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A possible role for cyclins in the zinc requirements during G1 and G2 phases of the cell cycle

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Zinc has been shown to be required for the passage of cells through the mid-G1 phase of the cell cycle and for differentiation of myoblasts. However, it has been suggested that zinc has other roles during the cell cycle. The experiments reported here indicate that readily available zinc is not required for DNA synthesis per se but is needed for a process contemporaneous with the S phase and required for subsequent progress of the cells through G2 and mitosis. The G1 and S/G2 requirements for zinc showed virtually identical sensitivities to zinc deprivation. Each of the above requirements for zinc coincides with the induction of specific cyclin mRNAs, and the concentrations of these mRNAs have now been shown to decrease in the absence of adequate zinc. This is the first study to indicate a possible common factor underlying the requirement for available zinc during both cell replication and differentiation. (J. Nutr. Biochem. 10:279–290, 1999)

Keywords: zinc; cell cycle; cyclin; G2

Introduction

Zinc is essential for growth and development of many organisms, ranging from *Euglena* to humans[1,2] and numerous investigations have indicated impaired entry of cells into the S phase in the absence of adequate zinc.[3–5] The decreased rates of DNA synthesis in zinc deficient animals and cell cultures have led to the concept that DNA synthesis is a zinc dependent process; however, there appears to be little evidence that DNA synthesis *per se* is sensitive to lack of zinc. Instead, the experimental data point to a zinc dependent step during the mid-G1 phase that, if not satisfied, blocks subsequent progress through to the S phase.[6] Therefore, some investigators doubt the requirement for readily available zinc during the actual synthesis of DNA.

The situation with regard to zinc sensitive steps during G2 and mitosis is equally unclear. Pioneering work by Falchuk et al.[1] indicated that when *Euglena* were allowed to grow in low zinc medium until lack of zinc inhibited growth, the cells mainly arrested in the G2 phase. However, lack of zinc produced widespread metabolic changes in *Euglena*[7] that have not been observed with higher organisms, and the possibility of a zinc dependent process in G2 does not appear to be have been investigated in higher eukaryotes. The present study of a mammalian cell line has shown that zinc is required to permit passage through the G2/M phase of the cell cycle but not for replication of DNA.

Myoblasts can be induced to differentiate in cell culture by transfer of the cells to medium restricting cell growth. Because lack of zinc restricts cell growth, it might have been expected that restricting zinc availability would initiate myoblast differentiation. However, previous studies have indicated that zinc is required for induction of myoblast differentiation,[8] a process initiated during mid-G1.[9–11] Because the zinc requirement for entry into the S phase also is located in mid-G1,[6] it appeared possible that these usually mutually exclusive processes shared an early zinc dependent event essential for both. Comparison of the timing of the zinc requirements and the induction of cyclins, which are cell cycle specific proteins, indicated a close temporal similarity. In addition, although most events involved in myoblast differentiation are the antitheses of those leading to cell replication, elevation of cyclin D3 mRNA concentration occurs both during the mid-G1 phase in cells traversing the replication cycle and during the initiation of myoblast differentiation.[12,13] Therefore the impact of zinc

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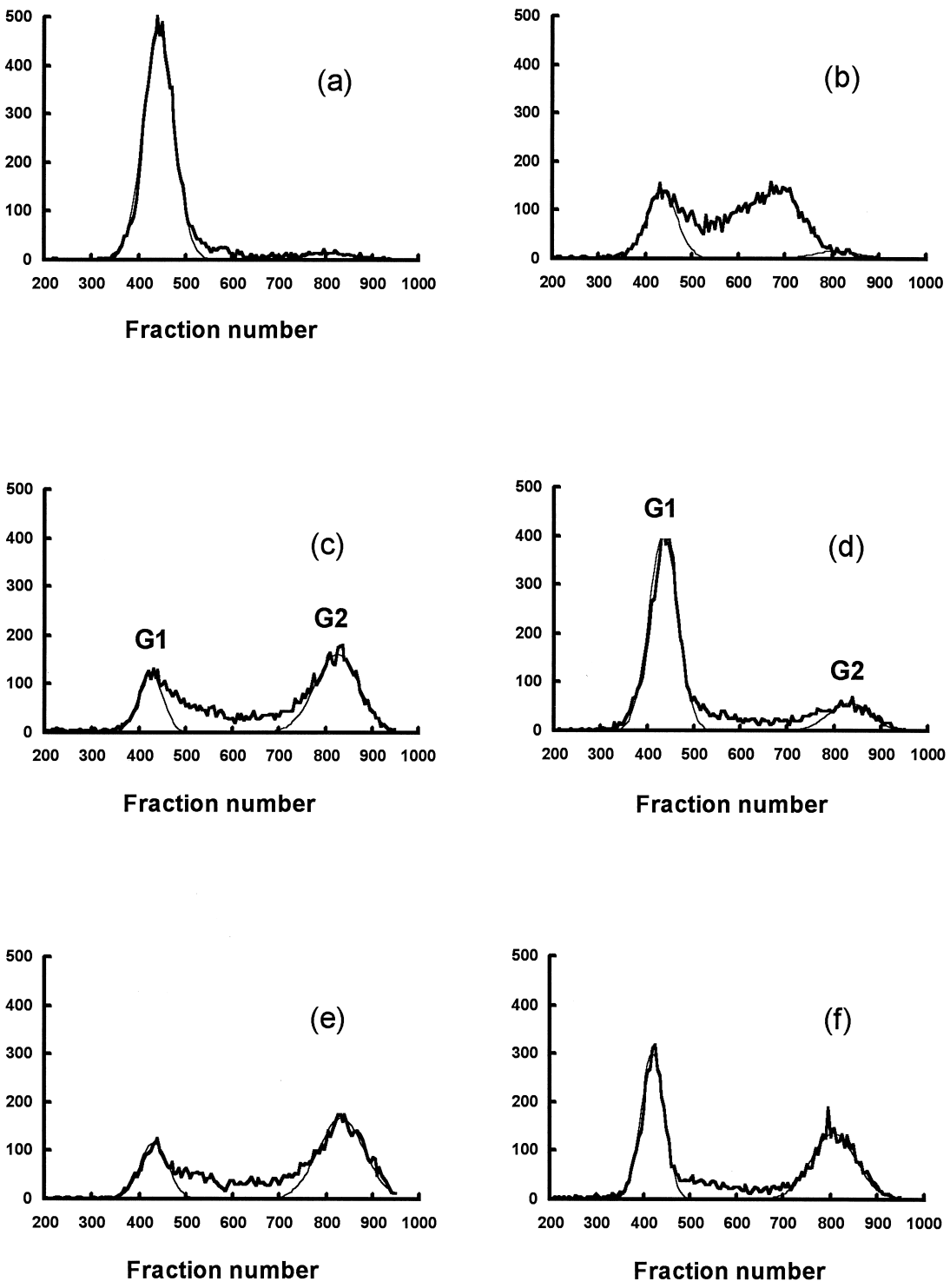

Figure 1 Flow cytometric analysis of 3T3 cells after release from synchronization at the G1/S boundary. The cells were subjected for 70 hours to medium containing only 0.1% fetal calf serum (FCS) and then transferred for an additional 20 hours to medium containing 12% FCS but supplemented with 0.5 mM hydroxyurea. (*a*) Cells were harvested without release from hydroxyurea; (*b*), (*c*), and (*d*) cells were harvested 4, 8, or 12 hours, respectively, after release into medium containing 12% FCS but no hydroxyurea. (*e*) and (*f*) Cells were harvested 8 or 12 hours, respectively, after release into medium containing 12% FCS, no hydroxyurea but with diethylenetriaminepentaacetic acid (DTPA)/Fe added. The fainter curves illustrate the Gaussian distributions used to assess the proportions of the cells in G1 and G2. These peaks are labelled in graphs (*c*) and (*d*); the S phase lies between them.

