Nuclear and Mitochondrial Inheritance in Yeast Depends on Novel Cytoplasmic Structures Defined by the MDM1 Protein

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Abstract. The *mdm1* mutation causes temperature-sensitive growth and defective transfer of nuclei and mitochondria into developing buds of yeast cells at the nonpermissive temperature. The *MDM1* gene was cloned by complementation, and its sequence revealed an open reading frame encoding a potential protein product of 51.5 kD. This protein displays amino acid sequence similarities to hamster vimentin and mouse epidermal keratin. Gene disruption demonstrated that *MDM1* is essential for mitotic growth. Antibodies against the MDM1 protein recognized a 51-kD polypeptide that was localized by indirect immunofluorescence to a novel pattern of spots and punctate arrays distributed throughout the yeast cell cytoplasm. These structures disappeared after shifting *mdm1* mutant cells to the nonpermissive temperature, although the cellular level of MDM1 protein was unchanged. Affinity-purified antibodies against MDM1 also specifically recognized intermediate filaments by indirect immunofluorescence of animal cells. These results suggest that novel cytoplasmic structures containing the MDM1 protein mediate organelle inheritance in yeast.

CYTOPLASMIC organelles proliferate by the growth and division of preexisting organelles (Wilson, 1925; Palade, 1983). Consequently, daughter cells must inherit an allotment of each of these subcellular structures before the completion of cell division (Yaffe, 1991*b*). This organelle inheritance ensures the continuation of the organelle populations and provisions daughter cells with adequate numbers of organelles to supply metabolic functions early in the cell cycle. This inheritance of organelles is an essential component of cell proliferation, yet little is known of its molecular basis or regulation.

Observations with a number of cell types have suggested roles for the cytoskeleton in organelle movement and distribution. Mitochondria, ER, and other organelles are often found in specific intracellular locations that correlate with positions of microtubules (Franke et al., 1978; Ball and Singer, 1982; Lee and Chen, 1988) or of intermediate filaments (Hirokawa, 1982; Chen, 1988). Organelles have been observed to exhibit saltatory motion characteristic of transport along cytoskeletal components (Adams, 1982; Aufderheide, 1977). Other investigations have revealed that regions of active organellar streaming in animal cells correlate with areas of change in the structure of actin-like filament bundles (Wang and Goldman, 1978), and the actin-mediated transport of ER and other organelles has been described for characean algal cells (Kachar and Reese, 1988). Studies in higher eukaryotic cells have characterized kinesin and cytoplasmic dynein as molecular motors capable of transporting organelles along microtubules (Vale, 1987; Schroer et al., 1989). Additionally, the function of nonmuscle myosin in the translocation of organelles along actin microfilaments has been described (Adams and Pollard, 1986). However, the role of any of these proteins or cytoskeletal systems in the intracellular movements required for organelle inheritance during mitotic growth is obscure. In particular, studies in the yeast *Saccharomyces cerevisiae* have demonstrated that microtubules are not essential for the transfer of mitochondria and most other cytoplasmic structures into developing buds (Huffaker et al., 1988; Jacobs et al., 1988), and evidence for a role of actin or intermediate filaments in organelle inheritance is lacking.

The yeast *mdm1* mutant is defective for the transfer of nuclei and mitochondria into developing daughter buds during incubation at 37°C (McConnell et al., 1990). This mutant is one of several temperature-sensitive strains displaying conditional defects in mitochondrial inheritance and an uncoupling of mitochondrial movement from bud growth (McConnell et al., 1990; Stewart and Yaffe, 1991). *mdm1* cells incubated at the nonpermissive temperature develop large buds devoid of nuclei and mitochondria. Successful nuclear division does occur in a fraction of *mdm1* mutant cells incubated at 37°C; however, the mitotic spindle appears misoriented, resulting in two nuclei in the mother portion of the cell. Other cellular structures including the actin- and tubulin-based cytoskeletons appear normal in *mdm1* cells. Genetic analysis demonstrated that the mutant phenotypes displayed by *mdm1* cells are due to a single, recessive, nuclear mutation (McConnell et al., 1990). In the present study we report the isolation and analysis of the *MDM1* gene. We demonstrate that *MDM1* is an essential yeast gene encoding a protein localized to novel punctate structures distributed throughout the yeast cytoplasm.

Materials and Methods

Strains and Genetic Methods

The parent strain A364A (*MATa adel ade2 ural his7 lys2 tyrl gall*) and isolation of the *mdml* mutant has been described previously (McConnell et al., 1990). Strains MYY290, MYY291, and MYY298 were described previously (Smith and Yaffe, 1991). Strain MYY403 (*MATa, mdml, ura3, leu2*) was derived as a haploid spore from a cross of the original *mdml* mutant, MYY275 (*MATa, mdml, adel, ade2, ural, his7, lys2, tyrl, gall*), with strain HR125 (*MATa, ura3, leu2, trpl, his3*) (Russell et al., 1986). Strain SEY6210 (*MATa, ura3, leu2, his3, trpl, ade2, suc2*) was obtained from S. Emr (University of California, La Jolla, CA). Plasmid DNA was prepared in *Escherichia coli* strains DH5α and MH6. Standard yeast techniques and genetic analysis were as described previously (Sherman et al., 1979).

Isolation of the MDM1 Gene

The *MDM1* gene was isolated by genetic complementation of the *mdml* temperature-sensitive lethal mutation. The *mdml* strain MYY403 was transformed with a yeast genomic plasmid library in the vector YEp13 (Broach et al., 1979) as described by Ito et al. (1983). Leu^+ transformants were selected at 23°C and screened for growth at the nonpermissive temperature by two successive replica platings at 37°C. Complementing plasmids (YEp13-1, YEp13-2, and YEp13-3) were analyzed by restriction analysis. Portions of the complementing DNA were subcloned into plasmid YCp50 (Rose et al., 1987), and the ability of these constructs to complement the temperature-sensitive defect in *mdml* cells was analyzed.

Integrative Transformation

A 5.0-kbp HindIII-SphI fragment isolated from YEp13-1 was inserted into the yeast integrating plasmid YIp5 (Struhl et al., 1979) to yield plasmid YIp5-MDM1. This plasmid was linearized at the unique KpnI site (see Fig. 1) and transformed into strain SEY6210. Stable Ura^+ transformants were crossed to strain MYY403, and the diploids were sporulated. The meiotic progeny were tested for growth at 37°C and for growth on media lacking uracil. Analysis of this cross revealed no recombination between *mdml* and *URA3* in any of the 40 tetrads tested. All tetrads were of the parental ditype (2 Ura^+, 2 ts^-: 2 Ura^-, 2 ts^+; ts^- = normal growth at 37°C). These results indicated that the plasmid YIp5-MDM1 had integrated at a site within 1.2 centimorgans of the *MDM1* locus.

Sequence Analysis of the MDM1 Gene

The complementing HindIII-XbaI fragment (see Fig. 1 *B*) was subcloned into the polylinker region of Bluescript SK (Stratagene, La Jolla, CA) to create plasmid BS-MDM1. Templates for sequencing were obtained using Exonuclease III to create a series of nested deletions (Henikoff, 1984) from both ends of the yeast DNA insert. The nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase 2.0 DNA sequencing kit (U. S. Biochemical Corp., Cleveland, OH). The deduced MDM1 protein sequence was compared with sequences in the GENBANK data base using the FASTA program (Lipman and Pearson, 1985) and with sequences in the NEWAT data base using the SCANA program (Doolittle, 1986).

