A requirement for Zn^{2+} for the induction of thymidine kinase but not ornithine decarboxylase in 3T3 cells stimulated from quiescence

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In 3T3 cells stimulated from quiescence by serum, impaired thymidine incorporation caused by inadequate supply of Zn^{2+} was associated with both decreased thymidine kinase activity and a comparable decrease in its mRNA concentration. In contrast, the amount of mRNA for ribosomal protein S6 was not affected, nor was the earlier increase in the activity of ornithine decarboxylase.

INTRODUCTION

In the absence of adequate Zn^{2+} , the normal increase in DNA synthesis which occurs approximately 16 h after serum stimulation of quiescent 3T3 cells is greatly decreased (Chesters *et al.*, 1989). However, this requirement for Zn^{2+} is largely restricted to a period from about 8 h after serum addition until 3 h before DNA synthesis starts. Completion of the Zn^{2+} -dependent process has been shown to require continuing mRNA and protein synthesis (J. K. Chesters & R. Boyne, unpublished work) and immediately precedes or overlaps with the induction of the enzymes necessary for DNA synthesis.

An increase in thymidine kinase (EC 2.7.1.21) activity has proved a convenient marker for the induction of these enzymes in a variety of systems (Schlosser *et al.*, 1981; Johnson *et al.*, 1982; Liu *et al.*, 1985; Coppock & Pardee, 1987), and dietary deficiencies of zinc have resulted in lower activities of thymidine kinase in foetuses from both rats and sheep (Dreosti & Hurley, 1975; Duncan & Hurley, 1978; Record & Dreosti, 1979; Masters & Moir, 1983). The latter observations led to the suggestion that thymidine kinase is a Zn^{2+} -dependent enzyme (Prasad, 1982), but there does not appear to be any evidence that the enzyme itself contains zinc. We report here on investigations of the effects of a lack of Zn^{2+} on the induction of thymidine kinase in cultures of 3T3 cells. Our results indicate that lower activity of the enzyme in Zn^{2+} -deficient cultures was associated with a corresponding decrease in the concentration of its mRNA. It thus appears likely that Zn^{2+} is necessary for the induction of thymidine kinase rather than for activation of the enzyme protein.

METHODS

Swiss 3T3 cells (European Collection of Animal Cell Cultures, Porton Down, Wilts., U.K.) were subcultured once and then stored frozen in liquid N_2 until required. Cells for experiment were recovered from liquid N_2 and allowed to grow without reaching confluence for one to two subcultures before use.

The cells were grown in Dulbecco's minimal essential medium with additional penicillin (50 units/ml), streptomycin (50 $\mu\text{g}/\text{ml}$) and fungizone (2.5 $\mu\text{g}/\text{ml}$). Initially this medium was supplemented with 12% (v/v) foetal bovine serum (FBS) and the cells were grown for 3 days. They were then transferred to medium containing only 2% (v/v) FBS for 48 h to induce quiescence. At all times the cells were grown in Petri dishes at 37 °C in a

humidified atmosphere containing air/ CO_2 (9:1). All medium ingredients were obtained from Gibco (Paisley, U.K.).

Experiments were initiated by returning quiescent cells to medium containing 12% (v/v) FBS. Where appropriate, the cultures were immediately supplemented with diethylenetriaminepenta-acetic acid (DTPA) at a final concentration of 600 μM and FeSO_4 at 400 μM . This treatment has been shown to decrease Zn^{2+} availability without impairing significantly the availability of other divalent cations (Chesters *et al.*, 1989). Other cultures were further supplemented with ZnSO_4 to a final concentration of 400 μM , which is sufficient to reverse the inhibition of thymidine incorporation caused by DTPA/ Fe^{2+} .

Assay of thymidine kinase activity

This was based on the method of Stuart *et al.* (1985). Cells were harvested in phosphate-buffered saline (8.1 mM- Na_2HPO_4 /1.9 mM- NaH_2PO_4 /150 mM- NaCl , pH 7.4), collected in micro-centrifuge tubes and broken in 0.25 ml of Nonidet P40 reagent [50 mM-Tris/HCl, pH 8, 3.6 mM-2-mercaptoethanol and 0.5% (v/v) Nonidet P40] by ultrasonication for three 10 s intervals separated by 1 min in ice. A sample was taken for determination of total protein (Lowry *et al.*, 1951) and the remainder was centrifuged at 11 000 *g* for 30 min at 4 °C. The supernatant was then used for the thymidine kinase assay.

