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Zinc and the initiation of myoblast differentiation

Linda Petrie, Jean N. Buskin,* and John K. Chesters

*Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK, and *Department of Biochemistry, University of Washington, Seattle, WA USA*

Previous studies have indicated that a lack of available zinc inhibited myoblast differentiation as shown by a failure of the cells to fuse and low expression of creatine kinase mRNA and activity. However, the nature of the requirement for zinc and its relationship to the events leading to differentiation have been unclear. The current studies with C_2C_{12} cells indicated that the muscle-specific enhancer present in the 5'-flanking region of the creatine kinase gene contributed to the zinc sensitivity of this enzyme. Because this enhancer can be activated by expression of the myogenic factors MyoD and myogenin, their sensitivity to zinc was investigated. The concentrations of both MyoD and, particularly, myogenin mRNA, were decreased by zinc deficiency. In vitro translation experiments suggested that these changes closely corresponded with alterations in their rates of synthesis. Further experiments failed to indicate a major effect of zinc on the stabilities of these mRNAs. Because an induction of myogenin mRNA is one of the earliest known events in myoblast differentiation, its particular sensitivity to lack of zinc suggests that zinc may be required before or during the initiation of myoblast differentiation. © Elsevier Science Inc., 1996 (J. Nutr. Biochem. 7:670–676, 1996.)

Keywords: zinc; myoblasts; differentiation; myogenin; myoD

Introduction

The effects of zinc deprivation in eukaryotes have been postulated to result from an impaired ability of cells to alter their gene expression in the absence of adequate zinc.[1] During cell replication, this manifests as reduced induction of several enzymes associated with DNA synthesis and an associated failure of zinc-deficient cells to enter S phase.[2,3] To assess whether a similar impairment of enzyme induction occurred during the changes in gene expression associated with differentiation, the induction of creatine kinase (EC 2.7.3.2) was investigated during the conversion of myoblasts to contractile myotubes.[4] This process is accompanied by a loss of the cells' ability to replicate and is initiated in culture by reducing the growth-promoting characteristics of the medium by lowering its serum content. Because reducing the ability of the cells to replicate by removal of serum induces myoblasts to differentiate, removal of zinc might also have been expected to trigger differentiation of the cells. In practice, however, the cells failed to fuse and exhibited only low levels of creatine kinase mRNA and activity when the availability of zinc was restricted by addition of a chelator to the differentiation medium. This failure to differentiate was consistent with the hypothesis that zinc is required for expression of new enzyme patterns, rather than specifically for initiation of DNA synthesis.

However, investigation of the dependence of DNA synthesis on zinc showed that zinc was required some hours earlier than the point at which the enzymes involved in DNA synthesis were induced.[5] Thus, the failure to increase the activity of these enzymes in zinc-deficient cultures appeared to be secondary to a zinc-requiring step situated approximately midway through G1. This is also the point in the cell cycle at which cultured myoblasts appear to commit to differentiation when exposed to a medium containing a low concentration of serum.[6,7] The question, therefore, arose whether the loss of creatine kinase induction seen previously in zinc-deficient cultures of myoblasts resulted from direct effects on induction of the enzyme or was a consequence of an inability of the cells to enter the differentiation pathway in the absence of zinc.

Address reprint requests to Dr. J.K. Chesters at Rowett Research Institute, Greenburn Rd., Bucksburn, Aberdeen AB2 9SB, UK.

Nutritional Biochemistry 7:670–676, 1996

655 Avenue of the Americas, New York, NY 10010

0955-2863/96/$15.00
PII S0955-2863(96)00129-5

To address these questions, the role of the creatine kinase 5′-flanking region in mediating the enzyme's sensitivity to zinc deprivation has been investigated using the fusing myoblast cell line C_2C_{12}. Elimination of the muscle-specific enhancer from the creatine kinase 5′-flanking region was shown to decrease markedly the enzyme's response to zinc deprivation. Activation of this enhancer depends on binding sites for a number of myogenic factors[8–10] and further experiments have now indicated the lack of zinc greatly reduced the synthesis of mRNA for myogenin, a myogenic factor characteristically induced during the earliest stage of myoblast differentiation.[11,12] A preliminary report of the importance of the muscle-specific enhancer in the sensitivity of creatine kinase to zinc deprivation has been previously published.[13]

