Localisation of metallothionein isoform mRNAs in rat hepatoma (H4) cells

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Abstract The localisation of metallothionein isoform mRNAs in rat hepatoma (H4) cells was investigated using two approaches, namely Northern hybridisation of total RNA extracted from free, cytoskeletal-bound and membrane-bound polysomes isolated by a sequential detergent/salt extraction procedure and in situ hybridisation. The cytoskeletal-bound polysomes were enriched in metallothionein-I (MT-I) and c-myc mRNAs but showed a significantly lower enrichment in MT-II mRNA. These findings indicate that the MT-I mRNA is localised to the cytoskeleton during translation. In situ hybridisation using a biotin-labelled oligonucleotide probe revealed a predominantly perinuclear localisation for the MT-I mRNA.

Key words: Cytoskeleton; Polysome; mRNA; Metallothionein; Metal; Compartmentation

1. Introduction

The localisation of certain mRNAs to distinct subcellular sites has been well documented in oocytes and early embryos of Xenopus and Drosophila, and there is increasing evidence that, similarly, mRNAs for some intracellular proteins are localised in differentiated somatic mammalian cells (for reviews see [1,2]). For example, the mRNA coding for β-actin is present in the peripheral cytoplasm in spreading fibroblasts [3], whilst in muscle vimentin mRNA is found close to the costameres [4]: in such cases it appears that the mRNAs are localised close to the site of function of the encoded protein, suggesting that localisation of some mRNAs may play a role in facilitating subsequent protein targeting. There have been a large number of reports which demonstrate an association of mRNA and polyribosomes (polysomes) with the cytoskeleton [5–8], and in addition recent evidence suggests that cytoskeletal elements are involved in the localisation process [9,10].

Metallothioneins (MTs) are a group of cysteine-rich, low molecular weight intracellular proteins which have a high affinity for divalent metals such as zinc (Zn^{2+}), copper (Cu^{2+}), cadmium (Cd^{2+}) and mercury (Hg^{2+}) and monovalent metals such as copper (Cu^+) and silver (Ag^+). These metals, as well as other endogenous factors such as glucocorticoid and certain cytokines, are also capable of inducing MT gene expression [11]. In mammals, there are multiple MT genes giving rise to a family of isoforms with highly-conserved cysteine-rich regions. The number of isoforms varies between species, there being two major isoforms in the rat. Several functions have been proposed

for MT, such as protection against toxicity of heavy metal ions [12], an antioxidant role [13] and in the regulation of hepatic zinc and copper metabolism [14–16]; however, at present it is not known whether the isoforms of this protein are conferred with different functions in any cell type. Neither is it known if the isoforms possess different subcellular distribution, although, interestingly, under certain circumstances MT has been found in the nucleus [17,18]. This paper provides evidence that in rat hepatoma cells MT-I mRNA is translated in cytoskeletal-bound polysomes (CBP) and present predominantly in the perinuclear cytoplasm.

2. Materials and methods

2.1. Cell culture

Rat liver hepatoma (H4-II-E-C3, ECACC number 85061112) cells were grown in 90-mm Petri dishes in Dulbeccos minimal Eagle's medium supplemented with 12% fetal calf serum at 37°C in an atmosphere of 5% CO_2. MT was induced by addition of zinc chloride and the concentration of zinc in aliquots of medium removed prior to cell fractionation or fixation was determined by atomic absorption spectrophotometry.

2.2. Cell fractionation

Cells were pre-treated with emetine (100 ng/ml medium) for 30 min in order to maintain mRNA-ribosome interactions [19], rinsed 3 times with PBS (10 mM phosphate buffer, pH 7.4 containing 0.9% NaCl) and then scraped into buffer A (10 mM Tris containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$). Cytosolic, cytoskeletal and membrane fractions were then isolated by a sequential detergent/salt extraction procedure, essentially as described previously [20]: treatment with 2.5 ml of buffer A containing 0.1% nonidet-P-40 (10 min, 4°C) lysed the cells and released a fraction containing free polysomes (FP); extraction with 2.5 ml of buffer A containing 160 mM KCl for 10 min at 4°C released cytoskeletal-bound polysomes (CBP) and treatment with 2.5 ml of buffer A containing 130 mM KCl, 0.5% N-P40 and 0.5% deoxycholate solubilized the endoplasmic reticulum and released membrane-bound polysomes (MBP). Aliquots of fractions were taken for enzyme and protein assays and the remainder layered on a 40% sucrose cushion and polysomes separated from monosomes and lighter material by centrifugation at $32,000 \times g$ for 17 h [21,22]. All buffers and cushion solutions were autoclaved as appropriate; extraction buffers contained RNAse inhibitor RNAsin (133 units/ml; Promega, UK).