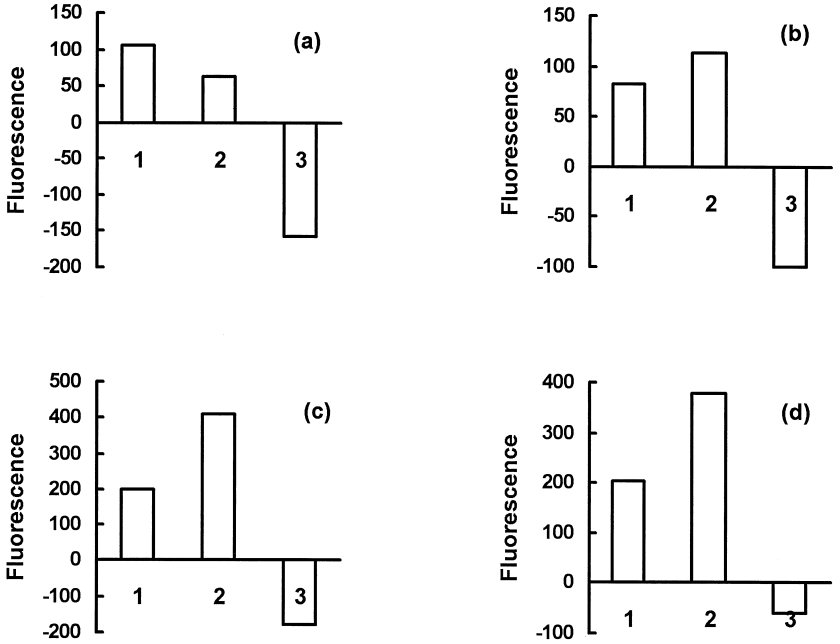

Figure 2 Relative alterations in fluorescence of Mag-fura-2 upon addition of (a) Ca^{2+}, (b) Mg^{2+}, or (c) Zn^{2+}. (d) The changes in fluorescence induced by adding 400 μM Zn^{2+} to diethylenetriaminepentaacetic acid (DTPA)/Fe-treated 3T3 cells loaded with Mag-fura-2. Fluorescences were estimated at excitation and emission wavelengths of: 1, 320 and 515 nm; 2, 315 and 460 nm; 3, 370 and 505 nm.

deprivation on cyclin mRNA expression also was investigated.

Materials and methods

Cell culture

Mouse 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (50 units/mL), streptomycin (50 units/mL), and 12% (v/v) fetal calf serum (FCS; Life Technologies, Paisley, UK). The cells were maintained in petri dishes at 37°C in an atmosphere containing air:CO_2 (9:1). Quiescent cells were prepared by transferring the cultures to medium containing FCS at only 0.1% for 68 to 72 hours. To synchronize cells at the G1/S boundary, quiescent cells were transferred to 12% FCS medium containing 0.5 mM hydroxyurea for 20 hours. In each case, the synchronized cells were re-incubated in standard 12% FCS medium during investigation of their zinc requirements.

The effects of zinc deprivation were investigated after restricting zinc availability by addition of the metal chelator diethylenetriaminepentaacetic acid (DTPA; 600 μM). Previous experiments had indicated that although Fe^{2+} was unable to replace Zn^{2+} in reversing the effects of DTPA, supplementation of the DTPA with Zn^{2+} and Fe^{2+} resulted in higher thymidine kinase activities than addition of Zn^{2+} alone, thus indicating a secondary requirement for iron.[6] Therefore, in most experiments, $FeSO_4$ (200 μM) was in addition to the DTPA (DTPA/Fe). In general, one of the DTPA-treated groups received no further supplementation (−Zn) whereas another received 400 μM $ZnSO_4$ (+Zn). Although it was not possible to estimate the free Zn^{2+} concentrations present under these conditions, it should be remembered that the basic medium contained the competing ions Ca^{2+} and Mg^{2+} at two and three times the molar concentration of DTPA, respectively. In addition,

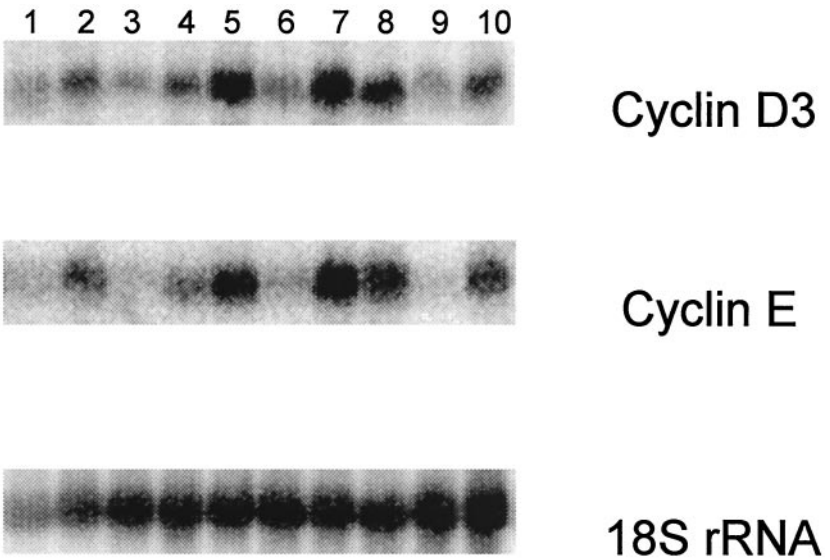

Figure 3 Visualization of cyclin mRNAs and 18S ribosomal RNA on Northern blots of total RNA from 3T3 cells progressing through G1. The images were obtained and quantified using the Instantimager described in Materials and methods. The RNA was prepared from: lane 1, quiescent control cells; lanes 2, 5, and 8, control cells 4, 8, and 12 hours, respectively, after release from quiescence; lanes 3, 6, and 9, same as lanes 2, 5, and 8 but with diethylenetriaminepentaacetic acid (DTPA)/Fe added to the medium at time zero; lanes 4, 7, and 10, same as lanes 2, 5, and 8 but with DTPA/Fe and 400 μM Zn^{2+} added to the medium at time zero.

