Cloning and sequencing a novel metallothionein I isoform expressed in human reticulocytes

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Abstract Total RNA from human reticulocytes was purified and reverse-transcribed into cDNA using an oligo-dT primer. This cDNA was used as a template for a polymerase chain reaction (PCR) with a primer specific for the N-terminal sequence of mammalian metallothioneins (MT) and a universal primer. A single amplified fragment was thus generated which when cloned and sequenced revealed two distinct MT cDNAs of almost identical molecular weights. One sequence was identical to that previously reported for human MT II and the other encoded a novel MT I isoform (MT I_R). The DNA sequence of MT 1_R is distinct from those documented for other MT I isoforms.

Key words: Metallothionein; Zinc status; Reticulocyte; Polymerase chain reaction

1. Introduction

Zinc is a trace element essential for the activity of numerous enzymes in all areas of metabolism. Zinc deficiency leads to a variety of features including anorexia, growth retardation, abnormal immune function, abnormal nitrogen metabolism, hypogeusia, impaired reproductive capacity and behavioural defects [1]. Assessment of zinc status is therefore important in the early diagnosis of zinc deficiency.

Present methods for assessing zinc status include measurement of serum zinc, erythrocytes, leucocytes, urine and hair. However, it has been demonstrated that serum, leucocyte and hair zinc concentrations are affected by other conditions not associated with zinc depletion [2]. Leucocyte zinc does not consistently decrease during zinc deficiency and urinary zinc increases during trauma. These methods, which represent those presently available for assessing zinc status are not satisfactory.

Metallothioneins (MT) are low molecular weight, cytosolic proteins with high affinity for Zn^{2+}, Cu^{2+} and Cd^{2+}. Functions of metallothionein include heavy metal homeostasis and detoxification [3]. Thirteen MT genes have been identified, which encode at least 10 functioning proteins [4]. MT isoforms may be classified by their retention times on ion-exchange columns, which is dictated by their overall charge. The major human MT isoforms are MT I and MT II, resolved by ion-exchange by virtue of the nature of the amino acid at position 11. Recently described is a third charge-separable isoform, MT 0 [5]. To date, sequence analysis has identified only one MT II isoform, with aspartic acid at position 11. In humans, there are at least 10 MT I isoforms, with heterogeneous peptide sequences, and having either a valine or a glycine at position 11 [6].

It has been suggested that zinc–MT I in human tissues constitutes a metabolic buffer pool of zinc which is less susceptible to induction by factors other than zinc. It has been shown that the concentration of MT I within erythrocytes decreases in response to zinc but not copper deficiency [7]. By contrast, MT II responds to other factors such as infection and disease. Erythrocyte MT I concentrations, therefore, may be an important index of zinc status. However, the isoforms of MT expressed in red blood cells (RBC) is not known. In this study we have determined the MT I isoforms expressed in RBCs, using a molecular biology approach.

2. Materials and methods

2.1. Isolation of reticulocytes

Reticulocytes were isolated from 15 ml of blood from a patient with polycythaemia vera (PCV). Platelets were removed by activation with ADP (Sigma, 0.5 mg ADP/ml of blood) and passage through a glass bead column [8]. Blood was then applied to a cellulose fibre column (Whatman CF-11). Blood eluting from the column, now free from white blood cells and platelets was centrifuged at $1200 \times g$, for 5 min. The top third of the RBC pellet, enriched with the less dense reticulocytes, was immediately plunged into liquid nitrogen.

2.2. Synthesis of cDNA

Total RNA was isolated from approx. 5×10^9 reticulocytes using the TRI Reagent protocol (Sigma Chemical Co., UK). To synthesise first strand cDNA, 1 μg total RNA was denatured by heating to 70°C for 3 min. To the denatured RNA was added 15 mM of dNTPs, 20 U of RNase inhibitor (RNAsin, Promega, UK), 50 pmol oligo(dT) adaptor primer (5′-CGG AGA TCT CCA ATG TGA TGG GAA TTC (T)17-3′), (synthesized by Pharmacia Biotech., Sweden) and 200 U of MMLV reverse transcriptase (Promega) in a volume of 30 μl, with MMLV reverse transcriptase buffer (Promega). The mixture was incubated for 2 h at 42°C and the reaction stopped by heating to 65°C for 5 min.