Lactic dehydrogenase was measured spectrophotometrically [23]. Actin and vimentin were assayed by enzyme-linked immunosorbent assay [8,24] using actin and vimentin standards and anti-actin from Sigma Chemical Co., Poole, Dorset, UK and anti-vimentin from Amersham International Ltd, Amersham, UK.

2.3. RNA extraction and hybridisation

Total RNA was extracted from cells or polysome pellets by the acid/guanidinium/phenol/chloroform method of Chomczynski and Sacchi [25] and assessed by the A_{260}/A_{280} absorbance ratio. RNA species were then separated by electrophoresis through a 2.2 M formaldehyde, 1.2% agarose gel and then transferred to a positively charged nylon membrane (Genescreen, NEN Dupont Ltd.) by capillary blotting. RNA was fixed to the membrane by exposure to UV light and the

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membranes were stored dry until required. The membranes were pre-hybridised for 6 h at 42°C with 10% dextran sulphate, 0.2% bovine serum albumin, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.1 mg/ml salmon sperm DNA, 50% formamide, 0.1% sodium pyrophosphate, 1% SDS and 50 mM Tris-HCl, pH 7.5.

The c-myc probe was a 1.8 kbp HindIII fragment derived from a cDNA corresponding to the three exons of the c-myc gene (a gift from Dr. M. Cole, Princeton University, NJ, USA). The MT-I and MT-II oligonucleotide probes were a 28-mer and 30-mer complementary to 5′-untranslated region sequences and specific to the MT-I and MT-II isoforms (Vasconcelos, Beattie and Hesketh, unpublished observations): they were synthesized by the cyanoethyl phosphoramide method in a Cruachem PS 250 automated DNA synthesizer. The glut-1 probe was a 2.0 kb BglI fragment from the rat cDNA (a gift from Dr. R. Knott, Dept. of Opthalmology, University of Aberdeen). 100 ng of the c-myc and Glut-1 probes were labelled with [^{32}P]dCTP by random priming (Amersham Multiprime kit) and the labelled probe was separated from free nucleotides by gel filtration through a Sephadex G-50 column. The MT-I probe was end-labelled with [^{32}P]ATP by incubation with 4 units T4-kinase (New England Biolabs Ltd.) and purified using Clontech chroma spin-10 columns. The labelled probes were then added to the pre-hybridisation mix and the membranes hybridized for 20 h at 42°C. The membranes were then washed to remove non-specifically bound probe. In the case of c-myc and glut-1 hybridizations the membranes were first washed in $2 \times$ SSC twice at room temperature for 10 min, followed by 0.5% SSC, 1% SDS at 65°C for 1 h twice. In the case of MT-I hybridisations, the membranes were initially washed with $6 \times$ SSC at room temperature for 10 min twice, followed by 0.1% SSC, 0.1% SDS at 48°C for 20 min. Specific hybridization was then detected either by autoradiography using Hyperfilm-MP (Amersham International, UK) at −70°C or with a Packard instantimager which was used to quantify the hybridisation signal from the specifically-bound probe. Loading errors were corrected by a control hybridisation with a 1.4 kb probe for 18S rRNA [26].

2.4. In situ hybridisation

Cells were grown on glass chamber slides and washed three times with PBS before fixation with 4% paraformaldehyde (PFA) in PBS. After partial dehydration in 70% ethanol for 1 h at 4°C cells were permeabilised by treatment with 0.2% Triton X-100 in 4% PFA/PBS (4°C). RNAse controls were then treated with 1000 µg/ml RNase A in 10 mM Tris-HCl, pH 7.5 containing 0.5 M NaCl for 30 min at 37°C and a further fixation performed. After incubation in 50% formamide, $2 \times$ SSC at room temperature for 10 min the cells were hybridised overnight with 0.2 ng/µl biotinylated MT-I probe (identical in sequence to the probe used for northern blot hybridizations) at 37°C; control patches were incubated with the in situ hybridization mix alone and contained no probe. After hybridization non-specifically bound probe was removed by washing in increasingly stringent SSC solutions and the bound probe was detected by incubation with alkaline phosphatase linked to streptavidin (Amersham International, UK) and then addition of 4-nitro blue tetrazolium for 2–3 h.

