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Metallothionein isogene transcription in red blood cell precursors from human cord blood

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The *in vitro* transcription patterns for 10 functional metallothionein (MT) isogenes have been investigated in red blood cell (RBC) precursors from human cord blood. Active transcription status of the isogenes, MT-0, MT-1A, MT-1B, MT-1E, MT-1G, MT-1X, and MT-2A, was detected in both *ex vivo* expanded RBC precursors (burst-forming unit-erythroid) and glycophorin A^+ and $CD71^+$ cells separated by magnetic cell sorting. Transcription patterns of these isogenes were analyzed at different times of incubation with the addition of Zn supplement. In neither the *ex vivo* expanded precursors nor glycophorin A^+ and $CD71^+$ cells could MT-1F and MT-3 be detected. Transcripts of MT-4 were detected in glycophorin A^+ and $CD71^+$ cells. Erythropoietin-responsive constitutive transcription of MT-1X and possible interleukin-3-responsive downregulation of MT-2A in *ex vivo* expanded precursors reveal their effect on MT biosynthesis. Biosynthesis and induction of MT at the protein level in the RBC precursors was also demonstrated by immunoblotting.

Keywords: erythropoietin; glycophorin A; interleukin-3; metallothionein; zinc status.

The occurrence and biosynthesis of metallothioneins (MTs), a family of cysteine-rich low-M_r metal-binding proteins, has been reported in different human tissues, e.g. liver [1–3], kidney [4], brain [5], and blood cells, e.g. lymphocytes [6,7], monocytes [8], platelets [9], and erythrocytes [10]. Since its discovery [11], MT has been recognized for its physiological importance in detoxification of toxic heavy metals, such as cadmium [12], homeostasis of essential heavy metals, such as Zn [13], and radical scavenging [14,15]. Its role in cell proliferation and differentiation has also been recognized in different cell/tissue types [16–19].

Based on the difference in the charge-separable elution pattern in HPLC, human MTs were first grouped into two isoforms, MT-1 and MT-2 [1]. A third charge-separable fraction of MTs from human fetal liver was reported and termed MT-0 by Soumillion *et al*. [20]. Human MT-3 expression was reported to be brain specific [5]. However, MT-3 mRNA has been reported in human kidney [21], lymphocytes [7], and the reproductive system [22]. So far, MT-4 expression has only been reported in stratified squamous epithelia in both human and mouse [23]. Besides these five isoforms, subisoform-specific expression of MT in different human tissues has been reported at both the protein and mRNA level [3,7,20,24]. Despite the tissue specificity of some of the members of the MT protein family, some isoforms such as MT-2A are reported to be ubiquitous in human tissues. The differential expression patterns of MT isoforms are attributed to their control at the molecular level [25]. Inducer-specific differential expression patterns have also been reported [26,27].

The inability of anucleated RBCs to synthesize protein has provoked speculation on the origin of MT therein. Lambert *et al*. [28] have shown the presence of MT mRNA in reticulocytes and speculated on its possible role in differentiation. Several investigators have suggested different possibilities. Early bone marrow precursors have been hypothesized to be the source of MT in murine RBCs because of the change in MT concentration in murine erythroid progenitor cells in response to dietary Zn intake and status [29]. MT originated in organs such as the spleen has also been suggested to be transferred to RBCs [30]. In mice, proerythroblasts have been hypothesized as the site of MT biosynthesis, which are in turn present in mature RBCs [31]. A study of cadmium-adminstered mice has shown that the Cd-MT present in mature RBCs is not transferred from plasma [32]. In a recent review, the induction, regulation, and biological significance of mammalian MTs have been described [33].

In our recent report [34], we demonstrated Zn induction of MTs in human nucleated RBC precursors (nRBCs) from umbilical cord blood. As an extension of this investigation, we here report the isoform-specific transcription pattern in human RBC precursors in the presence and absence of Zn, a potent MT inducer. In addition, we also investigated the effect of erythroid growth proliferation/differentiation-promoting factors such as erythropoietin (EPO) and interleukin-3 (IL-3) on MT isogene transcription. Confirmation of the precursor origin of MTs in RBCs using the

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Abbreviations: BFU-E colony, burst-forming unit erythroid colony; EPO, eythropoietin; gly A, glycophorin A; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-3, interleukin-3; MACS, magnetic cell sorting; MT, metallothionein; MNC, mononuclear cell; RBC, red blood cell; nRBC, nucleated precursor of RBC.