Methods and materials

Cell culture

The mouse myoblast cell line C_2C_{12} obtained from the American Type Culture Collection was used for all experiments. The cells were grown under an atmosphere of air/CO_2 (9:1) and in Dulbecco's modified Eagle's medium supplemented with penicillin (50 units/mL), streptomycin (50 units/mL), fungizone (2.5 μg/mL), and 12% (v/v) foetal calf serum (FCS) (all supplied by Life Technologies Ltd). Differentiation was induced at confluence by replacing FCS in the medium with 2% (v/v) horse serum (Sigma Chemical Co. St. Louis, MO USA). The cells were routinely seeded at a cell density of 2×10^5 cells per 60-min petri dish (chloramphenicol acetyltransferase (CAT) activity experiments) or 6×10^5 cells per 90-mm petri dish (RNA and in vitro translation experiments). On day 4, as the cells reached confluence, they were transfected if appropriate for the experiment, but in all cases they were transferred to differentiation medium. The required zinc status was also imposed by the addition as necessary of a metal chelator diethylenetriaminepentaacetic acid (DTPA) together with one of four levels of zinc: 600 μM DTPA + either 50, 100, 200, or 400 μM $ZnSO_4$. The cells were then left for 4 more day to allow fusion to occur before being harvested.

Plasmid constructs and probes

Most of the plasmids used have been previously described.[10,14,15] Basically, they contained the CAT coding region drive by various portions of the 5′-flanking region from mouse muscle creatine kinase (*Figure 1*). A plasmid lacking the putative binding site for the Sp1 transcription factor that lies between BamH1 sites at −800 and −1050 was prepared from the 1256MCKCAT plasmid by excision of the BamH1 fragment. SV40CAT was the pCAT control plasmid (Promega Ltd, Southampton, UK) containing the SV40 early promoter and SV40 enhancer.

The cDNAs for the 1.8-kb MyoD probe[16] and the 1.4-kb myogenin probe[11] were excised with EcoR1 from plasmids generously provided by Dr. H. Weintraub and Dr. W.E. Wright, respectively. The cDNA probes for myogenic factor 5 (Myf5, ATCC No. 59915) and myogenic factor 6 (Myf6, ATCC No. 61527) were EcoR1 fragments from plasmids purchased from American Type Culture Collection. The probes for creatine kinase and 18S rRNA were obtained as previously described.[4]

RNA extraction and Northern blots

RNA was extracted by the method of Chomczynski and Sacchi[17] and subjected to Northern blot analysis as previously described.[12]

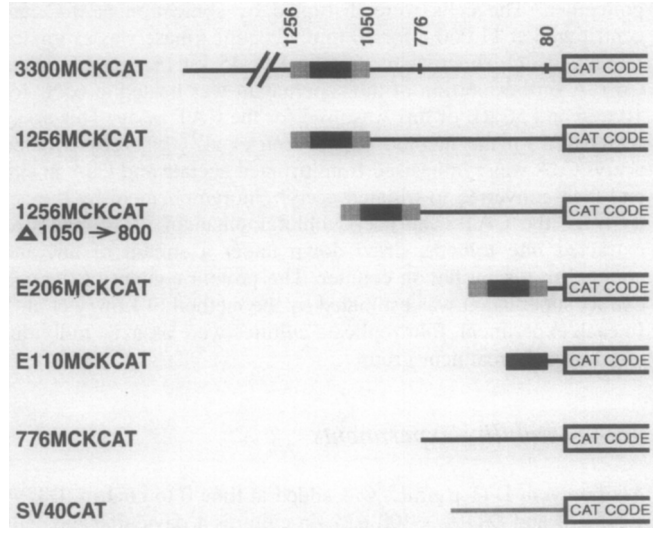

Figure 1 Diagrammatic representation of plasmid constructs used to transfect C_2C_{12} cells. The constructs, most of which have been previously described,[10,14,15] were based on pUC118 with insertion of modifications of the muscle creatine kinase (MCK) 5′-flanking region placed upsteam of the CAT coding sequence. The boxes illustrate the position of the muscle-specific enhancer with the truncated 110-bp enhancer (solid box) located within the 206 bp enhancer (shaded box).