3. Results

3.1. Induction of metallothionein

MT-I mRNA levels are low in cells grown in normal culture medium containing only 0.5 µg zinc/ml (Fig. 1a). In order to study MT-I mRNA distribution it was necessary to increase MT expression and this was achieved by exposing the cells to zinc for up to 18 h prior to extraction. Northern hybridisation showed an increasing induction in MT-I mRNA expression as the zinc concentration was increased up to 6 µg/ml. After correction of the hybridisation signal for that achieved with a probe for glyceraldehyde 3-phosphate dehydrogenase mRNA there was approximately a 5-fold increase in transcript levels in comparison to control cells (results not shown). All subsequent experiments were carried out using cells in Zn-supplemented medium (final concentration 6 µg/ml) for 18 h prior to assessment of MT-I mRNA distribution.
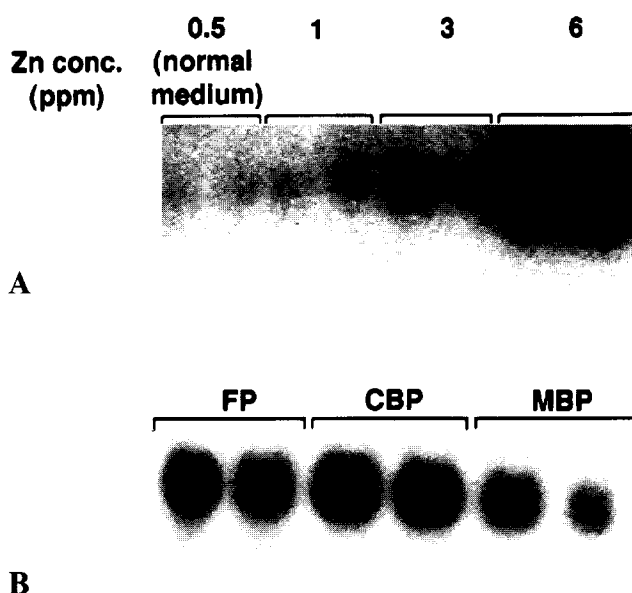

Fig. 1. Induction and distribution of MT-I mRNA studied by Northern hybridization. (A) Northern hybridization with the MT-I probe showing induction of MT-I mRNA by the presence of zinc for 12 h. Each lane was loaded with 20 µg of total RNA, and each Zn treatment was performed in duplicate. As can be seen from this autoradiograph there is an increase in MT-I mRNA levels with increasing amounts of zinc present in the medium with the largest induction clearly being produced with a zinc concentration of 6 µg/ml. (B) Northern hybridization showing the distribution of the MT-I message in the three polysome fractions from cells in which MT was induced by incubation with 6 µg/ml Zn for 18 h. FP, free polysomes; CBP, cytoskeletal-bound polysomes; MBP, membrane-bound polysomes. Each lane was loaded with 20 µg total RNA which was extracted from a polysome preparation of each fraction. As can be seen the hybridization signal is most intense for the CBP.

3.2. Fractionation of H4 cells to yield three polysome populations: free, cytoskeletal- and membrane-bound

Free, cytoskeletal and membrane-bound polysome fractions were isolated from H4 cells using a sequential detergent/salt extraction procedure [20]. A concentration of 0.1% NP-40 in low salt extraction buffer was sufficient to release 87% of lactic dehydrogenase activity and on this basis the fraction produced by such treatment was judged to be a cytosolic fraction containing free polysomes (FP). This concentration of NP-40 is somewhat higher than that required with 3T3 fibroblasts but less than that required for HepG2 and ascites cells: it emphasises that the concentration needed to release the bulk of the cytosolic material varies between different cell lines [7,8,20,22]. Enzyme-linked immunoassay of vimentin and actin in fractions from three replicate experiments showed an enrichment of these proteins in the high salt extract (Fig. 2) suggesting that, as in other cell lines, the salt treatment caused breakdown of the cytoskeleton. Thus, by virtue of the effect of the increased salt on release of cytoskeletal proteins this fraction was deemed to contain material (including polysomes and mRNA) associated with the cytoskeleton.

