

## Lymphocyte metallothionein mRNA responds to marginal zinc intake in human volunteers

Adrian K. Allan<sup>1</sup>, Gabrielle M. Hawksworth<sup>2</sup>, Leslie R. Woodhouse<sup>3</sup>, Barbara Sutherland<sup>3</sup>, Janet C. King<sup>3</sup>, and John H. Beattie<sup>1\*</sup>

<sup>1</sup>*Nutrient-Gene Interaction Group, Rowett Research Institute, Aberdeen, UK*

<sup>2</sup>*Departments of Medicine and Therapeutics and Biomedical Sciences, University of Aberdeen, Aberdeen, UK*

<sup>3</sup>*Department of Nutritional Sciences, University of California at Berkeley, Berkeley, CA, USA*

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Marginal Zn deficiency is thought to be prevalent in both developed and developing countries. However, the extent of Zn deficiency is not known, due to the lack of a reliable diagnostic indicator. Blood plasma and erythrocyte concentrations of metallothionein (MT) reflect Zn status, but measurement of MT is dependent on the availability of sensitive immunoassays. Our aim was to show whether measurement of T lymphocyte MT-2A mRNA, using a competitive reverse transcriptase (RT)–polymerase chain reaction (PCR) assay, could indicate Zn status in human subjects in a residential Zn-depletion study. In the study, the Zn intake of seven volunteers was maintained at 13.7 mg/d for 5 weeks (baseline) followed by 4.6 mg/d for 10 weeks (marginal intake) and then 13.7 mg/d (repletion) for 5 weeks. The quantitative assay was developed using standard techniques and concentrations of MT-2A mRNA were normalized by reference to  $\beta$ -actin mRNA which was also measured by competitive RT–PCR assay. An alternative method of measuring the PCR product using capillary electrophoresis with laser-induced fluorescence detection was also evaluated. There was considerable inter-individual variation in MT-2A mRNA concentration and the mean level at the end of the baseline period was 10.3 (SE 3.7) fg MT-2A mRNA/pg  $\beta$ -actin mRNA, which then decreased by 64 % during the low Zn intake period. After repletion, MT-2A mRNA returned to baseline concentrations. In contrast, plasma Zn was unchanged by marginal Zn intake or repletion. The effect of low Zn in all individuals was consistent. We conclude that this assay is a sensitive method of evaluating marginal changes in dietary Zn intake.

### Metallothionein: RT–PCR: Zinc deficiency: Capillary electrophoresis

As Zn is essential for the structure and function of many enzymes and transcription factors, it is not surprising that severe Zn deficiency can elicit a wide range of pathological effects (Aggett, 1989; Vallee & Falchuk, 1993). Severe deficiency is relatively uncommon in developed countries, but it has long been suspected that marginal Zn deficiency is prevalent worldwide, chronically affecting growth, development and immune function. Indeed, Hambidge *et al.* (1972) noted that short stature, low weight and poor appetite in preschool infants and children in Denver, Colorado, USA, could be ameliorated by Zn supplementation. Subsequent studies in different countries demonstrated a similar response to Zn supplementation (Walravens *et al.* 1983; Chen *et al.* 1985; Smit-Vanderkooy & Gibson, 1987). Zn deficiency has also been attributed to poor

bioavailability due to the presence in the diet of chelating agents, such as phytate (Sandström *et al.* 1980), or an abundance of antagonists of absorption, such as Ca (Davies *et al.* 1985).

Marginal Zn deficiency in the free-living human population is not easily diagnosed because many independent factors can influence the biochemical variables that correlate with Zn status under controlled experimental conditions. For example, plasma and urinary Zn are unreliable indicators of Zn status because they are also markedly affected by stress and infection (Solomons, 1979). Measurement of other Zn-dependent factors, including thymulin, alkaline phosphatase and 5'-ectonucleotidase have been proposed for the diagnosis of Zn deficiency, but they are of varying sensitivity and

**Abbreviations:** CE, capillary electrophoresis; MT, metallothionein; PCR, polymerase chain reaction; RT, reverse transcriptase.

\* **Corresponding author:** Dr John H. Beattie, fax +44 1224 716629, email J.Beattie@rri.sari.ac.uk

specificity (Baer *et al.* 1985; Prasad *et al.* 1988; Meftah *et al.* 1991). Thus, the prevalence of Zn deficiency in human populations is not well defined and there is a clear need for better diagnostic indicators.

On a low-Zn diet, deficiency occurs rapidly because the readily-exchangeable body pool of Zn, located in liver, plasma and possibly elsewhere, is quickly depleted and is not sufficiently replenished from body Zn pools with a slower turnover. Hence, stable-isotope techniques have been used to estimate the exchangeable Zn pool size and therefore the apparent level of deficiency (Miller *et al.* 1994). However, analysis of stable isotopes requires expensive instrumentation and a more readily quantifiable indicator of this exchangeable pool would be useful.

Metallothionein (MT) has a high Zn-binding capacity; indeed, it is one of the strongest biological binding ligands for Zn. MT gene expression is also regulated by Zn, and the close correlation between Zn and MT in tissues such as liver and pancreas is well documented (Sato *et al.* 1984; Bremner, 1987). Since the concentration of MT in plasma is closely related to liver MT concentrations, it appears to reflect changes in the metabolic Zn pool and has been proposed as a diagnostic indicator of Zn deficiency (Golden, 1989). Unfortunately, plasma MT concentrations are low and there are no commercially-available immunoassays that are suitable for its analysis in human blood. In rat studies, plasma MT concentrations are also influenced by, for example, stress and infection. The utility of measuring MT concentrations in erythrocytes has therefore been evaluated. Although erythrocyte MT concentrations decrease in Zn deficiency and are less influenced by Zn-independent factors, MT is concentrated in the reticulocyte fraction and can therefore change in response to factors influencing the rate of erythropoiesis, such as Fe deficiency (Bremner, 1993). Attempts have been made to analyse leucocyte MT by an ELISA system, but interferences have proved problematic (Branca, 1989).