Hybridizations for muscle creatine kinase, MyoD, and myogenin mRNAs and for 18S rRNA were performed as previously described.[4] After hybridization, the blots were analyzed with a microchannel array detector (InstantImager, Canberra Packard, Bracknell, UK). This provided a high-resolution, two dimensional quantitative image of the blot which was analysed to determine the radioactivity associated with the individual bands. Successive probes were removed from the blots by treatment with 0.1% (w/v) SDS at 95°C for 10 min.

Transfection experiments

The cells were transfected using a modification of the calcium phosphate precipitation method.[18] The transfection mix contained 137 mM NaCl, 21 mM HEPES pH 7.1, 0.7 mM NaH_2PO_4, 0.7 mM Na_2HPO_4, 125 mM $CaCl_2$, and 40 μg of the plasmid per mL. This mix was combined with an equal volume of 12% FCS medium before its addition to the aspirated plates, 2 mL of the final preparation being added per 60-min petri dish and 6 mL per 90-mm dish. For each experiment, sufficient mixture was prepared in bulk to treat all the cells transfected with a particular plasmid. They were then incubated in an atmosphere containing air/CO_2 (19:1) for 5-hr before being subjected to glycerol shock for 2 min with a solution containing 15% (v/v) glycerol in 10 mM Tris pH 7.4, 2.7 mM KCl, 137 mM NaCl, and 1.5 mM KH_2PO_4. Following this, the cells were transferred to differentiation medium and DTPA and zinc were added as required. The cultures were incubated in an atmosphere of air/CO_2 (9:1) for 4 days to allow fusion to occur. The cells were harvested and used for mRNA analysis or CAT and creatine kinase assay.

CAT and creatine kinase assays

The cells were rinsed several times with CAT wash buffer (40 mM Tris-HCl pH 7.4, 0.15 M NaCl, and 1 mM EDTA) and then scraped off the plate in 0.5 mL of CAT sonication buffer (250 mM Tris pH 7.8 containing 20% (v/v) glycerol) with a silicone rubber

policeman. The cells were disrupted by sonication at 0°C and centrifuged at 11,000 g for 10 min. Creatine kinase was estimated directly in the supernatant using a UV 45 kit (Sigma Chemical Co.). A further portion of the supernatant was heated at 65°C for 10 min and 50 μL of this was used for the CAT assay. This assay was based on the method of Nordeen et al,[19] whereby tritiated acetyl CoA was synthesised from tritiated acetate and CoA in situ and then converted to tritiated acetyl chloramphenicol by the activity of the CAT. The acetyl chloramphenicol so formed was extracted into toluene, dried down under a stream of air, and counted in a scintillation counter. The protein content of the cell extract supernatant was estimated by the method of Lowry et al.[20] In each experiment, four replicate cultures were assayed individually for each treatment group.

mRNA stability experiments

Actinomycin D (5 μg/mL) was added at time 0 to control, DTPA + 50 μM and DTPA + 400 μM Zn cultures 4 days after the cells had been transferred to differentiation medium. Cultures were then harvested at 0, 2, 4, 8, and 16 hr and the RNA was extracted and subjected to Northern blotting as described.

In vitro transcription

Four days after a switch to differentiation medium, nuclei were prepared in the cold by collecting the cells in PBS then resuspending the cells in hypotonic buffer (10 mM Tris pH 8, 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM DTT) containing 0.1% NP40. The cells were allowed to swell for 5 min before being homogenized in a Dounce homogeniser with a B pestle (Burkard Scientific, Uxbridge). The nuclei were pelleted through a 70% sucrose cushion by centrifuging in a swing-out rotor at 30,000 g for 45 min. The nuclei were resuspended in storage buffer (50 mM Tris pH 8, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT, and 40% glycerol) and immediately frozen in liquid nitrogen. A portion of the nuclei was used to estimate the DNA content and, hence, the number of nuclei and the final results were expressed as counts per hr/10^6 nuclei. The supernatant was used for the preparation of cytoplasmic RNA for Northern blot analysis as described.

