Manganese Effectively Supports Yeast Cell-Cycle Progression in Place of Calcium

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Abstract. Metal ion requirements for the proliferation of *Saccharomyces cerevisiae* were investigated. We used bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), a relatively acid tolerant chelator, to reduce the free metal ion concentrations in culture media. Chelatable metal ions were added back individually and in combination. In addition to a requirement for ~10 pM external free Zn^{2+} we found an interchangeable requirement for either 66 nM free Ca^{2+} or only 130 pM free Mn^{2+}. Cells depleted of Mn^{2+} and Ca^{2+} arrested as viable cells with 2 N nuclei and tended to have very small minibuds. In the absence of added Mn^{2+}, robust growth required ~60 μM total internal Ca^{2+}. In the presence of added Mn^{2+}, robust growth continued even when internal Ca^{2+} was <3% this level. Chelator-free experiments showed that $MnCl_2$ strongly and $CaCl_2$ weakly restored high-temperature growth of *cdc1*^{ts} strains which similarly arrest as viable cells with 2 N nuclear contents and small buds. Its much greater effectiveness compared with Ca^{2+} suggests that Mn^{2+} is likely to be a physiologic mediator of bud and nuclear development in yeast. This stands in marked contrast to a claim that Ca^{2+} is uniquely required for cell-cycle progression in yeast. We discuss the possibility that Mn^{2+} may function as an intracellular signal transducer and how this possibility relates to previous claims of Ca^{2+}'s roles in yeast metabolism.

DESPITE biochemical and genetic evidence for metal ion use in *Saccharomyces cerevisiae* (Goscin and Fridovich, 1972; Bragg, 1974; Busse, 1984; Baum et al., 1986; Eisen et al., 1988; Anraku et al., 1991), a rigorous survey of nutritional metal ion requirements of yeast has never been undertaken. The recipe of yeast synthetic medium (SD)[1] in common use today was formulated over 40 years ago as a general medium for a diverse array of fungi (Wickersham, 1951). The ingredients of this medium include, besides those of Mg^{2+} and monovalents, salts of Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+}. These five metal ions ranked essential, because they were dietary requirements of mammals and were present in trace amounts in yeast ash. Withholding from the medium the supplements of all five of these essential metal ions, including Ca^{2+}, does not stop growth, though. Such a culture presumably thrives on residual metal ions contaminating the medium. Even the addition of as much as 10 mM of the metal-ion chelator EGTA to synthetic medium containing only contaminating (<1 μM) Ca^{2+} did not prevent growth (Kovac, 1985; Iida et al., 1990*a*).

Iida et al. (1990*a*) blocked yeast growth in a Ca^{2+}-free synthetic medium with 10 mM EGTA only after a further addition of 10 μM A23187, an ionophore, which presumably was required to deplete internal pools of metal ions. $CaCl_2$, but not $MgCl_2$, restored growth in the presence of EGTA and A23187. However, chlorides of Mn^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, as well as other metals also rescued, often at lower free concentrations. It was concluded that these metal ions rescued by lowering the free [chelator] by 10% (Iida et al., 1990*a*). However, as pointed out by Youatt (1993), lowering free [chelator] >10% with 100 mM Mg^{2+} in these experiments did not rescue. In EGTA and A23187, cells were first held up at G1 phase (1N and unbudded), but became 2 N and randomly budded several hours later. More recently, Youatt and McKinnon (1993) found that EGTA alone could block suboptimal yeast growth in a synthetic medium, buffered to pH 7.0, designed for *Allomyces macrogynus*. They did not report the arrest phenotypes but found that $MnCl_2$, but not $CaCl_2$, restored yeast growth in such a medium.

References to Ca^{2+} being necessary for budding yeast proliferation are common (Anraku et al., 1991; Creutz et al., 1991; Ohya et al., 1991; Bertl et al., 1992; Belde et al., 1993; Payne and Fitzgerald-Hays, 1993; Riedel et al., 1993; Dunn et al., 1994; Iida et al., 1994). Whether Ca^{2+} or other metal ions are indeed essential for cell-cycle progression

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1. *Abbreviations used in this paper:* BAPTA, bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid; CFU, colony-forming units; EPR, electron paramagnetic resonance; MES, 2-[*N*-Morpholino]ethanesulfonic acid; SD, yeast synthetic medium; YEPD, yeast extract/peptone/dextrose medium.

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needs to be more rigorously established. We reasoned that one should avoid the complication of ionophores and should not culture this acidophillic organism at a pH above 7 where it grows poorly. EGTA's Ca^{2+} affinity weakens dramatically below pH 7, decreasing 100-fold from pH 7 to pH 6. Yeast are quite acidogenic, containing plasma membrane H^+ pumps (Serrano et al., 1986) which may further exacerbate EGTA's ineffectiveness in the critical region near the cell surface. We have therefore used a related, relatively acid tolerant chelator, bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) (Tsien, 1980). Whereas BAPTA and EGTA possess equivalent Ca^{2+} affinity at pH 7.1, BAPTA's Ca^{2+} affinity is 37-fold higher than that of EGTA at pH 6.1.

We undertook a series of experiments using BAPTA and no ionophore. BAPTA was always present in excess and the free [BAPTA] was held constant. This design kept all the background trace free metal ions constant, and therefore simplifies interpretation of metal ion supplementation experiments. Contrary to a previous claim (Iida et al., 1990*a*), we found free Mn^{2+} to be far more effective than free Ca^{2+} in supporting cell-cycle progression.

Materials and Methods

Strains and Culture Media

The common haploid laboratory strain X2180-1A (*MATa, SUC2, mal, mel, gal2, CUP1*) was used in all chelation experiments. Strains 2-12A (*MATa, ade1⁻, ura3⁻, leu2⁻, trp1⁻, his3⁻, cdc1-1^{ts}*) and 373-14C (*MATa, ade1⁻, ura3⁻, leu2⁻, trp1⁻, his3⁻, cdc1-6^{ts}*) were used in the *CDC1* experiments (gifts of Dr. Stephen Garrett, Duke University Medical Center, Durham, NC). All solutions and media were made with doubly distilled water and stored in extensively rinsed plastic ware.

YEPD6.5 medium was YEPD (Sherman, 1991) buffered to pH 6.5 with 50 mM potassium 2-[*N*-morpholino]ethanesulfonic acid (MES). The metal ion contents of YEPD6.5 medium are given in Table I. We found that YEPD cultures had an initial pH between 5 and 6 and became increasingly acidic as cells proliferated. Yeast grew at the same rate in YEPD6.5 medium as in YEPD, but the pH remained at 6.5 throughout logarithmic growth. The pH of YEPD6.5 medium decreased to ~6.2 as cells approached stationary phase. Only log-phase cells were used.

Medium A was derived from the standard defined synthetic medium SD (Wickersham, 1951). SD contains 4 mM $MgCl_2$, 700 μM $CaCl_2$, 3 μM $MnCl_2$, 2 μM $ZnCl_2$, 0.3 μM $CuSO_4$, and 1.2 μM $FeCl_3$. All but $MgCl_2$ were omitted in medium A. In addition, Ca^{2+} pantothenate was replaced with the Na^+ salt here. Medium A was buffered to pH 6.5 with 50 mM potassium MES. See Table I for metal analyses of media used. In liquid cultures, cells grew in medium A at nearly the same rate as in SD, but entered stationary phase at about half the cell density.

Culture Conditions and Cell Counts

Cells were cultured in fresh plastic ware at 28°C on a rotator. They were first cultured for 16-24 h to a density of ≤10^7 cells/ml in BAPTA-free YEPD6.5 medium or medium A. Such cells were then used as inocula for growth tests in variously modified YEPD6.5 media or media A. Unless otherwise stated, cultures were inoculated ≤10^4 cells/ml to prevent excessive metal ion carryover.

Cells were counted with an electronic particle counter (Particle Data Inc., Elmhurst, IL). Diluted samples were briefly sonicated before counting using a probe sonicator (Branson Ultrasonics Corp., Danbury, CT) to disperse unabscised cells. Cultures containing dextran-conjugated BAPTA were grown in small volumes because of its high cost and counted using a hemocytometer. Viable cells were determined as colony-forming units (CFU) on YEPD plates, counted after 3 d incubation at 28°C. Percent viability is the [CFU]/[particle] divided by 0.75. This is because [CFU] was only 75% of [particle] in control cultures. The discrepancy is likely due to unabscised cells in the plating aliquots which were not sonicated to avoid damage. Cell clumping was not obvious in any of the cultures.

Table I. Total Metal Contents of Experimental Solutions

	Calcium	Copper	Iron	Manganese	Zinc	Magnesium
				μM		
YEPD 6.5 medium	560	<0.3	12	0.3	20	240
Medium A	0.6	<0.3	<1	<0.3	<3	4,000
10 mM $CaCl_2$	—	nm	<1	<0.3	nm	nm
10 mM $FeCl_3$	1.5	nm	—	30	nm	nm
10 mM $MnCl_2$	0.12	nm	<1	—	nm	nm

Listed are total metal contents in two growth media and three metal ion stocks. <, limits of detection in the case of undetectability. n.m., not measured.

Chemicals and Their Analyses

Chemicals of the highest purity available were used in cases where significant metal ion contamination was likely to be introduced. Ultrapure $MnCl_2$, $ZnCl_2$, $CuCl_2$, $MgCl_2$, $CaCl_2$, potassium phosphate, and ammonium sulfate were from Aldrich Chemical Co. (Milwaukee, WI); $FeCl_3$ from Fluka ACT (Buchs, Switzerland); potassium salt of BAPTA or dextran-conjugated BAPTA (10 kD average molecular mass) from Molecular Probes (Eugene, OR).