Further characterisation was carried out by Northern hybridisation to study the distribution of two marker mRNAs in the three polysome fractions. Firstly, c-myc, which has consistently been found to be present in the cytoskeletal-bound polysome fraction in a number of cell lines [20,22,27] was found to be enriched in the presumptive cytoskeletal fraction in H4
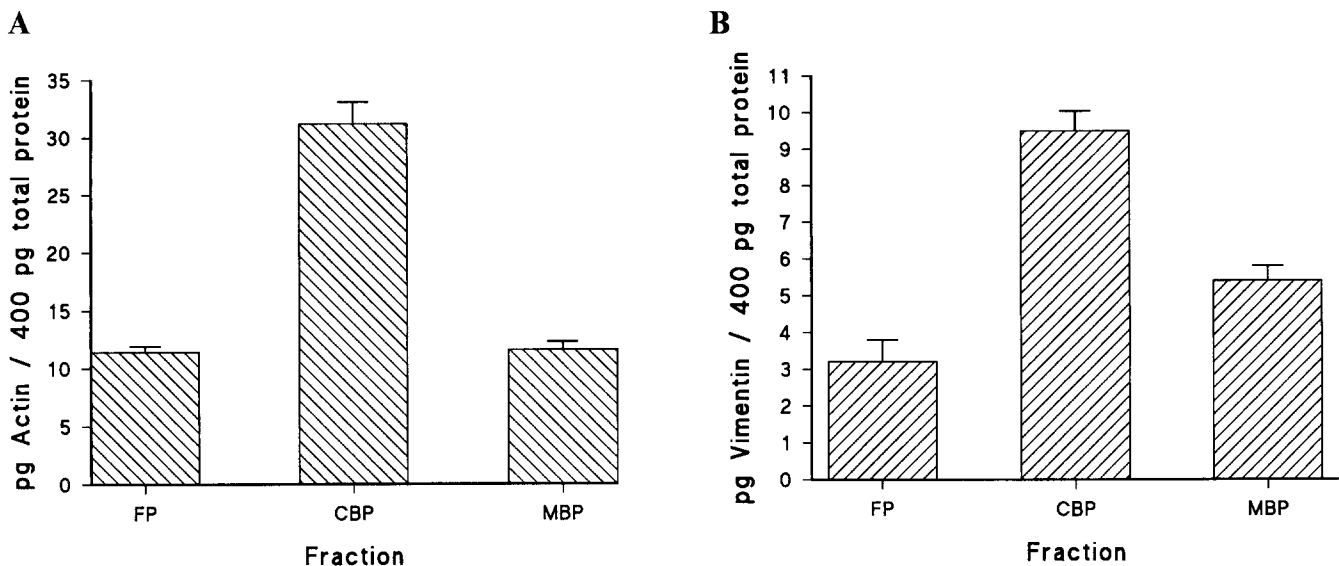
Fig. 2. Determination of the concentration of actin and vimentin present in the cytosolic, cytoskeletal and membrane fractions by ELISA. Results are shown as means ± S.E.M. from three fractionation experiments. ELISA was performed using 400 pg total protein from each fraction the amount of actin present calculated by comparison with absorbance produced by 3–100 pg actin and 1.5–50 pg vimentin standards; the results expressed in terms of picograms of actin (A) and vimentin (B) per 400 pg total protein.

cells (Table 1). Secondly, the mRNA which codes for the integral membrane protein glucose transporter 1 (glut-1), and which is therefore a marker for the membrane-bound polysomes, was found to be highly enriched in the MBP fraction.

To determine if the total RNA fractions obtained from the polysome pellet contain the same proportion of $poly(A^+)$ mRNA in relation to total RNA, a slot blot was prepared in which 2 μg of total RNA from each fraction was loaded in duplicate onto a nylon membrane. After hybridisation with a 25-mer polyT-oligonucleotide end-labelled with $[^{32}P]ATP$, quantification of the specifically-bound DNA indicated that comparable amounts of the oligonucleotide were bound by FP, CBP and MBP (759, 961 and 1005 counts bound respectively). This indicates that a given amount of total RNA from each polysome fraction contained approximately the same amount of mRNA.