In vitro transcription assays were performed by thawing out the nuclei (200 μL containing around 2×10^7 nuclei) in an equal volume of 2× reaction buffer containing 10 mM Tris pH 8, 5 mM $MgCl_2$, 300 mM KCl, 5 mM DTT, 1 mM each of ATP, CTP and GTP, and 100 μCi of 800 Ci/mmol ^{32}P-UTP (NEN-Dupont). They were then incubated at 30°C for 30 min and the reaction was stopped by the addition of 15 μL 20 mM $CaCl_2$ and 20 μL 1 mg/mL RNase-free DNase I and the incubation continued for 5 min. RNA was recovered by the standard Chomcznski and Sacchi method and used for hybridization after denaturing at 100°C for 7 min.

Each sample of RNA was hybridized to a separate filter containing individual slot blots of 1 μg of each of denatured myoD probe, myogenin probe, and non-specific DNA (bluescript vector) cross-linked to the nylon membrane. The hybridization was carried out at 65°C in 2 mL Church buffer (0.5 M sodium phosphate pH 7.1, 7% SDS, and 0.1 mM EDTA) overnight. The filters were washed in 2 × SSC for 1 h at 65°C, 2 × SSC containing 15 μg/mL RNase A for 30 min at 37°C and then 2 × SSC for a further 1 hr at 37°C. Under these condition the signal obtained was linear, with increasing amounts of counts added. The specifically bound transcripts were detected and quantified using a micro-channel array detector (InstantImager, Canberra Packard). *Figure 2* illustrates a typical image obtained. Bluescript vector was used as a negative control and all counts were corrected for this.

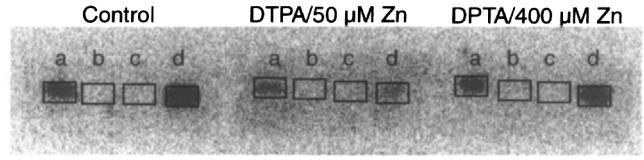

Figure 2 Typical image obtained from in vitro translation assays of MyoD and myogenin mRNA expression in nuclei derived from control cultures and those treated with DTPA and either 50 or 400 μM Zn. a) MyoD b) Blank c) Bluescript d) Myogenin.

Statistical analysis

Transfection experiments. The use of a bulk preparation of the transfection mix to treat all cells and random allocation of cultures to treatment groups largely eliminated systematic variations between replicates within an experiment, but absolute values differed substantially between experiments because of variations in transfection efficiency and degree of fusion. The results for the experimental groups were, therefore, expressed as percentages of the mean of control values obtained in each experiment from transfected cultures that had not been supplemented with either DTPA or zinc. This removed most of the between experimental variation and the data are presented as means and SEM for individual groups. The significance of differences between treatments and between plasmids was assessed by analysis of variance.

Stability experiments. For each treatment the ratio of the counts in the mRNA to those in the 18S rRNA was calculated at 0, 2, 4, 8, and 16 hr for duplicate samples and then the experiment was duplicated. This ratio was used for subsequent analyses rather than the absolute counts to correct for any variations in loading of RNA in the gel lanes. The decay was modeled with generalized linear models[21] using a logarithmic link function and error variance proportional to the mean (*i.e.*, quasi-Poisson). It was assumed that log (mRNA/rRNA) was linearly related to elapsed time and that the slope (but not the initial value) was the same for both replicates of any one treatment. These average slopes and their derived half lives were compared to detect differences between treatments.

In vitro transcription experiments. The results (counts per hr/2×10^7 nuclei) are expressed as means and SEM for eight experiments and the significance of the differences between the groups was assessed by Student's *t*-test.