Total calcium, copper, iron, manganese, and zinc in culture media or stock solutions were determined using an inductively coupled plasma spectrophotometer (ICP 2.5; Leeman Laboratories, Inc., Lowen, MA). To measure manganese in the $FeCl_3$ stock, an atomic absorption spectrophotometer (3030; Perkin-Elmer Corp., Norwalk, CT) was used. Absorption at two wavelengths both indicated a 1/300-fold contamination of manganese in the $FeCl_3$ stock. Both instruments were calibrated with dilute aqueous solutions of each metal salt. In samples where a metal could not be detected, the lowest detectable level of that metal was spiked into the sample for verification.

Determination of the K_d of $MnBAPTA^{2-}$

The K_d of $MnBAPTA^{2-}$ was unknown and had to be determined. Free Mn^{2+} in BAPTA solutions was measured by electron paramagnetic resonance (EPR) spectroscopy using an EPR spectroscope (3C; Varian Analytical Instruments, Sunnyvale, CA) (Cohn and Townsend, 1954). All measurements were done at 20°C in 0.1 M KCl, 10 mM KOAc, pH 4.00, buffer. This low pH was used to lower the Mn^{2+} affinity of BAPTA to produce measurable free Mn^{2+}. (The detection limit was >1 μM.) EPR spectra were taken of 0-100 μM $MnCl_2$ standard solutions with the following parameters: microwave power (50 mW), modulation amplitude (10 G), modulation frequency (100 kHz) (Fig. 1 *A*). A calibration curve correlating free [Mn^{2+}] and peak heights (second derivative of microwave absorbance) that are directly proportional to free [Mn^{2+}] was generated (data not shown). Accurately pH-buffered solutions containing either 750 μM BAPTA or 750 μM BAPTA with 720 μM $MnCl_2$ were mixed to create solutions of varying free [Mn^{2+}], all at exactly pH 4.00. EPR peak heights of these solutions were measured (Fig. 1 *B*) to determine free [Mn^{2+}] from the calibration curve generated above. Scatchard analysis of free vs. BAPTA-bound Mn^{2+} indicated that the affinity of Mn^{2+} for $BAPTA^{4-}$ at pH 4.00 was 92×10^3 M^{-1} (Fig. 1 *C*). Using the formula from Portzehl et al. (1964) of $K_{Mn}/K'_{Mn} = 1 + [H^+]K_{H1} + [H^+]^2K_{H1}K_{H2}$, where K_{Mn} is the absolute and K'_{Mn} the apparent affinity of Mn^{2+} for BAPTA at the given pH, and K_{H1} is the first and K_{H2} the second H^+ K_d's (Tsien, 1980), the absolute affinity of $MnBAPTA^{2-}$ (20°C, 0.1 N) was calculated to be 6.3×10^8 M^{-1} (p*Kd* 8.8). The third and fourth H^+ associations with BAPTA, and metal ion binding to other than the $BAPTA^{4-}$ form, are insignificant here (Tsien, 1980).

Calculation of Free Ion Concentrations

Free cations in BAPTA-containing solutions were calculated using the program Maxchelator written by Chris Patton of the Stanford University Hopkins Marine Station. Calculations used the known K_d's of $CaBAPTA^{2-}$, $MgBAPTA^{2-}$, $HBAPTA^{3-}$, and H_2BAPTA^{2-} (Tsien, 1980), the K_d of $MnBAPTA^{2-}$ determined above, and cation and proton affinity constants of phosphate (Perrin, 1979) and sulfate (Martell and Smith, 1974). The K_d's of $ZnBAPTA^{2-}$, $CuBAPTA^{2-}$, $Fe(II)BAPTA^{2-}$, and $Fe(III)BAPTA^{-}$ were approximated by assuming that the ratios of the K_d's of M-chelator/Ca-chelator would be similar between BAPTA and related chelators for

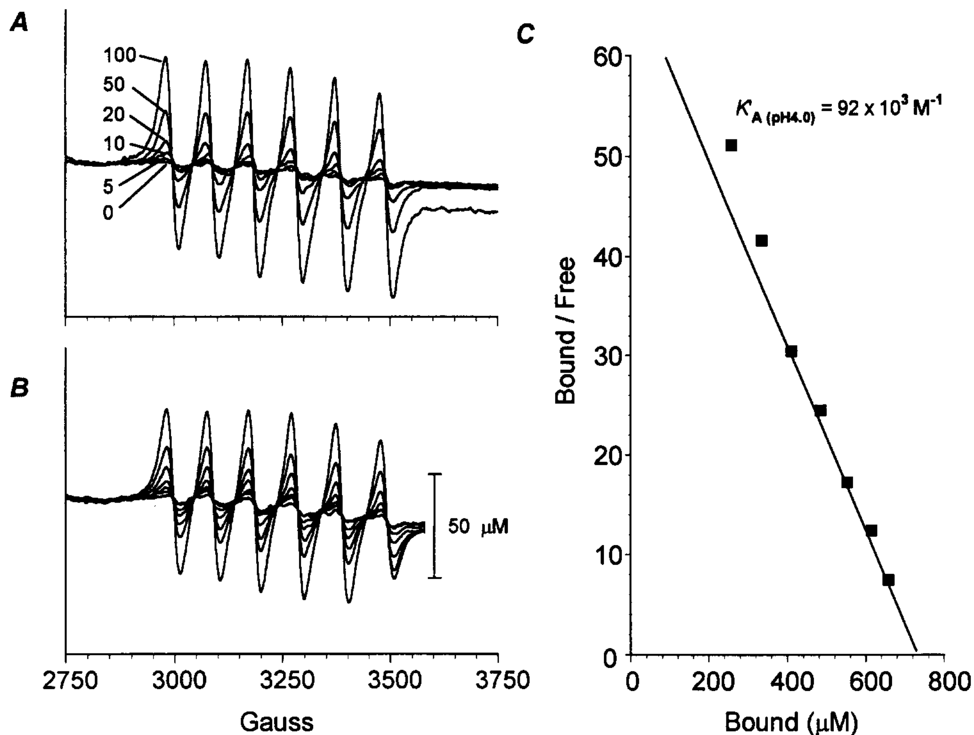

Figure 1. Determination of Mn^{2+}-$BAPTA^{4-}$ affinity constant. (*A*) EPR spectra of 0, 5, 10, 20, 50, and 100 μM $MnCl_2$ (labeled 0–100 respectively) in 100 mM KCl, 10 mM KOAc, pH 4.00 buffers. The heights of the peaks (second derivative of microwave absorbance) are directly proportional to the free Mn^{2+} concentration. (*B*) EPR spectra of similarly pH buffered solutions containing 750 μM BAPTA and from 720 μM (*largest peaks*) through 216 μM (*smallest peaks*) $MnCl_2$. Calibration of peak heights to free [Mn^{2+}] is derived from *A*. (*C*) Scatchard plot calculated from free Mn^{2+} measured in *B* and remaining BAPTA-bound Mn^{2+} indicates an apparent Mn^{2+}-$BAPTA^{4-}$ affinity constant of 92×10^3 M^{-1} at pH 4.00 corresponding to an absolute affinity of 63×10^7 M^{-1} (pK_d 8.8) (0.1 N, 20°C).

which the affinities for these metal ions are known (Martell and Smith, 1974; Grynkiewicz et al., 1985; Metcalfe et al., 1985). As an example, since EGTA, 5F-BAPTA and Fura-2 all have similar ratios of $K_{dZnChelator}/K_{dCaChelator}$ of ~1/100, the K_{dZn} of BAPTA was estimated to be 1/100 of its K_{dCa}, or 1 nM (Table II). Since the affinities of these cations for BAPTA have not been directly measured, all estimates of the free concentrations of Cu^{2+}, Fe^{n+}, and Zn^{2+} should be considered only order-of-magnitude approximations and are stated as such.

Table II lists the absolute K_d's, measured or extrapolated, that were used to generate apparent dissociation constants. Apparent K_d's accounted for the presence of protons (see above) and the ionic strength of the media (0.2 N contributed primarily by sulfate, phosphate, MES, and ammonium and adjusted for protonation at pH 6.5). The binding of Ca^{2+}, Cu^{2+}, Fe^{n+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and H^+ to BAPTA, phosphate, and sulfate were simultaneously accounted for in all calculations. Calculations ignore the potential presence of secreted cation-binding metabolites or cell-surface ion binding since the critical free concentration determination experiments were all done with low cellular inocula.

Measurement of Internal Ca^{2+} and Mn^{2+}

Medium A spiked with $^{45}CaCl_2$ (NEX013; New England Nuclear, Boston, MA) (1–10 mCi/ml diluted into 1.2–10 μM $^{40}CaCl_2$) was used to grow $^{45}Ca/^{40}Ca$-equilibrated inocula. These cultures were used to inoculate test media of similar $^{45}Ca/^{40}Ca$ ratios. For test media containing <1 μM free Ca^{2+} (those experiments left of dashed line in Fig. 8), free [Ca^{2+}] was held by buffering with 2.2–3.2 mM BAPTA and 1.8 μM–1 mM $CaCl_2$. The [BAPTA] and [$CaCl_2$] were chosen to keep free [BAPTA] at 1.9 mM while titrating the free [Ca^{2+}] from 0.8 to 200 nM. $ZnCl_2$ and $FeCl_3$ were also added at 100 μM each. In 1.9 mM free BAPTA, addition of 100 μM $FeCl_3$ was far from toxic (see Results). In cultures labeled "manganese" (see Fig. 8, *open circle*), 700 μM $MnCl_2$ (and an additional 700 μM BAPTA) was added to BAPTA-containing medium so that the free Mn^{2+} was 2 nM. (2 nM free Mn^{2+} is more than sufficient to support growth without free Ca^{2+}; see Results.) Test cultures were inoculated at 10^3–2×10^6 cells/ml so that they would reach a density of ~2×10^7 cells/ml in 48 h as judged by control cultures. At 48 h, three 2-ml aliquots of cultures were individually filtered under suction and the filters (HA25; Millipore Corp., Bedford, MA)were rinsed five times, each for 20–25 s with 5 ml each of 5 mM unlabeled $CaCl_2$ and 0.3 M sorbitol to remove surface-associated Ca^{2+}. The radioactivity of the rinsed filters was measured using a liquid scintillation counter (Packard Instrument Co., Inc., Meriden, CT). Three 1-ml samples of the same cultures were microcentrifuged in tared tubes and the pellets dried overnight at 37°C before weighing with an analytical balance.