Note: a web page is available at http://www.chem.kuleuven.ac.be/research/bio/mbsten.html.

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active MT isogene transcription pattern would be a significant step in the assessment of Zn status by erythrocyte Zn-MT content. The functional MT isogene transcription patterns in the RBC precursors in the presence or absence of Zn, in combination with EPO and IL-3, support the possible correlation between RBC differentiation and MT biosynthesis.

MATERIALS AND METHODS

Separation of RBC precursors

Human cord blood was collected in anticoagulant (acid/citrate/dextrose solution) and kept at 4 °C for not more than 12 h before use. Mononuclear cells (MNCs) were separated from human cord blood by density-gradient centrifugation on lymphoprep™ (Nycomed AS, Oslo, Norway) as described by Bøyum [35]. Finally, RBC precursors were obtained from the MNCs in two ways.

Ex vivo expansion of burst-forming unit erythroid (BFU-E) colonies in semisolid cell culture medium. Briefly, non-adherent MNCs were recovered by removing the plastic adherent cells and washed three times with NaCl/P_i followed by incubation at a density of $\approx 5 \times 10^4$ cells·mL^{-1} in methylcellulose semisolid cell culture medium (MethoCult™; StemCell Technologies, Vancouver, BC, Canada) supplemented with EPO (3 U·mL^{-1}) and IL-3 (5 ng·mL^{-1}) in 24-well tissue culture plates; BFU-E colonies were picked up that were grown in a humidified 5% CO_2 incubator at 37 °C; the purity of these erythroid precursors, hereafter named nRBCs, was checked by May-Grünwald-Giemsa staining (Fig. 1A).

Magnetic cell sorting (MACS) separation of glycophorin A^+ (gly A^+) and $CD71^+$ cells. Briefly, MNCs separated from human cord blood were washed in NaCl/P_i containing 2 mM EDTA and 3% (w/v) BSA, and incubated for 15 min at 6 °C with glycophorin A and CD71 (transferrin receptor) microbeads; positive-selection columns (LS^+/VS^+ type) were used according to the manufacturer's instructions (reagents and equipment from Miltenyi Biotech, Bergisch Gladbach, Germany); further enrichment of the positively selected gly A^+ and $CD71^+$ cells was achieved by a second pass of positive selection using fresh reagents and columns. The morphology of the MACS-separated gly A^+ and $CD71^+$ cells was also checked by May-Grünwald-Giemsa staining (Fig. 1B,C).

In vitro Zn induction and extraction of polyadenylated mRNA

Enriched populations of nRBCs or MACS-separated gly A^+ and $CD71^+$ cells were used for *in vitro* Zn induction of MT biosynthesis. Zinc induction was carried out at different concentrations of zinc acetate (as indicated) in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 0.005% (w/v) gentamicin, 2 mM glutamine (all from Gibco–BRL, Life Technologies Ltd, Paisley, Scotland, UK), 1% (w/v) glucose (Sigma, St Louis, MO, USA), 10 μM 2-mercaptoethanol (Fluka, Neu-Ulm, Germany); similar control cultures were maintained without Zn supplement. EPO (3 U·mL^{-1}) or IL-3 (5 ng·mL^{-1}) was added in parallel control and induced cultures as indicated.

Poly(A)-rich mRNA was extracted from either the cultured cells at different times as indicated or the freshly MACS-separated gly A^+ and $CD71^+$ cells using the commercially available mRNA extraction kit MICROPOLY(A)PURE™ (Ambion, Austin, TX, USA) according to the manufacturer's instructions. To obtain a more concentrated solution of mRNA, three different extracted mRNA pools from similar cultures were combined.

cDNA synthesis and MT-isoform-specific PCR

For the synthesis of cDNA, we used the GeneAmp® RNA PCR Core kit (PerkinElmer, Roche Molecular System, Branchburg, NJ, USA); briefly, for a final reaction volume of 20 μL, 1 μg mRNA and 50 pmol oligo(dT) as a first-strand cDNA primer were initially heated at 72 °C for 5 min followed by snap cooling on ice; from a single master mix preparation containing 1 × PCR buffer II, 2 mM each dNTP (dATP, dCTP, dTTP, dGTP), 5 mM $MgCl_2$, 20 U RNase inhibitor, and 50 U murine leukemia virus reverse transcriptase were added to the oligo(dT) annealed mRNA; first-strand cDNA synthesis was carried out at 37 °C for 50 min in a thermocycler (PHC-3; Techne, Cambridge, UK) followed by the heat inactivation of the enzyme at 99 °C for 5 min.