Triplicate reaction mixtures containing 40 μl of reaction buffer (75 mM-Tris/HCl (pH 8)/30 mM- NaF /5.4 mM-2-mercaptoethanol/5 mM- MgCl_2 /160 μM -thymidine) and 20 μl of [^3H]thymidine (40 $\mu\text{Ci}/\text{ml}$, 24 $\mu\text{Ci}/\text{nmol}$; Amersham International) were prepared, and one (sample A) was also supplemented with 5 mM-ATP. To two of the samples (A and B) was added 20 μl of the enzyme preparation and the samples were incubated at 37 °C for 30 min. At the end of the incubation the third reaction mixture (C) also received 20 μl of enzyme preparation, and all three samples were heated at 100 °C for 2 min followed by transfer to ice. The samples were then stored frozen until required.

After centrifugation, samples (20 μl) of the supernatants from B and C and two from A were spotted on to individual 2.5 cm discs of DE81 cellulose and left to air dry for 10 min. One disc from each of the three samples was then shaken gently in 10 ml of ammonium formate (1 mM) for 30 min at room temperature. The fluid was discarded and the filters were washed with three further portions of ammonium formate. The washed filters and

Abbreviations used: FBS, foetal bovine serum; DTPA, diethylenetriaminepenta-acetic acid.

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the second unwashed filter from sample A (D) were transferred to 20 ml scintillation vials and extracted for 30 min with 1 ml of 100 mM-HCl/200 mM-KCl before addition of 9 ml of liquid scintillation fluid (NE265; Nuclear Enterprises, Sighthill, Edinburgh, U.K.). Radioactivity in the samples was then determined by liquid scintillation counting.

The fraction of the thymidine converted to thymidine monophosphate was estimated from the following:

$$\text{Conversion} = \frac{\text{Counts in A} - \text{counts in B}}{\text{Counts in D} - \text{counts in C}}$$

and then the thymidine kinase activity was calculated by:

$$\text{Activity (nmol/min per mg of protein)} = \frac{\text{Conversion} \times 6.43}{30 \times \text{protein per assay (mg)}}$$

where the protein per assay refers to the equivalent amount of total cellular protein per assay.

Measurement of thymidine kinase and S6 ribosomal protein mRNA

Cells were prepared as described above, and 20 h after serum stimulation total RNA was isolated by the method of Chomczynski & Sacchi (1987). After quantification by absorption at 260 nm, 25 µg portions of total RNA were fractionated in triplicate by electrophoresis in 1.2% (w/v) agarose gels run under denaturing conditions in the presence of 6.7% (w/v) formaldehyde. The RNA was then transferred to a nylon membrane (Genescreen; NEN Research Products, Boston, MA, USA) by capillary blotting and fixed by short-wavelength u.v. irradiation.

The blots were hybridized with mouse thymidine kinase cDNA isolated from pMTK4 (ATCC no. 37556; Lin *et al.*, 1985) and labelled with [³²P]dCTP by random priming (Amersham). After suitable washing, the blots were then subjected to autoradiography.

After development of the initial autoradiographs, the blots were prepared for re-probing by heating in 0.1% (w/v) SDS at 90 °C for 5 min and were then rehybridized with a probe for the S6 ribosomal protein (pHS6-2B; Lott & Mackie, 1988). After washing, the blots were again subjected to autoradiography.

Quantification of autoradiographs

The absorbance of individual spots on autoradiographs was quantified by image analysis using a Triple X computer (Torch Computers, Cambridge, U.K.) linked to a Hitachi KP-140 video camera to determine the average decrease in light intensity over a predetermined rectangle set to cover just the area of the largest spot on each autoradiograph. The size and shape of the rectangle were held constant while the density of each of the spots on the autoradiograph was measured, thus allowing direct comparison of the intensities of the spots on a film.