Results

Creatine kinase

Creatine kinase activity serves as a convenient marker of myoblast differentiation and as expected from earlier studies with chick myoblasts,[4] it decreased when zinc availability was impaired by addition of DTPA during differentiation of C_2C_{12} cells. When various divalent cations were added individually to DTPA-treated cultures and creatine kinase activity determined 4 days later, only zinc fully reversed the effect of adding DTPA (*Table 1*).

Addition of 600 μM DTPA to cultures of C_2C_{12} cells also abolished the normal fusion of the myoblasts during a 4-day step down into a medium containing 2% horse serum. However, when added alone, it also tended to result in a partial loss of cells and, therefore, in all subsequent experiments, at least 50 μM Zn was added along with the DTPA

Table 1 Effects of addition of DTPA (600 μM) alone or with one of various divalent cations (400 μM) on creatine kinase activity in C_2C_{12} cells

Treatment	Creatine kinase activity
	(U/mg protein)
Control	0.93 ± 0.01
+DTPA	0.07 ± 0.01***
+DTPA + Cu^{2+}	0.11 ± 0.02***
+DTPA + Fe^{2+}	0.21 ± 0.06***
+DTPA + Mn^{2+}	0.12 ± 0.01***
+DTPA + Ni^{2+}	0.15 ± 0.01***
+DTPA + Zn^{2+}	0.92 ± 0.11

Values are means and SE for four replicates per treatment. Statistical significance of differences with respect to control cells without DTPA addition.
***$P < 0.001$.

Table 2 Effects of Zn^{2+} addition to DTPA-treated cultures of C_2C_{12} cells on CAT activities from plasmids transfected into the cells

Plasmid	Activity in control (mU/mg protein)	Activity of treated cells relative to untreated controls (%) Concentration of Zn^{2+} added along with DTPA (μM)		
		50	100	400
3300MCKCAT	660	5 ± 1***	17 ± 2***	89 ± 6
1256MCKCAT	201	10 ± 1***	42 ± 6***	87 ± 10
1256MCKCAT Δ−1050 − 800	119	9 ± 1***	34 ± 10***	141 ± 16**
E206MCKCAT	681	5 ± 1***	29 ± 3***	89 ± 4
E110MCKCAT	160	11 ± 3***	22 ± 3***	87 ± 6**
776MCKCAT	49	21 ± 2***	69 ± 9**	99 ± 7
SV40CAT	34	44 ± 6***	61 ± 5***	96 ± 6

The structures of the plasmids investigated are those illustrated diagrammatically in Figure 1. The treatment groups each contained data from two experiments with four replicates per experiment except for the 776MCKCAT and SV40CAT groups that related to four and three experiments, respectively each consisting of four independent replications per group. Statistical significance of differences with respect to control cells without DTPA addition.
**$P < 0.01$, ***$P < 0.001$.

to minimize cell loss. This addition of 50 μM Zn permitted a limited degree of cell fusion and expression of creatine kinase which, however, averaged only 30% of that in the control cultures or in those supplemented with 400 μM Zn.

Transfection experiments

The extent of differentiation and cell fusion achieved in the 4 days after transfer of the myoblasts to differentiation medium varied to some extent from experiment to experiment with consequent inevitable variations in creatine kinase activity. However, the relative effects of DTPA were independent of the extent of differentiation and the responses of individual transfection constructs to lack of zinc were, therefore, assessed in relation to the activity in transfected control cultures not supplemented with either DTPA or zinc.

As found previously with chick myoblasts,[4] variations in creatine kinase activity were highly significantly correlated ($P < 0.015$) with corresponding differences in creatine kinase mRNA, suggesting that mRNA concentration was the prime determinant of enzyme activity. A similar dependence on mRNA concentration was seen in the transfected CAT activity with those constructs inducing detectable levels of CAT mRNA. However, in the absence of a muscle-specific enhancer, CAT mRNA concentrations derived from the 776MCKCAT and SV40CAT plasmids were too low to permit assessment of the mRNA response to zinc deprivation. Activity estimates were, therefore, used to assess the sensitivity of various promoters to zinc in C_2C_{12} cells transfected with CAT plasmids.