In contrast to protein analysis by immunoassay, mRNA can be measured with the required sensitivity using commercially-available reagents. In this study, we investigated the utility of reverse transcriptase (RT)-polymerase chain reaction (PCR) for the analysis of MT-2A mRNA concentrations in human T lymphocytes and the responsiveness of this indicator to changes in Zn status in volunteers on a controlled marginal Zn intake and repletion study. We describe a double competitive PCR technique in which  $\beta$ -actin is also quantified to correct for any RNA degradation.

## Materials and methods

### *Human subjects and experimental design*

A 20-week human marginal Zn intake study was performed at the Metabolic Research Unit of the Western Human Nutrition Research Centre in San Francisco, CA, USA. This study was approved by the Human Subjects Institutional Review Boards of the University of California, Berkeley, CA, USA and the US Department of Agriculture. Seven healthy well-nourished adult Caucasian men were recruited for the study. Their ages ranged from 27 to 47

years, they weighed an average of 77 kg, were on average 1.79 m tall, and had a BMI of 20–31 kg/m<sup>2</sup>. During a 5-week baseline period, volunteers lived at home for 3 weeks and then in the metabolic unit for a further 2 weeks. There was then a 10-week marginal Zn-intake period and a 5-week Zn-repletion period, during which the volunteers remained in the metabolic unit. They were fed conventional foods (3-d rotating menu), giving a daily Zn intake from food of 4.6 mg. A Zn supplement of 9.1 mg/d, as the gluconate salt, was given orally during the baseline and repletion periods so that the subjects consumed a total of 13.7 mg Zn/d. This total intake was similar to that consumed by volunteers prior to starting the study. The supplements were given by capsule in three equal parts with breakfast, lunch and dinner. During the low-Zn period, the subjects received placebo capsules. Energy intake during the study was 12.6–14.6 MJ (3014–3493 kcal)/d (160–240 kJ (38.3–57.5 kcal)/kg body weight per d), protein intake was 61.6–73.9 g/d (0.8–1.1 g/kg body weight per d). Mg, Ca, and Fe supplements were added to the diet to meet the recommended dietary allowance for those minerals; the intake of all other nutrients either met or exceeded the recommended dietary allowance (Food and Nutrition Board, National Research Council, 1989) from the diet alone.

Fasting venous blood samples were obtained at the end of each dietary period. Blood was drawn into Sarstedt syringes (Sarstedt, Inc., Newton, NC, USA) containing ammonium heparin-coated beads (15 IU, heparin/ml blood). All blood samples were placed on ice for no more than 1 h and then centrifuged in a Sorvall RC-5C centrifuge (Sorvall Instruments, Dupont Corp., Wilmington, DE, USA) under refrigeration for 10 min at 1145 g. The plasma was transferred using polypropylene transfer pipettes into new polypropylene tubes. Samples were stored at –70°C and were then prepared for Zn analysis by 8-fold dilution with 0.125 M-HNO<sub>3</sub> (trace metal grade; Fisher Scientific, Pittsburgh, PA, USA) and centrifugation. The Zn concentrations in the supernatant fractions were analysed by atomic absorption spectrophotometry using a Smith-Hieftje-22 Atomic Absorption Spectrophotometer (Thermo Jarrell Ash Corporation, Franklin, MA, USA) with background correction. A standard plasma sample was analysed with every batch of samples as a quality control and all-plastic ware used throughout sample preparation and analysis was trace-element-free. T-cell samples were also obtained at the end of each dietary period by resuspending the buffy coat in PBS and following the procedure described later (p. 751).

For development of the competitive RT-PCR assay, blood samples were obtained from healthy volunteers in the Aberdeen (UK) area. Venous blood (10–20 ml) was withdrawn into 9 ml 'Vacuette' tubes (Greiner Labortechnik, Gloucester, Glos., UK) containing lithium heparin. The collection tubes were stored on ice for no more than 1 h. The blood was diluted with an equal volume of ice-cold PBS and T-cells were separated as described later (p. 751).

### *Separation of human T lymphocytes*

Buffy coat suspended in PBS or diluted blood was carefully

layered onto an equal volume of Histopaque 1077 (Sigma Chemical Company, Poole, Dorset, UK) in a 30 ml Universal tube. After centrifugation at 700 g for 30 min, the layer of leucocytes at the plasma–Histopaque interface was removed using a Pasteur pipette and transferred to a fresh tube. The volume was made up to 10 ml with sterile PBS and the tubes were centrifuged at 250 g for 10 min to pellet the cells, which were then resuspended in 1 ml sterile PBS. M-450 Pan T Dynabeads (CD-2; Dynal, Wirral, Merseyside, UK) were added to the buffy coat, as specified by the manufacturer. Approximately 2.5 beads per T lymphocyte were used. The samples were then gently mixed for 20 min at 2–4°C on a rotary mixer. The rosetted cells were isolated by placing them in a magnetic particle concentrator (Dynal MP; Dynal) for 2–3 min. The supernatant fraction was aspirated and the tubes were removed from the rack. The beads were resuspended in 1 ml ice-cold fetal calf serum (20 ml/l) in PBS and the tubes were replaced in the magnetic particle concentrator. This washing step was repeated four times. The beads with cells attached were then either quick-frozen in liquid N<sub>2</sub> and stored at –80°C, or RNA extraction was performed directly. Samples prepared at the Western Human Nutrition Research Centre, San Francisco, CA, USA were transported in dry ice to the Rowett Research Institute, Aberdeen, UK for extraction of RNA and RT–PCR analysis.