MT-I mRNA was found to be enriched in the CBP fraction (Fig. 1b). Quantification of the data, and correction for hybridisation to a ribosomal RNA probe to correct for any differences in gel loading, confirmed that the distribution of MT-I mRNA paralleled that of c-myc with the an enrichment in the

CBP fraction (Table 1): approximately 55% of both MT-I and c-myc mRNAs was recovered in the CBP fraction. The recovery of c-myc and MT-I in these polysomes occurs under conditions where there are comparable amounts of total mRNA in FP, CBP and MBP, thus indicating that the observed enrichment of MT-I and c-myc mRNAs in CBP is not due to a some non-specific effect. Reprobing these filters with a second specific oligonucleotide showed that there was a significantly lower ($P < 0.05$) enrichment of MT-II mRNA in CBP, and a small but significant reduction in the proportion of the MT-II mRNA recovered in CBP (Table 1).

3.3. In situ hybridization

In situ hybridization of H4 cells was carried out using the same oligonucleotide as was used in the Northern blot analysis, but in this case labelled with biotin. As shown in Fig. 3 visual inspection showed a much greater staining in cells in which MT-I was induced by 6 μg/ml zinc (Fig. 3b) compared to uninduced cells (Fig. 3a), as expected from the results of Northern hybridisation showing a marked (five-fold) increase in MT mRNA under these conditions (Fig. 1). At higher magnification

Table 1
Quantification of the distribution of c-myc, glut-1, MT-I and MT-II mRNA in free polysomes (FP), cytoskeletal-bound polysomes (CBP) and membrane-bound polysomes (MBP)

mRNA	Transcript abundance (n = 3)			Percentage of total transcript (n = 3)		
	FP	CBP	MBP	FP	CBP	MBP
c-myc	100	202 ± 19	139 ± 15	15 ± 4	56 ± 2	28 ± 1
glut-1	100	169 ± 59	390 ± 90	14 ± 4	27 ± 1	58 ± 5
MT-I	100	170 ± 9*	99 ± 12	18 ± 3	55 ± 2	25 ± 2
MT-II	100	131 ± 7*	108 ± 17	23 ± 4	49 ± 1	28 ± 5

The transcript abundance values are expressed per unit 18s rRNA and were normalized taking the abundance of FP as 100 for each experiment. The 'percentage of total transcript' values were calculated by firstly determing the total counts present in each fraction for each transcript (knowing the volume of fraction loaded and the total volume of fraction) and then summing these values and obtaining the percentage of this sum which was attributable to each fraction. Errors are expressed as S.E.M. Enrichments in CBP were compared using a Student's t-test, *$P < 0.05$.
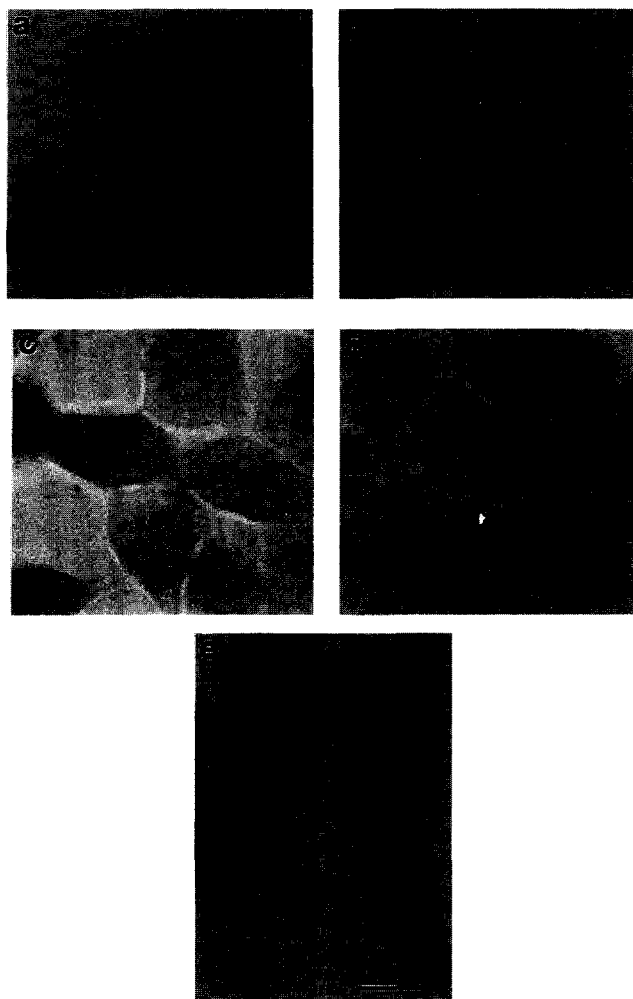
Fig. 3. In situ hybridisation showing the distribution of MT-I mRNA in rat hepatoma (H4) cells. Cells were hybridised with an biotin-labelled oligonucleotide probe specific for MT-I mRNA. Specific labelling was detected using steptavidin linked to alkaline phosphatase and 4-nitroblue tetrazolium as substrate. (A) cells in which MT was induced with 6 μg/ml Zn for 18 h. (B) uninduced cells grown in normal medium which was not supplemented with additional Zn. (C) picture at higher magnification of cells in which MT was induced with 6 μg/ml Zn for 18 h. (D) control cells which were not hybridized with the biotinylated MT-I probe. (E) control cells which were treated with RNAse A before hybridisation with the probe. Bar represents 10 μm.