2.3. Amplification of MT cDNA

cDNAs encoding all MT isoforms were amplified using PCR performed with a non-specific oligonucleotide primer corresponding to the oligo(dT) primer used in the first strand synthesis but lacking the oligo(dT) tail (5′-CGG AGA TCT CCA ATG TGA TGG GAA TTC-3′), and a specific primer encoding the N-terminal seven amino acids which are conserved throughout all human MTs (5′-ATG GAC/T CCC AAC TGC TCI TG-3′), where I is inosine. The annealing temperature of the primer was estimated to be 56–60°C.

PCR amplification of ss-cDNA derived from 0.1 μg total RNA was performed with Taq polymerase (5 U, *Thermus aquaticus* strain YT1(1), Promega), 50 pmol of each the above primers and 0.25 mM dNTPs (final concentration) buffered with Promega PCR storage buffer A (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100, containing 1.25 mM $MgCl_2$). The PCR programme, performed on a Perkin Elmer DNA Thermal cycler 480 machine, consisted of 30

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Abbreviations: MT(s), metallothionein(s); PCR, polymerase chain reaction

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cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, ending with a single cycle of 15 min at 72°C.

Parallel amplifications with Vent (exo-) DNA polymerase (New England Biolabs. Ltd., UK) were performed using an identical PCR programme but within a reaction mix consisting of ss-cDNA derived from 0.1 μg total RNA, Vent (exo-) DNA polymerase (2 U), 50 pmol each of the above primers and 0.25 mM dNTPs (final concentration) in New England Biolabs buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100).

2.4. Cloning and sequencing of PCR products

MT fragments amplified by Taq polymerase were sub-cloned using the pGEM-T Vector system according to the manufacturer's protocol (Promega, UK). MT fragments amplified by Vent (exo-) DNA polymerase were sub-cloned by ligation into a pCR-Script SK(+) vector according to the manufacturer's protocol (Stratagene Ltd., UK). *Escherichia coli* cells (strain JM109 for pGEM-T vector and MC1069 for pCR-Script SK(+) vector) were transformed with MT-DNA-vector ligation products.

Using the standard technique of insertional inactivation of plasmid *Lac-Z* gene, recombinant clones were identified. To confirm the presence of MT DNA in recombinant clones, plasmid DNA (purified using Wizard DNA preparation system, Promega) was digested with the restriction enzyme *Nco*I and run on an agarose gel. Plasmid DNA and excised MT DNA were visualised in the gel using ethidium bromide (results not shown). Clones were sequenced by T7 sequencing (Pharmacia Biotech.) using the Sanger dideoxy method using ^{35}S-α-dATP from denatured ds-DNA template. Multiple clones (10) were sequenced in both directions.

3. Results

In the 15 ml of whole blood sample, approx. 10% of total erythrocytes were reticulocytes. Total RNA (10 μg) was successfully isolated from 5×10^6 reticulocytes. The integrity of the RNA was confirmed by visualising the ribosomal RNAs on an ethidium bromide-stained agarose gel (results not shown).

