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

Stephen Loukin* and Ching Kung**

*Laboratory of Molecular Biology, and †Department of Genetics, University of Wisconsin–Madison, Madison, Wisconsin 53706

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 *cdc15* 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\(^{2+}\). 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. 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

---

\(^1\) 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.

© The Rockefeller University Press, 0021-9525/95/11/1025/13 $2.00
The Journal of Cell Biology, Volume 131, Number 4, November 1995 1025-1037 1025
needs to be more rigorously established. We reasoned that one should avoid the complication of ionophores and should not culture this acidophilic 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., 1990a), 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, 1p1) and 373-14C (MATa, ade1, ura3, leu2, trp1, his3, 1p1) 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 YEPlD (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 1. 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 YEPlD, but the pH remained at 6.5 throughout logarithmic growth. The pH of YEPlD6.5 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 phosphate (Perrin, 1979) and sulfate (Martell and Smith, 1974). The ion contents of YEPD6.5 medium are given in Table I. We found free Mn\(^{2+}\) to be far more effective than free Ca\(^{2+}\) in supporting cell-cycle progression.

**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 medium A. Unless otherwise stated, cultures were inoculated ~10\(^7\) 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 unabsorbed cells. Cultures containing dextran-conjugated BAPTA were grown in small volumes because of its high cost and counted using a hemocytometer. Viability of cells was determined by 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 unabsorbed cells in the plating aliquots which were not sonicated to avoid damage. Cell clumping was not obvious in any of the cultures.

**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 (3035; 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 (JES-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%), modulation frequency (100 kHz) (Fig. 1A). A calibration curve correlating free [Mn\(^{2+}\)] and peak heights (second derivative of microwave absorption) 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. 1B) 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\(^{2-}\) at pH 4.00 was 92 × 10\(^{4}\) M\(^{-1}\) (Fig. 1C). Using the formula from Portzehl et al. (1964) of K_d = [Mn\(^{2+}\)]/[MnBAPTA\(^{2-}\)] = 1 + [H\(^+\)]K_Mn + [H\(^+\)]\(^2\)K_HI K_HO, where K_Mn is the absolute and K’Mn, the apparent affinity of Mn\(^{2+}\) for BAPTA at the given pH, and K_HI, the first and K_HO, the second H\(^+\) K_d (Tsien, 1980), the absolute affinity of MnBAPTA\(^{2-}\) (20°C, 0.1 N) was calculated to be 6.3 × 10\(^{6}\) M\(^{-1}\) (pKd 8.8). The third and fourth H\(^+\) associations with BAPTA, and metal ion binding to other than the BAPTA\(^{4-}\) form, are insignificant (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\(^{2-}\), HBBAPTA\(^{2-}\), and H\(_2\)BAPTA\(^{2-}\) (Tsien, 1980), the K_d’s 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\(^{2-}\) 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...
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, 5-F-BAPTA and Fura-2 all have similar ratios of $K_{BAPTA}/K_{CaCl2}$ of ~1/100, the $K_{Ca}$ of BAPTA was estimated to be 1/100 of its $K_{Ca}$, 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$^{3+}$, and Zn$^{2+}$ should be considered only order-of-magnitude approximations and are stated as such.

Table II lists the absolute $K_{Ca}$, measured or extrapolated, that were used to generate apparent dissociation constants. Apparent $K_{Ca}$ 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$^{3+}$, 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 $^{45}$CaCl$_2$) was used to grow $^{45}$Ca$^{2+}$-equilibrated inocula. These cultures were used to inoculate test media of similar $^{45}$Ca$^{2+}$/Ca$^{2+}$ 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 x 10$^{6}$ cells/ml so that they would reach a density of ~2 x 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 $^{58}$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**

| Complex         | $pK_{Ca}$ | Reference |
|-----------------|-----------|-----------|
| MgBAPTA$^{2+}$  | 1.77      | a         |
| CaBAPTA$^{2+}$  | 6.96      | a         |
| HBAPTA$^{2+}$   | 6.36      | a         |
| H$_2$BAPTA$^{2+}$| 5.47      | a         |
| MnBAPTA$^{2+}$  | 8.8       | This work |