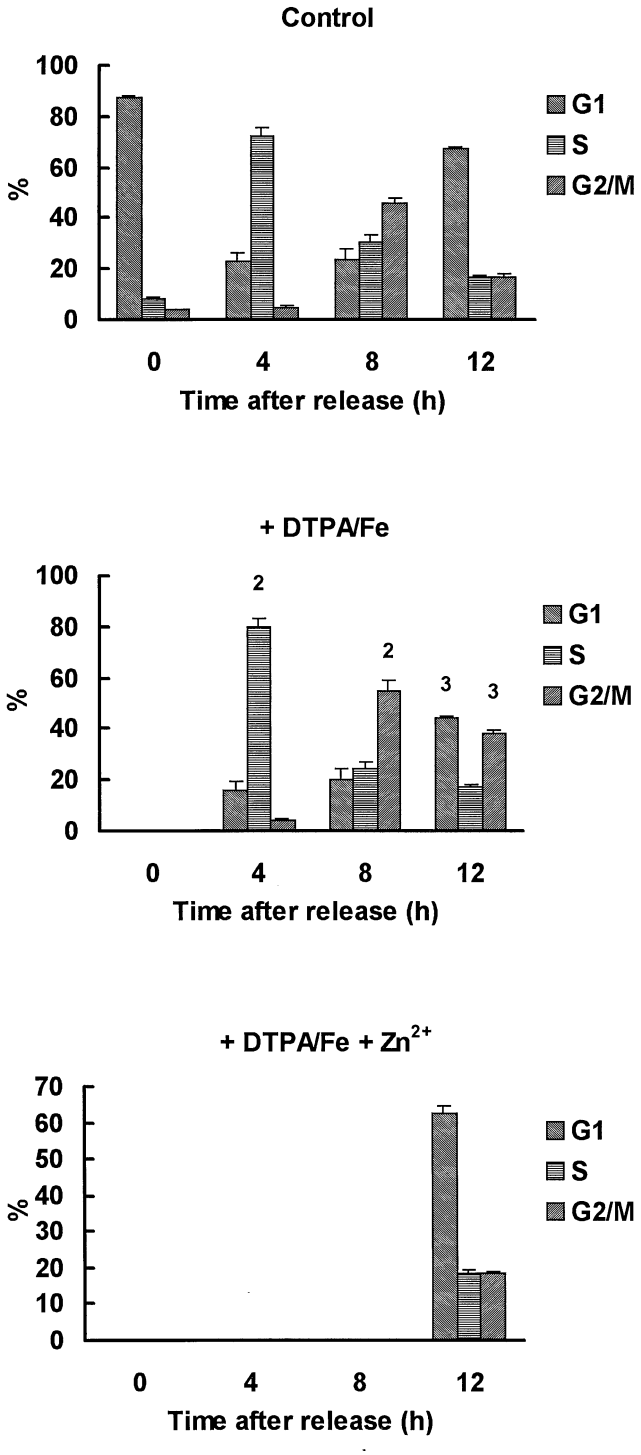

Figure 4 Influence of diethylenetriaminepentaacetic acid (DTPA)/Fe on the passage of 3T3 cells through the S and G2/M phases of the cell cycle. Quiescent cells were synchronized at the G1/S boundary by exposure to 0.5 mM hydroxyurea for 20 hours and then transferred to standard Dulbecco's modified Eagle's medium (DMEM) containing 12% FCS with or without DTPA (600 μM) and Fe (200 μM). Zinc-supplemented cultures received 400 μM $ZnSO_4$ immediately after the DTPA/Fe. The cells were harvested at the times indicated and the values for the proportions of the cells in each of the phases of the cell cycle are means and SEM for three replicates. The significances of the differences between means were determined by analysis of variance. At each time point, significance of the difference from the control value is indicated by: $^1P < 0.05$; $^2P < 0.01$; $^3P < 0.001$.

much of the Fe^{2+} probably would have been oxidized to its trivalent form and become bound to transferrin.

In later experiments, another chelator [diamsar (1,8 diamino-3,6,10,13,16,19 hexa-azabicyclo-6,6,6 eicosane, 600 μM)] was used in place of DTPA to restrict divalent cation availability.

Flow cytometry

Cells (approximately 6×10^4) for cell cycle analysis were grown in six-well plates and then the medium was removed from each well and stored individually. The cells were rinsed with phosphate buffered saline (PBS), each aliquot being added to its corresponding medium. The cells were released from the dishes by trypsinization and resuspended in their own stored medium/PBS, thus retaining any cells that had become detached from the substrate. The cells were collected by brief centrifugation at 300 g and then resuspended in 25 μL citrate buffer [40 mM sodium citrate, pH 7.6, 250 mM sucrose, 5% v/v dimethylsulphoxide (DMSO)] containing 2.5×10^5 chicken erythrocytes per milliliter, which acted as a calibration control for the flow cytometer. The cells then were supplemented with 120 μL 30 μg/mL trypsin in buffer A [0.5 mM Tris-HCl, 3.4 mM trisodium citrate, 1.5 mM spermine, 0.1% (v/v) Nonidet P40, pH 7.6] and incubated for 10 minutes at room temperature to release the nuclei. Further tryptic digestion was inhibited and RNA contamination minimized by addition of 100 μL 0.5 mg/mL trypsin inhibitor (Sigma Chemical Co, T9253, St. Louis, MO USA), 0.1 mg/mL RNAase A in buffer A and incubation at 37°C for 10 minutes. The preparations then were stored on ice until required (generally overnight). One hundred microliters 0.4 mg/mL propidium iodide, 1.15 mg/mL spermine in buffer A was added to the samples 10 to 15 minutes before their analysis by flow cytometry in an EPICS Profile II instrument (Coulter Electronics Ltd, Luton UK).

The data were smoothed by combining the counts from successive sets of five channels and these values were then analyzed by fitting Gaussian curves to the peaks of cells in the G1 and G2 phases (*Figure 1*). Cells lying between these two curves were taken to have been in the S phase.

Assessment of intracellular zinc concentration

Mag-fura-2 (Molecular Probes, Eugene, Oregon USA) is a fluorescent chelator that exhibits a characteristic spectrum when associated with any of a range of metal ions including Zn^{2+}. The compound was supplied as its acetoxymethyl ester, which is readily taken up by cells and then hydrolyzed intracellularly to liberate the free chelator. The latter is membrane impermeable, which allows the cells to be washed free of nonabsorbed probe while retaining that which has been taken up by the cells.[14]

The 3T3 cells were loaded with Mag-fura-2 essentially as recommended by the supplier. Mag-fura-2 acetoxymethyl ester was dissolved in anhydrous DMSO, and 4 μL of a 5 mM solution was dispersed in 1 mL of buffer B (140 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, pH 7.4, 0.2% polyethylene glycol 4000). The cells were washed with PBS, trypsinized, and collected by centrifugation at 200 g. They then were loaded with Mag-fura-2 by suspension in 750 μL buffer B, addition of 250 μL of the suspension of the Mag-fura-2 in buffer B, and incubation at 37°C for 1 hour. The cells were washed twice with buffer B to remove excess nonabsorbed Mag-fura-2, and their fluorescence was investigated in a Perkin-Elmer Luminescence spectrophotometer fitted with a cell housing thermostated to 37°C. Because of the sensitivity of Mag-fura-2 to light, all operations involving the compound were carried out under reduced lighting intensity. All fluorescence spectra obtained with the cells were corrected for the minor fluorescence or light scattering