MDM1 Gene Disruption

A 3.0-kbp SphI-KpnI fragment containing the entire *MDM1* gene (see Fig. 1 *A*) was isolated from plasmid YEp13-2 and ligated into the SphI and KpnI sites of pUC19 creating the plasmid pUC19-MDM1. A 2.2-kbp SalI-XhoI fragment containing the *LEU2* gene was isolated from YEp13 (Broach et al., 1979), the DNA ends were filled in with Klenow polymerase, and the fragment was blunt-end ligated into the filled EcoNI and NheI sites within the *MDM1* gene (see Fig. 1 *A*) in plasmid pUC19-MDM1. The resulting plasmid pUC19-MDM1::LEU2 was cut with StuI and PvuI. The resulting StuI-PvuI fragment was used to replace one copy of *MDM1* in the diploid strain MYY298. Leu^+ transformants were isolated and the meiotic products from six independently isolated diploid colonies were analyzed at 23°C. Of 38 tetrads, 37 gave rise to only two viable spores, both of which were Leu^-. One tetrad gave rise to only one viable spore which was Leu^-. Southern analysis (Maniatis et al., 1982) of the Leu^+ diploid strains, using a 1.9-kbp EcoRI probe (see Fig. 1), confirmed that one of the two copies of *MDM1* had been replaced by the *LEU2* gene (see Fig. 4). Growth of cells containing the gene disruption was rescued by transformation with a single wild-type copy of *MDM1* in the plasmid YCp50. This complementary DNA extended from the SphI site, 350 bp upstream from the *MDM1* ORF to an EcoRV site located 281 bp downstream from the ORF.

Preparation and Characterization of Antisera

Antibodies against the MDM1 protein were raised against a β-galactosidase-MDM1 fusion protein. Specifically, a 726-bp HaeIII fragment (see Fig. 1 *A*) containing the COOH-terminal 418 bp of the 1,329-bp *MDM1* ORF was isolated from plasmid BS-MDM1, the ends were filled using Klenow, and the fragment was inserted into the filled BamHI site of the vector pTRBO (Burglin and DeRobertis, 1987). The fusion protein was expressed in the *Escherichia coli* strain 71-18, purified by SDS-PAGE and electro-elution, and used to immunize rabbits (Harlow and Lane, 1988).

Antibodies raised against the β-galactosidase-MDM1 fusion protein were affinity-purified on nitrocellulose blots of either the fusion protein or the 51-kD band (see below) separated from a preparation of total yeast proteins as described by Pringle et al. (1991) with the following modification: antibody was eluted for 3 min at 4°C using 200 mM glycine and immediately neutralized in 1/10 vol 1 M Tris, pH 8.0. Antibodies were concentrated using centricon 30 microconcentrators (Amicon Corp., Danvers, MA) according to the manufacturer's instructions.

Antibodies were analyzed by Western blotting (Towbin et al., 1979) of protein extracted from whole yeast cells (Yaffe, 1991*a*) or from isolated subcellular fractions (Daum et al., 1982; Schauer et al., 1985).

Immunofluorescence Microscopy

All fluorescence and indirect immunofluorescence microscopy on yeast was as previously described (McConnell et al., 1990). Affinity-purified anti-MDM1 antibodies were used at a final concentration of 25 μg/ml. Actin was visualized with rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR). For indirect immunofluorescence in animal cells, CV-1 (African green monkey kidney) cells were grown at low density on 12-mm round glass coverslips in alpha MEM supplemented with FCS, penicillin, and streptomycin at final concentrations of 10%, 200 U/ml, and 200 μg/ml, respectively, at 37°C. Coverslips were washed in PBS (0.15 M NaCl, 0.05 M KPO_4, pH 7.4), and cells were fixed by immersion in methanol at −20°C for 10 min. Coverslips were washed three times with PBS and incubated with 1 ml blocking buffer (2.5% FCS in PBS) for 15 min at 23°C. After washing three additional times with PBS, primary antibody (diluted in blocking buffer) was added, and coverslips were incubated at 23°C for 30 min. Coverslips were washed as before, followed by incubation with secondary antibody (FITC or rhodamine-conjugated goat anti-mouse IgG, goat anti-rat IgG, or goat anti-rabbit IgG; Jackson Immunoresearch Inc., West Grove, PA) for 30 min at 23°C. After washing in PBS (3×), coverslips were mounted on slides in mounting medium (Pringle et al., 1991), sealed with clear nail polish, and viewed on a Leitz Laborlux 12 microscope equipped for epifluorescence. Intermediate filaments were visualized using mAbs (purchased from Amersham Corp., Arlington Heights, IL) against vimentin and cytokeratin. Antibodies against tubulin were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Antibodies against MDM1, vimentin, cytokeratin, and tubulin were used at final concentrations or dilutions of 25 μg/ml, 1:200, 1:500, and 1:100, respectively. In some experiments, cells were treated with nocodazole (Sigma Chemical Co., St. Louis, MO) at 5 μM for 1 h at 37°C.

Results

Cloning of the MDM1 Gene

Yeast cells with the recessive *mdml* mutation display temperature-sensitive defects in growth and in the transfer of both nuclei and mitochondria into developing buds (McConnell et al., 1990). To determine the molecular basis of these defects, the wild-type *MDM1* gene was cloned by complementation of the temperature-sensitive growth phenotype of *mdml* cells. Out of ~8,000 Leu^+ transformants, three were isolated which contained plasmids conferring growth at 37°C. These plasmids also complemented the mitochondrial inheritance defect of *mdml* cells. Restriction analysis

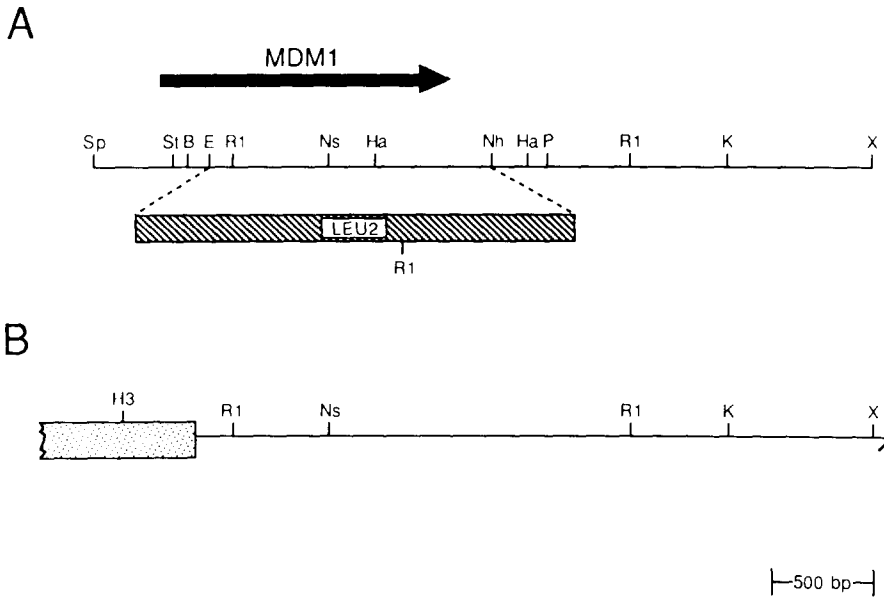

Figure 1. Localization of the *MDM1* gene. (*A*) Restriction map for several different enzymes was determined for the complementing SphI–XbaI DNA fragment. Location of the *MDM1* open reading frame is indicated by the solid arrow. The region replaced by the yeast *LEU2* gene for disruption of *MDM1* is indicated. (*B*) Yeast DNA insert and adjacent vector regions (*stippled*) of plasmid YEp13-1. Restriction sites: *B*, BglII; *E*, EcoNI; *Ha*, HaeIII; *H3*, HindIII; *K*, KpnI; *Ns*, NsiI; *Nh*, NheI; *P*, PvuI; *RI*, EcoRI; *Sp*, SphI; *St*, StuI; *X*, XbaI.

revealed that the three plasmids contained inserts of yeast DNA which partially overlapped in their sequences. Subcloning and re-introduction of the yeast DNA fragments into *mdm1* cells demonstrated that a 5.0-kbp HindIII–SphI fragment from the plasmid YEp13-1 (the complementing plasmid containing the smallest insert of yeast DNA) complemented the mutant phenotypes.

To verify that complementation by the isolated DNA reflected the presence of the wild-type *MDM1* gene (rather than another gene that could suppress the *mdm1* defect), the 5.0-kbp HindIII–SphI fragment of complementing DNA was inserted into the plasmid YIp5, integrated into the yeast genome, and the site of integration was mapped. This fragment directed integration of plasmid DNA to the *MDM1* locus, indicating that the plasmid contained the authentic *MDM1* gene (see Materials and Methods). Additionally, the *MDM1* gene was mapped to chromosome XIII by hybridization of a 3.7-kbp HindIII-XbaI probe to a yeast chromosomal OFAGE blot (data not shown).