Cellular Mn^{2+} was assayed in a similar manner using $^{54}MnCl_2$ (NEZ040; New England Nuclear), $MnCl_2$/sorbitol rinses, and radioactivity was measured with a dry scintillation counter (Packard Instrument Co., Inc.).

Determination of Nuclear Content and Bud Size

To determine nuclear contents, cells were sonicated, fixed with ethanol, and stained with propidium iodide (Aldrich Chemical Co.) according to

Table II. Dissociation Constants of Metal BAPTAs

A **Measured**

Complex	pK_d	Reference
$MgBAPTA^{2-}$	1.77	a
$CaBAPTA^{2-}$	6.96	a
$HBAPTA^{3-}$	6.36	a
H_2BAPTA^{2-}	5.47	a
$MnBAPTA^{2-}$	8.8	This work

B **Extrapolated**

	Model Chelator		
Metal (M)	Name (ref.)	$p(K_{dM}/K_{dCa})$	Estimated $pK_{d\,M\text{-}BAPTA}$
Cu^{2+}	EGTA (b and c)	5.7–6.7	~13
Fe^{2+}	EGTA (b)	0.94	~8
	FURA-2 (d)	0.5–1	
Fe^{3+}	EGTA (b)	9.6	~17
Zn^{2+}	EGTA (b)	1.7	~9
	FURA-2 (d)	2	
	5F-BAPTA (e)	2	

All constants are absolute dissociation constants at 0.1 N and 20–22°C. Apparent dissociation constants used in all calculations of free metal concentrations account for the presence of protons and an ionic strength of 0.2 N. In *B*, dissociation constants were extrapolated as described in Materials and Methods. References are a: Tsien, 1980; b: Martell and Smith, 1974; c: Perrin, 1979; d: Grynkiewicz et al., 1985; e: Metcalfe et al., 1985.

the method of Hutter and Eipel (1979). The fluorescence intensity of 10^4 cells was determined using a flow cytometer (FACScan; Becton Dickinson and Co., Mountain View, CA) and data analyzed using LYSYS software.

Relative bud diameters were determined by measuring the parent and bud diameters from slide projections of phase micrographs. For ellipsoidal cells, diameters midway between the long and short axes were used.

Results

BAPTA Arrests Growth in Rich Media

Like other investigators (Kovac, 1985; Iida et al., 1990*a*), we failed to block yeast multiplication by adding EGTA alone to the standard rich medium YEPD (~pH 5.8). We found that EGTA did block growth when added to YEPD buffered at pH 7.0. However, cells displayed no consistent terminal phenotypes and the blockage could not reliably be removed by additions of metal salts. It should be noted that yeast cells acidified this medium in spite of the 100 mM pH buffer. Since the efficacy of EGTA would be diminished by this acidification and cells grew noticeably slower at pH 7.0, we instead studied the effect of the relatively acid-tolerant chelator BAPTA added to media at pH 6.5.

Yeast grew normally in YEPD6.5 medium, which is the conventional rich medium YEPD (Sherman, 1991) buffered at pH 6.5 with 50 mM potassium MES. BAPTA addition had little effect at 1 mM but arrested growth ≥5 mM (Fig. 2 *A*). After BAPTA addition, growth proceeded at the normal rate for several generations but stopped abruptly within one doubling time (~2 h). This lag before growth arrest was inversely proportional to the [BAPTA] added, being about four doublings for 5 mM, and three doublings for 12 mM (Fig. 2 *A*, △ and ◇).

The long delay to growth arrest indicated that either BAPTA must gradually enter cells or a cellular metal ion(s) must gradually be depleted. We tested the effectiveness of BAPTA conjugated to high molecular weight dextran, which should dramatically slow if not completely prevent entry. As shown in Fig. 3, dextran conjugation did not even weaken, let alone abolish the ability of BAPTA to block growth. Therefore, growth during the lag seems to rely on a reserve of internal metal ions in the inoculum. As this reserve is depleted by growth dilution and possibly also by efflux, external BAPTA hampers its replenishment and growth is eventually arrested.

Since electronic particle counting did not distinguish live from dead cells, we also assessed viability by counting CFU. Treated cells were as viable as untreated controls when sampled at the beginning of the growth arrest (6 h for the 5 mM, 4 h for the 12 mM BAPTA addition), though only ~30% survived 2 h later (Fig. 2 *B*). Thus growth arrest apparently precedes and leads to death, a conclusion supported by the arrest phenotypes (see below).

BAPTA Arrest Is Cell-Cycle Specific

We used a flow cytometer to assess the nuclear content of cells stained with the quantitative nucleic acid stain propidium iodide. Cells with 2 N nuclei increased from 60% at the time of 12 mM BAPTA addition to 74% by 4 h, and to 90% by 6 h (Fig. 4). Microscopic examination revealed very few cells with double or elongated nuclei after 6 h in

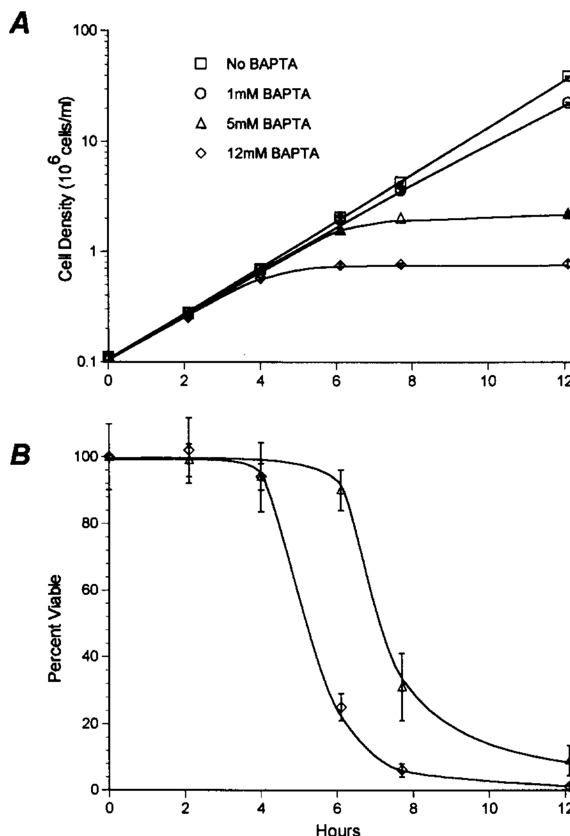

Figure 2. BAPTA blocks yeast proliferation in rich growth media. Cells of *S. cerevisiae* strain X2180-1A were grown in BAPTA-free YEPD6.5 medium and reinoculated into fresh YEPD6.5 medium containing either no additions (□) or 1 mM (○), 5 mM (△), or 12 mM (◇) BAPTA. At the indicated times aliquots were removed and assayed in triplicate either for (*A*) cellular density using an electronic particle counter or (*B*) cell viability as measured by colony-forming ability on YEPD plates. All measurements were done in triplicate and error bars represent standard deviation of the means, which are smaller than the symbols in *A*.

12 mM BAPTA, indicating the nuclear arrest to be in G2 or early M phase. There was a 38% decrease in the 1 N population between 0 and 4 h after BAPTA addition. Thus at least 38% of the cells manifested directly scorable nuclear-division arrest at a time they were nearly 100% viable (Fig. 2 *B*). Iida et al. (1990*a*) also observed a nuclear arrest with the combined application of EGTA and A23187. In that experiment though, cells were initially arrested in G1 followed by an increase in the fraction of 2 N cells 6–12 h after arrest and the viability of the cells was not assayed. In the present experiment, 2 N nuclei became manifest nearly concomitantly with the arrest of viable cells.

Bud development is tightly coordinated with other aspects of cell-cycle progression. A bud normally emerges shortly after the initiation of DNA synthesis and enlarges to about two-thirds the parent's diameter before postmitotic abscission (Brewer et al., 1984). BAPTA treatment resulted in a fourfold increase in the fraction of cells with minibuds of <20% the parent cell diameter (Fig. 5, *filled*

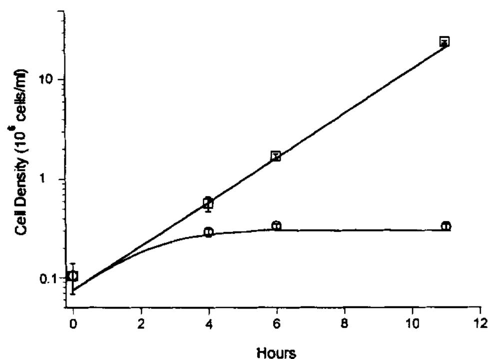

Figure 3. BAPTA does not need to enter cells to block growth. Cells were inoculated into 100-μl cultures as in Fig. 2 either with (○) or without (□) 20 mM BAPTA-dextran conjugate (10,000 kD average molecular mass). 20 mM BAPTA-dextran corresponds to an unconjugated BAPTA concentration of ~11 mM since the Ca^{2+} dissociation constant (K_d) of the BAPTA-dextran conjugate is 1.75 times that of unconjugated BAPTA. Cells were counted in triplicate using a hemocytometer because of the small culture volumes. Error bars represent standard deviation of the mean. □, no BAPTA; ○, BAPTA 10k.

bars). This budding phenotype was not as tight as the nuclear phenotype. 6 h after BAPTA application, 14% of the population remained unbudded and 40% had substantially developed buds although even these buds tended to be smaller than those of the untreated cells (Fig. 5 *C*, *open bars*). For brevity's sake, we will nonetheless refer to this arrest as 2 N minibudded from here on. Since cells in G2 or M phase (i.e., 2 N cells) normally have well-developed buds, BAPTA apparently blocks two independent pathways, the nuclear and the morphogenic pathways (Pringle and Hartwell, 1981), at different points in the cell cycle. Note that a substantial portion were already minibudded by 4 h, when all cells were still viable, confirming the previous conclusion that cell-cycle arrest precedes cell death.

