Nuclear Import of Metallothionein Requires Its mRNA to Be Associated with the Perinuclear Cytoskeleton

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The influence of mRNA localization on metallothionein-1 protein distribution was studied by immunocytochemistry. We used Chinese hamster ovary cells that had been transfected with either a native metallothionein-1 gene construct or metallothionein-1 5'-untranslated region and coding sequences linked to the 3'-untranslated region from glutathione peroxidase. The change in the 3'-untranslated region caused the delocalization of the mRNA with a loss of the perinuclear localization and association with the cytoskeleton. Clones were selected which expressed similar levels of metallothionein-1 protein, as assessed by radioimmunoassay. The results showed that loss of metallothionein-1 mRNA localization was associated with a loss of metallothionein-1 protein localization, most notably with a lack of metallothionein-1 protein in the nucleus of synchronized cells which were beginning to synthesize DNA. This indicates that the association of metallothionein-1 mRNA with the cytoskeleton around the nucleus is essential for efficient shuttling of the protein into the nucleus during the G1 to S phase transition. This is the first demonstration of a physiological role for perinuclear mRNA localization and we propose that such localization may be important for a wide range of nuclear proteins, including those that shuttle between nucleus and cytoplasm in a cell cycle dependent manner.

Delivery of proteins to their site of function could theoretically be achieved by either polypeptide targeting motifs, by targeting of the mRNAs and local protein synthesis, or a combination of these two processes. It is becoming increasingly clear that in a wide range of cells from yeast to mammals, certain mRNAs are localized in specific cytoplasmic regions and/or associated with the cytoskeleton (1–4). This phenomenon is due to targeting signals within their 3'-untranslated region (3'-UTR). For example, the mRNA for β-actin is present in the peripheral cytoplasm of spreading fibroblasts (5) whereas the mRNA coding for c-Myc has a perinuclear localization (6); in both cases targeting is achieved by a 3'-UTR signal (7, 8). It has been suggested that mRNA localization could provide a mechanism to synthesize proteins close to their site of function. Such mRNA targeting and protein synthesis could provide a critical mechanism in cell organization and development.

Metallothioneins (MTs) are a family of low molecular mass proteins characterized by a high cysteine content and a high binding capacity for transition metals (9). There are several MT genes giving rise to different protein isoforms which all have in common a highly conserved coding region. The number of isoforms differs from one species to the other. Several functions have been attributed to MTs, such as detoxification of heavy metals (10) and regulation of zinc and copper metabolism in the liver (11). More recently, it has been proposed that MTs could have a role in protecting DNA against oxidants (12) and may play a role in cell proliferation (13) as well as in apoptosis (14); however, the mechanisms involved remain unclear.

Although MTs were originally considered to be largely cytoplasmic proteins, more recent evidence shows that MT-1 and/or MT-2 are found localized in the cell nucleus under certain physiological circumstances, for example, during the G1 to S phase transition of the cell cycle (15–17). This suggests that mRNA localization and association with the cytoskeleton is important in allowing the protein to be synthesized in a location which allows efficient nuclear import when required. The aim of the present study was to investigate whether perinuclear localization of MT-1 mRNA promotes subsequent compartmentalization of MT-1 protein as well as its shuttling into the nucleus during the G1 to S phase transition of the cell cycle. Since the targeting of MT-1 mRNA depends upon its 3'-UTR (18), our approach has been to study MT-1 mRNA distribution in cells transfected with gene constructs in which the MT-1 coding sequence is linked either to its native 3'-UTR or to that from glutathione peroxidase which does not contain a perinuclear localization signal.

EXPERIMENTAL PROCEDURES

Materials—Mouse anti-MT (E9) was purchased from DAKO Ltd. (Cambridge, United Kingdom) and FITC goat anti-mouse IgG was from AMS Biotechnology (Oxford, UK). Both normal mouse and goat sera were from the Scottish Antibody Production Unit (Carluke, UK). [3H]Thymidine was purchased from Nycodem-Amersham. The Ham's F-12 medium was from ICN Biomedicals (Basingstoke, UK) and all other chemicals were purchased from Sigma (Poole, UK).