Sequencing of the MDM1 Gene

The nucleotide sequence of a 3.7-kbp HindIII–XbaI fragment from plasmid YEp13-1 which complemented *mdm1* was determined by dideoxy sequencing. A single, long open reading frame (ORF)[1] was identified; however this ORF was initiated by a methionine located within adjacent vector sequences (Fig. 1 *B*). Nucleotide sequence analysis of the insert from a second complementing plasmid, YEp13-2, revealed the full-length *MDM1* gene (Fig. 1 *A*). These results indicate that the HindIII–XbaI fragment from plasmid YEp13-1 can complement the *mdm1* mutation even with 49 amino acid residues from the amino terminus of the protein replaced by unrelated residues encoded in vector sequences (Fig. 1 *B*).

The full-length ORF of 1,329 bp encodes a putative protein of 443 amino acids with a predicted size of ~51.5 kD (Fig. 2). The length of this ORF correlated well with the appearance of a single, 1.6-kbp band on Northern blots of isolated yeast RNA (probed with a 1.6-kbp StuI–PvuI fragment, data not shown). In addition, the ORF and adjacent sequences do not contain conserved elements characteristic of introns or spliced genes.

Comparison of the predicted protein sequence of MDM1 with sequences in the GENBANK database using the FASTA program (Lipman and Pearson, 1985) revealed no significant similarity to any previously described proteins. However, analysis with the SCANA program (Doolittle, 1986) indicated a relationship between the MDM1 protein and hamster vimentin (Quax-Jeuken et al., 1983) and mouse epidermal keratin (Steinert et al., 1983) with identities of 14 and 12%, respectively, over the entire MDM1 protein sequence (Fig. 3). In addition, MDM1 and vimentin are essentially the same size (443 and 448 amino acids, respectively), show 32% similarity of amino acid residues, share identical and similar residues throughout the length of their sequences, and possess very similar amino acid compositions. Keratin and MDM1 exhibit 22% similarity, and the two proteins display 27% similarity when the extremely glycine-rich amino- and carboxy-terminal domains of keratin are excluded from the comparison. Analysis of predicted secondary structure by two independent programs (Chou and Fasman, 1974; Garnier et al., 1978) suggested that the MDM1 protein possesses a high percentage of α-helical structure (48 and 50%, respectively). Sequence of the MDM1 protein revealed no hydrophobic domains typical of membrane spanning regions or characteristic targeting signals for intracellular protein sorting. These results demonstrate that *MDM1* encodes a previously unidentified protein possessing modest sequence similarity to several vertebrate intermediate filament proteins.

MDM1 Is an Essential Gene

Cells with the *mdm1* mutation show a temperature-sensitive

1. *Abbreviation used in this paper*: ORF, open reading frame.

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-90  AACATTTTCA AAGATAAACT CTTCGACGAT GAAAACGATA ATACCTCAGA AATTTCTGTT GTCGAGGATC AGTTGGATCA CCCACGTAAT

1    ATG GAG AAA GTA AGT GTT TCC AGC GGG AAT TCA GGC CTA AAT CCT AGC CAA TTT TAT GGG TCA AAC AAC TTT AGG
1    MET Glu Lys Val Ser Val Ser Ser Gly Asn Ser Gly Leu Asn Pro Ser Gln Phe Tyr Gly Ser Asn Asn Phe Arg

76   GAT AAT ATA GCA TCC TTG ACT ATT TCA ATT GAT CAG ATT GAG AAA GAG CTT GAA CTT TTA AGG CAT TTG ATT TTG
26   Asp Asn Ile Ala Ser Leu Thr Ile Ser Ile Asp Gln Ile Glu Lys Glu Leu Glu Leu Leu Arg His Leu Ile Leu

151  AAG GCA GAT CTG ACC AAC AAT CAA ATG CAG TTG AAA ATT TTA AAA AAG TCA CAA AGA ACC CTA TTA AAG GAA CTA
51   Lys Ala Asp Leu Thr Asn Asn Gln MET Gln Leu Lys Ile Leu Lys Lys Ser Gln Arg Thr Leu Leu Lys Glu Leu

226  GAA ATG AAG GAA CTG TTG AAA CAG CAA TAT ATG GTA CGA GAA AAT GGT AAT AGT TTG TTC AGA AAA ACT AAG ATA
76   Glu MET Lys Glu Leu Leu Lys Gln Gln Tyr MET Val Arg Glu Asn Gly Asn Ser Leu Phe Arg Lys Thr Lys Ile

301  TAC ATT AGG TCG TAC TTC AGT GAG AAT TCT AGT AAT GGC CTA AAA GAA ATA ACT TAT TAT ATC ATC AAT ATC CAT
101  Tyr Ile Arg Ser Tyr Phe Ser Glu Asn Ser Ser Asn Gly Leu Lys Glu Ile Thr Tyr Tyr Ile Ile Asn Ile His

376  CAT TTT AAC AAT GGG CAG GTA AGT TCT TGG GAT ATG GCA AGG AGA TAT AAT GAA TTT TTT GAA TTA AAT ACA TAT
126  His Phe Asn Asn Gly Gln Val Ser Ser Trp Asp MET Ala Arg Arg Tyr Asn Glu Phe Phe Glu Leu Asn Thr Tyr

451  TTG AAA AAG AAT TTT AGG GAT CTG ATG AGG CAG TTG CAA GAT TTA TTC CCG TCA AAA GTG AAA ATG TCA TTG AAA
151  Leu Lys Lys Asn Phe Arg Asp Leu MET Arg Gln Leu Gln Asp Leu Phe Pro Ser Lys Val Lys MET Ser Leu Lys

526  TAT CAT GTT ACA AAA ACA TTG CTT TAT GAG GAA CGA AAG CAA AAA CTT GAA AAG TAC TTG CGA GAA CTA TTA TCT
176  Tyr His Val Thr Lys Thr Leu Leu Tyr Glu Glu Arg Lys Gln Lys Leu Glu Lys Tyr Leu Arg Glu Leu Leu Ser

601  ATT TCT GAA ATA TGC GAA GAT AAC ATT TTC AGA AGA TTT TTG ATA GAT CCT ACC CCA TTC AAA CTG AAC AAG GAA
201  Ile Ser Glu Ile Cys Glu Asp Asn Ile Phe Arg Arg Phe Leu Ile Asp Pro Thr Pro Phe Lys Leu Asn Lys Glu

676  TAT ATG CAT GAT GAT ATT CTG GAG GAG CCA CTT CAT GAA CCT ATT GGT AGT AGT AAT AGC ACA AGT AAT AGC AGT
226  Tyr MET His Asp Asp Ile Leu Glu Glu Pro Leu His Glu Pro Ile Gly Ser Ser Asn Ser Thr Ser Asn Ser Ser

751  TCT GTT GTT GAT TTA CAA AGC TCT GAA GAC GGC GGC GAA TTG AAT TTC TAT GAA GAT GAA AGA CAT TTT TTC ACC
251  Ser Val Val Asp Leu Gln Ser Ser Glu Asp Gly Gly Glu Leu Asn Phe Tyr Glu Asp Glu Arg His Phe Phe Thr

826  GAT TCT GGC TAT CCT TTT TAT TCG CAA AAT AAA TCT TTT GTT AAA CAG ATA TGT GAC TTA TTT ATT TCG CTC TTT
276  Asp Ser Gly Tyr Pro Phe Tyr Ser Gln Asn Lys Ser Phe Val Lys Gln Ile Cys Asp Leu Phe Ile Ser Leu Phe

901  GCT TTA AAT AAG GCC AAC GCG GGT TGG TTA AGA GGA AGG GCT ATA ATT ACC GTT TTA CAA CAG CTT CTT GGG AGC
301  Ala Leu Asn Lys Ala Asn Ala Gly Trp Leu Arg Gly Arg Ala Ile Ile Thr Val Leu Gln Gln Leu Leu Gly Ser