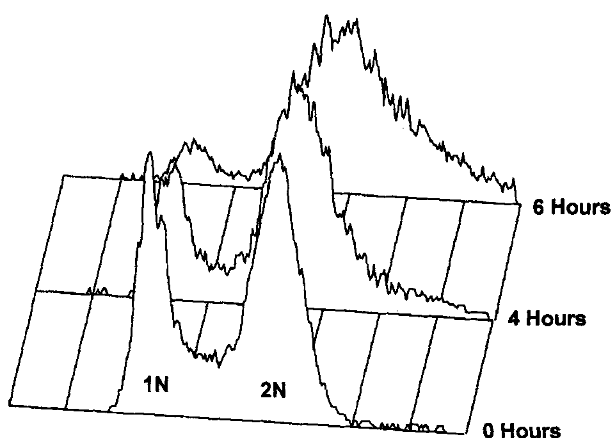

Figure 4. BAPTA blocks nuclear division. At the indicated times, aliquots of cells from the 12-mM BAPTA culture described in Fig. 2 were fixed, stained with propidium iodide, and the fluorescence intensity of stained cells were measured using a flow cytometer. The left and right peaks represent cells containing 1 N and 2 N nuclear contents, respectively. 10,000 stained cells were assayed for each time point.

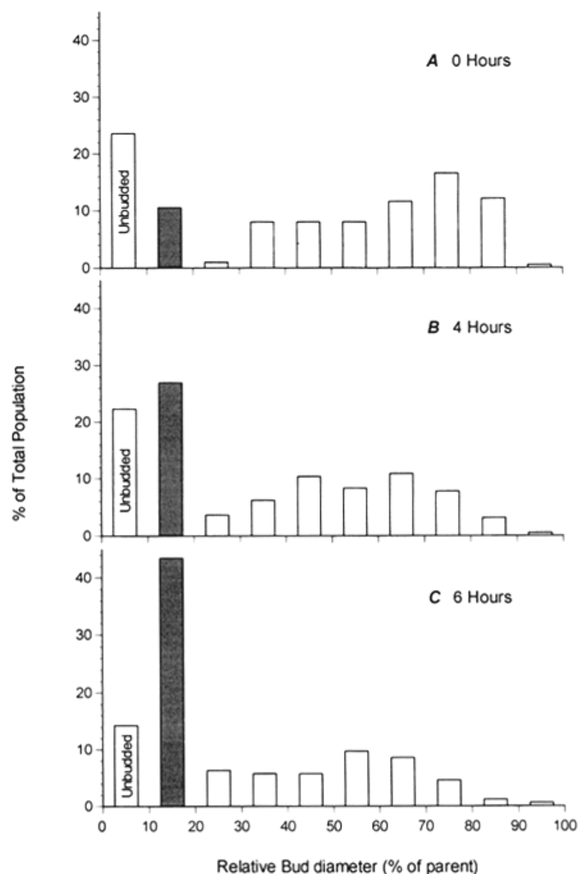

Figure 5. BAPTA inhibits bud development. Phase micrographs were taken of cells from the 12-mM BAPTA culture described in Fig. 2 at 0, 4, and 6 h (*A*, *B*, and *C*, respectively). Bud diameters relative to the parent cell diameter were determined by measuring slide projections of these micrographs. BAPTA caused a dramatic increase in the portion of cells with barely emerging minibuds which have diameters <20% of those of the parent cells (*filled bars*). 200 cells were measured for each time point.

Arrest of Bud and Nuclear Development Is Due to Depletion of Free Mn^{2+} and Ca^{2+}

Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{n+} are present in YEPD6.5 medium (Table I) and BAPTA chelates all of these metal ions. To test whether or which ion's depletion causes the BAPTA arrest, we added back metal chlorides one at a time. To prevent the added metal ion from replacing and releasing other ions bound to BAPTA, we adjusted total [BAPTA] to keep the free [BAPTA] constant among experiments. When added at 4 mM, chlorides of either Mn^{2+} or Ca^{2+}, but not Zn^{2+}, Cu^{2+}, Fe^{3+}, restored growth in YEPD6.5 medium containing 11.4 mM free BAPTA (16 mM total BAPTA) (Fig. 6). The combination of $FeCl_3$ and BAPTA at these concentrations immediately stopped growth, indicating a toxicity (discussed below). Thus cell-cycle arrest by BAPTA in YEPD is apparently due to chelation of both free Mn^{2+} and Ca^{2+}, and the two ions appear to function interchangeably.

Chlorides of Sr^{2+}, Ba^{2+}, Sn^{2+}, Hg^{2+}, Ni^{2+}, Cd^{2+}, Fe^{2+}, La^{3+}, Lu^{3+}, Er^{3+}, Tb^{3+}, or Nd^{3+} were similar tested individually. Only $SrCl_2$ relieved the BAPTA arrest (data not

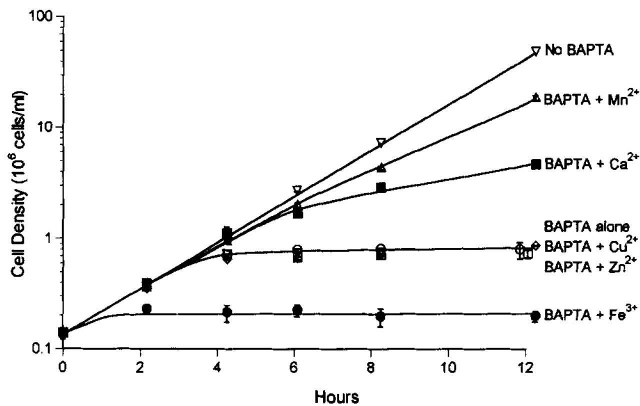

Figure 6. Mn^{2+} or Ca^{2+} individually restore growth in BAPTA containing rich media. Cells were inoculated as in Fig. 2 into YEPD6.5 medium containing either no additions (▽), 12 mM BAPTA (○), or 16 mM BAPTA plus 4 mM $CaCl_2$ (■), $MnCl_2$ (△), $CuCl_2$ (□), $FeCl_3$ (●), or $ZnCl_2$ (◇). All measurements were done in triplicate and the error bars represent the standard deviation of the mean.

shown). Sr^{2+} is chemically very similar to Ca^{2+} and often acts as a Ca^{2+} surrogate in situ (Fatt and Ginsborg, 1958; Silinsky and Mellow, 1981) and was not investigated further.

Quantification: K_d's of M-BAPTAs and the Use of a Synthetic Media

As stated, YEPD6.5 medium contains substantial amounts of chelatable metal ions (Table I). To assess more stringently metal ion requirements we investigated the effects of BAPTA added to a defined medium depleted of these metals. This medium A is derived from the standard synthetic medium SD (Wickersham, 1951), buffered to pH 6.5 with 50 mM potassium MES, and lacking salts of Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{3+} specified in the SD formula (the 4 mM $MgSO_4$ was not omitted). The levels or the upper limits of contaminating metals are listed in Table I. The only measurable contaminating metal in medium A was 600 nM calcium.

Yeast grew indefinitely at near normal rates in medium A, presumably on the contaminating metals that are mostly immeasurable. We did find, however, that cells in medium A entered stationary phase at about one-half the density of those in standard SD medium. Unlike the 5 mM BAPTA required to arrest growth in YEPD6.5 medium, <100 μM BAPTA arrested growth in medium A.

The affinities of BAPTA for Ca^{2+}, Mg^{2+}, H^+, but not Mn^{2+} have been measured (Tsien, 1980). We used EPR spectroscopy (Cohn and Townsend, 1954) to determine the pK_d of $MnBAPTA^{2-}$ to be 8.8 (Fig. 1; see Materials and Methods). The pK_d's of $ZnBAPTA^{2-}$, $CuBAPTA^{2-}$, Fe(II)$BAPTA^{2-}$, and Fe(III)$BAPTA^-$ were estimated from their relative affinities for four model chelators (see Materials and Methods). Note that the Cu^{2+}, Fe^{n+}, and Zn^{2+} concentrations are not crucial to our conclusion on Mn^{2+} and Ca^{2+}, the focus of this study. Because the BAPTA K_d's for Cu^{2+}, Fe^{n+}, and Zn^{2+} are derived from extrapolations and not direct determinations, the calculated free concentrations of these cations are stated as order-of-magnitude approximations and should not be overinterpreted. All free ion concentrations are calculated from effective K_d's derived from absolute K_d's (Table II), the pH (6.5), and the estimated ionic strength of the media (0.2 N).