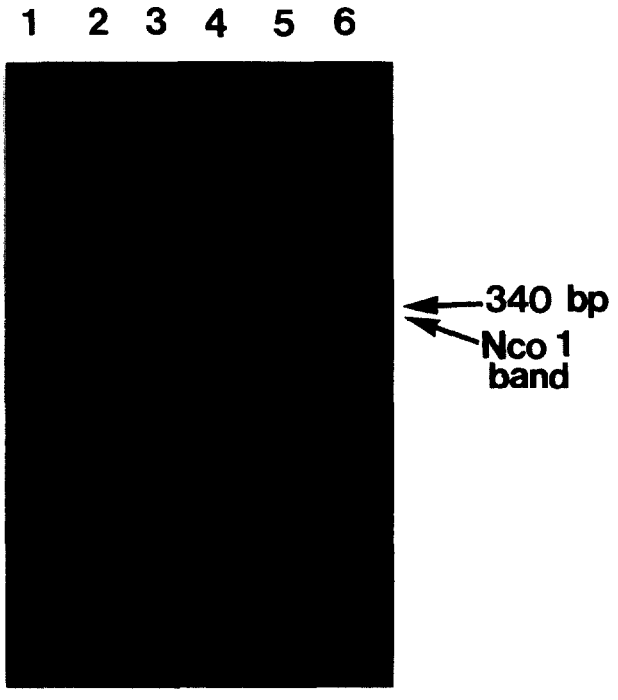

Fig. 1. Electrophoretic analysis of products of MT-specific RT-PCR in reticulocytes. Lane 1: φX174 DNA/*Hae* III markers (Promega); lane 2: Taq DNA polymerase-amplified MT; lane 3: Vent (exo-) DNA polymerase-amplified MT; lane 5: Taq DNA polymerase-amplified MT digested with *Nco*I; lane 6: Vent (exo-) DNA polymerase-amplified MT digested with *Nco*I. Electrophoresis was carried out using a 2% agarose gel in a 1×TBE buffer and DNA visualised using ethidium bromide.

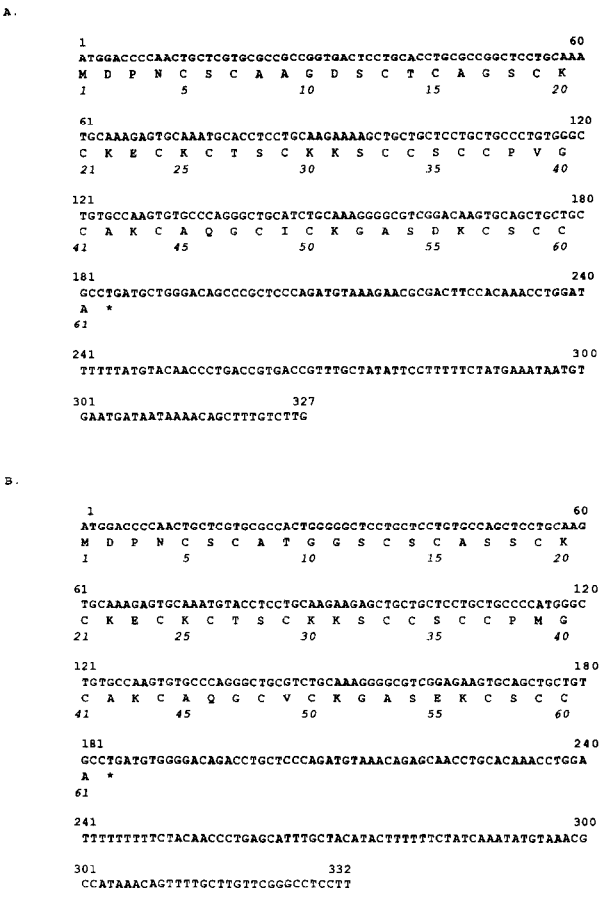

Fig. 2. Nucleotide sequences with protein translations (below) of reticulocyte MT II (A) and MT I_R (B). PCR products generated using an MT-specific RT-PCR protocol were cloned and the nucleotide sequence of the cloned products determined using dsDNA T7 sequencing. Three clones of each MT isoform from Taq RT-PCR reactions were sequenced.

MT-specific PCR produced one major band at the predicted molecular weight for the coding and 3′-untranslated region of the MT gene, ~340 bp (Fig. 1). This MT cDNA was cloned, and 10 separate clones sequenced. From these sequences eight were identical to the sequence already reported for human MT II (Genbank accession number J00271, GenEMBL database, Fig. 2) whilst two clones contained a novel MT which, although its sequence showed a great homology to other MTs, was clearly unique (Fig. 2). Furthermore, sequence analysis showed that the novel MT contained an *Nco*I restriction site (using the Wisconsin DNA manipulation package, through Seqnet, Daresbury, UK), absent in the MT II cDNA sequence which allowed clones containing MT I_R to be easily identified. Digestion with *Nco*I enabled the ratio of MT II/MT I_R expressed in reticulocytes to be estimated as 1 : 1.