976  ACT ATT GAA AAG TAT ATA AAG GTC AGC ATT CAA AAA TTG AGA TCA GAG GAT CAA GTA TTT GAA GCA ATA GTT ACA
326  Thr Ile Glu Lys Tyr Ile Lys Val Ser Ile Gln Lys Leu Arg Ser Glu Asp Gln Val Phe Glu Ala Ile Val Thr

1051 TTC AAG AAT ATG CTA TGG GGT GAT AAC GGT CTG TTC GAA AGA AAA AGA AAT GAA ACG GCA GAG GCA ACA AGA AGC
351  Phe Lys Asn MET Leu Trp Gly Asp Asn Gly Leu Phe Glu Arg Lys Arg Asn Glu Thr Ala Glu Ala Thr Arg Ser

1126 GAA GGT GAA AAG TTA CGG ACG GAA CAA CTC GCT TTA ACC AGT TTG CAA AGA CTC TTC GCA GAC TCT TGT GGT CGT
376  Glu Gly Glu Lys Leu Arg Thr Glu Gln Leu Ala Leu Thr Ser Leu Gln Arg Leu Phe Ala Asp Ser Cys Gly Arg

1201 GTA GTT GGT CTA AGA GAT TCT CAT GAA GCT GCT CGC CGG GTC CAT GCC ATG CTA CAG AAT CCG TAT TTA AAT GCC
401  Val Val Gly Leu Arg Asp Ser His Glu Ala Ala Arg Arg Val His Ala MET Leu Gln Asn Pro Tyr Leu Asn Ala

1276 AGC TTA CTG TTG GAA GCT CTT GAT GCA ATT TTA TTG GAC ATA ATA TGT AAT GAC TGA
476  Ser Leu Leu Leu Glu Ala Leu Asp Ala Ile Leu Leu Asp Ile Ile Cys Asn Asp ***

1333 AAATAAAACT TGACTAAGTA CCGAACAATC TAAAAAAAAA GTGTAATTGT CACAATAGCA AATAAATTGG TACACTTCTT CAGCCGCTTT
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Figure 2. Sequence of the *MDM1* gene and its predicted protein product. These sequence data are available from EMBL/GenBank/DDBJ under accession number X66371.

lethal phenotype, suggesting that *MDM1* encodes an essential gene product or one that is needed for growth at elevated temperatures. To determine whether *MDM1* is essential for cell viability at all temperatures, a null mutation in *MDM1* was constructed. Most of the coding region of one of two copies of *MDM1* in a diploid cell was replaced with the yeast *LEU2* gene (see Materials and Methods and Fig. 1 *A*). This gene replacement was confirmed by Southern analysis (Fig. 4 *B*). When the resulting diploid cells were sporulated and the haploid progeny cultured at 23°C, only two spores from each tetrad developed into colonies (Fig. 4 *A*). All viable spores were Leu$^-$, indicating the presence of an intact *MDM1* gene. Additionally, the wild-type *MDM1* gene, on a single-copy yeast plasmid (see Materials and Methods), res-

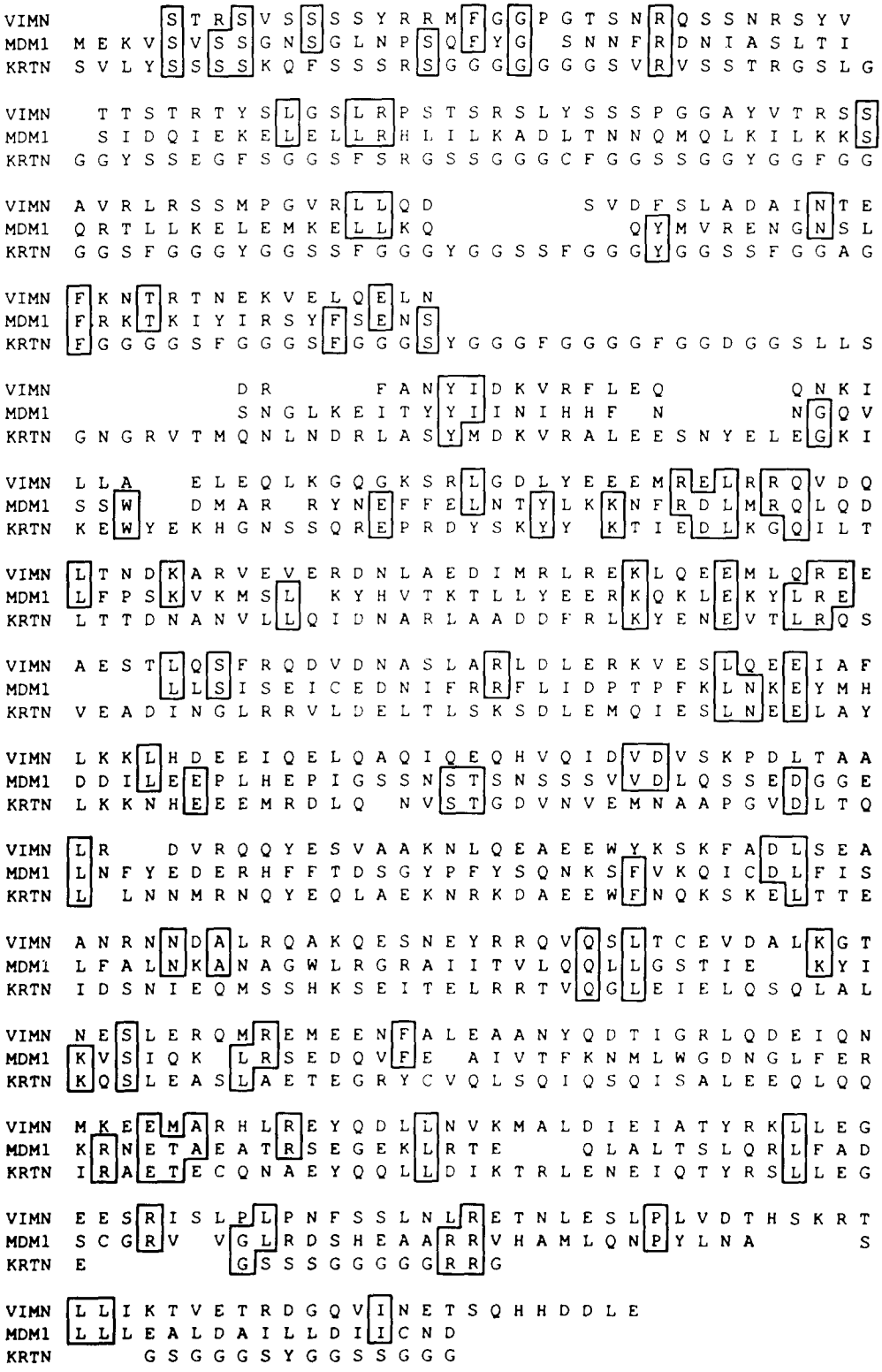

Figure 3. Sequence comparison of MDM1 with vimentin and keratin. Protein sequences were aligned as described by Feng and Doolittle (1987). Amino acids identical between MDM1 and hamster vimentin (*VIMN*) and between MDM1 and mouse epidermal keratin (*KRTN*) are boxed. Shown are the entire amino acid sequences of MDM1 (443 residues) and vimentin (448 residues), and the first 479 residues of keratin.

cued growth in haploid spores containing the gene disruption. These results demonstrate that *MDM1* is a gene essential for normal mitotic growth and cell viability.