#### *Extraction of RNA*

In the case of freshly-prepared or frozen T lymphocytes attached to Dynabeads, 1 ml RNazol B (Biogenesis, Poole, Dorset, UK) was added to each tube, and RNA extraction was performed according to the manufacturer's instructions. The absorbance of diluted aliquots from the solubilised RNA solutions was measured at 260 and 280 nm using a GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK). The concentration of RNA was calculated assuming that 40 µg RNA/ml has an absorbance of 1 at 260 nm, and  $A_{260}:A_{280}$  was calculated to evaluate RNA purity. The RNA samples were stored at –80°C until required.

#### *Reverse transcription of RNA*

A sample of solution of known RNA concentration (1–5 µg) was added to 125 ng random primers (Promega, Southampton, Hants., UK), followed by the addition of sterile water to give a total volume of 12 µl. The mixture was heated to 70°C for 10 min to release any RNA secondary structure and was then chilled on ice for 30 s. The following reagents were added to each tube: 2 µl 0.1 M-dithiothreitol, 4 µl first-strand buffer (250 mM Tris HCl, 375 mM-KCl, 15 mM-MgCl<sub>2</sub>), 1 µl 10 mM-dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH; Promega), and 1 µl 200 U Superscript II reverse transcriptase (BRL, Paisley, Strathclyde, UK)/l. The mixture was heated at 42°C for 50 min and the reaction was then inactivated by heating at 70°C for 15 min. The cDNA reaction solution was diluted to give a known

concentration of reverse-transcribed RNA and was either stored at –80°C or used directly for PCR.

#### *Preparation of human metallothionein-2A competitive standard*

Quantification of reverse-transcribed mRNA by non-competitive PCR is unreliable, because measurement of the PCR product must be made over the linear phase of amplification, which has to be determined empirically. Additionally, when target mRNA is present at low concentrations and the complementary DNA is amplified within the linear range, measurement of the product may require sensitive detection techniques (e.g. radioactive probes). A further limitation is that between-sample differences can vary greatly (up to 6-fold) in duplicate reactions (Gilliland *et al.* 1990).

These problems can be avoided using competitive RT–PCR, which involves the addition of a standard cDNA that competes with the target for PCR amplification. The standard has the same forward and reverse primer-binding sequences, but contains a deletion or insertion so that the PCR product size is different from that of the target, which makes them separable by electrophoresis. With the primers used in the present study the target product size for human MT-2A was 278 bp, and a standard containing an 80 bp deletion was made using an MT-2A cDNA template (supplied as an insert in a pSVL plasmid vector), and an ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK) with the following primers:

forward primer

5'-GCC CCG CTG GGT CAT GTA AAG AAC 3'

reverse primer (5'-phosphorylated)

5'-TGT CCC GTC GTG GAG CAG CAG C 3'

The method for PCR-directed mutagenesis was followed according to instructions supplied with the kit, using twelve cycles, an annealing temperature of 72°C and a buffer composition of 100 mM-Tris HCl (pH 8.8), 35 mM-MgCl<sub>2</sub> and 250 mM-KCl. *Epicurian coli* XL-1-Blue supercompetent cells (included with the kit) were transformed with the mutated plasmid before selection on Luria-Bertani (LB)–ampicillin plates. Transformants were selected and grown in LB–ampicillin medium for plasmid preparation. The plasmids were purified using silica columns (Qiagen, Crawley, West Sussex, UK) and PCR analysis, using the MT-2A primers and the conditions described later (p. 753), was used to identify plasmids containing the deletion. The mutant MT-2A cDNA was ligated into the pGEM-T vector using the pGEM-T Easy Vector System I (Promega). The pGEM plasmid containing the MT-2A deletion insert was purified on silica columns (Qiagen) and quantified spectrophotometrically.

#### *Competitive polymerase chain reaction for human metallothionein-2A*

After reverse transcription, MT-2A cDNA (target) was

amplified by PCR. The primer sequences for MT-2A amplification were:

forward primer 5'-GCA ACC TGT CCC GACTCTAG 3'

reverse primer 5'-ATC CAG GTT TGT GGA AGT CG 3'

PCR reaction mixtures were prepared in a PCR workstation and reagents were dispensed using positive-displacement pipettes. A 25 µl PCR reaction contained 0.2 mM dNTP mix, 10 mM-Tris HCl, pH 8.3, 50 mM-KCl, 1.5 mM-MgCl<sub>2</sub>, 0.25 µM-MT-2A primer mix, 1.25 units Taq DNA polymerase (Amplitaq Gold; Perkin Elmer, Warrington, Ches., UK), 2.5 µl of each cDNA sample and 2.5 µl competitive DNA standard in the pGEM vector. The tubes were centrifuged to concentrate the contents and a drop of mineral oil (Promega) was added to overlay the reaction contents and prevent evaporation. A Hybaid Omn-E thermal cycler (Hybaid, Ashford, Middx, UK) was used for PCR according to the following conditions: 95°C for 10 min, 1 cycle; 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, 35 cycles; 72°C for 5 min, 1 cycle.