Zn^{2+} Is Required

Unlike the case with YEPD6.5 medium, supplementation of a single individual metal chloride failed to restore growth in medium A containing BAPTA. Chlorides of Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, or Fe^{3+} were added at 200 μM in all possible combinations to BAPTA-containing medium A (Table III). Total [BAPTA] was adjusted in each case so that the free [BAPTA] never varied significantly from 1.9 mM. Only those cultures supplemented with $ZnCl_2$ supported proliferation. Zn^{2+} was apparently necessary but not sufficient. $MnCl_2$ or a combination of $CaCl_2$ and $FeCl_3$ were also needed (see below). Calculations showed that as little as ~10 pM free Zn^{2+} satisfies the requirement (in 10 μM $ZnCl_2$, 200 μM $MnCl_2$, 2.2 mM total BAPTA) (see Table VII); ~1 pM free Zn^{2+} does not (in 1 μM $ZnCl_2$, 200 μM $MnCl_2$, 2.2 mM total BAPTA). Zn^{2+} cannot be replaced here by any other metal ion, even when they are added at 3 mM (with 200 μM $MnCl_2$, in 5.2 mM total BAPTA). Elemental analysis shows that the rich YEPD6.5 medium has 20 μM total Zn^{2+} (Table I). Therefore, after the addition of 12 mM BAPTA, between ~1 and 10 pM free Zn^{2+} should remain in YEPD6.5 medium, which apparently is sufficient, since Zn^{2+} supplementation was not required in YEPD6.5 medium (Fig. 6).

Deprivation of Zn^{2+} left cells unbudded and with 1 N nuclei (Fig. 7 *A*), distinctly different from the phenotype elicited by Mn^{2+} and Ca^{2+} deprivation (Figs. 4, 5, and 7, *B* and *C*). Because of the Zn^{2+} requirement, all subsequent investigations were performed in medium A containing BAPTA and $ZnCl_2$. These media are referred to as X mM BAPTA-Zn Medium, where X is the free [BAPTA] maintained, and X/10 is the total [$ZnCl_2$] added.

Fe^{3+} Potentiates Ca^{2+}

Besides the requirement for $ZnCl_2$, Table III also shows that growth in medium A with 1.9 mM free [BAPTA] required 200 μM $MnCl_2$. Unlike the case in YEPD6.5 medium, replacing this $MnCl_2$ with $CaCl_2$ failed to support growth. However, the combined application of 200 μM $CaCl_2$ and 200 μM $FeCl_3$ restored growth in BAPTA-Zn medium.

To investigate further this dual requirement for free

Table III. Growth in BAPTA-added Medium A Supplemented with Metal Chlorides

	None	Cu^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+} Mn^{2+}	Cu^{2+} Zn^{2+}	Mn^{2+} Zn^{2+}	Cu^{2+} Mn^{2+} Zn^{2+}
None	−	−	−	−	−	−	+	+
Ca^{2+}	−	−	−	−	−	−	+	+
Fe^{3+}	−	−	−	−	−	−	+	+
Ca^{2+}, Fe^{3+}	−	−	−	+	−	+	+	+

Chlorides of the stated metal ions were added at 200 μM each. Extra BAPTA was added accordingly so that free [BAPTA] was 1.9 mM throughout. + cultures that grew to $>5 \times 10^7$ cells/ml in 48 h. −, cultures that showed no visible signs of growth (< approximately 10^6 cells/ml) in 48 h.

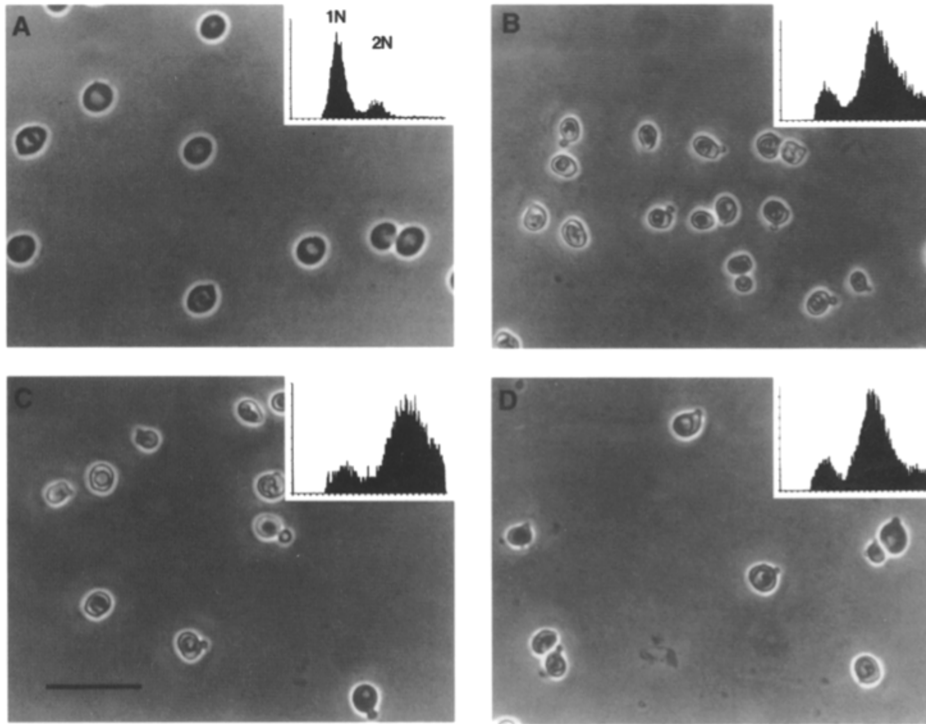

Figure 7. Terminal phenotypes of cells lacking essential metal ions. Cells logarithmically growing in medium A were used to inoculate medium A containing 1.9 mM free BAPTA with 100 μM $MnCl_2$ (*A*); with 100 μM $ZnCl_2$ (*B*); with 100 μM $ZnCl_2$ and 100 μM $FeCl_3$ (*C*); with 100 μM $ZnCl_2$ and 100 μM $CaCl_2$ (*D*). At 12 h, cells were photographed and assayed for nuclear content (*insets*) as in Fig. 4. A lack of Zn^{2+} left cells 1 N and unbudded (*A*). A lack of Mn^{2+}, Ca^{2+}, and Fe^{n+} (*B*), Mn^{2+} and Ca^{2+} (*C*), or Mn^{2+} and Fe^{n+} (*D*) all left cells 2 N minibudded. Bar, 10 μm.

Ca^{2+} and Fe^{3+}, we tested combinations of $CaCl_2$ and $FeCl_3$ ranging from 0 to 3 mM in 1.9 mM BAPTA-Zn media (Table IV *A*). $CaCl_2$ alone supported growth at the highest concentration tested, 3 mM in 1.9 mM BAPTA-Zn medium (5.2 mM total BAPTA). However, as little as 100 μM $CaCl_2$ supported growth in 1.9 mM BAPTA-Zn medium when 100 μM $FeCl_3$ was also added (1/30 the amount of Ca^{2+} required in the absence of Fe^{3+} supplementation) (Table IV *A*). 100 μM $FeCl_3$ alone (Fig. 7 *C*) or 100 μM $CaCl_2$ alone (Fig. 7 *D*) left yeast with a similar 2 N minibudded arrest in BAPTA-Zn media. Thus it appears that Fe^{3+} is potentiating the ability of free Ca^{2+} to mediate bud and nuclear development.

The rich YEP6.5 medium used above contained 12 μM total iron, explaining why the potentiating effect of additional Fe^{3+} was not detected there. There was measurable manganese contaminating our $FeCl_3$ stock (Table I). We verified that this contaminating Mn^{2+} was not the agent potentiating free Ca^{2+} rescue here (data not shown). Fe^{3+} did not substantially potentiate Mn^{2+} in similar tests (Table IV *B*). The ability of Fe^{3+} to potentiate free Ca^{2+} was not investigated further, but Fe^{3+} was added to most of the subsequent growth tests.

Supplementation of Fe^{3+} is complicated by the fact that it is toxic at higher concentrations in the presence of BAPTA. All cultures in 1.9 mM BAPTA-Zn media with ≥1 mM $FeCl_3$ failed to grow, even in the presence of rescuing amounts of free Mn^{2+} or Ca^{2+} (Table IV, *A* and *B*). It was the combination of Fe^{3+} and BAPTA that was toxic since 1 mM $FeCl_3$ alone was not. This same toxicity was observed in YEPD6.5 medium (Fig. 6). Neither Ca^{2+}, Cu^{2+}, Mn^{2+}, nor Zn^{2+} showed this kind of toxicity. Chelated Fe^{3+} is known to react with hydrogen peroxide to generate toxic levels of free OH· radicals (Sutton and Winterbourn, 1984). At ≤300 μM $FeCl_3$ this toxicity was not observed. In 1 mM $FeCl_3$ and 3 mM BAPTA, cells became unbudded with 1 N nuclei, distinctly different from the 2 N minibudded arrest caused by BAPTA alone. We did not investigate this toxicity further since it is not central to our study.

Free Mn^{2+} Is a Much More Efficient Mediator of Cell-Cycle Progression Than Free Ca^{2+}

We tested the effects of different free [Mn^{2+}] and [Ca^{2+}] in medium A with BAPTA ($ZnCl_2$ and $FeCl_3$ were also added). Between 53 and 66 nM free Ca^{2+} was sufficient without added Mn^{2+} (Table V, rows *1–4*) but only between 66 and 130 pM free Mn^{2+} was sufficient without added Ca^{2+} (rows *5–8*). Free Mn^{2+} thus supports bud and nuclear

Table IV. Effects of Fe^{3+}

	Fe^{3+}						
	None	10 μM	30 μM	100 μM	300 μM	1 mM	3 mM
A. Ca^{2+}							
None	−	−	−	−	−	−	−
10 μM	−	−	−	−	−	−	−
30 μM	−	−	−	−	−	−	−
100 μM	−	−	−	+	+	−	−
300 μM	−	+	+	+	+	−	−
1 mM	−	+	+	+	+	−	−
3 mM	+	+	+	+	+	−	−
B. Mn^{2+}							
None	−	−	−	−	−	−	−
10 μM	−	−	−	−	−	−	−
30 μM	−	+	+	+	−	−	−
100 μM	+	+	+	+	+	−	−
300 μM	+	+	+	+	+	−	−
1 mM	+	+	+	+	+	−	−
3 mM	+	+	+	+	+	−	−

Chloride salts were added at the given concentrations to 1.9 mM BAPTA-Zn medium. Equimolar BAPTA was included with the chlorides to maintain the 1.9 mM free [BAPTA]. Growth was scored as in Table III.