The MDM1 Protein Is Localized in a Novel Cytoplasmic Pattern

To determine the intracellular location and distribution of the MDM1 protein, antibodies were raised against a β-galactosidase-MDM1 fusion protein (see Materials and Methods). This antiserum specifically recognized a 51-kD polypeptide by Western blot analysis of total yeast proteins (Fig. 5). The level of the 51-kD band was increased in wild-type cells harboring a multicopy plasmid containing the *MDM1* gene (Fig. 5), indicating that the antibody specifically recognized the

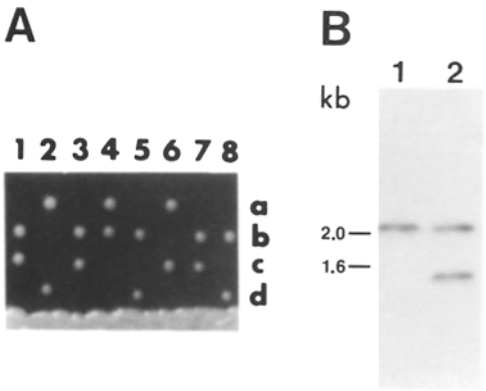

Figure 4. MDM1 is essential for cell viability. (*A*) One of two wild-type copies of *MDM1* was replaced by the yeast *LEU2* gene in the diploid strain MYY298 as described in the text. The diploid strain was sporulated, and the spores of eight tetrads were separated and cultured on an agar slab. The four spores from each tetrad (#1–8) were placed at positions a–d. (*B*) Southern analysis of diploid cells containing the *MDM1* gene disruption. (Lane *1*) Wild-type (MYY-298) chromosomal DNA cut with EcoRI and probed with a labeled fragment of *MDM1* (see Materials and Methods). (Lane 2) Chromosomal DNA from a strain in which one copy of *MDM1* was replaced with the yeast *LEU2* gene, cut with EcoRI, and probed as in lane 1.

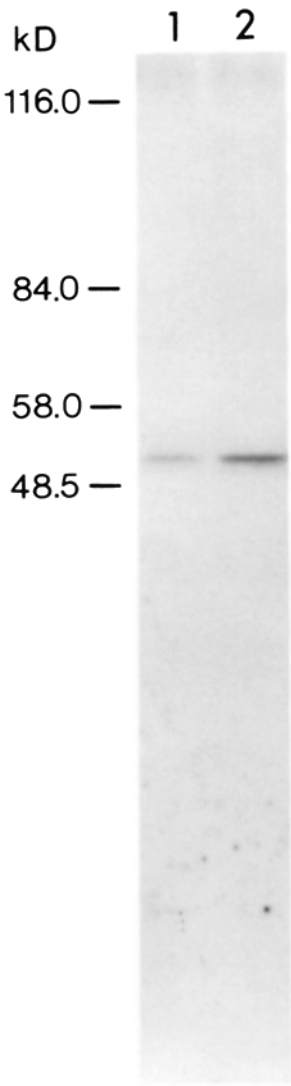

Figure 5. Antiserum against a β-galactosidase-MDM1 fusion protein recognizes the MDM1 protein on Western blots. Total proteins (100 μg) were extracted from yeast strain MYY290 (wild-type) carrying either plasmid YEp13 (lane *1*) or plasmid YEp13-2 (*MDM1*, lane *2*). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, decorated with antiserum and [^{125}I]protein A, and analyzed by autoradiography.

MDM1 protein. The 51-kD band also was specifically recognized by antibodies affinity purified on the fusion protein (not shown).

Western blotting of proteins extracted from subcellular fractions isolated from a yeast homogenate revealed the presence of MDM1 in all but the cytoplasmic fraction (data not shown). This finding suggested that the MDM1 protein exits in an aggregated or filamentous form, or is associated with membraneous structures.

Distribution of the MDM1 protein in intact cells was characterized further by indirect immunofluorescence using affinity-purified antibodies. This approach revealed localization of MDM1 to a unique pattern of spots, sometimes appearing in linear arrays, distributed throughout the cytoplasm (Fig. 6). This punctate staining was most distinct and intense in unbudded cells, and appeared less abundant and less intense in cells with small daughter buds, particularly within the buds (Fig 6). Double-label indirect immunofluorescence analysis revealed no particular correlation of MDM1 spots and cytoplasmic organelles: MDM1 spots were distributed throughout the cytoplasm, some coincident with positions of nuclei and mitochondria while others localized in regions distinct from the organelles (not shown). Antibodies affinity-purified on either the β-galactosidase-MDM1 fusion protein (purified from *E. coli*) or the 51-kD band excised from a nitrocellulose blot of whole yeast proteins revealed an identical pattern of MDM1 distribution. Preimmune sera produced no specific cellular staining (data not shown).

Double-label microscopic analysis indicated that the pattern of MDM1 distribution is unrelated to the distribution of actin and tubulin (Fig. 7), major components of the two well-characterized cytoskeletal networks in yeast (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Thomas et al., 1984). These results from microscopic analysis and subcellular fractionation demonstrate that the MDM1 protein is extensively distributed in the yeast cell cytoplasm in a novel pattern of punctate structures.

Antibodies to the MDM1 Protein Recognize the Intermediate Filament Network in Animal Cells

The pattern of MDM1 distribution detected by indirect immunofluorescence was unlike any previously reported for yeast proteins but suggested a cytoskeletal network. Since the cytoskeleton of animal cells has been highly characterized (Bershadsky and Vasiliev, 1988), we examined possible cross-reaction of anti-MDM1 antibodies with animal cell proteins by indirect immunofluorescence. When CV-1 (African green monkey kidney) cells were stained with affinity-purified anti-MDM1 antibody, a striking filamentous pattern was observed (Fig. 8, *d* and *f*; Fig. 9, *d* and *f*). The web-like fluorescent pattern was characteristic of that described previously for intermediate filaments (Ball and Singer, 1981; Bershadsky and Vasiliev, 1988), and was absent when preimmune sera subjected to affinity purification was employed (data not shown). In addition, a similar indirect immunofluo-

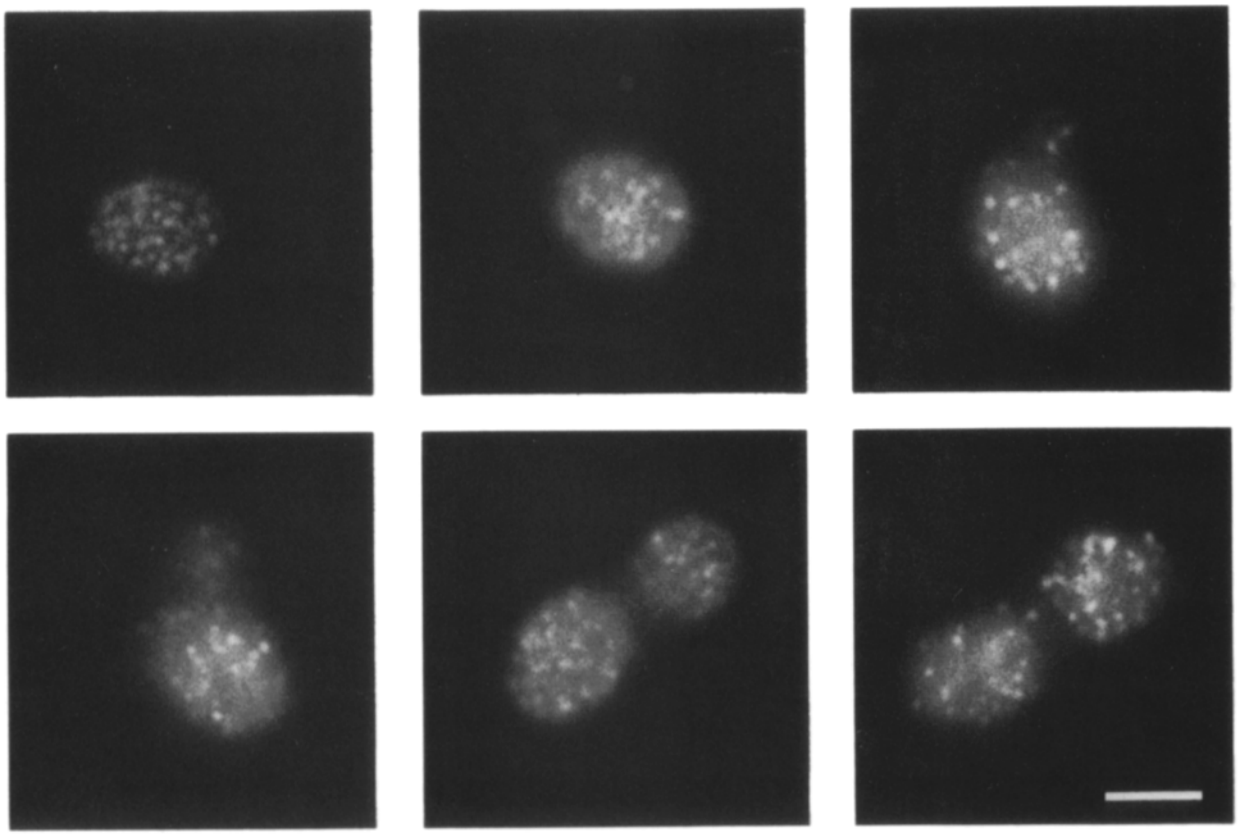

Figure 6. Distribution of the MDM1 protein in wild-type yeast cells. Wild-type cells (MYY290) were grown to mid-log phase in YPD medium at 30°C. Cells were fixed with formaldehyde and processed for indirect immunofluorescence using affinity-purified anti-MDM1 antiserum as described in Materials and Methods. Bar, 2 μm.