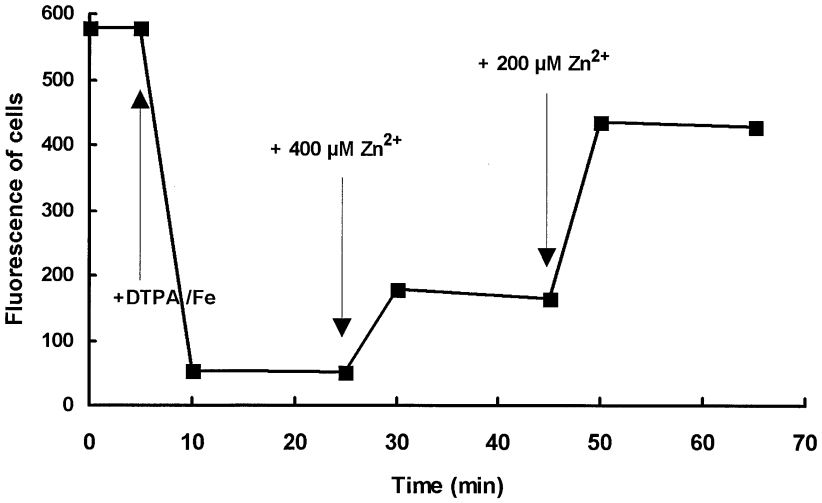

Figure 5 Time course of the response of the fluorescence of 3T3 cells loaded with Mag-fura-2 to the addition of diethylenetriaminepentaacetic acid (DTPA)/Fe and subsequent additions of Zn^{2+}. The fluorescence of the cells was measured with an excitation wavelength of 315 nm and emission wavelength of 460 nm to give maximum sensitivity to changes in intracellular zinc. All values were corrected for the corresponding minor alterations in fluorescence in a parallel sample of cells that had not been loaded with Mag-fura-2.

obtained with duplicate samples treated identically apart from omission of Mag-fura-2.

Free Mag-fura-2 for spectral studies was obtained by alkaline hydrolysis of its ester, and its fluorescence in buffer B either alone or in combination with a range of divalent cations was determined over a range of excitation and emission wavelengths. Addition of any of the redox ions Cu^{2+}, Fe^{2+}, or Mn^{2+} resulted in a progressive decrease in Mag-fura-2 fluorescence across all wavelengths with increasing ion concentration. In contrast, addition of Ca^{2+}, Mg^{2+}, or Zn^{2+} produced characteristic changes in the pattern of fluorescence. From pairs of wavelengths ranging from 300–340 nm for excitation and from 450–520 nm for emission, three combinations of excitation and emission wavelengths were chosen as giving best differentiation of the metal ion induced changes: for Ca^{2+} and Mg^{2+} excitation at 320 nm and emission at 515 nm; Zn^{2+} 315 nm and 460 nm; and free Mag-fura-2 370 nm and 505 nm. *Figure 2a–c* show the patterns of change of fluorescence at these wavelengths when saturating concentrations

Table 1 Timing of the requirement for zinc during passage of 3T3 cells through the S and G2 phases of the cell cycle

				Distribution within the cell cycle (%)					
	Period of adequate zinc			G1		S		G2/M	
Harvest	0–4 h	4–8 h	8–12 h	Mean	SEM	Mean	SEM	Mean	SEM
Time zero				82.4	1.6	13.3	1.5	4.3	0.1
12 h	■	■	■	63.0	1.3	17.3	0.4	19.7	0.2
12 h	□	□	□	42.6	0.5[c]	17.8	1.2	39.6	0.9[c]
12 h	■	□	□	57.8	0.2[b,f]	16.1	1.5	26.1	1.2[b,f]
12 h	□	■	□*	49.9	0.6[c,f]	22.4	2.1[a,d]	27.8	3.2[c,f]
12 h	□	□	■	46.4	0.7[c,d]	17.9	1.8	35.7	1.0[c]
12 h	■	■	□	58.4	0.2[a,f]	18.7	1.1	22.8	0.7[f]
12 h	□	■	■	59.8	2.6[f]	16.3	0.4	23.9	1.5[a,f]
12 h	■	□	■	60.1	0.7[f]	15.6	1.5	24.3	1.6[a,f]

Cells were synchronized at the G1/S boundary by incubation for 20 hours in the presence of medium containing 0.5 mM hydroxyurea. The cells were then released into fresh medium without hydroxyurea and periods of low zinc availability (open bars) were induced by addition of DTPA/Fe. When appropriate, the effects of DTPA/Fe were reversed by subsequent addition of 400 µM $ZnSO_4$.

*With this group, the zinc-supplemented medium was replaced after 8 hours with fresh medium containing DTPA/Fe. Values are means and SEM for three replicates. The significance of differences are indicated as follows:

Significantly different from 0–12 h zinc adequate: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; significantly different from 0–12 h zinc deficient: [d]$P < 0.05$; [e]$P < 0.01$; [f]$P < 0.001$.

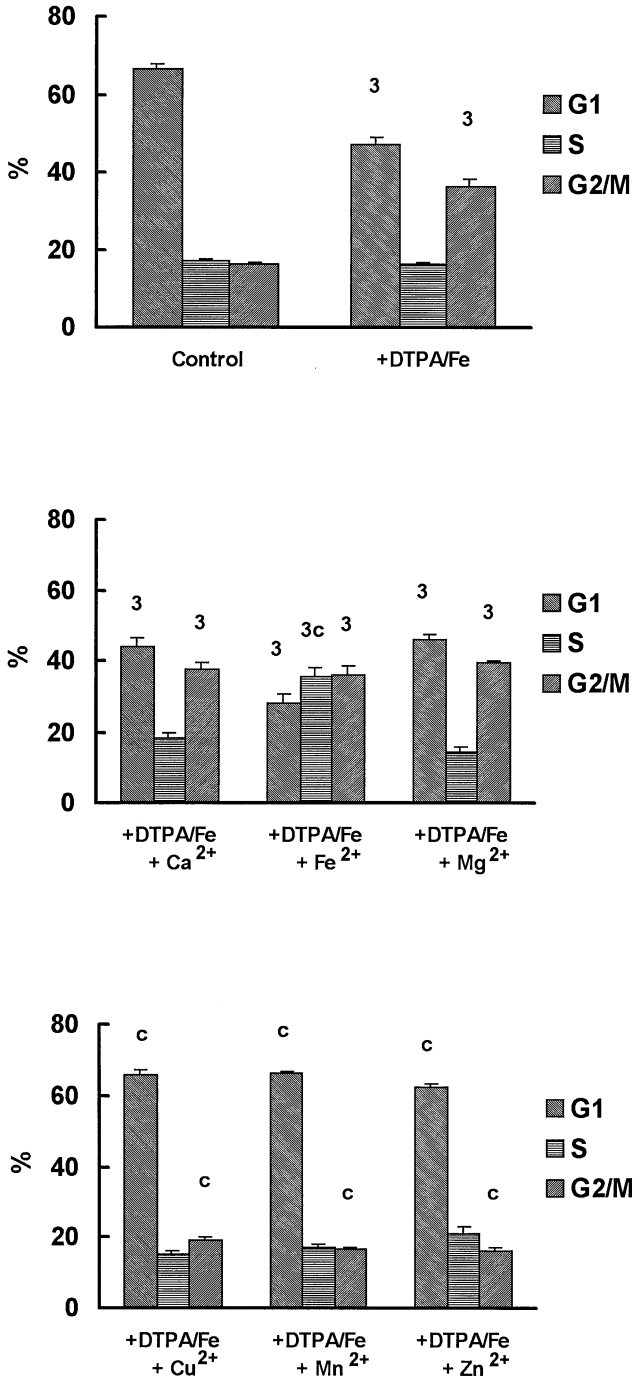

Figure 6 Impact of diethylenetriaminepentaacetic acid (DTPA)/Fe and various divalent cations on the cell cycle distribution of 3T3 cells 12 hours after release from synchronization with hydroxyurea. Cultures released from synchronization with hydroxyurea received DTPA/Fe as indicated either with or without an additional 400 μM of the divalent cation listed. Values are means and SEM for three replicates and the significances of the differences between means were determined by analysis of variance. Significance of difference from control cells: $^{1}P < 0.05$; $^{2}P < 0.01$; $^{3}P < 0.001$. Significance of difference from cells given DTPA/Fe alone: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

of individual divalent cations were added to 100 nM Mag-fura-2, 600 μM DTPA in buffer B from which the Ca^{2+} and Mg^{2+} had been omitted. The corresponding changes induced by addition of Zn^{2+} to cells loaded with Mag-fura-2 in buffer B and then treated with DTPA/Fe are illustrated in *Figure 2d*. Clearly, the changes induced by adding Zn^{2+} to the DTPA/Fe-treated cells were very similar to those obtained with Zn^{2+} in the absence of cells.