Samples were then kept on ice or stored at 4°C until required.

#### *Competitive polymerase chain reaction for human β-actin*

The amount of amplified product from PCR of targeted cDNA can be normalised by reference to a standard 'housekeeping gene', such as glyceraldehyde-3-phosphate dehydrogenase and β-actin. However, the rate of amplification of target and housekeeping cDNA can be variable because their sequence lengths may be quite dissimilar and the primers and optimal PCR conditions used will be different. In order to overcome these potential problems and to control for any RNA degradation in the samples, we used a competitive PCR assay to quantify human β-actin mRNA.

The competitive standard for β-actin, containing a 151 bp deletion was supplied in a pCRII vector (In Vitrogen NV, Leek, The Netherlands). The PCR product from this competitive standard using β-actin primers was 392 bp, compared with the target PCR product of 543 bp. The primer sequences for β-actin PCR were as follows:

forward primer ATC GTG GGG CGC CCC AGG CAC

reverse primer CTC CTT AAT GTC ACG CAC GAT TTC.

Reaction mixtures were prepared as described earlier for MT-2A, and PCR conditions were as follows: 95°C for 10 min, 1 cycle; 95°C for 1 min, 60°C 1 min, 72°C 1 min, 35 cycles; 72°C for 5 min, 1 cycle. The competitive standard plasmid was serially diluted to produce a series of known concentrations and these were added to a constant amount of cDNA before PCR, as described.

#### *DNA gel electrophoresis of polymerase chain reaction products*

The PCR reactions were analysed by electrophoresis in

1.8 % agarose gels with Tris acetate-EDTA buffer containing 0.5 µg ethidium bromide/ml. After electrophoresis, the gels were examined under u.v. light and photographed or scanned using a UVP Image store 5000 system (UVP, Cambridge, UK). Band intensity was quantified using image analysis software (Phoretix 1D, version 3.0; Phoretix, Newcastle, Tyne and Wear, UK).

#### *Capillary electrophoresis of polymerase chain reaction products*

Capillary electrophoresis (CE) is a capillary-based high-resolution electrophoretic technique which has previously been applied to the separation of PCR products. In addition to improved resolution CE has advantages over gel electrophoresis for quantification of separated products, and we therefore compared the utility of these two separation techniques. A P/ACE 5000 capillary electrophoresis instrument equipped with a laser-induced fluorescence detector (Beckman Coulter, Fullerton, CA, USA) was used in the reverse polarity mode (negative potential at the injection end of the capillary). The laser-induced fluorescence detector has an off-board Ar ion source and excitation was at 488 nm with emission at 520 nm. A 570 mm or 270 mm DB-17-coated capillary was used (500 mm or 200 mm to detector, 100 µm i.d.; J & W Scientific, Folsom, CA, USA). The temperature of the cartridge was set at 25°C and CE separations were performed using a field of 175 V/cm. DNA separation buffer for CE (Sigma Chemical Co.), which consisted of a proprietary sieving medium prepared in buffer (89 mM-Tris, 89 mM-boric acid, 2 mM-EDTA, pH 8.5), was used as electrolyte. Before CE separations, SYBR I green (Molecular Probes Europe, Leiden, The Netherlands) was added to the buffer at a dilution of 1:30 000 (v/v) and mixed for 15 min. The 100 bp DNA ladder (Promega) was diluted to the required concentration before separation by CE. The PCR reaction mixtures were analysed directly after amplification, without further sample preparation. The samples were either electrokinetically injected at a field of 35 V/cm, or pressure injected (10 s). Before sample injection the capillaries were washed with methanol and sterile water. A voltage was then applied to the capillary containing separation buffer until a stable baseline was achieved. Data were collected and analysed using System Gold software (Beckman Coulter High Wycombe, Bucks., UK).

#### *Statistics*

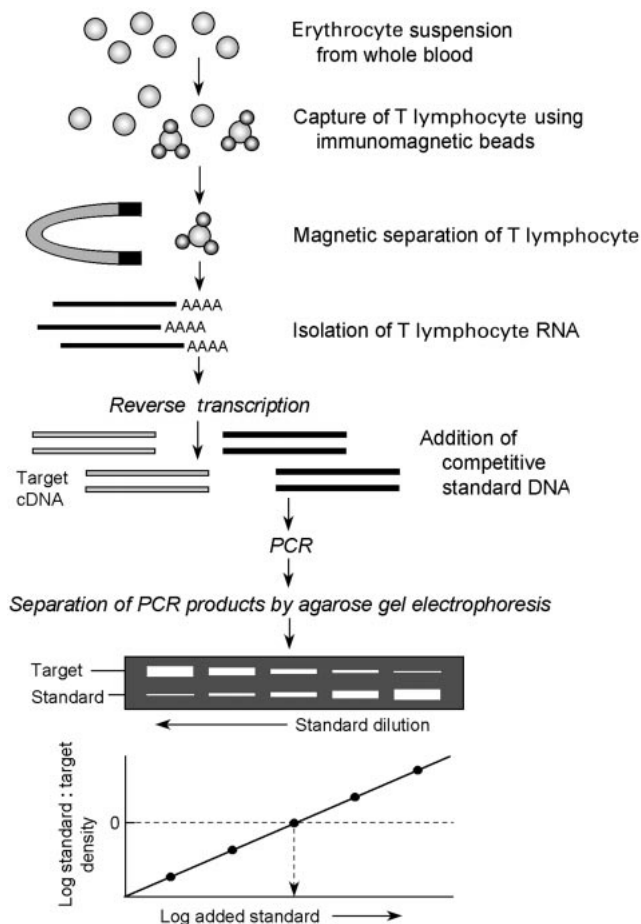
Differences between two dietary periods were assessed by a paired sign test, in case the data were not normally distributed.