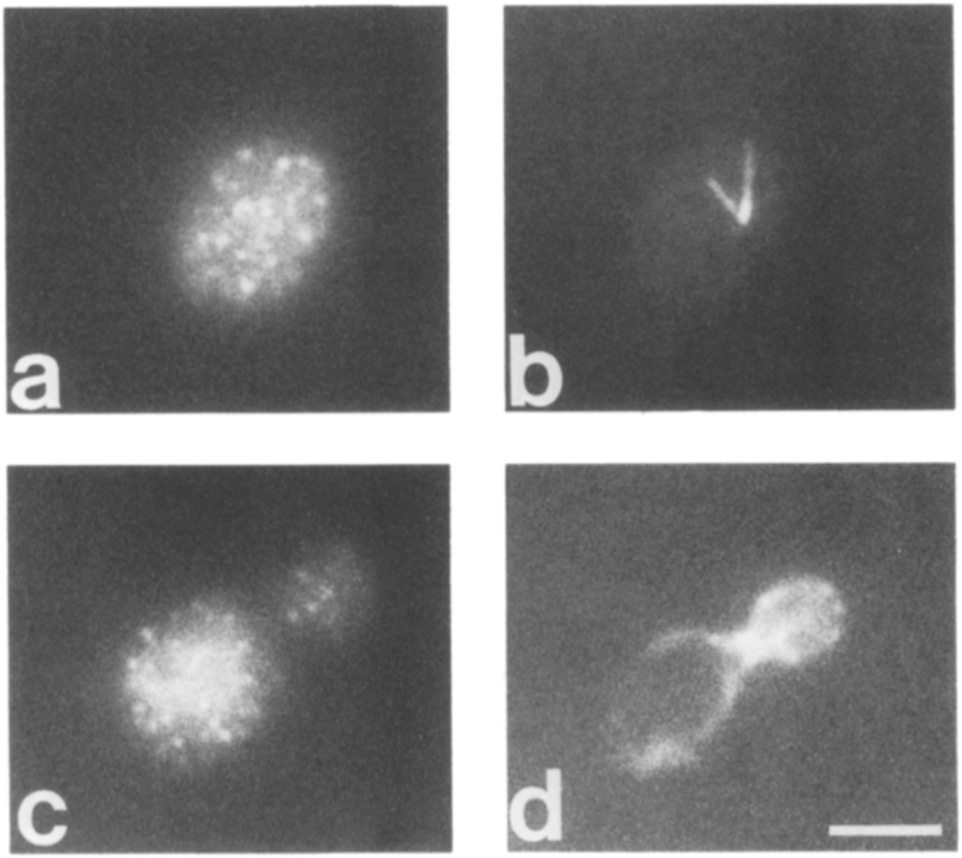

Figure 7. The MDM1 distribution is distinct from that of actin and tubulin. Wild-type yeast (MYY290) were grown at 30°C to mid-log phase and analyzed by microscopy as described for Fig. 6. Actin was detected with rhodamine-conjugated phalloidin, and microtubules and MDM1 were detected by indirect immunofluorescence. Cells were simultaneously stained for MDM1 (*a*) and microtubules (*b*) or for MDM1 (*c*) and actin (*d*). Bar, 2 μm.

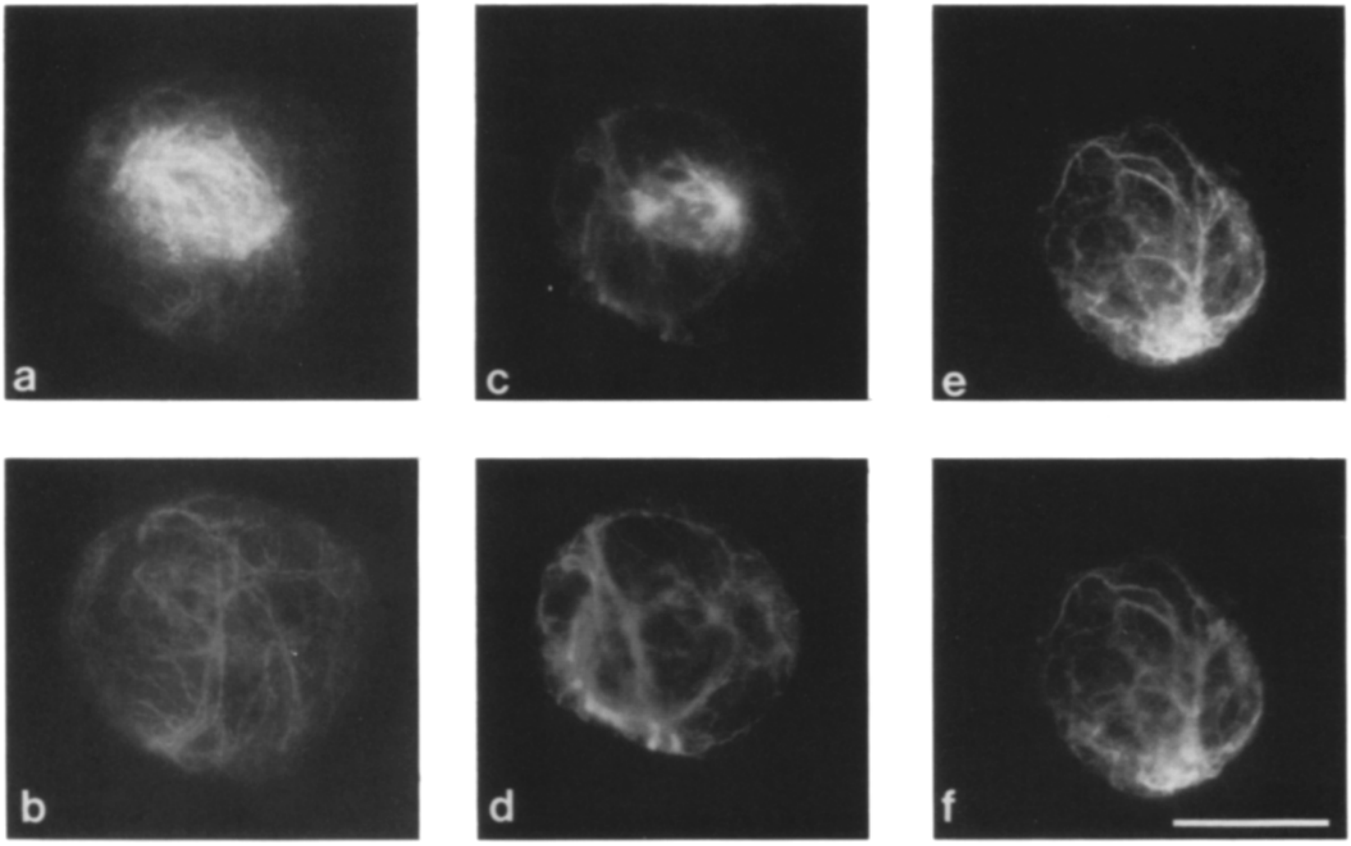

Figure 8. Antibodies to the MDM1 protein localize to intermediate filaments in mitotic CV-1 cells. Double indirect immunofluorescence labeling was performed on mitotic CV-1 cells. Mitotic cells were harvested from supernatants of CV-1 cells grown as monolayers in tissue culture. Cells were fixed and processed for indirect immunofluorescence as described in Materials and Methods. Double-label analysis of single cells (*a* and *b*, *c* and *d*, *e* and *f*) are shown. (*a*) Microtubules; (*b*) vimentin intermediate filaments; (*c*) microtubules; (*d*) MDM1; (*e*) vimentin intermediate filaments; (*f*) MDM1. Bar, 40 μm.

rescence pattern was detected with anti-MDM1 antibody in CHO cells, XR-1 glial cells, and BHK-21 (baby hamster kidney) cells (data not shown).