Hot start PCR was performed with the cDNA using published MT-isoform-specific primers [24]. For a final reaction volume of 50 μL, equal amounts (2 μL) of

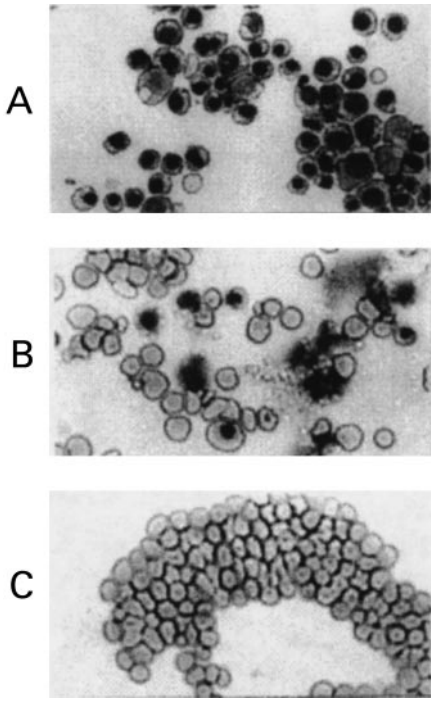

Fig. 1. Nucleated RBC precursors from human cord blood. Photomicrographs after May-Grünwald-Giemsa staining of (A) RBC precursors obtained from BFU-E colonies consisting mainly of proerythroblasts, (B) MACS-separated gly A^+ cells consisting of orthocromatic/polychromatic erythroblasts and reticulocytes, and (C) MACS-separated $CD71^+$ populations consisting of reticulocytes.

reverse-transcribed cDNA and 15 pmol of each upstream and downstream primer for each MT isoform were first heat denatured at 95 °C for 3 min; from a single master mix, 2 mM each dNTP, 1 × PCR buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$), and 2.5 U *Taq* polymerase (Gibco-BRL, Life Technologies) were immediately added to the heat-denatured cDNA; amplification was carried out for not more than 35 cycles of heat denaturation at 95 °C for 1 min, primer annealing at 63 °C (55 °C for MT-1B), and extension at 75 °C for 1 min followed by one cycle of final extension at 75 °C for 5 min. Sample variations for either cDNA synthesis of PCR were minimized using the same master mix from the same batch of reagents. A negative control (to check for genomic DNA contamination) was tested for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) according to the published sequence [36] using the extracted mRNA from each sample directly in PCR, hereafter indicated as '−'. To check the progress of the PCR amplification in exponential phase, primers for G3PDH were also analyzed by RT-PCR, hereafter indicated as '+'. Constitutive transcription was assumed when retro-transcripts were found only in control cultures, and inducible transcription refers to transcription observed only after Zn treatment. Upregulated transcription was judged by the increased band intensity of the cDNA pools from the Zn-induced cultures compared with that from the control cultures. Comparisons were made only among amplified retro-transcripts started from the same amount of mRNA and amplified from the same volume of cDNA.

Agarose gel electrophoresis

Amplified retro-transcripts were resolved by agarose (typically 2.2%) gel electrophoresis. Care was taken when different samples were applied to the gel for comparison with each other using equal volumes (typically 6 μL) of PCR products. Gels were photographed under UV light after ethidium bromide staining.

Extraction of total proteins and detection of MT

Total proteins were extracted by cell lysis in 10 mM Tris/HCl (pH 7.4) for three cycles of freezing (in a CO_2/ethanol bath at −72 °C for 5 min) and thawing (at 37 °C in a water bath for 5 min). Lysed cell suspensions were centrifuged at 14 000 ***g*** for 15 min; supernatant was collected in a fresh microcentrifuge tube and heated in a boiling water bath for 2 min followed by another round of centrifugation at 14 000 r.p.m. for 15 min. Total protein from the final supernatant was measured by the spectrophotometric method of Bradford [37]. BSA was used as the reference for the calibration. Immunoblotting was used to detect MT, as described by Aoki *et al*. [38] using equal amounts of heat-denatured total protein (from control and induced cultures) after electrophoresis on SDS/PAGE; E9 (Dako, Carpinteria, CA, USA), mouse anti-MT, was used as the primary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG was used as the secondary antibody, and immune reactivity was observed using 4-nitrophenyl phosphate (disodium salt; $6H_2O$; Sigma, Steinheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrate.