Table V. Comparison of the Growth-supporting Concentrations of Free Mn^{2+} and Ca^{2+}

	Added total			Calculated free		
	BAPTA	$CaCl_2$	$MnCl_2$	Ca^{2+}	Mn^{2+}	Growth
	mM	*μM*	*μM*	*nM*	*pM*	
Ca^{2+} supported growth	0.24	25	0	53	<10	−
	0.25	40	0	**88**	<10	+
	2.3	200	0	44	<1.0	−
	2.4	300	0	**66**	<1.0	+
Mn^{2+} supported growth	0.21	0	2.0	1.3	66	−
	0.22	0	5.0	1.3	**160**	+
	2.1	0	20	0.13	65	−
	2.1	0	40	0.13	**130**	+

Cells were inoculated to a density of 10^3 cells/ml into medium A containing the stated total [$MnCl_2$], [$CaCl_2$] and [BAPTA]. All cultures contained $FeCl_3$ and $ZnCl_2$ each at either 5 μm (~0.2 mM BAPTA cultures, rows *1*, *2*, *5*, and *6*) or 50 μM (~2 mM BAPTA cultures, rows *3*, *4*, *7*, and *8*). These amounts of $FeCl_3$ are far below the level that are toxic (Table IV). Note that the [Zn^{2+}] is one-fourth that used in BAPTA-Zn medium but more than sufficient to support growth. The free [cation] supporting growth is bold.

development at <1/500 the concentration of free Ca^{2+}. The fact that either ~70 nM free Ca^{2+} or ~150 pM free Mn^{2+} was required at the two different chelator concentrations (~0.2 or 2 mM) demonstrates that growth is dependent on free, not total Mn^{2+} or Ca^{2+}. In the rich YEPD6.5 medium (Fig. 2), the critical free [Ca^{2+}] calculated from the total (Table I) was between 53 and 570 nM, agreeing with the findings in medium A (Table V). Free Mn^{2+} was well below its critical concentration in YEPD6.5 medium with 1 mM BAPTA.

Zn^{2+}, Fe^{3+}, and Cu^{2+} all failed to substitute for Mn^{2+} or Ca^{2+} at any concentration tested. However, even when added at 3 mM in 1.9 mM free BAPTA (5.2 mM total BAPTA), free Cu^{2+} was only raised to ~10^{-13} M and free Fe^{3+} to ~10^{-16} M (Table VI) since they both bind BAPTA tenaciously (Table II). Whether one can use equimolar free Cu^{2+} or Fe^{3+} to replace free Mn^{2+} or Ca^{2+} cannot be tested here (but see BAPTA free experiments below).

There was <1.0 pM free Mn^{2+} contaminating the Ca^{2+} test (Table V, line *4*) and only 130 pM free Ca^{2+} in the Mn^{2+} test (Table V, line *8*), judging from the measured total metal contaminants (Table I). These concentrations set the upper limits of free Mn^{2+} or Ca^{2+} required for noninterchangable functions, if there are any. These are ~1/500 the free Ca^{2+} and <1/100 the free Mn^{2+} concentrations required for their interchangeable cell-cycle functions (Table VII, rows *2–5*).

Table VI. External Free [Metal Ion] Supporting Bud and Nuclear Development

	Total [Metal chloride]	Total [BAPTA]	Free [Metal ion]
A. Supported			
Ca^{2+}	3×10^{-4}	2.4×10^{-3}	66×10^{-9}
Mn^{2+}	4×10^{-5}	2.1×10^{-3}	13×10^{-11}
B. Did not support			
Cu^{2+}	3×10^{-3}	5.2×10^{-3}	~10^{-13}
Zn^{2+}	3×10^{-3}	5.2×10^{-3}	~10^{-9}
*Fe^{3+}	6×10^{-4}	8.2×10^{-4}	~10^{-16}

(*A*) The minimal [metal ion] (molar) required to overcome the 2 N minibudded arrest in medium A with BAPTA, Zn^{2+}, and Fe^{3+} (from Table V). (*B*) The maximum free [cations] tested which failed to overcome this arrest in similar media as *A*.
*The combination of 3×10^{-3} M $FeCl_3$ and 5×10^{-3} M BAPTA was toxic. 6×10^{-4} M $FeCl_3$ and 8×10^{-4} M BAPTA did not support growth but was not toxic, since the further addition of 2×10^{-4} M $MnCl_2$ (and equimolar BAPTA) restored growth. All concentrations are given in molar.

Copper and Iron Requirements

In the 1.9 mM BAPTA-Zn medium where growth was supported solely by Mn^{2+} (Tables III and IV *B*), the free Cu^{2+} should be less than ~10^{-17} M (Table VII). This estimate is based on the limit of detectable Cu^{2+} in medium A (Table I) and the extrapolated K_d of $CuBAPTA^{2-}$ (Table II). Like Cu^{2+}, we could not detect an absolute requirement for free Fe^{n+}, which was at most ~10 pM as a contaminant in growing cultures (Table VII). Note that this is the conservative high estimate based on the unlikely complete reduction of any Fe^{3+} by extracellular ferric reductase (Dancis et al., 1980), since Fe^{2+} binds BAPTA with lower affinity than Fe^{3+} does (Table II).

Table VII summarizes both the free ion concentrations required (*A*) and the upper limits of free metal ion requirements (in the case where a requirement could not be detected) (*B*) as deduced from our BAPTA studies. Our inability to detect a requirement for either free Cu^{2+} or Fe^{3+} should not be taken as proof of their nonessentiality (see Discussion).

Intracellular Ca^{2+} and Mn^{2+}

By spiking cultures with trace amounts of $^{45}CaCl_2$ or $^{54}MnCl_2$, intracellular Ca^{2+} and Mn^{2+} contents were determined. Note that this method measures the total cellular [cation], most of which likely resides in organelles, not free in the cytosol (Ohsumi and Anraku, 1983; Nieuwenhuis et al., 1981). In BAPTA-free medium A, internal Ca^{2+} traced with ^{45}Ca was found to be ~800 pmol/mg, or ~250 μM averaged over cell volume (Fig. 8). Using BAPTA to reduce the free Ca^{2+} in the medium from 100 μM to 100 nM (a thousandfold drop) only reduced the cell Ca^{2+} from 823 to 354 pmol/mg (a 2.3-fold drop). Thus yeast cells can effectively extract Ca^{2+} from the medium. However, we found this scavenging capability to be limited. Total cellular Ca^{2+} dropped sharply with the decrease of medium free Ca^{2+}

Table VII. External Free [Metal Ion] Required for General Proliferation

Metal Ion	Free [M^{2+}]
A. Minimum required (requirement detected)	
Zn^{2+} (with Mn^{2+})	~10^{-11}
Mn^{2+} (with Zn^{2+})	1.3×10^{-10}
Ca^{2+} (with Zn^{2+})	6.6×10^{-8}
B. Maximum required (no requirement detected)	
Mn^{2+} (with Zn^{2+}, Ca^{2+})	$<1.0 \times 10^{-12}$
Ca^{2+} (with Zn^{2+}, Mn^{2+})	$<1.3 \times 10^{-10}$
Cu^{2+} (with Zn^{2+}, Mn^{2+})	less than ~10^{-17}
*Fe^{2+} (with Zn^{2+}, Mn^{2+})	less than ~10^{-11}

(A) The minimum free [metal ion] (molar) required for proliferation in medium A containing BAPTA (plus stated additionally required cation) was calculated from the dissociation constants of BAPTA (Table II) and the total [cation] required for growth. (*B*) Maximal free [cation] present in cases where its necessity could not be detected. The upper limit of free [cation] in 2 mM BAPTA-Zn medium (with 200 μM Mn^{2+} or 300 μM Ca^{2+} as stated) was calculated as above from the maximal concentration of that cation contaminating the media (Table I).
*The more conservative (higher) estimate of free [Fe^{n+}] based on Fe^{2+} is used here due to the potential reduction of the added Fe^{3+} by extracellular ferric reductases.

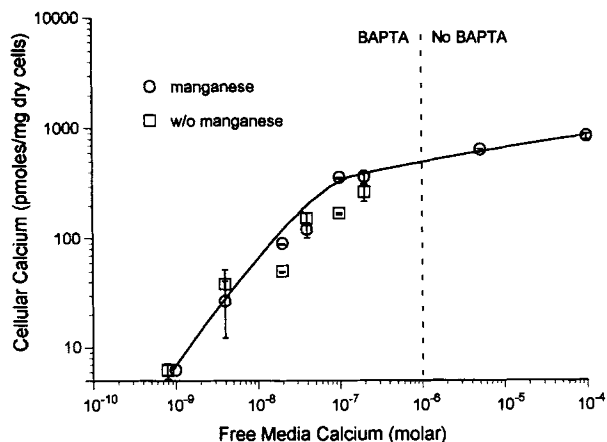

Figure 8. Correlation of total internal Ca^{2+} with external free $[Ca^{2+}]$. Cells were inoculated into ^{45}Ca containing medium A of varying free $[Ca^{2+}]$ either with (○) or without (□) 2 nM free Mn^{2+} supplementation. Left of dashed line, 1.9 mM free BAPTA was maintained to lower free $[Ca^{2+}]$ in the media. After 48 h growth, aliquots from cultures were either microfuged, dried, and weighed in tared tubes or filtered and rinsed with unlabled $CaCl_2$. The radioactivities of the filtered cells were measured to determine cellular Ca^{2+} contents. All measurements were done in triplicate and error bars represent the standard deviation of the mean. See Materials and Methods for a more detailed description of the experimental protocol.