The fluorescence pattern observed with anti-MDM1 antibodies resembled that of intermediate filaments, but in many animal cell types microtubules colocalize with intermediate filament during interphase (Ball and Singer, 1981; Bershadsky and Vasiliev, 1988). To determine whether anti-MDM1 labeled the microtubule or intermediate filament network, double-label indirect immunofluorescence analysis was performed on mitotic cells. In such cells, microtubules redistribute to form the mitotic spindle, while the intermediate filaments remain in an array extending throughout the cytoplasm (Lazarides, 1980; Bershadsky and Vasiliev, 1988). In the mitotic CV-1 cells, anti-MDM1 antibodies recognized a protein that colocalized with intermediate filaments and not with tubulin (Fig. 8). Control experiments in which cells were treated with only a single antibody revealed no crossover fluorescence (not shown).

To confirm that the cytoskeletal protein recognized by anti-MDM1 was associated with intermediate filaments, and not with microtubules, CV-1 cells were treated with nocodazole (to disrupt microtubules) before immunofluorescence analysis. Although microtubules were essentially absent after treatment with nocodazole (Fig. 9 *c*), filaments detected with the anti-MDM1 antibody persisted (Fig. 9 *d*). Furthermore, the anti-MDM1 pattern remained identical to the vimentin filament pattern (Fig. 9, *e* and *f*).

The pattern of anti-MDM1 fluorescence also was identical to that detected with antibodies against a second intermediate filament protein, cytokeratin (data not shown). This result is consistent with the colocalization of vimentin and cytokeratin in many cultured cells (Steinert and Roop, 1988; Lazarides, 1980). These results, taken together, demonstrate that antibodies against MDM1 recognize the intermediate filament network in a variety of animal cells.

MDM1 Distribution Is Greatly Altered in mdm1 Mutant Cells

Nuclei and mitochondria are not transferred into developing buds when *mdm1* cells are shifted to 37°C (McConnell et al., 1990). To explore relationships between the mutant phenotype and the distribution of MDM1, indirect immunofluorescence was performed on *mdm1* and wild-type cells that had been incubated at the nonpermissive temperature. Wild-type cells incubated at 37°C for 4 h retained the pattern of spots and punctate arrays (Fig. 10). However, this pattern was essentially absent from mutant cells exposed to the nonpermissive temperature (Fig. 10). Instead, weak, diffuse fluores-

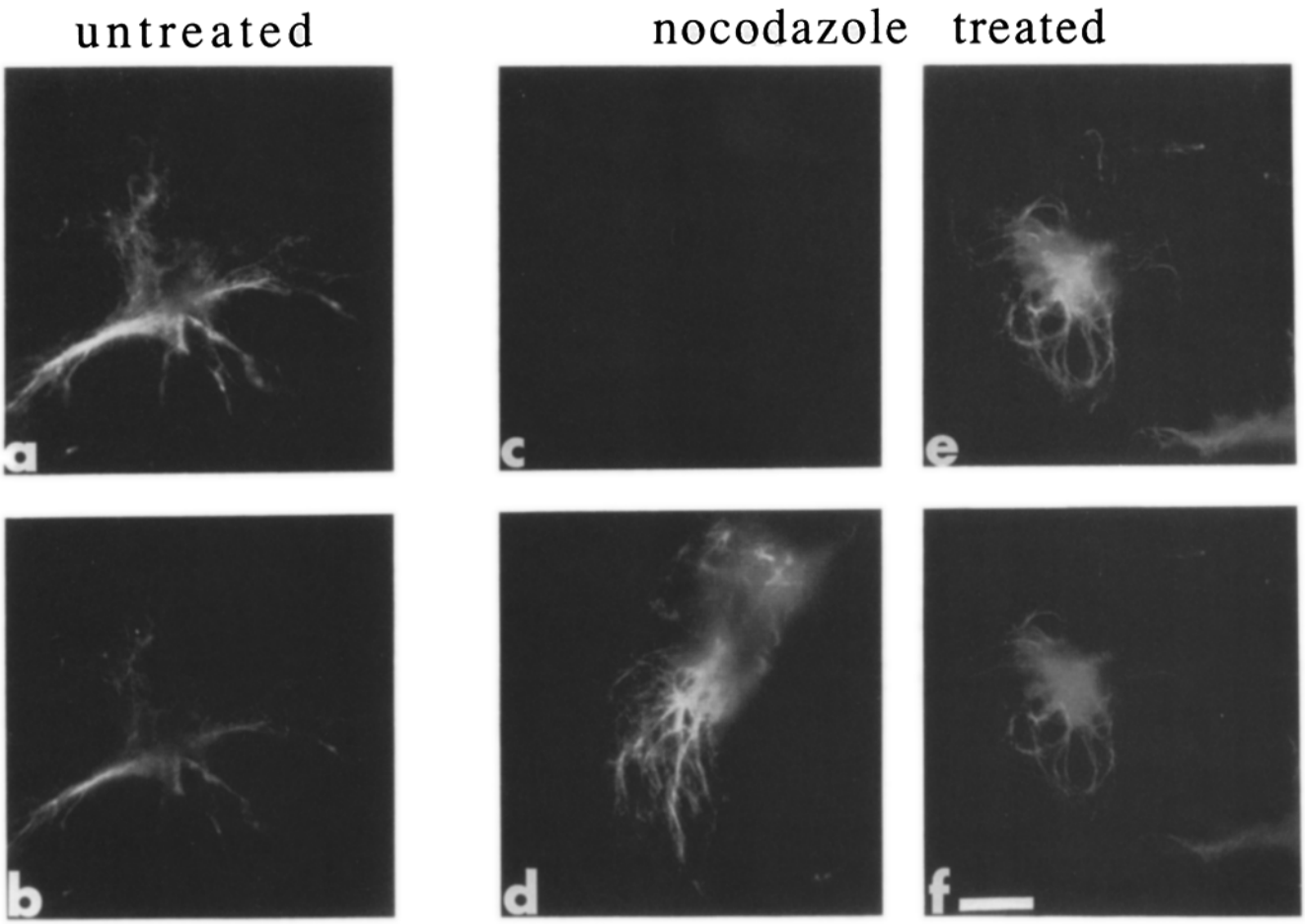

Figure 9. Antibodies to the MDM1 protein localize to intermediate filaments in nocodazole-treated CV-1 cells. Cells were grown to semi-confluency, treated with nocodazole (Materials and Methods), and analyzed by indirect immunofluorescence microscopy as described for Fig. 8. Double-label analysis of single cells (*a* and *b*, *c* and *d*, *e* and *f*) is shown. Untreated panel: (*a*) microtubules; (*b*) vimentin intermediate filaments. Nocodazole-treated panel: (*c*) microtubules; (*d*) MDM1; (*e*) vimentin intermediate filaments; (*f*) MDM1. Bar, 40 μm.

cence was apparent throughout the mutant cell cytoplasm. Additionally, *mdm1* cells grown at the permissive temperature displayed the characteristic wild-type pattern of MDM1 distribution (data not shown).