The CAT activity promoted by both the 3300 and 1256 MCKCAT plasmids was very sensitive to lack of zinc (*Table 2*). The region between −1050 and −800 bp relative to the transcription start site contains a putative binding site for the zinc finger protein Sp1, but removal of this section of the 5′-flanking region did not reduce its zinc sensitivity compared with that of the parent 1256MCKCAT plasmid. Similarly, those plasmids whose 5′-flanking region contained solely the full or truncated versions of the muscle-specific enhancer and the region from −80 to +7 bp relative to the transcriptional start site (E110MCKCAT and E206MCKCAT) were also fully sensitive to lack of zinc. In each case, the effects of DTPA were reversible by further supplementation with zinc. The absolute activities of the 776MCK and SV40 constructs that lacked a muscle-specific enhancer were low, but both were still inhibited by reduced availability of zinc. However, when the inhibitions of CAT activity in cultures treated with DTPA and only 50 μM or 100 μM Zn were compared between plasmids, the inhibition of CAT activity by low zinc availability was statistically greater with the constructs containing the muscle-specific enhancer than in those without it.

Myogenic factors

Muscle cell differentiation is associated with synthesis of myogenic transcription factors[22,23] and an inability to induce them could underlie the inhibition of differentiation of C_2C_{12} cells by lack of zinc. Furthermore, since activation of the muscle-specific enhancer in the 5′-flanking region of the creatine kinase gene partially depends on binding sites for these transcription factors within the enhancer, impaired production of the factors could explain the additional sensitivity to lack of zinc of the enhancer-containing constructs.

Northern hybridization of RNA from fused C_2C_{12} cells failed to detect mRNAs for myf5 and myf6, even in control cells; that agrees with the previous observation that these factors play little part in the differentiation of C_2C_{12} cells.[24,25] In contrast, the mRNAs for MyoD and myogenin were both present. The ratio of myoD to 18S ribosomal RNA was much less affected by lack of zinc during differentiation than that for myogenin, which was greatly reduced (*Table 3*). This marked decrease in myogenin mRNA concentration in zinc-deficient cells could result from either a reduction in the stability of the message or a failure in the transcription mechanism and these possibilities were investigated.

Table 3 Effects of Zn^{2+} addition to DTPA-treated cultures of C_2C_{12} cells on the concentrations of mRNA for MyoD and myogenin

Concentration of Zn^{2+} added along with 600 μM DTPA	MyoD mRNA (% of control)	Myogenin mRNA (% of control)
50 μM	83 ± 14	9 ± 2***
400 μM	125 ± 14	96 ± 11

MyoD and myogenin mRNA values were initially calculated as ratios to 18S rRNA and then these ratios for the DTPA-treated cultures were expressed as a percentage of the corresponding values in the control cultures. The results represent means ± SE of eight replicates. Statistical significance versus control cells without DTPA addition.
***$P < 0.001$.

Stability of MyoD and myogenin mRNAs

The stabilities of MyoD and myogenin mRNAs were assessed in control, DTPA/50 μM Zn and DTPA/400 μM Zn-treated cells (*Figure 3*) and their half lives calculated as described in the methods section. Zinc deficiency had little if any effect on the stability of the MyoD message. The calculated half lives were 3.3 hr in control cultures and 2.1 and 2.2 hr in DTPA-treated cultures with 400 or only 50 μM added zinc respectively. The corresponding values of myogenin were 8 and 5 hr for the control and DTPA/400 μM Zn cultures. Restricting the availability of zinc by adding only 50 μM Zn to DTPA-treated cultures appeared to stabilize the myogenin mRNA. Whether this apparent stabilization was of biological significance is questionable in view of the errors inherent in estimating a slow decline from a low initial mRNA concentration. However, there was no suggestion of an increased rate of degradation of the myogenin mRNA with zinc deficiency and the observed decrease in myogenin mRNA concentration cannot, therefore, be attributed to an increase in the degradation rate of the message.