**B Extrapolated**

**Model Chelator**

| Metal (M) | Name (ref.) | $p(K_{Ca}/K_{M})$ | Estimated $pK_{Ca}/M$-BAPTA |
|-----------|-------------|-------------------|-----------------------------|
| Cu$^{2+}$ | EGTA (b and c) | 5.7-6.7           | ~13                         |
| Fe$^{3+}$ | EGTA (b) | 0.94              | ~8                          |
| Fe$^{2+}$ | FURA-2 (d) | 0.5-1             | ~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: Perrin, 1979; c: Grynkiewicz et al., 1985; d: Metcalfe et al., 1985.
BAPTA Arrest Is Cell-Cycle Specific

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., 1990a), 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, Δ 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 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. (1990a) 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 minibuuds of <20% the parent cell diameter (Fig. 5, filled
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 2N minibudded from here on. Since cells in G2 or M phase (i.e., 2N cells) normally have well-developed buds, BAPTA apparently blocks two independent pathways, the nuclear and the morphogenetic 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.

Arrest of Bud and Nuclear Development Is Due to Depletion of Free Mn²⁺ and Ca²⁺

Ca²⁺, Mn²⁺, Zn²⁺, Cu²⁺, and Fe³⁺ 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 Mn²⁺ or Ca²⁺, but not Zn²⁺, Cu²⁺, Fe³⁺, restored growth in YEPD6.5 medium containing 11.4 mM free BAPTA (16 mM total BAPTA) (Fig. 6). The combination of FeCl₃ 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 Mn²⁺ and Ca²⁺, and the two ions appear to function interchangeably.

Chlorides of Sr²⁺, Ba²⁺, Sn²⁺, Hg²⁺, Ni²⁺, Cd²⁺, Fe²⁺, La³⁺, Lu³⁺, Er³⁺, Tb³⁺, or Nd³⁺ were similarly tested individually. Only SrCl₂ relieved the BAPTA arrest (data not
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 $K_a$'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

---

**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 (V), 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.

---

**Quantification: $K_a$'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 (Tsin, 1980). We used EPR spectroscopy (Cohn and Townsend, 1954) to determine the p$K_a$ of MnBAPTA$^{2-}$ to be 8.8 (Fig. 1; see Materials and Methods). The p$K_a$'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$^{3+}$, and Zn$^{2+}$ concentrations are not crucial to our conclusion on Mn$^{2+}$ and Ca$^{2+}$, the focus of this study. Because the BAPTA $K_a$'s for Cu$^{2+}$, Fe$^{3+}$, 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 $K_a$'s derived from absolute $K_a$'s (Table II), the pH (6.5), and the estimated ionic strength of the media (0.2 N).
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 \(\mu\)M CaCl\(_2\) supported growth in 1.9 mM BAPTA-Zn medium when 100 \(\mu\)M FeCl\(_3\) was also added (1/30 the amount of Ca\(^{2+}\) required in the absence of Fe\(^{3+}\) supplementation) (Table IV A). 100 \(\mu\)M FeCl\(_3\) alone (Fig. 7 C) or 100 \(\mu\)M CaCl\(_2\) alone (Fig. 7 D) left yeast with a similar 2 N minibudded arrest in BAPTA 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 \(\mu\)M 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 \(\geq 1\) 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 YEP6.5 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 \(\leq 300\) \(\mu\)M 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 \([\text{Mn}^{2+}]\) and \([\text{Ca}^{2+}]\) 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 development.

### Table IV. Effects of Fe\(^{3+}\)

| Fe\(^{3+}\) | None | 10 \(\mu\)M | 30 \(\mu\)M | 100 \(\mu\)M | 300 \(\mu\)M | 1 mM | 3 mM |
|------------|-----|-----|-----|-----|-----|-----|-----|
| A. Ca\(^{2+}\) |       |     |     |     |     |     |     |
| None       | –   | –   | –   | –   | –   | –   | –   |
| 10 \(\mu\)M | –   | –   | –   | –   | –   | –   | –   |
| 30 \(\mu\)M | –   | –   | –   | –   | –   | –   | –   |
| 100 \(\mu\)M | –   | –   | –   | +   | +   | +   | –   |
| 300 \(\mu\)M | –   | +   | +   | +   | +   | +   | –   |
| 1 mM       | –   | +   | +   | +   | +   | +   | –   |
| 3 mM       | +   | +   | +   | +   | +   | +   | +   |
| B. Mn\(^{2+}\) |       |     |     |     |     |     |     |
| None       | –   | –   | –   | –   | –   | –   | –   |
| 10 \(\mu\)M | –   | –   | –   | –   | –   | –   | –   |
| 30 \(\mu\)M | –   | +   | +   | +   | +   | +   | –   |
| 100 \(\mu\)M | +   | +   | +   | +   | +   | +   | –   |
| 300 \(\mu\)M | +   | +   | +   | +   | +   | +   | –   |
| 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.
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 noninterchangeable 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).