Cyclin mRNA

Quiescent and hydroxyurea-treated 3T3 cells for mRNA isolation were grown in 90-mm petri dishes as described above and mRNA was isolated by the method of Chomczynski and Sacchi[15] as described previously.[8] To examine effects on G1 cyclins, quiescent cells were transferred to 12% FCS medium for 0, 4, 8, or 12 hours prior to harvest. mRNA for estimation of G2 cyclins was harvested from hydroxyurea-treated cells after release into fresh 12% FCS medium for either 0 or 8 hours. Fused C2C12 myoblasts were obtained as described by Petrie et al.[8]

mRNA concentrations were determined by Northern blotting using cDNA probes as previously described.[8] Radioactivity from the probes was detected and quantified with a high-resolution, microchannel array detector (Instantimager, Canberra Packard, Bracknell, UK). The cyclin cDNA probes were obtained by reverse transcription polymerase chain reaction (RTPCR) using specific primers based on the known mouse sequences for cyclin D3,[16] cyclin E,[17] and cyclin B.[18] The identity of these probes was confirmed by sequencing in both directions and the probes were labelled by unidirectional PCR in the presence of ^{32}P-dCTP. mRNA concentrations for the cyclins were normalized for possible loading and transfer effects during Northern blotting by expressing the values relative to those for the corresponding 18S ribosomal RNA. Examples of the images obtained are shown in *Figure 3*.

Statistical analysis

The results are presented as means and their standard errors with the statistical significance of the differences between means determined by analysis of variance.

Results

S phase

When cells were subjected to 0.5 mM hydroxyurea for 20 hours following release from quiescence, most arrested at the end of G1 or the start of S phase (*Figure 1*). After release into fresh 12% medium for 4 hours, a majority were in the S phase, whereas by 8 hours they had passed into G2, and by 12 hours most of the cells had returned to G1. However, if the cells were released from hydroxyurea into a medium containing DTPA/Fe, the cells still appeared to complete the S phase normally but many became trapped in G2 (*Figure 4*). The addition of Zn^{2+} to the cells along with the DTPA/Fe abolished the effect of the chelator.

The ability of the cells to complete the S phase after release from hydroxyurea into DTPA/Fe-containing medium suggests that DNA synthesis per se was not sensitive to lack of zinc. Therefore, we investigated an alternative explanation that there was a slow change in the availability of zinc within the cells with the addition of DTPA/Fe to the medium. Cells loaded with Mag-fura-2 were transferred to a fluorimeter and treated with DTPA/Fe. Their fluorescence at the zinc specific excitation (315 nm) and emission (460 nm) wavelengths was determined immediately and at various time intervals after subsequent addition of Zn^{2+}. The change in fluorescence was characteristic of Zn^{2+} binding

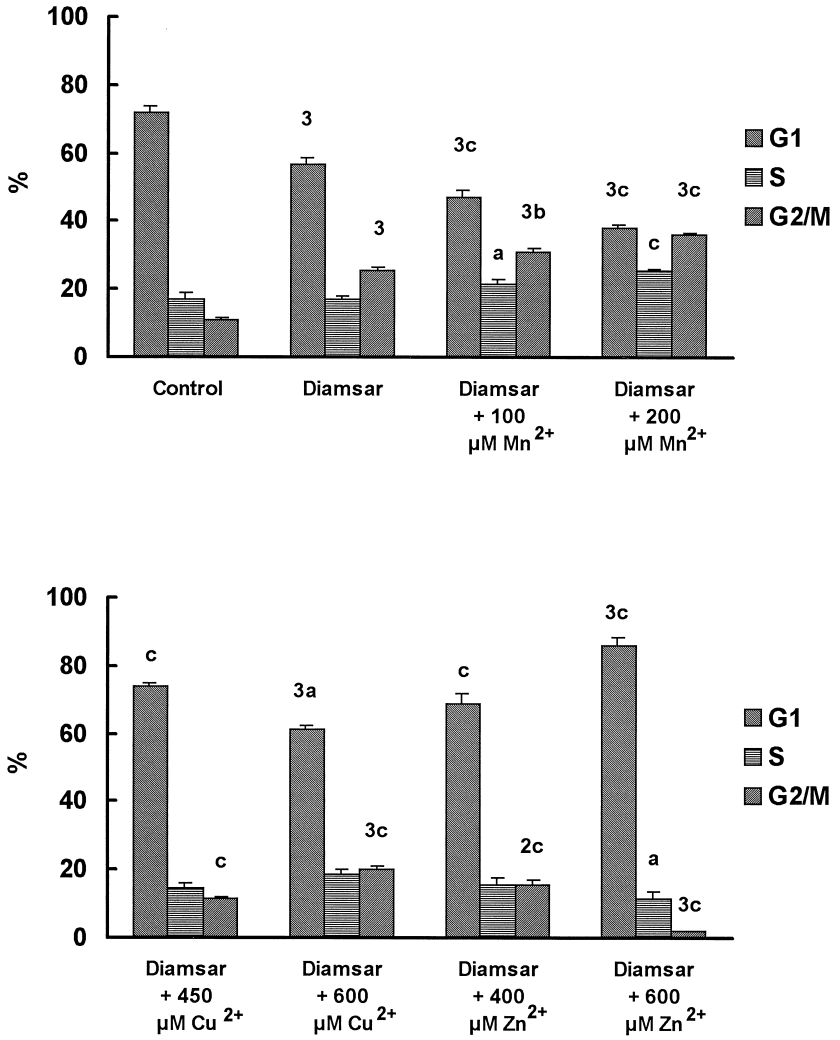

Figure 7 Impact of diamsar and various divalent cations on the cell cycle distribution of 3T3 cells 12 hours after release from synchronization with hydroxyurea. Cultures released from synchronization with hydroxyurea received diamsar as indicated either with or without a divalent cation as listed. Values are means and SEM for three replicates. The significances of the differences between means were determined by analysis of variance. Significance of difference from control cells: $^{1}P < 0.05$; $^{2}P < 0.01$; $^{3}P < 0.001$. Significance of difference from cells given diamsar alone: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

to Mag-fura-2 (*Figure 2*) and occurred within minutes of addition of the ion (*Figure 5*).

G2/M phases

Because the cells appeared to be able to synthesize DNA normally and accumulate in G2 after 8 hours even in the presence of DTPA/Fe, it seemed likely that the zinc dependent event that prevented them from dividing in the absence of adequate zinc had occurred between 8 and 12 hours. However, when the cells were subjected to restricted periods of zinc deprivation between 0 and 12 hours after release from hydroxyurea, it became apparent that lack of zinc between 8 and 12 hours did not restrict their passage through mitosis (*Table 1*). Instead, exit from G2/M was dependent on adequate zinc for at least 4 hours during the 0- to 8-hour period.