The possibility that the MDM1 protein was unstable in *mdm1* cells incubated at 37°C was analyzed by Western analysis of extracted cellular proteins. Similar levels of the 51-kD polypeptide were present in *mdm1* and wild-type cells at both room temperature and after a 4-h incubation at 37°C (Fig. 11). These results indicate that the *mdm1* lesion leads to a

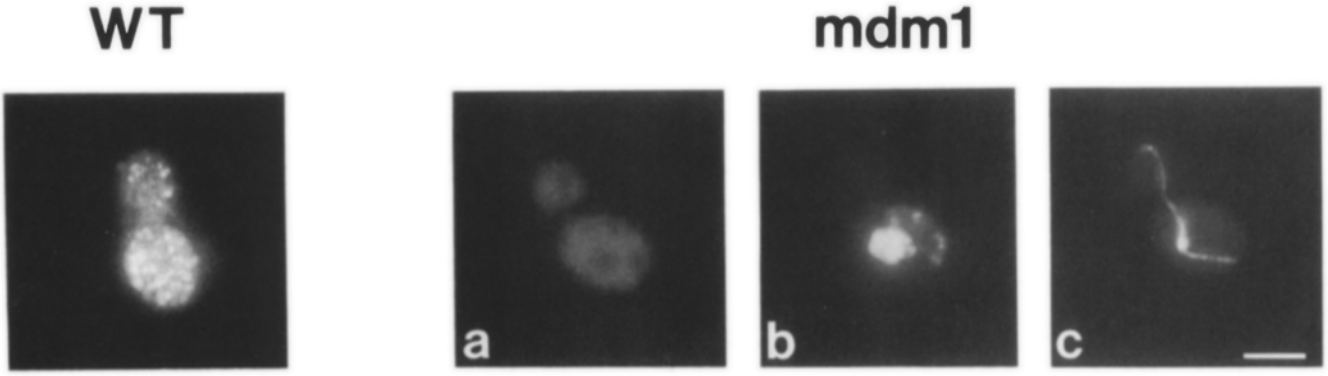

Figure 10. The MDM1 distribution is greatly altered in *mdm1* mutant cells. Wild-type (MYY290) and *mdm1* (MYY275) mutant cells were grown overnight at 23°C in YPD, diluted with fresh media, and incubated at 37°C for 4 h. Cells were fixed with formaldehyde, and processed for indirect immunofluorescence as described in Fig. 6. Wild-type panel shows MDM1 staining. *mdm1* panel: (*a*) MDM1; (*b*) DAPI staining showing mitochondrial and nuclear DNA; and (*c*) microtubules. Bar, 2 μm.

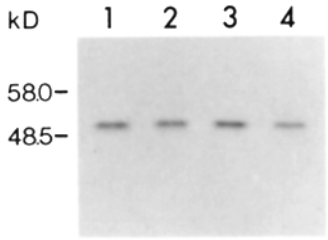

Figure 11. The MDM1 protein level is similar in wild-type and mutant cells after incubation at the nonpermissive temperature. Wild-type (MYY-290) and *mdm1* (MYY275) mutant cells were grown overnight at 23°C in YPD, diluted with fresh media, and incubated at either 23 or 37°C for 4 h. Total proteins were extracted from cells and equivalent amounts (135 μg) were separated by SDS-PAGE. The proteins were transferred electrophoretically to nitrocellulose, and MDM1 was detected as described for Fig. 5. (Lane *1*) *mdm1*, 23°C; (lane *2*) *mdm1*, 37°C; (lane *3*) wild-type, 23°C; (lane *4*) wild-type, 37°C.

temperature-dependent mislocalization of MDM1 or to instability or misassembly of the MDM1-containing structures.

Discussion

We have discovered a system of novel cytoplasmic structures that play a role in nuclear segregation and mitochondrial inheritance in yeast. This system was identified by indirect immunofluorescence using antibodies that recognize the product of the *MDM1* gene. A mutation in this gene (*mdm1*) causes temperature-sensitive growth and defects in the transfer of nuclei and mitochondria into developing buds in yeast cells incubated at 37°C (McConnell et al., 1990). In addition, gene disruption experiments demonstrated that *MDM1* is an essential gene, required for mitotic growth at all temperatures. After *mdm1* cells were shifted to the nonpermissive temperature, the pattern of intense punctate staining was no longer detectable, although the MDM1 protein was still present at normal levels in the cells. Taken together, these results suggest a direct role for the MDM1-containing structures in organelle inheritance.

A number of observations suggest a relationship between the MDM1 protein and intermediate filament proteins of animal cells. First, affinity-purified antibodies against MDM1 recognize the intermediate filament network by indirect immunofluorescence in a number of different types of animal cells. Consistent with this observation, the affinity-purified antibodies react weakly with purified vimentin and with several unidentified species on immunoblots of total proteins from animal cells (S. McConnell, preliminary results). Second, protein sequence comparisons indicate a similarity of MDM1 to both vimentin and cytokeratin. Third, structural predictions suggest that the MDM1 protein contains a high α-helical content, a property shared by many filamentous proteins. Finally, the MDM1 protein demonstrates solubility properties similar to those described for intermediate filament proteins (Steinert and Roop, 1988; Lazarides, 1980). In particular, MDM1 remained largely in the insoluble fraction upon extraction of disrupted yeast cells with the detergent Triton X-100 (S. McConnell, unpublished observations). Additionally, the MDM1 protein in cellular protein extracts appeared to aggregate readily, resulting in the frequent appearance of material of high molecular weight recognized by the anti-MDM1 sera on Western blots (not shown). These results may suggest that MDM1 is a distant homologue of intermediate filament proteins of animal cells. Alternatively, MDM1 and intermediate filament proteins may be unrelated evolutionarily, but only share certain traits such as common epitopes or solubility characteristics. A third possibility is that animal cells contain a (as yet, unidentified) homologue of MDM1 which colocalizes with intermediate filaments. The future purification and biochemical analysis of the MDM1 protein should lead to a greater understanding of its structural properties.

The distribution of MDM1 is distinct from that of all previously described structures in the yeast cytoplasm. The MDM1 pattern is dramatically different from the cellular distributions of actin and tubulin, major components of the two well-characterized cytoskeletal networks in yeast (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Thomas et al., 1984). Consistent with this observation, changes in the actin- or tubulin-based cytoskeletons have not been detected in *mdm1* mutant cells at the nonpermissive temperature (McConnell et al., 1990), suggesting no direct involvement of these structures in the mutant phenotypes. Interestingly, MDM1 appears similar to actin in that both proteins are localized to largely punctate structures in the cytoplasm (Adams and Pringle, 1984; Kilmartin and Adams, 1984). However, the actin "dots" are concentrated in small buds or localized to the cell cortex, whereas the MDM1 spots are distributed throughout the cytoplasm and appear least abundant in small buds. Another structure extending through the cytoplasm, termed "cytoplasmic matrices," was described previously in yeast (Wittenberg et al., 1987). This complex network of filamentous material was detected after yeast spheroplasts were extracted with the detergent Triton X-100. The biological significance, composition, and presence of these matrices in untreated cells remain to be demonstrated, although it is possible that the MDM1 protein (with its similar solubilization profile) is associated with such structures. With respect to other yeast cellular structures, however, there is no evidence for a relationship between the MDM1 protein and the 10-nm filaments localized to a ring surrounding the bud neck (Byers and Goetsch, 1976). MDM1 displays no specific localization to the bud neck, and the *MDM1* gene is distinct from four genes (*CDC3, CDC10, CDC11, CDC12*) whose function is required for the formation of the neck-associated structures (Kim et al., 1991).

The mechanistic role of the MDM1 protein in facilitating transfer of nuclei and mitochondria into developing buds remains to be determined. One model of the protein's function is that a network containing or composed of the MDM1 protein serves to organize the cytoplasm and provide a matrix along which organelles can be transported. Another role for this network might be to orient the mitotic spindle, a process defective in *mdm1* cells incubated at 37°C (even though cytoplasmic microtubules extend into buds in these cells; see Fig. 10 and McConnell et al., 1990). Intriguingly, such functions have been proposed for the intermediate filament network in animal cells (Lazarides, 1980; Geiger, 1987; Skalli and Goldman, 1991). Future studies will focus on characterizing molecular details of MDM1 function, its assembly into complex structures, and on the identification of other cellular components with which the MDM1 protein interacts.

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