## **Results**

#### *Competitive reverse transcriptase-polymerase chain reaction for metallothionein-2A*

An overview of the competitive RT-PCR assay for human MT-2A is shown in Fig. 1. PCR of the reverse-transcribed





**Fig. 1.** Schematic diagram of the procedure for T lymphocyte preparation and reverse transcriptase–polymerase chain reaction (PCR) analysis of metallothionein and  $\beta$ -actin mRNA.

RNA yielded a single product of approximately 300 bp (Fig. 2(A)), thus confirming the predicted product size of 278 bp. The competitive mutant standard gave a product of approximately 200 bp (Fig. 2(A)), which was also close to the predicted value of 198 bp. Both products were cloned and sequenced to demonstrate that the correct target DNA was being amplified (data not shown). Increasing the concentration of the competitive standard decreased the yield of product from PCR of MT-2A cDNA and the bands were quantified by densitometry. Plotting standard:target density *v.* the concentration of added standard, revealed a linear relationship from which the target concentration could then be determined by extrapolation from the equivalence point (a density ratio of 0).

Single products for  $\beta$ -actin (543 bp) and the  $\beta$ -actin competitive standard (392 bp) were obtained (Fig. 2(B)) and linear plots of their relative density in relation to increasing amounts of added standard permitted quantification of the target (Fig. 2(B)).

Concentrations of MT-2A and  $\beta$ -actin mRNA in T lymphocytes from volunteers of Scottish origin not on a controlled dietary regimen were found to be in the femtogram and picogram range per 200 ng of reverse-transcribed RNA respectively. When expressed as the ratio

MT-2A: $\beta$ -actin, the interassay variation was found to be <15 %.

#### Marginal zinc intake study

Concentrations of MT-2A mRNA in baseline T lymphocyte samples from human volunteers in the marginal Zn intake study were found to be 1–29 fg/pg  $\beta$ -actin mRNA. As found for MT-2A mRNA, the concentrations of  $\beta$ -actin mRNA were quite variable and correction of MT-2A data using quantitative data for  $\beta$ -actin was essential.

In contrast to plasma Zn, which was unaffected by marginal Zn intake or repletion, T lymphocyte MT-2A mRNA decreased in response to low dietary Zn in all seven volunteers (Table 1). As seen in Fig. 3, the percentage decrease in MT-2A mRNA from the baseline level was statistically significant ( $P = 0.0156$ ). Sufficient intact RNA for RT–PCR was obtained from only three volunteers following repletion but the concentrations of MT-2A mRNA recorded were all higher than those during the low-Zn period (Table 1).

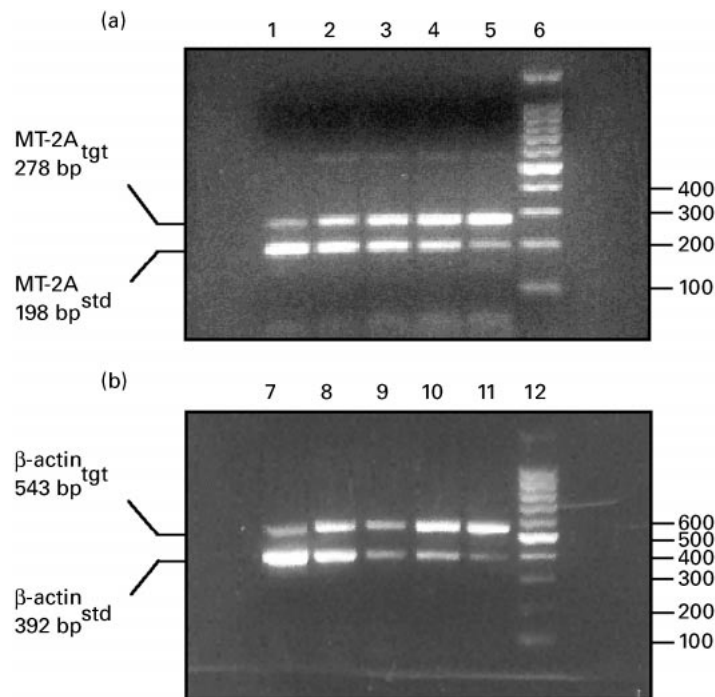
#### Separation and quantification of polymerase chain reaction products by capillary electrophoresis

Using normal polarity (cathode at outlet) with a P/ACE cartridge containing the DB-17 capillary, DNA ladder samples injected electrokinetically at the capillary outlet showed good separation and resolution of fragments between 100 and 1500 bp in under 6 min (Fig. 4(A)). Similar separation of PCR products demonstrated clear separation of MT-2A, the competitive standard and the primers in under 4 min (Fig. 4(B)). For evaluating quantification by this technique, samples were loaded by pressure injection and since the P/ACE 5000 does not permit this procedure at the capillary outlet, samples were injected at the inlet and separated using reverse polarity (anode at outlet). While the outlet-to-detector-length is 70 mm, the minimum inlet-to-detector length is 270 mm, so separation time was lengthened. Nevertheless, quantification was improved and a comparison of PCR product separation by CE and agarose gel is shown in Fig. 5. For both CE and densitometric data from scanned gels, construction of linear plots of target relative to the competitor *v.* the concentration of added standard, yielded similar values for the concentration of MT-2A mRNA.