### 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$^{3+}$, 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 $^{46}$CaCl$_2$ or $^{55}$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 pM averaged over cell volume (Fig. 8). Using BAPTA to reduce the free Ca$^{2+}$ in the medium from 100 pM 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 VI. External Free [Metal Ion] Supporting Bud and Nuclear Development

| Added total | Calculated free |
|-------------|-----------------|
| BAPTA | CaCl$_2$ | MnCl$_2$ | Ca$^{2+}$ | Mn$^{2+}$ | Growth |
| mM | mM | mM | pM | pM |
| Ca$^{2+}$ supported growth | 0.24 | 25 | 0 | 53 | <10 | - |
| 2.3 | 0 | 0 | 44 | <1.0 | - | + |
| Mn$^{2+}$ supported growth | 0.21 | 0 | 2.0 | 1.3 | 66 | <1.0 | - |
| 2.1 | 0 | 0 | 20 | 0.13 | 160 | + |
| 2.1 | 0 | 0 | 40 | 0.13 | 130 | + |

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

| Metal Ion | Free [M$^{2+}$] |
|-----------|----------------|
| A. Minimum required (requirement detected) | Mn$^{2+}$ (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 × 10$^{-12}$ |
| Ca$^{2+}$ (with Zn$^{2+}$, Mn$^{2+}$) | <1.3 × 10$^{-10}$ |
| Cu$^{2+}$ (with Zn$^{2+}$, Mn$^{2+}$) | less than ~10$^{-17}$ |
| *Fe$^{3+}$ (with Zn$^{2+}$, Mn$^{2+}$) | less than ~10$^{-11}$ |

*(A) The minimum [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 [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 pM Mn$^{2+}$ or 300 pM 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$^{3+}$] based on Fe$^{2+}$ is used here due to the potential reduction of the added Fe$^{3+}$ by extracellular ferric reductases.
Correlation of total internal Ca$^{2+}$ with external free [Ca$^{2+}$]. Cells were inoculated into 45Ca containing medium A of varying free [Ca$^{2+}$] either with (O) 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 unlabeled 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 8. Correlation of total internal Ca$^{2+}$ with external free [Ca$^{2+}$]. Cells were inoculated into 45Ca containing medium A of varying free [Ca$^{2+}$] either with (O) 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 unlabeled 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.

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 45Ca 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 45CaCl$_2$ 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 cdcl 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 cdcl$^{ts}$ mutants. We tested cdcl$^{ts}$ 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 Cdcl activity caused by mutation. Indeed, we found that addition of 100 mM CaCl$_2$ weakly restored and 5 mM MnCl$_2$ completely restored cdcl$^{ts}$ growth at restrictive temperatures in YEPD (Fig. 10). Similar results were obtained with cdcl$^{ts}$ (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 Cdcl 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-
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 2N, 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 2N small-budded viable arrest by \textit{cdc1-1ts} and \textit{cdc1-6ts} 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\(^{3+}\) may have accumulated into proliferating cells, away from the chelator. Both Cu\(^{2+}\) and Fe\(^{3+}\) 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 (Fratisto 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\(^{2+}\) (Osterberg, 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 case, oxygen- and nitrogen-based, cellular receptors with in-field stabilization or soft acid/base interactions. As such, cyclic nucleotide metabolism (Keller et al., 1980) in secretion has been shown to replace Ca^{2+} in the activation of calmodulin (Wolff et al., 1977; Mark 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; Lonergan, 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., 1990a). 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 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. CML1 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. Mn^{2+} is not scrupulously excluded from the cytoplasm and has uniquely rigid coordination requirements because of its small size (Frausto 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 cdc15 (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., 1990b). 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 morphogenetic 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+}, Mn^{2+}, and other multivalent cations.