Each gel was used to separate triplicate samples of two different RNA preparations for each of the three treatments: control, DTPA/Fe²⁺ and DTPA/Fe²⁺ plus Zn²⁺, and the averages for the triplicates were used for two-way analyses of variance, with treatment and gel as factors. Although there were significant differences between gels in the values for thymidine kinase and S6, these were not apparent when the data were analysed as the ratios of thymidine kinase/S6 mRNA for individual gel lanes.

Assay of ornithine decarboxylase (EC 4.1.1.17)

Cells harvested as for the thymidine kinase assay were dis-

rupted in 50 mM-phosphate buffer (pH 7.2)/5 mM-dithiothreitol/1 mM-pyridoxal phosphate using an UltraTurrax homogenizer (Janke & Kunkel, Staufen, Germany) run at full speed for 45 s. After removal of a sample for estimation of total cellular protein, the remainder was centrifuged at 11 000 *g* for 45 min at 4 °C and the supernatant was used for the enzyme assay.

The enzyme preparation (0.4 ml) was incubated with 0.1 ml of 1.05 mM-[1-¹⁴C]ornithine (5 µCi/ml; Amersham International) in a 3 ml glass specimen tube sealed with a rubber septum cap. Immediately before sealing, 0.2 ml of 1 M-Hyamine hydroxide contained in a small glass tube was inserted into the specimen tube to absorb CO₂. After incubation with shaking at 37 °C for 30 min, 0.2 ml of 50% (w/v) trichloroacetic acid was injected into the enzyme mixture and shaking was continued for a further 45 min. Absorbed ¹⁴CO₂ was determined by liquid scintillation counting of 100 µl of the Hyamine hydroxide in 4.5 ml of NE260 scintillation fluid. Total radioactivity in the enzyme assays was determined by counting 10 µl of the 1.05 mM-[¹⁴C]ornithine solution under similar conditions, and the activity present in a blank incubation (with buffer substituted for the enzyme preparation) was used to correct for any non-enzymic release of radioactivity. The fraction of the total radioactivity released by the enzyme and the ornithine concentration of the incubation mixture were used to calculate the rate of ornithine decarboxylation, which was expressed per mg of the original total cellular protein.

RESULTS AND DISCUSSION

Previous studies (Schlosser *et al.*, 1981; Coppock & Pardee, 1985) have indicated that rates of thymidine incorporation increase from about 14–16 h after serum stimulation of quiescent 3T3 cells, and our previous results showed that this rise in incorporation was inhibited in the absence of adequate Zn²⁺ caused by addition of the chelator, DTPA, even in cultures supplemented with Fe²⁺ (Chesters *et al.*, 1989). Full recovery of activity depended specifically on further addition of Zn²⁺. Since, as indicated above, dietary deficiencies of Zn²⁺ have been

Fig. 1. Time course of thymidine activity in quiescent 3T3 cells stimulated by transfer to serum-containing medium

Serum-deprived cells were incubated from time zero in medium containing 12% (v/v) FBS with DTPA/Fe²⁺, with (■) or without (□) Zn²⁺. Values are means of duplicate preparations from each of two experiments.

Table 1. Thymidine kinase (TK) and ribosomal protein S6 mRNA concentrations in total RNA isolated from 3T3 cells after serum stimulation in the presence or absence of adequate Zn²⁺

Cells were incubated with or without DTPA/Fe²⁺ and Zn²⁺ and RNA was extracted 20 h after serum stimulation. Values represent means for four RNA preparations for each treatment, each preparation being separated on duplicate gels. ** Significantly different from control ($P < 0.01$).

Condition	TK mRNA (absorbance units)	S6 mRNA (absorbance units)	TK/S6 ratio
Control	0.123	0.254	0.496
DTPA/Fe ²⁺	0.004**	0.241	0.025**
DTPA/Fe ²⁺ /Zn ²⁺	0.112	0.256	0.486
Within-column standard error of difference	0.024	0.054	0.093

associated with decreased thymidine kinase activity, the enzyme's activity was measured in 3T3 cells in the presence of DTPA/Fe²⁺ with and without adequate Zn²⁺ (Fig. 1). It is clear that lack of Zn²⁺ inhibited the usual increase in thymidine kinase activity and that this could be restored by supplementation with Zn²⁺. The time course of the increase in activity in the Zn²⁺-supplemented cultures was similar to that reported previously in control cultures (Schlosser *et al.*, 1981; Coppock & Pardee, 1985).