#### Discussion

MT mRNA concentrations in human T lymphocytes are very low and cannot be detected by Northern blotting, which is a relatively insensitive technique. Thus, a more sensitive competitive RT–PCR assay was developed to measure mRNA from the gene for MT-2A. Using human T lymphocytes, this assay gave results in the lower femtogram range per 200 ng reverse-transcribed RNA. Two important innovations are presented in the current work, i.e. the correction of MT-2A data by reference to quantitative  $\beta$ -actin mRNA values and the application of the assay to samples from Zn-deficient subjects.

mRNA values in T lymphocytes were very variable in



**Fig. 2.** Separation of (a) metallothionein (MT-2A) and (b)  $\beta$ -actin standard (std) and target (tgt) polymerase chain reaction products by agarose gel electrophoresis. Standard additions for lanes 1–5 were 1600, 800, 400, 200 and 100 fg and for lanes 7–11 were 400, 200, 100, 25 and 12.5 pg. Lanes 6 and 12 were both a 100 bp DNA ladder. For details of procedures, see Fig. 1 and p. 749.

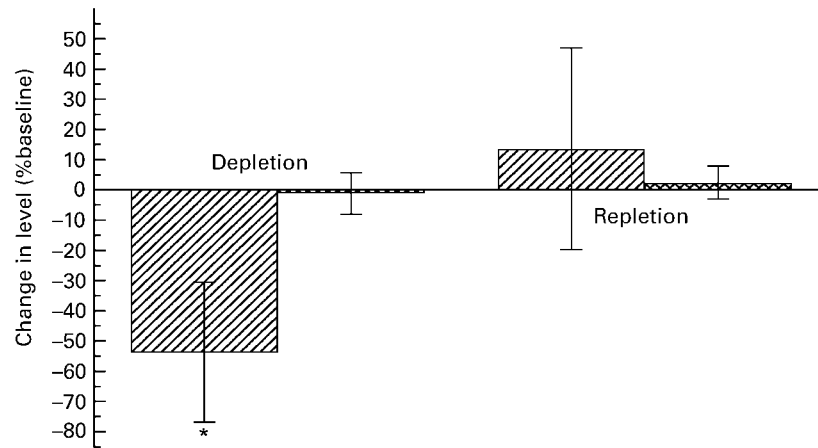
the human volunteers used in the present study, confirming results of a previous study (Harley *et al.* 1989). However, we noted that  $\beta$ -actin mRNA concentrations also tended to be variable, which suggested that some of the variation in MT-2A mRNA may be due to problems of RNA degradation. The use of Dynabeads for purification of T lymphocytes greatly accelerated the processing of blood

samples, although the efficiency of cell extraction was not monitored. RNA pellets obtained from purified cells were very small, and care was required to avoid their aspiration with the solvent. Spectrophotometric quantification of extracted RNA consumed much of the sample, and the procedure would benefit from a sensitive microquantification technique. Indeed, there is also criticism of using

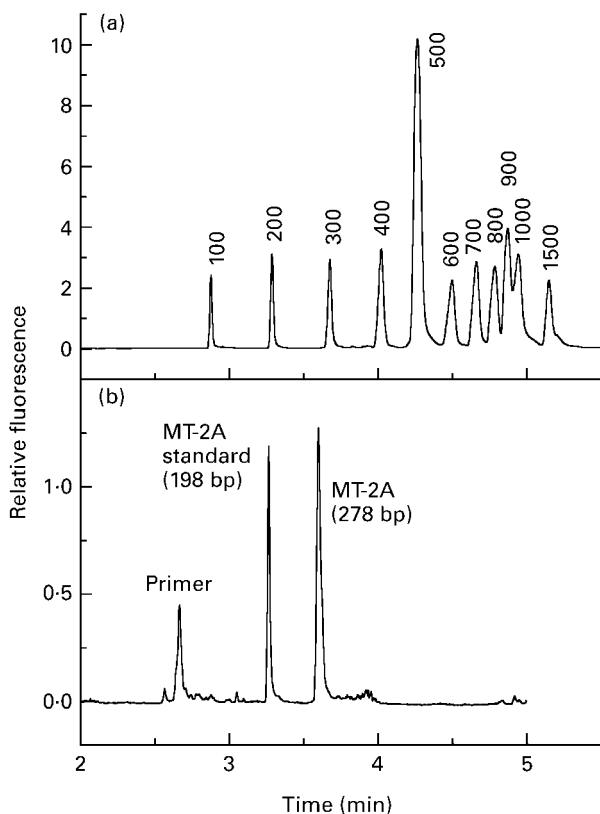
**Table 1.** Metallothionein-2A (MT-2A) mRNA in T lymphocytes and plasma zinc from human volunteers whose zinc intake changed from 13.7 (baseline) to 4.6 (depletion) and back to 13.7 (repletion) mg/d per 70 kg body weight during the 121 d study\*

Subject no.	T lymphocyte MT-2A mRNA (fg MT-2A mRNA/pg $\beta$ -actin mRNA)		
	Baseline	Depletion	Repletion
1	1.8	0.3	2.1
2	17.7	6.3	
3	28.8	9.0	15.5
4	3.4	2.6	5.8
5	2.1	1.7	
6	1.1	0.6	
7	17.3	5.5	
Mean	10.3	3.7	
SE	4.1	1.2	
Plasma Zn ( $\mu$ g/l): Mean	754	741	694
SE	35	27	44

\* Samples for baseline, depletion and repletion were obtained on days 20, 84 and 119 respectively. For details of subjects and procedures, see p. 748.