RESULTS

Transcription of MT isogenes in nRBCs in response to Zn

Transcripts of 10 functional MT isogenes were investigated by RT-PCR in both the control and Zn-treated cultures. Constitutive transcription of only MT-2A was observed in the control cultures at 48 h (Fig. 2A). Inducible transcription of MT-1E, MT-1G, and MT-1X was detected 48 h after treatment with 60 μM Zn (Fig. 2B), and MT-0 (also known as MT-1H), MT-1A, MT-1B, MT-1E, MT-1G, and MT-1X were inducibly transcribed in similar cultures treated with 100 μM Zn (Fig. 2C). Continuation of control cultures until 96 h resulted in constitutive transcription of three more isogenes, MT-1E, MT-1G, and MT-1X in addition to MT-2A (Fig. 2D). In the cultures treated with 100 μM Zn,

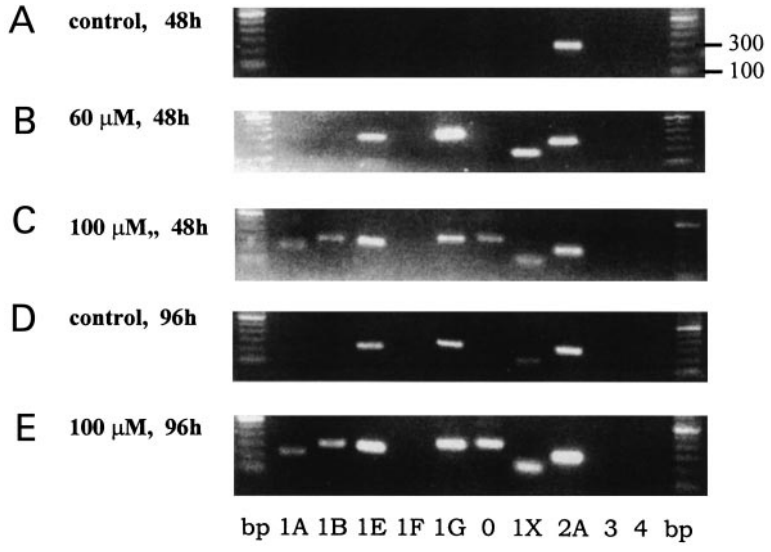

Fig. 2. Transcription of MT isogenes in *ex vivo* expanded nRBCs at different Zn concentrations. Only MT-2A was constitutively transcribed at 48 h (A) while MT-1E, MT-1G, MT-1X, and MT-2A were constitutively transcribed at 96 h (D). Inducible or upregulated transcription (as indicated in the text) of MT-1A, MT-1B, MT-1E, MT-1G, MT-0, MT-1X, and MT-2A in culture treated with 100 μM Zn are observed at both 48 h (C) and 96 h (E). The absence of any band for MT-1F, MT-3 and MT-4 indicates their transcriptionally inactive state. (B) Transcripts of MT-1E, MT-1G, MT-1X, and MT-2A were detected 48 h after treatment with 60 μM Zn. bp, DNA size marker, 100-bp ladder.

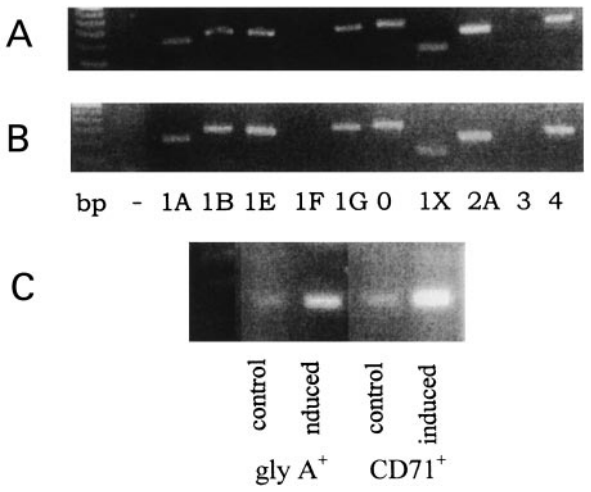

Fig. 3. Isogene-specific transcription of MT in MACS-separated gly A^+ and $CD71^+$ cells. In both the gly A^+ (A) and $CD71^+$ (B) cell populations, mRNA for MT-1A, MT-1B, MT-1E, MT-1G, MT-0, MT-1X, MT-2A, and MT-4 was detected. Zn induction for 48 h also caused the upregulated transcription of total MT isogenes compared with the control and induced cultures of both the gly A^+ and $CD71^+$ cells (C). bp, DNA size marker, 100-bp ladder.