Metal specificity

Previous studies have suggested that the ability to reverse the effects of DTPA/Fe on cell replication and differentiation is specific to zinc,[5,6,19] and the present experiments confirmed the ability of zinc to reverse these effects in cells previously synchronized at the G1/S boundary (*Figure 6*). However, when the metal specificity of the G2 block was investigated, Cu^{2+} and Mn^{2+}, but not Ca^{2+}, Fe^{2+}, or Mg^{2+}, were also able to reverse the block. The affinities of Cu^{2+} and trivalent Mn^{3+} for DTPA are several orders of magnitude greater than that of Zn^{2+}. In the presence of a chelator and under aerobic conditions, the divalent Mn^{2+} that was added probably oxidized to its much more strongly bound trivalent form, and thus the ability of Cu^{2+} and Mn^{3+} to activate the system could have resulted from displacement of endogenous Zn^{2+} from the chelator. Consistent with this was the observation that at molar concentrations of the divalent ions significantly below that of the DTPA, the chelator's effects were reversed by Zn^{2+} but not by the other ions (results not shown). Unfortunately, this did not provide definitive evidence for the primary importance of zinc because when added at subsaturating concentrations, the other ions' stronger binding to DTPA might not have reached a free concentration necessary to activate passage through mitosis.

To address further the question of the specificity of the zinc response, the effects of another chelator (diamsar), were investigated. Diamsar has a cage-like structure that

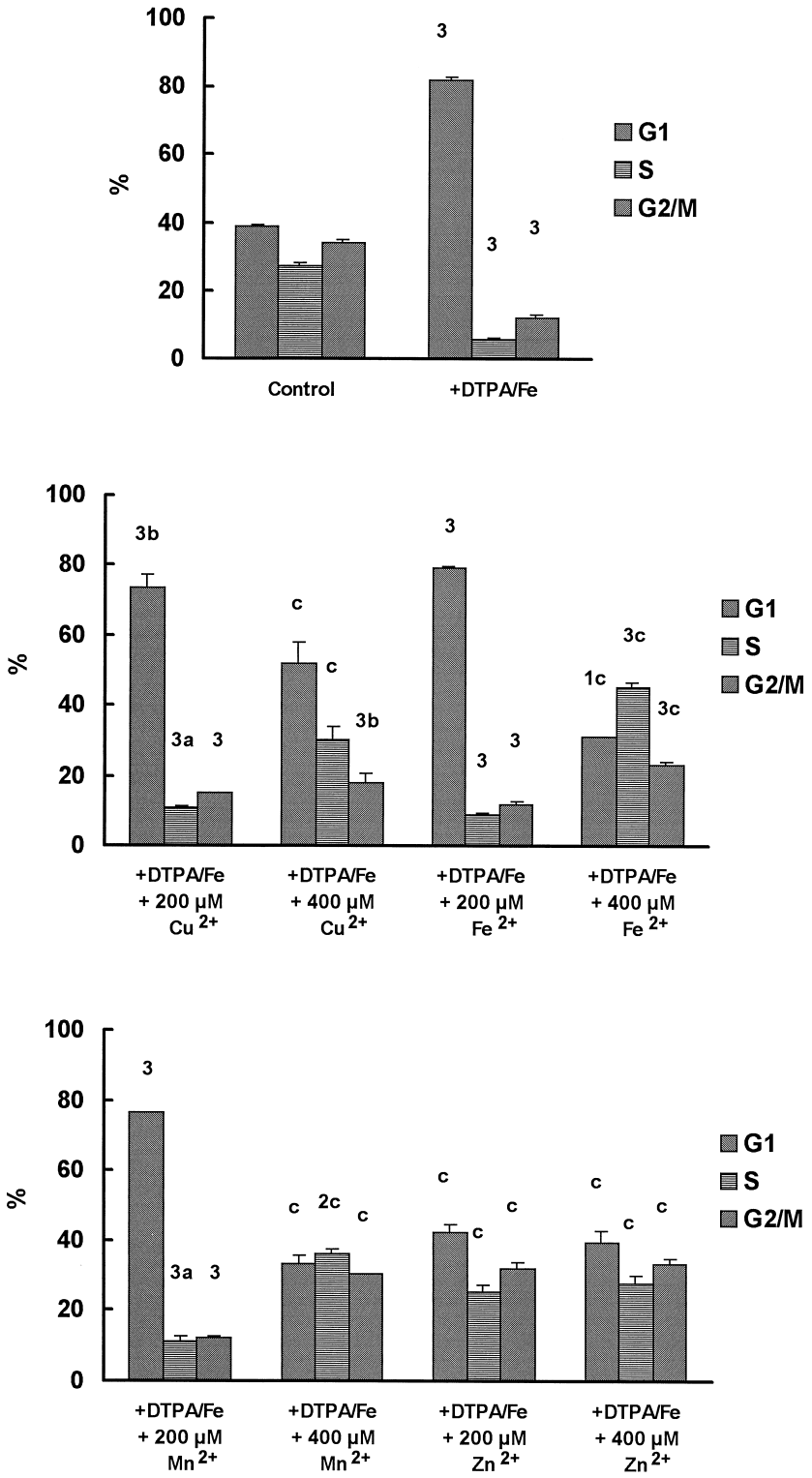

Figure 8 Impact of diethylenetriaminepentaacetic acid (DTPA)/Fe and various divalent cations on the cell cycle distribution of 3T3 cells during passage through G1 20 hours after release from quiescence. Cells were synchronized by incubation in Dulbecco's modified Eagle's medium (DMEM) containing only 0.1% fetal calf serum (FCS) for 70 hours prior to release into DMEM containing 12% FCS. Where appropriate DTPA/Fe with or without the indicated divalent cation was added at the time of release from quiescence. Values are means and SEM for three replicates and the significances of the differences between means were determined by analysis of variance. Significance of difference from control cells: $^{1}P < 0.05$; $^{2}P < 0.01$; $^{3}P < 0.001$. Significance of difference from cells given DTPA/Fe alone: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

surrounds the metal ion and forms relatively specific complexes with Zn^{2+} and Cu^{2+}[20]. The fluorescence spectrum of Mag-fura-2 alters characteristically when it binds Cu^{2+}, Fe^{2+}, Mn^{2+}, or Zn^{2+} and this was used as an indicator of the availability of free metal ions in the presence of diamsar. These experiments confirmed that diamsar bound Zn^{2+} and Cu^{2+} strongly but had a much lower affinity for Fe^{2+} and Mn^{2+}. In the presence of 600 µM diamsar and Ca^{2+} and Mg^{2+} at concentrations similar to those found in the culture medium, approximately 400 µM Cu^{2+} or Zn^{2+} had to be added to provide adequate free ions to half saturate 0.5 nM Mag-fura-2. In contrast, less than 50 µM Fe^{2+} or Mn^{2+} was

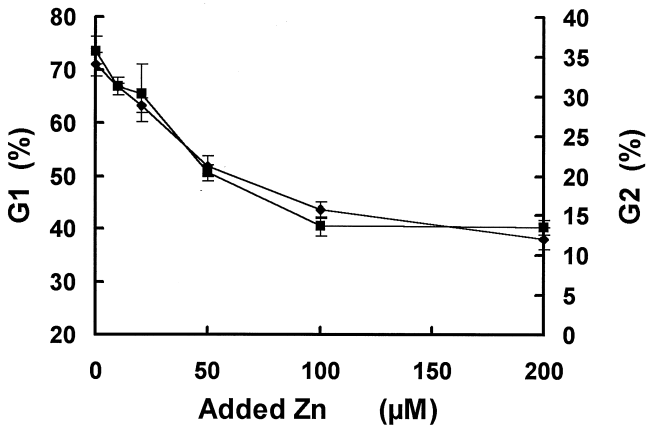

Figure 9 Zinc sensitivity of the exit of 3T3 cells from G1 20 hours after release from quiescence and of the exit from G2 of cells previously synchronized with hydroxyurea. In each case the cells were released into medium containing diethylenetriaminepentaacetic acid (DTPA) (600 μM)/Fe^{2+} (200 μM) with graded additions of Zn^{2+}. ◆—◆ G1 (%); ■—■ G2 (%).

sufficient to produce the same effect on the Mag-fura-2. Therefore, addition of diamsar was unlikely to influence Fe^{2+} availability significantly and this chelator was generally added without additional supplemental Fe^{2+}.