In vitro transcription assays

In vitro transcription assays were performed with extracts from control, DTPA/50 μM Zn, and DTPA/400 μM Zn-treated cells to assess whether the observed effects on the MyoD and myogenin mRNA concentrations were due to a failure to synthesize the message. The results shown in *Table 3* refer to cytoplasmic mRNA concentrations from the same cells. The rate of synthesis of MyoD message was reduced by zinc deficiency to 65% of the control value and was returned to normal by supplementation of the cultures with adequate zinc (*Table 4*). The rate of mRNA synthesis for myogenin in zinc-deficient cultures decreased to less than 10% of the control value but again returned to normal with further zinc supplementation. The reduction in myogenin transcription rate was very similar to the decrease seen *Table 3* and, therefore, could account fully for the decrease in its mRNA concentration.

Discussion

Previous studies[4] had indicated that lack of zinc inhibited the induction of creatine kinase and prevented fusion of

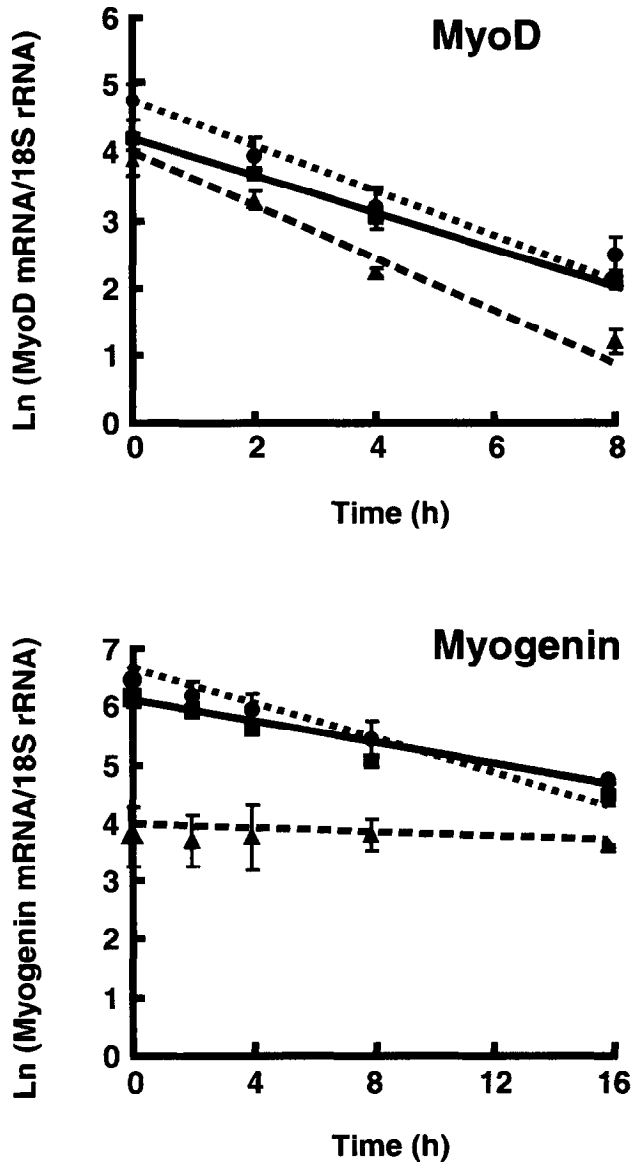

Figure 3 Effect of zinc status of C_2C_{12} cells on the breakdown of MyoD and myogenin mRNAs. The cellular concentrations of the mRNAs were determined at periods of up to 16 hr after actinomycin treatment of the cells.— control, ---- DTPA + 50 μM Zn, ------- DTPA + 400 μM Zn.

chick myoblasts. These observations, which have now been confirmed with the mouse C_2C_{12} cell line, pointed to a zinc requirement for the completion of differentiation of myoblasts, but gave no indication of the nature and timing of the requirement. Because the creatine kinase 5′-flanking region contains a consensus-binding site for the zinc-finger transcription factor Sp1, loss or inactivation of Sp1 due to the absence of adequate zinc could have resulted in decreased synthesis of creatine kinase mRNA. However, removal of the presumed Sp1 binding site from the creatine kinase 5′-flanking region failed to influence the loss of promoter strength associated with zinc deficiency when the 5′-flanking region was linked to the CAT reporter gene.