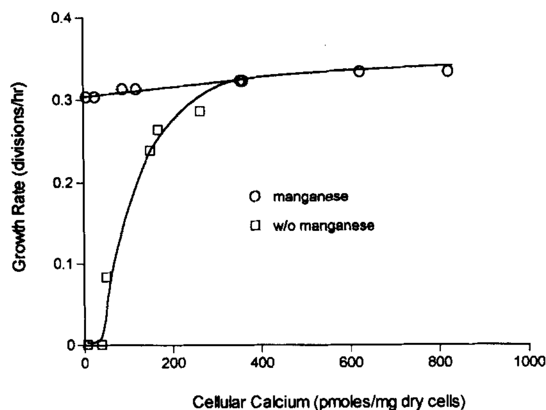

Figure 9. Mn^{2+} addition overrides a growth requirement for intracellular Ca^{2+}. Growth rates of the cultures in Fig. 8 were determined by measuring cellular density 8 h before and at the point of cellular Ca^{2+} determination. These growth rates were plotted against the internal total Ca^{2+} concentrations shown in Fig. 8.

below 100 nM (Fig. 8). This same trend was seen with (Fig. 8, *open circles*) or without (*open squares*) growth-supporting free Mn^{2+} in the medium. This observation shows that Mn^{2+} does not stimulate Ca^{2+} accumulation.

Without Mn^{2+} supplement, losing cell Ca^{2+} below ~200 pmol/mg sharply curtailed growth (Fig. 9, *open squares*). In contrast, when sufficient free Mn^{2+} was added in the medium (*open circles*), growth continued robustly even when cell Ca^{2+} was only 6 pmol/mg. This amount is derived from a barely detectable activity of ^{45}Ca retained on the filters. Even if this radioactivity is completely due to internal accumulation, it is no more than 3% the required amount when Ca^{2+} is the sole supporter of robust growth. Note that the trace $^{45}CaCl_2$ added here raises the free $[Ca^{2+}]$ ~10 times that in the Mn^{2+}-supported cultures shown in Tables III–V. In those cultures, cell-associated Ca^{2+} should be even less than this 3% estimate. Thus, growth in BAPTA-Zn medium without Ca^{2+} supplement is supported largely, if not solely, by Mn^{2+}. It may well be possible to grow yeast completely free of cell-associated Ca^{2+}, but we were unable to unequivocally demonstrate that here. Nonetheless, it is at least safe to conclude that yeast cells need to maintain much higher concentrations of internal Ca^{2+} in the absence of sufficient free Mn^{2+} than in its presence.

In reciprocal experiments, cell ^{54}Mn fell below detection limits long before external free Mn^{2+} became growth limiting. Therefore, this tracer cannot be used to plot the slowing of growth with the loss of internal total Mn^{2+}, as in Fig. 9 for internal Ca^{2+}. The detection limit for total cell Mn^{2+} is ~1 pmol/mg or ~300 nM averaged over cell volume. The critical average internal concentration must be lower. Note that a failure to define the critical internal level of Mn^{2+} does not deny its being required. Its being essential is judged by the need for a critical free external supply in the absence of sufficient free Ca^{2+} (Table V).

Mn^{2+} Rescues cdc1 Mutants from Cell-Cycle Arrest

There are complications in determining metal ion requirements through the use of chelators. In particular, the free $[Cu^{2+}]$ and $[Fe^{3+}]$ always remain extremely low in BAPTA media (Table VI), and therefore we cannot make strong claims from their inability to rescue (see above and Discussion). To circumvent these problems inherent to using chelators, we tested the ability of metal ions to rescue the temperature-induced growth arrest of *cdc1*^{ts} mutants. We tested *cdc1*^{ts} mutants because, at restrictive temperatures, they arrest with phenotypes nearly identical to Mn^{2+}/Ca^{2+} depletion: small budded (Hartwell, 1974), 2N, and viable at the time of arrest (Garrett, S., manuscript in preparation). This phenotypic identity suggested that *CDC1* may well function in a pathway that requires Mn^{2+} or Ca^{2+}. Adding high concentration of either ion to the growth medium may thus compensate for a reduction of Cdc1 activity caused by mutation. Indeed, we found that addition of 100 mM $CaCl_2$ weakly restored and 5 mM $MnCl_2$ completely restored *cdc1-6*^{ts} growth at restrictive temperatures in YEPD (Fig. 10). Similar results were obtained with *cdc1-1*^{ts} (not shown). $CuCl_2$, $FeCl_3$, or $ZnCl_2$ did not rescue at any concentration showing that these ions indeed cannot support bud and nuclear development as Mn^{2+} or Ca^{2+} can. The likely association between Cdc1 function and Mn^{2+}/Ca^{2+} metabolism is interesting but is beyond the scope of this work. The *CDC1* gene has been isolated and its deduced product bears no homology to any known proteins (Garrett, S., personal communication).

Discussion

Metal Ion Requirements for Yeast Proliferation

Using BAPTA to effectively control metal ion concentra-

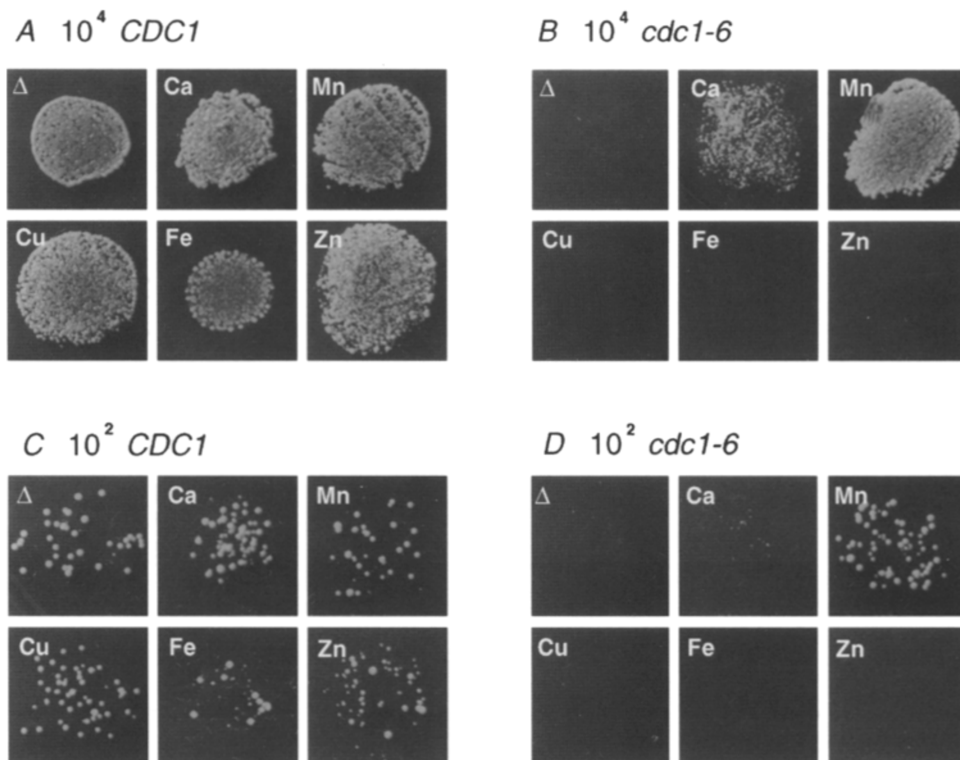

Figure 10. $MnCl_2$ effectively restores growth of *cdc1*^{ts} strains. Approximately 10^4 (*A* and *B*) or 10^2 (*C* and *D*) cells of the strain 373-14C (*cdc1-6*^{ts}) carrying either the plasmid YCP50 with a wild-type *CDC1* insert (*A* and *C*, *CDC1*) or YCP50 without an insert (*B* and *D*, *cdc1-6*) were inoculated onto YEPD plates containing chloride salts of the stated metal ion. $CaCl_2$ was added at 100 mM, $MnCl_2$, $FeCl_3$, and $ZnCl_2$ at 5 mM, and $CuCl_2$ at 2 mM. Δ indicates no chloride was added. Plates were incubated for 48–60 h at 35°C. $CaCl_2$ supported slow growth (*B* and *D*; note barely visible colony size in *D*) and $MnCl_2$ supported full growth (*B* and *D*) of *cdc1-6* at 35°C. Cells on all other media did not grow at 35°C (*B*).

tions, we have ascertained two metal ion requirements for culturing yeast. First, ~10 pM free Zn^{2+} is needed in the medium (Table VII). Zn^{2+} deficiency, like deficiency of organic nutrients, results in G1 arrest (Fig. 7 *A*). Second, 66 nM free Ca^{2+} or 130 pM free Mn^{2+} is needed (Table V). Ca^{2+} and Mn^{2+} deficiency causes a cell-cycle arrest. The arrested populations are viable, mostly 2 N, and ~50% minibudded (Figs. 2 *B*, 4, 5, and 7). The fact that addition of either ion alone prevents this arrest indicates that Mn^{2+} and Ca^{2+} interchangeably support both bud and nuclear development.

Because Cu^{2+} and Fe^{3+} bind BAPTA with very high affinity (Table II), we could not adequately test their abilities to replace Mn^{2+}/Ca^{2+} in the BAPTA experiments. Investigation of $FeCl_3$ was further complicated by its toxicity at high concentrations when applied with BAPTA (Table IV). However, addition of Cu^{2+}, Fe^{3+}, or Zn^{2+} failed to relieve the 2 N small-budded viable arrest by *cdc1-1*^{ts} and *cdc1-6*^{ts} as Ca^{2+} or Mn^{2+} did (Fig. 10). By this chelator-free test then, Cu^{2+}, Fe^{3+}, or Zn^{2+} cannot substitute for Mn^{2+} or Ca^{2+} for their cell-cycle functions.

