented Dictyostelium cells depended on which protein was being expressed (Fig. 7). Those cells complemented with the Dictyostelium cluA protein resembled wild-type Dictyostelium cells in that the mitochondria were well-dispersed and had no sign of clustering. For cells expressing yeast Clu1p, the mitochondria were no longer tightly clumped, but were scattered throughout the cytoplasm as small, loose aggregates. These aggregates sometimes appeared to be linked by a single elongated mitochondrion or possibly a line of interconnected mitochondria. Because Clu1p only partially restored normal mitochondrial dispersal in cluA− Dictyostelium cells, and because the phenotypic consequences of CLU1 disruption in yeast cells were relatively subtle, we did not undertake the reciprocal complementation experiment, expression of cluA in clu1Δ yeast cells.

**DISCUSSION**

**Consequences of CLU1 deletion**

In order to gain further insight into the function of cluA and its homologues, we have disrupted the CLU1 gene of *S. cerevisiae*, which encodes a protein approximately 50% similar to the Dictyostelium protein in primary structure. As had been found in Dictyostelium, the lack of Clu1p had minimal consequences on cell growth. The clu1Δ yeast cells grew at normal rates on both glucose and non-fermentable carbon sources, did not manifest temperature sensitivity, and survived as well as wild-type cells exposure to stress conditions such as heat shock and osmotic shock. They were also no more sensitive than wild-type cells to DiOC6. This lipophilic dye is accumulated by mitochondrial membranes in a manner dependent on the high trans-membrane potential of functional mitochondria (Johnson et al., 1981). High concentrations of the dye are toxic to cells growing on a non-fermentable carbon source such as glycerol (Koning et al., 1993). Thus, one might expect cells whose mitochondrial function had been compromised to be more sensitive to the dye; this was not the case for clu1Δ cells. In summary, a variety of assays detected no impairment of cell physiology in general and mitochondrial function in particular.
as a consequence of the disruption of CLU1. Nonetheless, as in Dictyostelium, there were clear changes in the morphology of the mitochondrial network in clu1Δ cells.

Other mutations affecting mitochondrial morphology

As described in the Introduction, there are many types of mutations known to affect the distribution of mitochondria in S. cerevisiae (also see review by Yaffe, 1996). Two general classes of mutations have been found, those that affect proteins of mitochondrial membranes and those that affect cytoskeletal elements with which the mitochondria interact. The identifying phenotype of both types of mutations has been an impaired transmission or absence of mitochondria in the bud; commonly, mitochondrial morphology is also aberrant. Few mutations have been identified that alter the morphology of the mitochondrial reticulum without affecting mitochondrial inheritance. Other than CLU1, we are aware of only DNM1. It was recently reported that disruption of DNM1, which encodes a dynamin-like protein (Gammie et al., 1995), causes the mitochondrial network to collapse to a tubular morphology, but does not impair its function or inheritance (Shaw et al., 1997). The basis of this effect is not yet known. Although CLU1 has no significant homology to DNM1, the similar phenotypes raise the intriguing possibility of a common pathway or a genetic interaction between the products of these genes.

Complementation analysis

Complementation studies have demonstrated that CLU1 encodes a functional homologue of the Dictyostelium cluA gene. This homology has interesting implications, especially in light of the many differences between these two organisms. Dictyostelium cells are highly motile amoebae, while yeast cells, encased by a cell wall, are nonmotile. Dictyostelium cells contain an extensive array of cytoplasmic microtubules, and, although this has not been directly demonstrated, it is likely that these function in organelle transport much as they do in mammalian cells (see Introduction). In yeast cells, microtubules are not required for transport of mitochondria into buds (Huffaker et al., 1988). The mitochondria in growing Dictyostelium cells are in the form of dozens of small, individual units distributed throughout the cytoplasm. In S. cerevisiae, exponentially growing cells possess a reticular, essentially continuous mitochondrial structure that surrounds the cell just beneath the plasma membrane; the mitochondrial mass is greater when cells are grown on a non-fermentable

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**Fig. 4.** Visualization of mitochondria in living CLU1, clu1Δ, and complemented clu1Δ yeast strains. Cells in log phase growth on YPG were stained with DiOC₆ and observed by standard fluorescence microscopy. (A-B) Wild-type CLU1 yeast cells (SFY562B). (C-D) clu1Δ yeast cells (SFY561A). (E-F) clu1Δ cells (SFY561A) complemented with YCpCLU1. Bar, 5 μm.

**Fig. 5.** SDS polyacrylamide gel showing expression in cluA⁻ Dictyostelium cells of proteins encoded by plasmid-borne copies of S. cerevisiae CLU1 and Dictyostelium cluA. Total cell lysates from 1×10⁶ cells were denatured and electrophoresed on an SDS gel containing 7.5% polyacrylamide; proteins in the first three lanes were visualized by staining with Coomassie Brilliant Blue. The cells were (a) cluA⁻ cells transformed with pDXA-CLU1, (b) cluA⁻ cells, (c) cluA⁻ cells transformed with pDM2 (= pDXA-cluA). The positions of the prestained molecular mass markers (Life Technologies) and their apparent size in kDa are indicated to the left, and the new polypeptides observed in lanes a and c are marked with arrowheads. Lane d, an immunoblot of pDXA-CLU1 cells stained with peroxidase-conjugated antibody to (His)₆. The antibody stained a single polypeptide, which co-migrated with the band marked in a, confirming its identity as Clu1p.
carbon source (Hoffman and Avers, 1973; Stevens, 1981). The branching interconnections of the mitochondrial reticulum are continually being rearranged by ongoing fusion and fission (Nunnari et al., 1997).