inducible transcription of MT-1A, MT-1B, and MT-0 and upregulated transcription of MT-1E, MT-1G, MT-1X, and MT-2A were observed at 96 h (Fig. 2E). Transcripts of three other functional isoforms, i.e. MT-1F, MT-3, and MT-4, could not be detected even after *in vitro* Zn treatment either at 48 h (Fig. 2B,C) or at 96 h (Fig. 2E), and are considered to be transcriptionally inactive.

MT isogenes in gly A^+ and $CD71^+$ cells

The precursor population from the RBC lineage harvested as MACS-separated gly A^+ and $CD71^+$ cells were investigated for the presence of different MT isogene transcripts. Transcripts of MT-0, MT-1A, MT-1B, MT-1E, MT-1G, MT-1X, and MT-2A isogenes were detected by RT-PCR at 0 h (i.e. mRNA was extracted just after the separation of these cells from the cord blood MNCs) in both gly A^+ (Fig. 3A) and $CD71^+$ (Fig. 3B) cell populations. To our surprise, transcripts of MT-4 were also detected by RT-PCR in both cell types. However, transcripts of MT-1F and MT-3 could not be detected. The effect on the changes in total MT transcripts (regardless of MT isogenes) 48 h after treatment with 100 μM Zn in both these cell populations was also investigated by RT-PCR. Upregulation of total MT transcription was clearly demonstrated by the increased amplified cDNA pools from Zn-induced cultures compared with those from control cultures of both the gly A^+ and $CD71^+$ cells (Fig. 3C).

Possible order of activation of MT isogenes in response to Zn

To determine the order of activation of the functional isogenes, concentration-dependent isogene transcription patterns were assessed. nRBC cell cultures incubated at different concentrations of Zn for 48 h were investigated. At a lower concentration (60 μM), four isogenes, namely MT-1E, MT-1G, MT-1X, and MT-2A, were found to be transcribed (Fig. 2B), whereas at a higher concentration (100 μM) seven isogenes, MT-0, MT-1A, MT-1B, MT-1E, MT-1G, MT-1X, and MT-2A, were transcribed (Fig. 2C). Therefore, the seven functional isogenes are possibly activated in response to Zn in the order: MT-2A > (MT-G/MT-1X/MT-1E) > (MT-1A/MT-1B/MT-0).

Relative abundance of MT isogenes in response to Zn

To check the relative abundance of the active isogenes, amplified retro-transcripts were assessed at different cycles during PCR amplification. To ensure homogeneity and to minimize sample variation, RT-PCR was carried out using the same master mixtures. Moreover, cDNA synthesized using mRNA extracted from three different nRBC cultures treated with 100 μM Zn for 48 h were combined for PCR amplification. The comparative abundance of the different isogenes was determined by the order of appearance of the respective retro-transcripts amplified for different numbers of PCR cycles analyzed on agarose gel. MT-2A was omitted from this experiment as it is constitutively present, and MT-1F, MT-3, and MT-4 were also omitted because they were found to be transcriptionally inactive. At the 26th cycle of PCR amplification, MT-1E, MT-1G, and MT-1X were detected along with a weaker signal for MT-0 (Fig. 4A). At the 28th cycle,