**Fig. 3.** Change in T lymphocyte metallothionein-2A mRNA (▨) and plasma zinc (▩) concentrations after marginal zinc intake and repletion in human volunteers. Data are expressed as the means with their standard errors, represented by vertical bars, for the percentage change from the baseline value for each volunteer. The depletion and repletion mean values can therefore be directly compared. Both metallothionein-2A mRNA and zinc values used to calculate the means are from the same volunteers (seven volunteers in the depletion phase and three in the repletion). \*Mean value was significantly different from 0.  $P = 0.0156$ . For details of procedures, see Fig. 1 and p. 748.

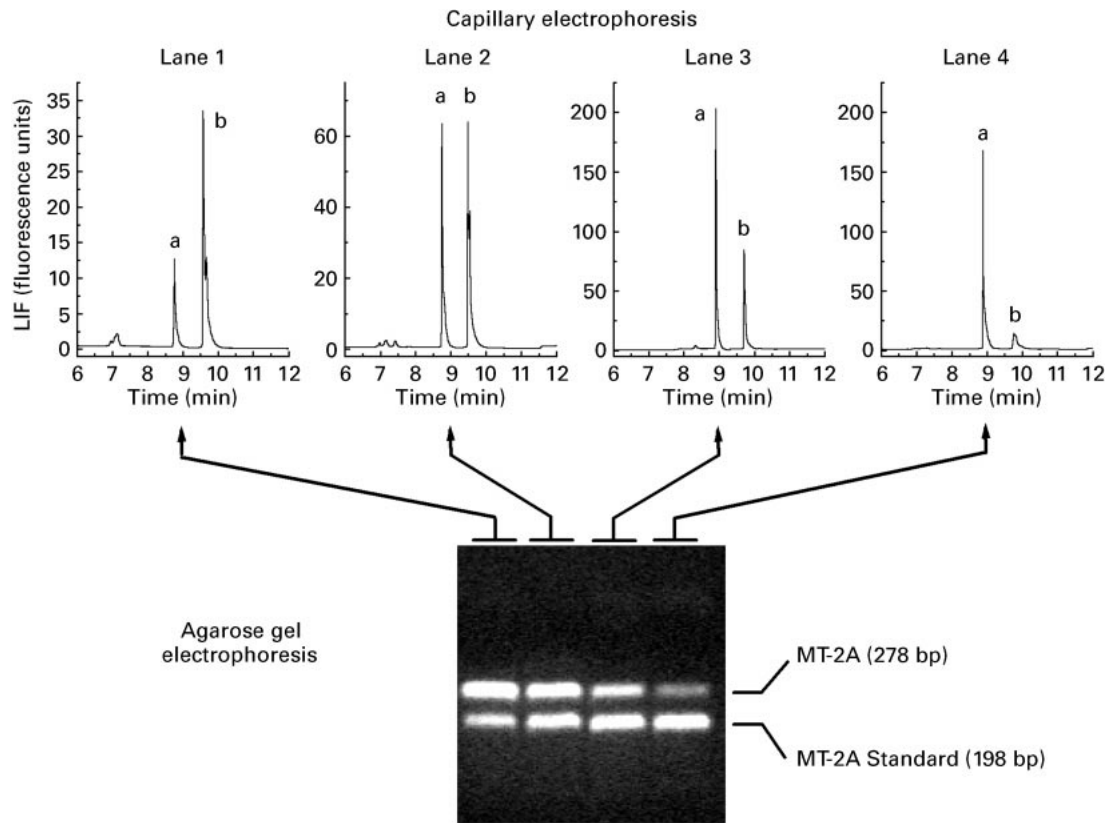


**Fig. 4.** Capillary electrophoresis of (a) a 100 bp DNA ladder and (b) polymerase chain reaction products of metallothionein-2A target and standard, detected by addition of SYBR green (Molecular Probes Europe, Leiden, The Netherlands) to the electrolyte (DNA buffer; Sigma Chemical Co., Poole, Dorset, UK) and laser-induced fluorescence. Samples were loaded electrokinetically at 35 V/cm into the output end of a 270 mm  $\times$  50  $\mu$ m i.d. DB17 capillary and were then separated at 175 V/cm. For details of procedures, see Fig. 1 and p. 750.

spectrophotometry (260:280 nm) to determine purity and concentration of nucleic acid preparations. It has been shown that a ratio of 1.8, which is usually accepted as a criterion for pure RNA preparations, could be obtained from a mixture of about 60 % protein and 40 % nucleic acids (Glasel, 1995). It has also been noted that normalisation of a specific gene using quantitative RT-PCR to that of a constitutively-expressed gene also allows correct relative mRNA quantification in degraded RNA samples (Tong *et al.* 1997). In view of the difficulties in working with small amounts of RNA and the possibility of degradation, it was essential to correct quantitative MT-2A mRNA data by reference to quantitative  $\beta$ -actin mRNA concentrations. Reproducibility of results was thereby greatly improved and the inter-assay variation for analysis of the same samples was <15 %.

The mutant standard for MT-2A was produced using a PCR-based mutagenesis kit, but in our hands this technique proved to be lengthy and costly, with a poor rate of success. A much easier method was subsequently used to generate a rat MT-1 mutant standard. Celi *et al.* (1993) showed that competitive standards can be rapidly synthesised from genomic DNA by amplification using an adapter PCR primer. This procedure eliminates the need for plasmids, specialised kits and modified primers. This approach was improved further by Forster (1994). In this protocol the competitive standard of a defined size for a chosen PCR product is generated in two consecutive amplifications of the PCR product to be quantified. We suggest that this method should be used instead of the mutagenesis kit. Recent real-time PCR techniques may ultimately replace the need for mutant standards, but their availability is currently limited because of the high instrumentation costs.