In order to confirm that this gene was not an artefact, the process was repeated by preparation of a second batch of total RNA, transcription into cDNA (as previously described) and amplification by PCR. To further ensure the authenticity of the novel MT I_R sequence, this second amplification procedure utilised Vent (exo-) DNA polymerase with its intrinsic proof-reading activity instead of Taq DNA polymerase. This

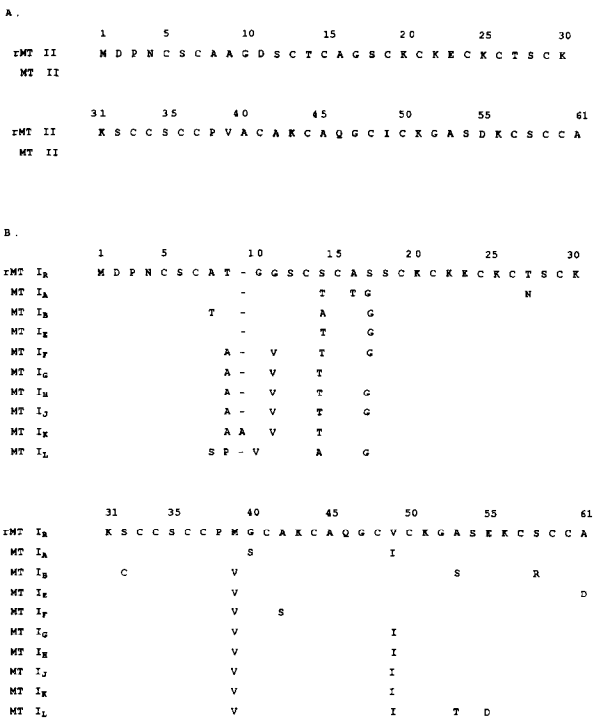

Fig. 3. Alignment of the peptide sequences of human MT isoforms. (A) Human MT II peptide sequence derived from the reticulocyte cDNA sequence and that of MT II previously reported [6]. (B) Human MT I_R peptide sequence and sequences of the other human MT I isoforms [6]. Where no residue is shown, sequence identity exists with rMT II (A) and MT I_R (B).

amplification produced a single band of ~340 bp which when digested with *Nco*I gave rise to two bands suggesting that a portion of the amplified product was the novel *MT* I_R gene (Fig. 1). This was confirmed by cloning the undigested fragment and sequencing clones which contained the *Nco*I site. These clones contained the novel MT I_R sequence. Sequencing three clones which did not contain an internal *Nco*I site, gave rise to the sequence of MT II (results not shown).

The DNA sequence of the novel *MT* gene predicted a unique amino-acid sequence. The presence of a glycine at position 11 qualifies it as an MT I isoform (Fig. 3). This novel *MT* gene has been denoted *MT* I_R, and may be specific to RBCs.

4. Discussion

New members of the human MT family have been described with each tissue whose MT content is examined. These novel MTs have two things in common: localisation of their expression to a particular tissue, and co-expression of other 'ubiquitous' MT isoforms, such as MT II. The expression profile of the MT family suggests that the 'ubiquitous' MT isoforms occupy a central position in cellular physiology, whilst the tissue-specific isoforms have precise biological functions relating to the tissue in which they are expressed.

Of the MT isoforms expressed tissue-specifically, MT III has only been detected in brain tissue [9,10] and has been called 'growth inhibitory factor', low levels of which are implicated in the onset of Alzheimer's disease. MT IV has only been detected in stratified squamous epithelia [11] and is thought to play a unique role in its differentiation. Previously thought to be a foetal liver isoform, MT 0 has been detected in stimulated adult monocytes [5,12]. Monocytes induced with zinc express among others MT I_G and MT 0, which in adults may be specific to these cells and have a role in monocyte activation and/or differentiation. We report here the MT mRNA profile of human reticulocytes – we found MT II and a novel MT I isoform, termed MT I_R. Isolation of the message encoding MT II was expected, as it is the predominant isoform in most tissues. The predicted amino-acid sequence of the novel MT I_R found in reticulocytes bears the closest homology to MT I_E, and is classified as an MT I isoform by virtue of the glycine residue at position 11 (Fig. 3) [5]. MT I_R has a novel serine at position 14: this serine and the unique order of amino acids throughout its sequence distinguish MT I_R from all other MT isoforms thus far identified (Fig. 3).

The observations reported here raise questions concerning the control of MT expression and the cellular role of the novel MT isoform detected. It is known that RBC MT is synthesised by immature RBCs in the marrow [13,14]. Such a protein may have a role in RBC proliferation and differentiation, delivering zinc to enzymes requiring it. Further experiments would examine the genomic locus of the MT I_R gene, looking for control regions such as metal-responsive elements, glucocorticoid-responsive elements, and the TATAA box.

It has yet to be shown whether the measurement of reticulocyte MT I_R may be used as a specific and sensitive index of zinc status.

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