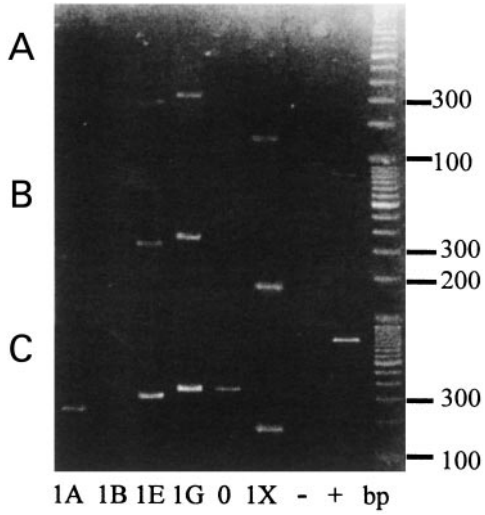

Fig. 4. Relative abundance of MT isogene transcripts in nRBCs 48 h after treatment with 100 μM Zn. (A) MT-1X, MT-1E, MT-1E, and MT-0 transcripts were detected in the earliest cycles (26 cycles). (B) Increased band intensities for MT-1X, MT-1E, MT-1E, and MT-0 transcripts and appearance of MT-1A were observed at 28 cycles. (C) Further increase in band intensities for MT-1X, MT-1E, MT-1E, MT-0, and MT-1A transcripts were again observed at 30 cycles. The band intensity for the housekeeping gene G3PDH (+) was also increased with the increase in number of PCR cycles; the absence of genomic DNA contamination was checked using extracted mRNA directly by PCR for the presence of G3PDH (–). bp, DNA size marker, 100-bp ladder.

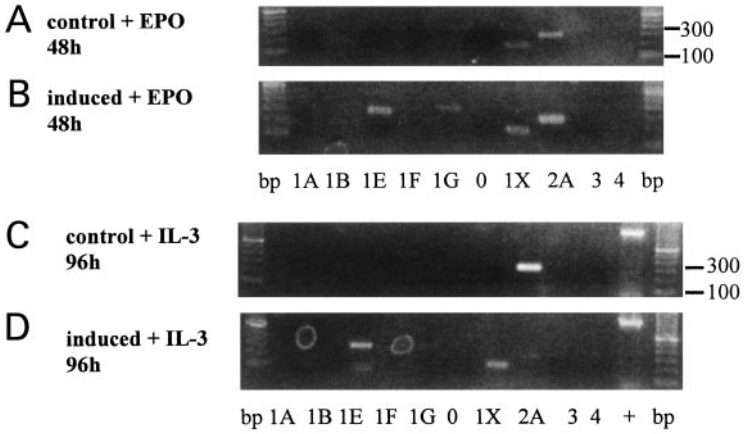

Fig. 5. Transcription of MT isogenes in response to EPO and IL-3 in nRBCs at 48 h. Transcription of MT-1X and MT-2A is observed in the EPO-containing control culture (A) while only MT-2A is transcribed in the IL-3-containing control culture (C). In cultures induced with 100 μM Zn, transcription of MT-1E, MT-1G, MT-1X, and MT-2A is observed with the addition of either EPO (B) or IL-3 (D). Transcripts for MT-1F, MT-3 and MT-4 (along with MT-1A, MT-1B and MT-0) could not be detected in any of these cultures. bp, DNA size marker, 100-bp ladder. + , G3PDH.

transcripts for MT-1A appeared along with increased band intensities for the MT-1E, MT-1G, MT-0, and MT-1X transcripts (Fig. 4B). A further increase in band intensity for MT-1A, MT-1E, MT-1G, MT-0, and MT-1X transcripts was observed after 30 cycles (Fig. 4C). After 30 cycles of PCR amplification, MT-1B had not yet appeared, although its active status with Zn treatment was confirmed elsewhere (Figs 2B, 2D, 3A, 3B). The band intensity of the housekeeping gene G3PDH also increased with increasing number of PCR cycles. Therefore, the relative abundance of the active isogenes in human RBC precursors is in the order: MT-2A > (MT-1G ≈ MT-1E ≈ MT-1X) > MT-0 > MT-1A > MT-1B.

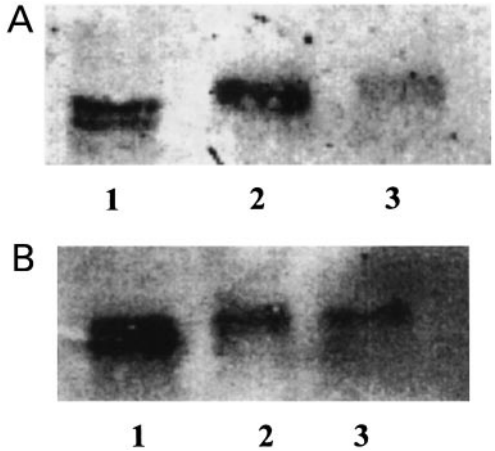

Fig. 6. Immunoblotting demonstration of MT in RBC precursors from human cord blood. Monoclonal mouse anti-MT (E9) was used as the primary antibody; alkaline phosphatase-conjugated goat anti-mouse IgG was used for indirect staining of the mouse anti-MT. (A) Zinc induction of MT in *ex vivo* expanded BFU-E cell population is shown by the difference in band intensity of MT from Zn-induced (lane 2) and control cultures (lane 3); Zn induction was carried out at 100 μM Zn for 72 h. (B) Presence of MT in total protein extract from $CD71^+$ (lane 2) and gly A^+ (lane 3) cell populations; total protein was extracted from the respective cell populations immediately after MACS separation. Lane 1 in both (A) and (B) contains purified total MT from human fetal liver.