When hydroxyurea-treated cells were released into medium containing diamsar, many of the cells completed the S phase but accumulated in G2/M as expected (*Figure 7*). Addition of Mn^{2+} at concentrations shown to provide available Mn^{2+} did not reverse the effect of diamsar, which suggests that its effects in DTPA-treated cultures were caused by displacement of Zn^{2+} from the DTPA. Adding Zn^{2+} or Cu^{2+} at concentrations previously shown with Mag-fura-2 to be just sufficient enough to provide available Zn^{2+} or Cu^{2+} reversed the effects of the chelator. However, when Cu^{2+} and Zn^{2+} were added to the diamsar-treated cultures at a concentration equimolar with the chelator, further Zn^{2+} stimulated the passage of the cells through mitosis whereas it was inhibited by extra Cu^{2+} (*Figure 7*).

G1 phase

When 3T3 cells were subjected to flow cytometry 18, 20, and 22 hours after release from quiescence, the number of cells remaining in the G1 phase reached a minimum after approximately 20 hours and this time was used in subsequent investigations to assess the divalent cation requirements for passage through G1. As expected, the addition of DTPA/Fe prevented the cells from leaving G1 and when divalent cations were added at concentrations insufficient to saturate the DTPA, only Zn^{2+} was able to reverse its effects (*Figure 8*). However, when the total concentration of divalent cation added was equimolar with the DTPA, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}, each resulted in a significant activation. When diamsar was substituted for DTPA/Fe in a parallel experiment, there was a similar decrease in the proportion of cells entering the S and G2 phases, but Fe^{2+} and Mn^{2+} no longer reversed the effects of the chelator (results not shown).

Relative zinc requirements of G1 and G2

The impact of zinc availability on the loss of cells from G1 20 hours after release from quiescence and from G2 12 hours after release from hydroxyurea synchronization is shown in *Figure 9*. From this it is clear that these two zinc dependent steps were equally sensitive to zinc deprivation.

Cyclin mRNA

Lack of zinc markedly inhibited the increase in both cyclins D3 (*Figure 10*) and E (*Figure 11*) mRNA following release of the 3T3 cells from quiescence. In each case, zinc supplementation reversed the inhibition by DTPA/Fe. A similar effect was observed with cyclin B mRNA, where zinc supplementation actually resulted in higher cyclin B mRNA concentrations than in the control cultures (*Figure 12*). With myoblasts, fusion resulted in the previously observed increase in mRNA for cyclin D3 in control cultures (*Figure 13*). In fused cultures lacking adequate zinc, cyclin D3 mRNA concentration actually fell below that observed in unfused myoblasts but zinc supplementation largely reversed the effect of DTPA.

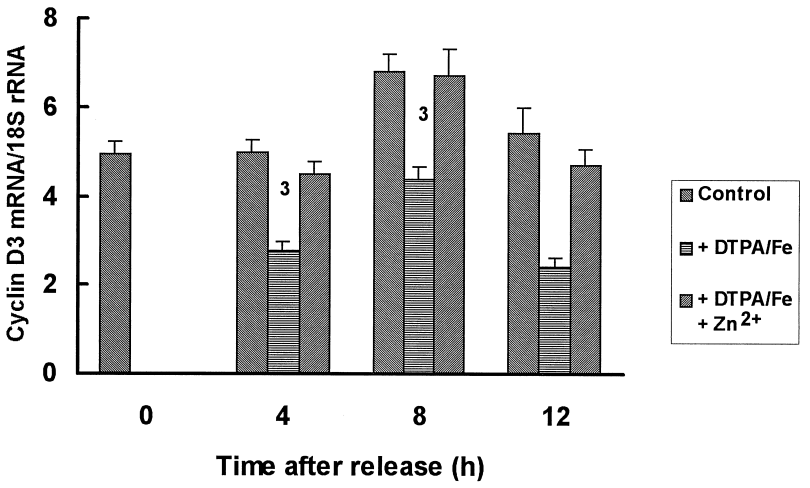

Figure 10 Influence of zinc availability on cyclin D3 mRNA:18S rRNA ratios at various times after release of 3T3 cells from quiescence. Where appropriate diethylenetriaminepentaacetic acid (DTPA)/Fe with or without 400 μM $ZnSO_4$ was added at the time of release from quiescence. Values are means and SEM for 12 replicates and the significances of the differences between means were determined by analysis of variance. Significance of difference from control cells at each time point: $^1P < 0.05$; $^2P < 0.01$; $^3P < 0.001$.

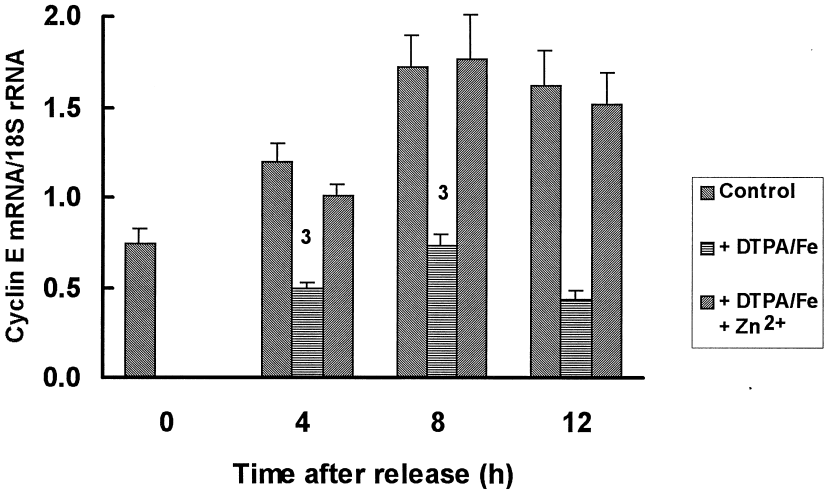

Figure 11 Influence of zinc availability on cyclin E mRNA:18S rRNA ratios at various times after release of 3T3 cells from quiescence. Where appropriate diethylenetriaminepentaacetic acid (DTPA)/Fe with or without 400 μM $ZnSO_4$ was added at the time of release from quiescence. Values are means and SEM for 12 replicates and the significances of the differences between means were determined by analysis of variance. Significance of difference from control cells at each time point: $^1P < 0.05$; $^2P < 0.01$; $^3P < 0.001$.