We could not detect an absolute need for free Cu^{2+} or Fe^{3+}. This is surprising, since the free concentrations of Cu^{2+} and Fe^{3+} should have been extremely low in several growing cultures (Table VII), possibly indicating that they are not required for vegetative growth. It should be noted, though, that chelation is not the same as omission. Since cells and BAPTA in essence compete for contaminating free metal ions, a sufficient amount of initially chelated Cu^{2+} and Fe^{n+} may have accumulated into proliferating cells, away from the chelator. Both Cu^{2+} and Fe^{n+} are known to bind intracellular receptors with very high affinities (see below) and thus this kind of irreversible partitioning may be expected here.

What Role Might Mn^{2+} and Ca^{2+} Interchangeably Assume?

It is beyond the scope of this work to define the interchangeable biochemical function(s) of Mn^{2+} and Ca^{2+}, but some general conclusions nonetheless can be drawn. The delayed onset of arrest by external BAPTA indicates that depletion of intracellular, not extracellular Mn^{2+} and Ca^{2+} is the direct cause of cell-cycle arrest (Figs. 2 *A* and 3). Their shared role(s) is not as a catalytic cofactor, since Ca^{2+} is not a strong Lewis acid and has only one physiological oxidation state (Fraústo da Silva and Williams, 1990). The principal role of cytoplasmic Ca^{2+} is as a signal transducer. The properties of Mn^{2+}, considered below, indicate that it may indeed have the potential to function like Ca^{2+} as a signal transducer. It must be emphasized that assigning a signaling role to Ca^{2+} or Mn^{2+} here is speculative since changes in cytoplasmic free [Ca^{2+}] or [Mn^{2+}] during mitotic cell-cycle progression have never adequately been demonstrated in yeast. Since there is evidence that transient increases in cytoplasmic Ca^{2+} regulate the cell cycles of other organisms (Hepler, 1992; Lu et al., 1993; Whitaker, 1995), it is not unreasonable to speculate that Ca^{2+} and Mn^{2+} may be functioning as signaling molecules here. On the other hand, their functioning as constitutive structural cofactors cannot be ruled out.

Mn^{2+} is often inappropriately grouped with softer transition cations such as Cu^{2+} and Zn^{2+}, and assumed to bind receptors too tightly to function as a signaling ion. The coordination chemistry of Mn^{2+} is in fact much closer to that of Ca^{2+} than to those of Cu^{2+} and Zn^{2+} (Österberg, 1974; Lawrence and Sawyer, 1978; Williams, 1982; Basolo and Johnson, 1986). Like Ca^{2+} and unlike other transition cations, Mn^{2+} (in the common high-spin configuration) has no contribution to receptor binding from either ligand

field stabilization or soft acid/base interactions. As such, both Ca^{2+} and Mn^{2+} bind to oxygen-based, and in Mn^{2+}'s case, oxygen- and nitrogen-based, cellular receptors with intermediate affinities, a property key for a transient signal transducer. As hard cations, neither Ca^{2+} nor Mn^{2+} binds with tenacious affinity to cysteine/histidine-based cellular receptors as Zn^{2+} and Cu^{2+} do, and therefore are not sequestered by these receptors. The ionic radius of Mn^{2+} is closer to that of Ca^{2+} than those of other cellular divalents are (Reed, 1986) and thus it comes as no surprise that Mn^{2+} induces conformational changes similar to those caused by Ca^{2+} in calmodulin and troponin C (Kawasaki et al., 1986; Sundaralingam, M., personal communication). Mn^{2+} has in fact been shown to replace Ca^{2+} in the activation of calmodulin (Wolff et al., 1977; Mørk and Geisler, 1989), in cyclic nucleotide metabolism (Keller et al., 1980) in secretion (Ritchie, 1979; Wilson and Kirshner, 1983; Drapeau and Nachshen, 1984), and to a limited extent, even in muscle contraction (Hoar and Kerrick, 1988; Lategan and Brading, 1988).

A strong electrochemical gradient of Ca^{2+} towards the cytoplasm is required for its signaling function. Ca^{2+} is actively pumped out of the cytoplasm by transporters that generally also transport Mn^{2+} (but not smaller cations such as Mg^{2+}) (Williams, 1982). Many Ca^{2+} channels also pass Mn^{2+} (Guerrero and Darszon, 1989; Lückhoff and Clapham, 1992). Thus, the distribution (Williams, 1982) and mobilization of Mn^{2+} may well mirror those of Ca^{2+}, if Mn^{2+} is available. Mn^{2+} is readily available to yeast, being present at 100 μM or more in the rotting plant material on which yeast flourishes (Reed, 1986; Clarkson, 1988; Loneragen, 1988). In summary, there is no clear reason why Ca^{2+} and Mn^{2+} could not function interchangeably as signal transducers in wild yeast.

Do These Results Contradict Previous Claims of the Roles of Ca^{2+} in Yeast Metabolism?

We found that free Mn^{2+} is at least 500-fold more effective than free Ca^{2+} in supporting yeast cell-cycle progression. This is contrary to a previous conclusion that Ca^{2+} exclusively mediates the cell cycle based on studies using EGTA and A23187 (Iida et al., 1990*a*). That conclusion was weakened by the observations in the same study that other metal ions also restored growth in EGTA and A23187. The use of an invasive ionophore may have effects besides metal ion depletion. Nonetheless, both EGTA-A23187 and BAPTA ultimately resulted in a 2 N arrest, indicating some commonality between the two experiments.

Several essential yeast genes encode putative or bona fide Ca^{2+}-binding proteins (Baum et al., 1986; Davis et al., 1986; Miyamoto et al., 1987; Levin et al., 1990; Payne and Fitzgerald-Hayes, 1993). The Ca^{2+} binding potential of these proteins is often entirely inferred from homology to canonical Ca^{2+}-binding motifs. The best characterized Ca^{2+}-binding motif is the E-F hand structure described by Kretsinger (1975), who later cautioned against its use as the sole indicator of Ca^{2+} binding (1987). In some cases, Ca^{2+} binding potential was inferred from homology to less well-characterized motifs. Even in cases where Ca^{2+} binding has been verified in vitro (Davis et al., 1986; Spang et al., 1993), the physiological relevance of this binding is not ensured in vivo. *CMD1* encodes the essential yeast calmodulin (Davis et al., 1986) yet directed mutations that completely abolish its ability to bind Ca^{2+} do not affect its essential functions (Geiser et al., 1991). Until the function of binding of Ca^{2+} and Mn^{2+} are directly tested, one needs to exercise caution in assigning physiological roles solely to Ca^{2+} based on the presence of putative and even genuine Ca^{2+}-binding proteins.

Some yeast mutants manifest their phenotypes in media containing high (~100 mM) concentrations of $CaCl_2$ (Ohya et al., 1986). It is not clear whether these phenotypes are truly Ca^{2+} specific. There are no adequate controls for these experiments. Most multivalent cations are toxic at much lower concentrations. Mg^{2+} is not toxic but is a poor control since it differs greatly from other multivalent cations. Mg^{2+} is not scrupulously excluded from the cytoplasm and has uniquely rigid coordination requirements because of its small size (Fraústo da Silva and Williams, 1991). Even if the phenotype is truly Ca^{2+} specific, one still needs to distinguish between a mutational loss of a physiological Ca^{2+} function and a defect in coping with Ca^{2+} toxicity (Kretsinger, 1990).

There are also mutants whose ability to grow is restored by the addition of hundreds of millimolar $CaCl_2$ (Ohya et al., 1984; Levin and Barlett-Heubusch, 1992). Given our results on *cdc1*^{ts} (Fig. 10), it would be of interest to test whether $MnCl_2$ can also rescue these mutants. Mn^{2+} rescue at low millimolar concentration may be a more specific indicator of defect in Mn^{2+}/Ca^{2+}-binding protein than rescue by much higher concentrations of Ca^{2+}, which may have general ionic or osmotic effects. We are currently testing Mn^{2+}'s ability to rescue these mutants.

An increase in Ca^{2+} accumulation occurs in response to mating pheromone (Iida et al., 1990*b*). Since most Ca^{2+} transport systems also pass Mn^{2+} (Williams, 1982; Guerrero and Darszon, 1989), it is likely that Mn^{2+} accumulation likewise increases, though only Ca^{2+} was tested. Recently *mid1* mutants have been isolated, which are hyper-sensitive to Ca^{2+} depletion during the mating response (Iida et al., 1994). Interestingly, it was found that Mn^{2+} also effectively restored viability here, but the authors discounted its effects since Mn^{2+} also prevented the normal morphogenic response to mating pheromone (shmooing). Our results may warrant a reinterpretation of this ability of Mn^{2+} to substitute for Ca^{2+} in rescuing *MID1*.

Conclusion

We found that free Mn^{2+} is 500- to 1,000-fold more effective than free Ca^{2+} in supporting bud and nuclear development, and are thus led to conclude that Mn^{2+} may indeed act as a physiological mediator of these two processes in yeast. To conclude that Ca^{2+} is the sole physiological agent would have been based on an a priori bias. It does not seem plausible that if yeast evolved to use Ca^{2+} exclusively an alternative ion would, by chance, work this much more efficiently.

We have yet to define the biochemical targets of these two ions. Nonetheless we hope this and other works will stimulate further investigation and encourage more rigorous standards for assigning physiological roles to Ca^{2+},

particularly in the case of plants and microbes that generally require at most trace amounts of Ca^{2+} and other metal ions (Burström, 1968; Youatt, 1993) and, at least in the former case, contain substantial cellular concentrations of Mn^{2+} (Reed, 1986; Clarkson, 1988; Loneragen, 1988).

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