The absolute level of CAT activity varied substantially in

Table 4 Rates of synthesis of MyoD and myogenin mRNAs during in vitro transcription in vitro

Treatment	MyoD	Myogenin
	(counts per h/2 × 10^7 nuclei)	
Control	116 ± 26	598 ± 80
DTPA (600 μM)/Zn^{2+} (50 μM)	74 ± 18	46 ± 12***
DTPA (600 μM)/Zn^{2+} (400 μM)	146 ± 14	590 ± 52

Values are means and SEM for eight replicates. Statistical significance of differences with respect to control cells without DTPA addition.
***$P < 0.001$.

the different transfectants in response to the modified 5′-flanking regions of creatine kinase used to activate the reporter gene. However, when the CAT activities in the zinc-deficient cultures were expressed relative to those in corresponding control cultures, some of the zinc responsiveness was clearly associated with the region containing the muscle-specific enhancer. Thus CAT activity in the DTPA-treated cultures supplemented with only 50 or 100 μM Zn was significantly more sensitive to lack of zinc with those constructs containing the enhancer than with the 776MCK-CAT and SV40CAT constructs that lacked it.

Clearly, there were multiple effects of zinc deprivation and even the constructs lacking the enhancer retained significant sensitivity to zinc deprivation. However, the influence of the muscle-specific enhancer in promoting zinc sensitivity pointed to a possible involvement of zinc in the expression of myogenic factors. A range of such factors are known to bind to this enhancer including helix-loop-helix complexes.[24,26–28] Only two myogenic helix-loop-helix proteins, MyoD and myogenin, were detected in the present cell line. Of these MyoD is present in replicating myoblasts as well as in differentiated myotubes.[22,29] In contrast, myogenin is expressed at very low levels, if at all, in myoblasts, but is a very early marker for myoblast differentiation with marked increase in both protein and mRNA concentration even before the cells lose the ability in replicate.[11,12] It was of particular interest, therefore, that, whereas the mRNA concentrations of both factors were affected by lack of zinc, myogenin mRNA was depleted to a much greater extent. This points to a role for zinc in initiating differentiation rather than in modifying overall mRNA concentrations. Furthermore, the relative sensitivity of myogenin mRNA to inadequate zinc supply appeared to result from a failure to induce synthesis of the mRNA rather than excessive degradation of it.

A possible pattern appears to be emerging in the functions of zinc. Thus, zinc is required for successful passage of cells through mid-G1 towards DNA replication.[5] It now appears that it may be necessary for initiation of myoblast differentiation, a process also reported to commence in mid-G1.[6,7] If one further considers that lack of zinc has been implicated in stimulating apoptosis,[30–32] it seems that zinc may be required for passage through a critical decision point in mid-G1 common to each of these cell fates. If zinc is present in adequate amounts, cells will proceed either to replicate their DNA and divide or differentiate depending on environmental influences. However, if zinc availability is reduced to critical levels, the cells may be diverted to programmed cell death. Clearly such a role for zinc could explain many of the observed effects of zinc deficiency including its teratogenic effects during embryo development.[32] At present, the hypothesis is highly speculative, but the potential importance of zinc in determining the fate of cells is such that it merits further experimental investigation.

Acknowledgments

The authors wish to acknowledge the encouragement provided by Professor S.D. Hauschka and thank Dr. M. Franklin for help with statistical analysis. The gifts of plasmids containing cDNAs for MyoD and myogenin by Dr. H. Weintraub and Dr. W.E. Wright, respectively were greatly appreciated. The authors also acknowledge the major support of the work by the Scottish Office Agriculture and Fisheries Department. Additional support came from National Institutes of Health (U.S.A.) Grant 2R01AR18860 to Professor S.D. Hauschka

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