MT-2A mRNA concentrations in T lymphocytes from subjects with a marginal Zn intake were consistently lower than those in baseline samples, and the marginal:baseline



**Fig. 5.** Comparison of agarose gel electrophoresis and capillary electrophoresis for the analysis of polymerase chain reaction products from metallothionein (MT)-2A target and standard cDNA. The T lymphocyte sample was from volunteer no. 7 after the marginal zinc phase of the study and competitive DNA standard additions before polymerase chain reaction were 12.5, 25, 50 and 100 fg (lanes 1, 2, 3 and 4 respectively). a, b, Capillary electropherogram peaks representing MT-2A standard and target respectively. For details of procedures, see Fig. 1 and p. 750.

concentration ratio ranged between 17 % and 81 %. Repletion of dietary Zn partially or more than fully restored MT-2A mRNA concentrations in the three subjects for whom data were available. In contrast, plasma Zn concentrations were unchanged throughout the low-Zn and repletion periods and this finding demonstrates the relative sensitivity of the lymphocyte MT-2A mRNA assay to a marginal intake of Zn, such as may result from a normal diet. The relative sensitivity of MT-2A mRNA to changing Zn intake has also been found for monocytes; monocyte MT-2A mRNA is elevated consistently in Zn-supplemented men (50 mg/d) while plasma Zn is not (Sullivan *et al.* 1998).

Decreased expression of MT during Zn deficiency is apparently not caused by changes in plasma Zn. Instead, the altered flux of the intracellular pools of Zn may provide the regulatory mechanism. It has been suggested that some subjects with low levels of Zn in their rapidly-exchangeable pools may be at risk of Zn deficiency. The Zn status of such individuals is not apparent using conventional indicators of Zn status such as plasma Zn measurements (Miller *et al.* 1994; Thomas, 1996). However, variations in MT mRNA concentrations may reflect the size of the exchangeable Zn pool in each individual.

The specificity of MT-2A mRNA as a diagnostic indicator of Zn status has yet to be evaluated. Plasma

concentrations of MT are affected by factors independent of Zn, such as stress and infection, and erythrocyte MT is dependent on the proportion of reticulocytes in the erythrocyte population (Bremner, 1993). Since T lymphocytes are composed of different cell types, variations in responsiveness of MT genes to Zn may depend on the balance of subtypes in the T lymphocyte population. *In vitro* studies have shown that different subpopulations of lymphocytes vary in their MT induction response to Cd (Yurkow & Makhijani, 1998). Also, since the promoter region of the MT-2A gene contains *cis*-acting elements for a variety of transcription factors (Hamer, 1986), it seems likely that the concentrations of the corresponding mRNA may change in relation to Zn-independent inducers. Together with the natural inter-individual variation in constitutive MT-2A expression and the relatively small response of lymphocyte MT-2A mRNA concentrations to nutritionally-relevant dietary intakes of Zn, these factors may diminish the diagnostic utility of this assay when health status is not also evaluated. However, the technique could be of considerable diagnostic value when used in association with tests for acute-phase cytokines and other indicators of Zn status. The assay is clearly of utility for controlled experimental trials that evaluate marginal Zn intakes. Selection of MT-2A for this assay, rather than those isoforms which are reported to be more selectively



## References

induced by Zn (Hamer, 1986), was made on the basis that this gene shows strong constitutive levels of expression in T lymphocytes, and sensitivity of detection is an important consideration when limited by the volume of blood which may be ethically removed for T-cell purification.

Competitive RT-PCR is becoming widely used in diagnostic procedures when expression of the gene of interest is low, or when tissue size or cell number is limited, such as with biopsies or needle aspirations. Using refinements to this technique, such as the CE-laser-induced fluorescence method described in the present paper, the assay sensitivity can be increased further and the RT-PCR procedure can be more automated. With further refinement it may be possible to use this approach with only a few cells. The type of blood cell (e.g. T lymphocyte) can be quickly and specifically extracted using magnetic beads coated with a selected antibody. Indeed, mRNA can be extracted from various blood cells using poly dT-coated magnetic beads for RT-PCR if desired. These and other developments in microanalytical techniques (Roberts *et al.* 2000) should make RNA-based nutritional assessment less technically demanding in the future, and allow simultaneous measurement of the expression of many genes, enabling a more accurate nutritional assessment than that based on a single biomarker.

In summary, using a sensitive RT-PCR assay, we have shown that T lymphocyte MT-2A mRNA concentration displayed large inter-individual variation. However, this mRNA consistently decreased in individual human subjects during marginal Zn intake and increased during subsequent repletion, unlike plasma Zn concentration which did not show any change. This finding suggests that measurement of T lymphocyte mRNA may be a sensitive index of Zn intake, and may be of use in studies where confirmation of changes in an individual's Zn intake over time is desired. The RT-PCR assay for MT-2A allows measurement of MT from tissues or cell specimens that are limited in size or have low MT expression, and with modifications could be used to monitor mRNA concentrations of other MT isoforms *in vivo* in response to various dietary treatments and disease states. More work is required to evaluate the MT RT-PCR assay as an index of Zn status on the basis of single T-cell analyses in 'free-living' volunteers. However, this assay is very suitable for using RNA-based diagnosis to evaluate changes in dietary Zn of individuals subjected to controlled marginal Zn intake or Zn supplementation.

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