Effect of EPO and IL-3 on MT isogene transcription in nRBCs

The effect of EPO and IL-3 on MT isogene transcription in nRBCs was shown using RT-PCR. In the EPO-supplemented control cultures, MT-1X and MT-2A (Fig. 5A) were constitutively transcribed, and in the IL-3-supplemented control cultures, only MT-2A (Fig. 5C) was constitutively transcribed. With the addition of 100 μM Zn, MT-1E, MT-1G, MT-1X, and MT-2A were found to be transcribed in cultures containing either EPO or IL-3 (Fig. 5B,D). Unlike in the IL-3-containing cultures and the control cultures without EPO/IL-3, transcription of MT-1X in addition to MT-2A was detected in the EPO-containing cultures. On the other hand, relatively weak transcription of MT-2A was observed in IL-3-containing Zn-induced cultures (Fig. 5D) compared with that in IL-3-containing control cultures (Fig. 5C). A weaker signal for MT-1G was also observed in IL-3-containing Zn-induced cultures (Fig. 5D). These observations confirm the effect of erythroid differentiation and/or proliferation growth factors such as EPO and IL-3 on specific MT isogene transcription.

Detection of MT at the protein level by immunoblotting

Biosynthesis of MT was demonstrated in *ex vivo* expanded BFU-E colonies and gly A^+ and $CD71^+$ cells by immunoblotting (Fig. 6A,B). The presence of MT on the blotting paper was detected by indirect immunochemical staining using monoclonal mouse anti-MT (E9) as the primary antibody. Purified total MT from human fetal liver was stained in the parallel lanes (lane 1) as the positive control. At 72 h after treatment with 100 μM Zn, upregulation of MT biosynthesis was shown by the difference in band intensity from Zn-induced and control cultures of nRBCs (Fig. 6A). The presence of MT in gly A^+ and $CD71^+$ cells (Fig. 6B) immediately after MACS separation was also confirmed even without Zn induction. MT induction was not attempted in these cell populations as they were found to be in the later stages of terminal differentiation.

DISCUSSION

Since the complete disclosure of the human MT gene family on chromosome 16q13 [39,40], isogene-specific expression of members of this protein family has received greater attention. The presence of MTs in human erythrocytes has been documented elsewhere [10,41]. The nucleated precursor origin of MTs in murine erythrocytes has long been hypothesized [31], and we have recently demonstrated Zn induction of MTs in nRBCs from human cord blood [34]. Using the RT-PCR developed by Mididoddi *et al*. [24], later modified by Vandeghinste *et al*. [7], we continued investigating the active MT isogenes in human nRBCs.

Seven functional isogenes, MT-1A, MT-1B, MT-1E, MT-1G, MT-0, MT-1X, and MT-2A, are confirmed to be at least transcriptionally active in human RBC precursors. This was shown either by *in vitro* Zn treatment of the *ex vivo* expanded RBC precursors or in MACS-separated gly A^+ and $CD71^+$ cells without *in vitro* Zn treatment. It is important to mention here that a higher percentage of more mature forms of nucleated precursors was found in the MACS-separated gly A^+ and $CD71^+$ cells (Fig. 1B,C) than in *ex vivo* expanded precursors (Fig. 1A). Considering tissue-specific or cell-specific MT isoform expression stringency as summarized above, the *in vitro* constitutive transcription of only MT-2A in nRBCs at 48 h and switching on of three more isogenes (MT-1E, MT-1G, and MT-1X) at 96 h suggest the transcriptional activation of different MT isogenes at different stages of erythroid differentiation, i.e. more isogenes are expected to be transcribed in more mature forms of precursors.

The peak MT expression at later stages of differentiation requires explanation, which could be an *in vivo* kinetic coincidence instead of a correlation between differentiation and MT biosynthesis. However, growing evidence on the possible involvement of MTs in differentiation in many cell types and its nuclear localization [19] suggest that it is not a kinetic coincidence.