it was evident that in the induced cells (Fig. 3e) the staining was predominantly perinuclear. Cells which were either not incubated with the labelled oligonucleotide (Fig. 3d) or which were pre-treated with RNase before hybridisation (Fig. 3e) showed a very low level of non-specific staining.

4. Discussion

The present results show that the MT-I mRNA is enriched in polysomes which are released, along with significant amounts of actin and vimentin proteins, from the cell matrix by salt; therefore, they indicate that MT-I mRNA is translated in association with the cytoskeleton. In addition, the in situ hybridisation data suggest that the MT-I mRNA is not found equally distributed throughout the cytoplasm but is predomi-

nantly in the cytoplasm around the nucleus and not in the peripheral cytoplasm. Further localisation is hindered by the limited volume of cytoplasm present in these cells.

In HepG2 cells c-myc, cyclinA and two ribosomal protein mRNAs [22] have been found to be enriched in CBP and, on the basis of these results and the perinuclear localisation of c-myc mRNA [27], it has been suggested that one class of mRNAs found on CBPs is that corresponding to the mRNAs encoding proteins which are transported into the nucleus. The finding that MT-I mRNA is enriched in CBP could be related to the observations that during cell proliferation [17], early mammalian development [18], and possibly after metal loading [28] MT is found in the nucleus. It is possible therefore that the positioning of the MT-I mRNA around the nucleus and in CBP results in synthesis of the protein in the perinuclear cytoplasm so that under appropriate conditions it can be transported into the nucleus. Furthermore, the translation of the MT-I mRNA in association with the cytoskeleton might suggest the possible involvement of cytoskeletal filaments in this translocation process. Alternatively, the cytoskeleton-associated translation of MT-I mRNA may reflect a role of the newly made MT protein in protecting the cytoskeleton since it has been shown that cultured cells exposed to cadmium and nickel have altered cytoskeletal organisation [29].

The observation that the mRNAs for β- and -isoforms of actin are differentially located within myoblasts [30] suggests that for intracellular proteins which exist as isoforms, the differential localisation of the isoform mRNAs may provide a means whereby cells can synthesise different isoforms in separate compartments for subsequent targeting of the proteins to specific subcellular sites; in turn this may confer the isoforms with different functions. Analysis of MT-II mRNA distribution in H4 cells indicates that the enrichment of the MT-II transcript in CBP is less than that of the MT-I transcript, although some 49% of the MT-II mRNA was recovered in CBP. These data suggest that the MT-II mRNA is associated with CBP but more weakly than MT-I mRNA and, in turn this implies that there is some compartmentation of MT-I and MT-II mRNAs. However, the present observations have been made on only a single cell line and further studies are required to determine whether MT isoform mRNAs are translated in different subcellular locations in order to functionally differentiate between MT isoforms.

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