The varied biochemical effects of lack of Zn²⁺ on cell replication and animal growth had led to the hypothesis that Zn²⁺ is involved in the regulation of the genetic expression of the enzymes required for DNA synthesis (Chesters, 1978, 1982; Vallee & Falchuk, 1981). The effects of low Zn²⁺ availability on the levels of thymidine kinase mRNA were therefore investigated at a time when the Zn²⁺-dependent differences in thymidine kinase activity were maximal (Table 1). It is clear that lack of Zn²⁺ lowered the concentration of thymidine kinase mRNA to an extent which was closely comparable with the corresponding depression in activity of the enzyme. This effect was fully preventable by addition of Zn²⁺ and was not associated with the death of the cells, since in previous investigations cells maintained in medium containing these levels of DTPA/Fe²⁺ could be stimulated to enter S phase even when addition of Zn²⁺ was delayed for 20 h after serum stimulation (Chesters *et al.*, 1989). Furthermore, lack of Zn²⁺ did not influence the levels of another mRNA, that for the ribosomal protein S6, which was selected for comparison since it appeared unlikely to be cell-cycle-dependent. The effect on thymidine kinase mRNA was not therefore the result of a generalized decrease in mRNA synthesis, such as might have been expected if the activity of RNA polymerase II had been decreased.

Another enzyme which increases in activity following serum stimulation of 3T3 cells is ornithine decarboxylase, its activity reaching a maximum between 4 and 6 h after stimulation (Stimac & Morris, 1987; Katz & Kahana, 1987; Gilmour & O'Brien, 1989). Previous experiments (Chesters *et al.*, 1989) suggested that Zn²⁺ supplementation of DTPA/Fe²⁺-treated cultures was unnecessary until 8 h after stimulation to permit normal initiation of S phase. It was of interest, therefore, to see whether the increase in ornithine decarboxylase activity, which has also been suggested to relate to increases in its mRNA, would be influenced by lack of Zn²⁺ during the first 4 h after stimulation of the cells. Average activities of ornithine decarboxylase (nmol/h per mg of total cellular protein) for two experiments were: control (un-

treated) before stimulation, 2.1; after 4 h stimulation, 9.6; after 4 h in the presence of DTPA/Fe²⁺, 10.8; after 4 h with DTPA/Fe²⁺/Zn²⁺, 12.4. This confirms the apparent insensitivity of 3T3 cells to lack of Zn²⁺ during the initial period after serum stimulation from quiescence.

The regulation of thymidine kinase activity is highly complicated, with evidence for effects at transcriptional, post-transcriptional and translational levels (Coppock & Pardee, 1987; Sherley & Kelley, 1988; Lipson *et al.*, 1989). However, neither thymidine kinase mRNA nor pre-mRNA was detectable in quiescent fibroblasts (Lipson & Baserga, 1989), and there is considerable evidence, including that in the present paper, for a link between the increase in mRNA concentration and activity of thymidine kinase in fibroblasts stimulated from quiescence (Stuart *et al.*, 1985; Coppock & Pardee, 1987). The origin of the increase in mRNA is less clear. Some authors have suggested that the rate of transcription, as determined by nuclear run-off experiments, increases only 2–4 fold, compared with a 10–20 fold increase in mRNA content (Coppock & Pardee, 1987). Others have demonstrated a 10-fold increase in thymidine kinase pre-mRNA under similar circumstances (Lipson & Baserga, 1989). Whether the Zn²⁺-dependent alterations in thymidine kinase activity relate to increased rates of transcription or to variations in survival of its mRNA awaits further experimental clarification, but it appears clear that under the present circumstances the changes in activity were correlated with alterations in mRNA concentration and were unlikely to have been related to direct activation of the enzyme protein.

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