Constitutive transcription of MT-1X in addition to MT-2A after EPO addition, unlike IL-3 addition, and without addition of either of these growth factors in nRBC cultures, reveals the effect of these erythroid proliferation and/or differentiation-promoting factors on MT isogene tanscription. Therefore, EPO is shown to be a new member in the growing list of MT inducers. A weaker signal for MT-1G and MT-2A in IL-3-containing Zn-treated cultures again indicates the effect of IL-3 on individual MT isogene transcription.

The functional MT isogenes were analyzed by RT-PCR (a) to investigate their transcription pattern in nRBCs at different concentrations of Zn, and (b) to assess their relative abundance and order of activation in response to Zn. In our experiments, the housekeeping gene G3PDH was always included for comparison and to select the number of cycles for the assessment of PCR products before amplification reached its plateau. Zinc-induction experiments were conducted at different concentrations of Zn ranging from 30 μM to 150 μM. Under the latter conditions, cell viability was reduced obviously because of the toxic level of Zn. However, no more than the seven mentioned isogenes were found to be transcribed. Under the former conditions, i.e. at 30–50 μM Zn, variation in the MT-1E transcripts was observed, and three other isogenes (MT-2A, MT-1G, and MT-1X) were found to be transcribed in all cases. The active MT isogenes in nRBCs from human cord blood are most likely in the order MT-2A > (MT-1G≈MT-1E≈MT-1X) > MT-0 > MT-1A > MT-1B. However, a more precise and quantitative correlation should be made using strictly quantitative PCR techniques.

The absence of MT-1F isogene transcription in nRBCs from human cord blood even after induction with 100 μM Zn at 96 h requires further investigation of tissue/cell-specific regulation of transcription. Unique differential expression of MT-1F, three times lower than MT-1G, was explained in response to Cd by the difference in the distinct TATA motifs in the promoter region of the two isogenes [25]. Among the other functional MT isoforms, MT-3 and MT-4 were also found to be transcriptionally inactive in nRBCs. Although the active status of the MT-4 isogene could not be demonstrated in the purest form of RBC precursors obtained by *ex vivo* expansion of BFU-E colonies, the presence of this transcript in gly A^+ and $CD71^+$ cells could not be rejected as a false-positive PCR product. The absence of genomic DNA contamination, the correct size of the amplified sequence, and the absence so far of any report of a pseudo-gene for MT-4 means that the only possible explanation of its origin is other cell type(s) separated along with the gly A^+ and $CD71^+$ cells as a result of non-specific selection during MACS.

Many workers have found it difficult to demonstrate MT in a single band at the correct M_r position (6000–7000) by immunoblotting. Polymerization due to oxidation or other unknown cause has been blamed for the smear-like appearance of MTs on blots. A special Western-blotting procedure was therefore designed [38] as described in Materials and methods. In our experience, with the use of this protocol, the smear-like appearance on the blots can be avoided, but getting the bands in the correct M_r position cannot always be guaranteed. However, the parallel positions of the purified human MTs and staining with mAbs confirm the presence of MTs in the nRBCs (Fig. 6A) and in the MACS-separated glyA$^+$ and $CD71^+$ cells (Fig. 6B). Because of the limited number of nRBCs harvested by *ex vivo* expansion of the precursors, differential expression of MT isoforms at the protein level was not attempted, but this is currently under investigation using a recent development in mass spectrometry.

The physiological and immunological significance of Zn was established as an integral component of this element in many enzymes, transcription factors and other proteins [42–44]. Nutritional Zn deficiency was found to be associated with many clinical symptoms [45,46]. Therefore, it was of clinical importance to find a reliable and easily monitored index of overall Zn status. Several investigators suggested erythrocyte Zn-MT as a potential index for overall Zn status [10,41,47]. Increases in erythrocyte Cd-MT content were found to correlate with the amount of Cd administered to mice [32]. However, to make the proper correlation between overall Zn status and respective MT in erythrocytes in a particular clinical/physiological condition, it is important to know the MT source in mature RBCs. Therefore, the observation of MT biosynthesis and upregulation at the protein level with Zn treatment as demonstrated by immunoblotting would

provide a significant parameter to consider erythrocyte Zn-MT as a measure of overall Zn status. It remains to be resolved whether receptor-mediated transfer of MT can take place, although at least one report has demonstrated the MT receptor on astrocytes [48].

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