Loukin and Kung Manganese in Yeast Cell Cycle

1035
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, 1968; Clarkson, 1988; Loneragan, 1988).

We thank Y. Saimi for advice, R. R. Preston, E. Spaulding, and others for critical reading of the manuscript. We also thank K. Schell and L. Morrissey for assistance with the flow cytometry, G. Reed for advice and assistance in EPR spectroscopy, S. Garrett for cdc1 strains, D. R. Prestwich and M. Sandler for discussion of unpublished results, and M. Sanders for assistance in elemental analysis.

This work was supported in part by National Institutes of Health grant GM22714 and the Vilas Foundation of the University of Wisconsin.

We are grateful to Y. Saimi for advice, R. R. Preston, E. Spaulding, and others for critical reading of the manuscript. We also thank K. Schell and L. Morrissey for assistance with the flow cytometry, G. Reed for advice and assistance in EPR spectroscopy, S. Garrett for cdc1 strains, D. R. Prestwich and M. Sandler for discussion of unpublished results, and M. Sanders for assistance in elemental analysis.

We are grateful to Y. Saimi for advice, R. R. Preston, E. Spaulding, and others for critical reading of the manuscript. We also thank K. Schell and L. Morrissey for assistance with the flow cytometry, G. Reed for advice and assistance in EPR spectroscopy, S. Garrett for cdc1 strains, D. R. Prestwich and M. Sandler for discussion of unpublished results, and M. Sanders for assistance in elemental analysis.

References

Arraku, Y., Y. Ohya, and H. Iida. 1991. Cell cycle control by calcium and calmodulin in Saccharomyces cerevisiae. Biochim. Biophys. Acta. 1093:169-177.

Basolo, F., and R. C. Johnson. 1986. Coordination Chemistry. Science Reviews, UK. 143 pp.

Baum, P., C. Furlong, and B. Byrn. 1986. Yeast gene required for spindle pole body duplication: homology of its product with Cdc2-binding proteins. Proc. Natl. Acad. Sci. USA. 83:5512-5516.

Belde, P. J. M., J. H. Vossen, G. W. F. H. Borst-Pauwels, and A. P. R. Thijssen. 1995. Inositol 1,4,5-trisphosphate releases Ca$^{2+}$ from vacuolar membranes of Saccharomyces cerevisiae. FEBS Lett. 353:113-118.

Bertl, A., G. Schmid, and C. Weinerm. 1992. Calcium and voltage-dependent channels in Saccharomyces cerevisiae. Philos. Trans. R. Soc. Lond. B Biol. Sci. 336:63-72.

Bragg, P. D. 1974. Non-heme iron in respiratory chains. In Microbial Iron Metabolism. A Comprehensive Treatise. J. B. Neilands, editor. Harcourt Brace Jovanovich, Inc., Orlando, FL. 303-348.

Brewer, B. J., E. Chiewbowski-Siedzicka, and W. L. Fangman. 1984. Cell cycle phases in the unequal mother-daughter cell cycles of Saccharomyces cerevisiae. Mol. Cell. Biol. 4:2529-2531.

Burnström, H. G. 1968. Calcium and plant growth. Biol. Rev. 43:287-316.

Busse, G. 1984. Cytochrome c oxidase. In Copper Proteins and Copper Enzymes. Vol. 3. R. Lontie, editor. CRC Press, Inc., Boca Raton, FL. 119-149.

Clarkson, D. T. 1988. The uptake and translocation of manganese by plant roots. Philos. Trans. R. Soc. Lond. B Biol. Sci. 336:63-72.

Creutz, C. E., S. L. Snyder, and N. G. Kambouris. 1991. Calcium and cell cycle control in Saccharomyces cerevisiae. Yeast. 7:229-244.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Davis, T. N., M. S. Urdea, F. R. Masiarz, and J. Thorner. 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein. Mol. Cell. Biol. 6:423-431.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Davis, T. N., M. S. Urdea, F. R. Masiarz, and J. Thorner. 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein. Mol. Cell. Biol. 6:423-431.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.

Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal. 1990. Genetic evidence that ferric reductase is required for iron uptake in yeast. J. Biol. Chem. 265:13391-13399.