Discussion

Previous studies with 3T3 cells indicated that zinc was required around the mid-G1 phase to allow normal entry into the S phase.[6] The present experiments strongly suggest that once the cells have passed the G1 decision point, there is no further requirement for readily available zinc to permit replication of DNA. This conclusion is based on the ability of cells released from hydroxyurea block at the start of the S phase to complete the S phase even in the presence of DTPA/Fe. An alternative explanation would be that under these conditions, the lowering of intracellular zinc concentration by external application of this membrane-impermeable chelator occurs only slowly. This seems unlikely in view of the rapid response of the fluorescence of Mag-fura-2 loaded cells to the addition of Zn^{2+} in the presence of DTPA/Fe (*Figure 5*). Although this technique is potentially subject to the criticism that the Mag-fura-2 could have remained bound to the outside of the cells, the same technique has been validated for measurement of intracellular magnesium concentrations[21] and inspection of Mag-fura-2 loaded cells by fluorescent microscopy gave no indication of preferential peripheral location of the dye.

This should not be taken to suggest that zinc is not a necessary component of one or more elements of the DNA replication complex because many proteins will be able to retain their essential zinc and metabolic function even when the extracellular availability of zinc is sharply reduced. Indeed, dietary deficiency of zinc can prevent totally the growth of young rats although they nevertheless retain near normal tissue zinc concentrations.[22]

The present evidence of an inability of cells to divide in the absence of zinc even when they appeared to have completed replication of their DNA points to a second requirement for zinc. The evidence available for the timing of this requirement suggests that zinc is necessary for an event that occurs contemporaneously with DNA synthesis or early in the G2 phase.

Thus far, the metabolic changes have been attributed to a deficit of available zinc. The data obtained with DTPA are open to various interpretations because of DTPA's lack of specificity as a chelator of divalent cations. The inability of additional Ca^{2+} and Mg^{2+} to reverse the effects of DTPA on the G2/M to G1 transition was to be expected because both were already present in the medium in molar excess over the chelator and they have substantially lower affinity for DTPA than Zn^{2+}. It seems probable that in the presence of DTPA and an aerobic medium, iron and manganese would have been converted to their trivalent forms, which have a higher affinity for DTPA than for Zn^{2+}. When diamsar was used to restrict availability of the divalent cations, neither Fe^{2+} nor Mn^{2+} was able to replace Zn^{2+}. Thus, their reversal of the inhibition in G2/M by DTPA probably was associated with the displacement of endogenous Zn^{2+} from the chelator.

However, the chelator studies introduced the possibility of copper as an alternative activator. The enhancement of the G2/M to G1 transition in the presence of diamsar, which occurred at the higher Zn^{2+} concentration in contrast to an impairment of the transition at the higher Cu^{2+} concentration, suggested that Zn^{2+} is the biologically active ion

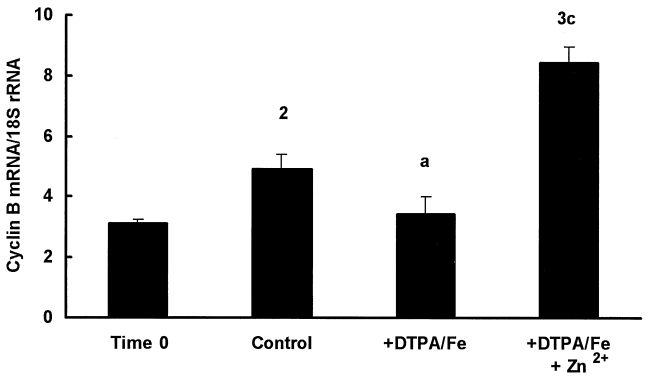

Figure 12 Ratios of cyclin B mRNA:18S rRNA in 3T3 cells 8 hours after release from initial synchronization at the G1-S boundary by exposure to hydroxyurea. Where appropriate, diethylenetriaminepentaacetic acid (DTPA)/Fe with or without 400 μM $ZnSO_4$ was added at the time of release. Values are means and SEM for eight replicates and the significances of the differences between means were determined by analysis of variance. Significance of difference from time zero control cells: $^1P < 0.05$; $^2P < 0.01$; $^3P < 0.001$. Significance of difference from 8-hour control cells: $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$.

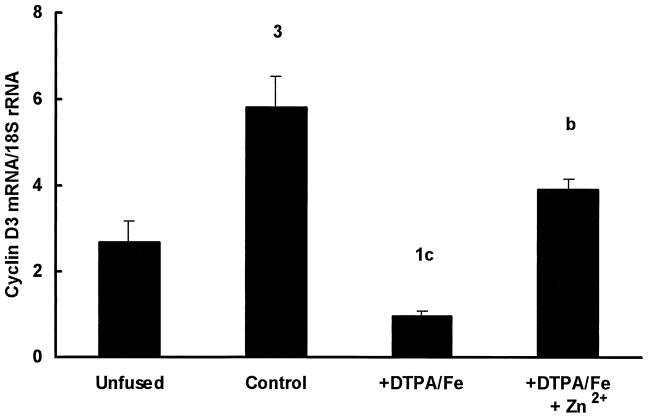

Figure 13 Influence of fusion and zinc availability on cyclin D3 mRNA: 18S rRNA ratios in C2C12 myoblasts. RNA was isolated from growing C2C12 myoblasts or from cultures that had been induced to fuse to form myotubes by exposure for 4 days to Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% horse serum. Where appropriate diethylenetriaminepentaacetic acid (DTPA) with or without 400 μM $ZnSO_4$ was added at the time the cells were transferred to the medium containing 2% horse serum. Values are means and SEM for four replicates and the significances of the differences between means were determined by analysis of variance. Significance of difference from unfused control cells: $^1P < 0.05$; $^2P < 0.01$; $^3P < 0.001$. Significance of difference from fused control cells: $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$.

(*Figure 7*). Previous studies with C2C12 cells indicated that Zn^{2+} but not Cu^{2+} could reverse the inhibition of myoblast differentiation by DTPA.[8] Further, because with both DTPA and diamsar the affinity for Cu^{2+} is significantly greater than that for Zn^{2+}, it is possible that Cu^{2+} could function by displacing trace amounts of Zn^{2+} from the chelator, but it is highly unlikely that the reverse could apply. Thus, it is improbable that the effects of Zn^{2+} could have resulted from its liberation of Cu^{2+} from the chelators. The concentrations of zinc that are present in tissues are also several-fold greater than those of copper whereas the tendency for copper to be more firmly bound than zinc is a general one. Assuming that the metal ion requirements during both G1 and G2 are transient, it appears more likely that zinc, not copper, would be available to fulfill these requirements. In agreement with this, the physiologic effects of dietary deficiency of zinc in animals are consistent with those seen here whereas those associated with dietary copper deficiency are not.[23,24] Therefore, all the evidence points to zinc as the metal required to activate passage through G1 and G2 *in vivo*.

The sensitivities to lack of zinc of the G1 and the G2/M requirements were indistinguishable and the timing of the G1 requirement corresponds with that of the zinc requirement for initiation of myoblast differentiation. Each of these processes is associated with induction of specific cyclin proteins that are essential regulators of cell cycle progression. Therefore, the effects of lack of zinc on cyclin mRNA concentrations were investigated. In each case, addition of DTPA lowered the concentration of cyclin mRNA and this effect was reversible by zinc supplementation. Further studies will be needed to clarify the role of zinc in cyclin expression, but these results provide the first evidence for a possible common role for zinc in its multiple effects on cell replication and differentiation.

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