In spite of these substantial differences between the two cell types, disruption of cluA in Dictyostelium or CLU1 in yeast produces a similar phenotype, clustering of mitochondria on the one hand, and compression of the mitochondrial mass on the other. In Dictyostelium cluA\(^-\) cells, the dozens of individual mitochondria became collected into a single aggregate. In yeast clu1\(\Delta\) cells, the reticular mitochondrial became condensed to one side of the cell. Importantly, transformation of cluA\(^-\) Dictyostelium cells with yeast CLU1 resulted in complementation of the phenotypic defects in the Dictyostelium mutant, indicating that the two gene products serve similar functions in their respective organisms. This result suggests that the aspect of mitochondrial structure or interaction with other cellular components that depends on cluA/CLU1 is common to both cell types.

Remarkably, complementation extended to an area in which the effect of the mutations differs in Dictyostelium and yeast cells. The cluA\(^-\) Dictyostelium cells are impaired in cytokinesis, yielding a population that contains many giant, multinucleated cells (Fig. 6C; also Zhu et al., 1997). No comparable defect was exhibited by yeast clu1\(\Delta\) cells, which grew normally and were able to separate the bud from the mother cell. Nonetheless, expression of yeast Clu1p in cluA\(^-\) Dictyostelium cells restored normal cytokinesis. This result is consistent with the possibility that the problem in cytokinesis in Dictyostelium is indirect, i.e. the contractile ring may sometimes be physically obstructed by the large mass of mitochondria, blocking completion of cleavage. If this were the case, dispersal of the mitochondrial aggregate would suffice to correct the problem.

In cluA\(^-\) Dictyostelium cells complemented with the Dictyostelium protein, the mitochondria were completely dispersed, as in wild-type cells. However, in cells complemented with yeast Clu1p, the dispersal was less complete, and the cells often contained loose clusters of mitochondria scattered throughout the cytoplasm. In both types of transformants, the gene carried by pDXA was overexpressed, and the protein accumulated to higher levels than found in wild-type cells. For the Dictyostelium protein, we have monitored protein levels on a cell-by-cell basis, either by staining fixed cells with a monoclonal antibody specific for the cluA protein (Zhu et al., 1997), or by observing living cells expressing a fusion of this protein with green fluorescent protein (unpublished data). Both methods have shown that there is great cell-to-cell variation (even in clonal populations) in the level of the cluA protein expressed from pDXA, and both methods have revealed a normal dispersed pattern of mitochondria in cells whose staining intensities ranged from undetectable (as is true of wild-type cells) to extremely bright. Those rare cells with clustered or only partially dispersed mitochondria displayed no detectable staining, arguing that such phenotypes resulted from inadequate levels of the cluA protein. Thus, the incomplete dispersal observed in cluA\(^-\) cells expressing high levels of yeast Clu1p is unlikely to be a consequence of overabundance of the yeast protein; instead, this result suggests that the yeast protein acts somewhat less effectively than the Dictyostelium protein in Dictyostelium cells. This result also raises an interesting possibility, to be explored in future ultrastructural studies, that the mitochondria in Dictyostelium cells expressing Clu1p may be interconnected, with a pattern of organization intermediate between that of Dictyostelium and that of yeast cells.

**Partitioning of mitochondria at cell division**

In general, cells use one of two mechanisms, stochastic or ordered, for distributing mitochondria at cell division (Warren and Wickner, 1996). For a cell that contains a large number of well-dispersed mitochondria, cleavage into two equally-sized daughter cells is sufficient to ensure that each daughter receives an approximately equal complement of mitochondria. This is normally the case for Dictyostelium amoebae. However, in
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cluA− Dictyostelium cells, the stochastic mechanism fails because the mitochondria are clustered into a single aggregate, and the cleavage plane (even when cleavage is successful) may not divide this aggregate into similar portions. Some daughters receive few (Fig. 6E) or no mitochondria, and others an overabundance. A cell that receives no mitochondria is probably not viable, but less extreme imbalances are presumably corrected during the cell cycle by the normal, albeit unidentified, regulatory mechanisms.

S. cerevisiae employs an ordered mechanism to ensure that the daughter cells receive an appropriate complement of mitochondria. A portion of the mitochondrial reticulum is actively transported into the bud, where it moves rapidly back and forth through the bud neck until cleavage occurs (Stevens,
1981). This process appears to remain intact in cluA cells, since mitochondrial inheritance is not impaired. (The dynamics of mitochondrial movement have not yet been analyzed to determine whether kinetic differences exist between mutant and wild-type cells.) An implication of the normal transmission of mitochondria to buds is that the association of the yeast mitochondrion with components required for its movement, such as cytoskeletal elements and motor proteins, is still functional in the mutant. This hypothesis can be tested by in vitro assays of mitochondrial movement (Simon et al., 1995) and by time lapse observation of the behavior of mitochondria in living cells (see Nunnari et al., 1997).

In conclusion, although we still do not understand the mechanism of action of the proteins encoded by cluA and CLU1, a comparison of the effects of gene disruption in Dictostylium and yeast cells and of the properties of cluA-Dictostylium cells complemented with these two proteins, have suggested directions for future research. In addition, the present study has shown that the Dictostylium and yeast proteins, while only 50% similar in primary structure, are functional homologues. Searches of databases containing expressed sequences have detected possible homologues of this protein in organisms ranging from insects to mammals. The conservation of function between Dictostylium and yeast (in spite of their differences in mitochondrial structure and transport mechanisms) and the apparent presence of this protein in a diversity of species, argue that this protein family will be found to play an important and similar role in all eukaryotes.

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