Cell Culture and Synchronization—Chinese hamster ovary cells (CHO, ECACC number 85050302) were grown in Ham's F-12 modified medium supplemented with sodium bicarbonate (1.176 g/liter), 10%
A

MTMT

B

MTGSH

Fig. 1. Metallothionein distribution in exponentially growing transfected CHO cells. A, cells were transfected with gene constructs containing the MT-1 5′-UTR (black) and coding sequence (white) linked to either the MT-1 3′-UTR (blue) (MTMT) or the 3′-UTR from glutathione peroxidase (orange) (MTGSH). B, immunostaining of exponentially growing cells after incubation with anti-MT-1 antibody (E9) and FITC-labeled anti-mouse IgG. There was a distinct perinuclear staining in MTMT cells (1) but in MTGSH cells MT-1 was present throughout the cytoplasm and showed no localization (2). Untransfected cells (3) showed no staining. Note that in MTMT cells (1) the nucleus is surrounded by strong staining but as is evident from confocal analysis there is no staining within the nucleus (see Fig. 2). Bars represent 10 μm.

mRNA Localization Promotes Nuclear Import of Metallothionein

fetal calf serum (FCS), penicillin (50 IU/ml), streptomycin (50 μg/ml), and amphotericin B (Fungizone, 2.5 μg/ml) at 37 °C in an atmosphere of 5% CO₂. Cells were synchronized in Go phase by serum deprivation using culture in 0.5% FCS for 48 h. The subsequent release into the S phase was achieved by replacement of low serum medium, by medium containing 10% FCS and DNA synthesis was monitored by thymidine incorporation.

Transfected Cell Lines—The two transfected CHO cell lines studied have been previously described (18). MTMT cells expressed a gene construct consisting of the MT-1 cDNA (i.e. 5′-UTR, coding sequence, and 3′-UTR) digested with BamHI-ApalI and inserted into the polylinker region of pcDNA3. The MTGSH cells expressed a construct containing the 5′-UTR and coding region of MT-1 linked to the 3′-UTR of the glutathione peroxidase. This was inserted into the BamHI-NotI site of the vector pcDNA3 (Fig. 1, panel A). For each of the two transfected cell lines, clones were selected and those expressing the same amount of MT-1 protein, as assessed by radioimmunoassay (20), were used for further study.

Thymidine Incorporation—2 × 10⁴ cells were plated into 6-well plates and left to attach and grow for 16 h. They were then synchronized into Go phase of the cell cycle with culture medium containing 0.5% FCS, for a further 48 h. Serum was then added back to a final concentration of 10% and [³H]thymidine was added in each well and the cells were incubated for 30 min. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and scraped off in 0.2 M perchloric acid into a microcentrifuge tube. This suspension was incubated on ice for 30 min and then centrifuged at 14,000 rpm for 10 min. The pellets were washed twice with 0.2 M perchloric acid, before being resuspended in 0.5 M perchloric acid. DNA was extracted in hot perchloric acid by heating the suspension at 70 °C for 30 min. After brief cooling, the Microfuge tubes were centrifuged as before and the supernatant was transferred to scintillation counting vials. The amount of [³H]thymidine label was determined in Packard 1900TR liquid scintillation fluid. Incorporation was expressed per mg of total protein.

Immunocytochemistry—Cells were subcultured into 8-well chamber slides and left to attach and grow for 2 days. Prior to immunocytochemistry, cells were fixed in 4% formaldehyde at room temperature overnight. The cells were then washed three times in PBS (5 min each) and the nonspecific binding sites were blocked using 10% goat serum for 30 min at room temperature. Cells were then incubated with a monoclonal anti-MT antibody (E9, diluted 1:50 in PBS) for 12 h at 4 °C in moist chambers. This E9 monoclonal antibody recognizes a very well conserved epitope found in the MT-1 and MT-2 of several mammalian species. Slides were then washed 3 times (5 min each) with PBS and FITC-conjugated goat anti-mouse secondary IgG (diluted 1:50 in PBS) applied to the cells for 2 h at room temperature. After another 3 washes in PBS, the slides were mounted with Citifluor. The specificity of the staining was assessed with 3 different controls: 1) substitution of the primary antibody with normal mouse serum; 2) labeling of untransfected CHO cells which have a very low constitutive level of MT; and 3) omission of the primary antibody.

Microscopy and Image Analysis—Standard microscopy was performed using a Zeiss Axioplan microscope and cells were photographed under the ×100 oil immersion lens, using Kodak EliteII films. Cells were also examined under a Bio-Rad MRC 1024 confocal microscope, z-series images captured using lasersharp software and selected images converted into TIFF files using confocal assistant software. Staining distribution in the images from the confocal microscope were then quantified using these TIFF files and Freena/Cyclops image analysis software (Kinetic Imaging, Liverpool, UK). Quantification was carried out either by constructing a distribution profile across the cells or by measurements of staining intensity in cytoplasmic regions. Profiles were obtained by drawing an arbitrary line across the image of a cell; the Freena software was then used to display the intensity of staining all along this line. For measurements of staining intensity within discrete compartments 15 cells per cell line were analyzed as follows: eight squares of identical size were drawn, two over the perinuclear cytoplasm, two over the cell periphery, two over the nucleus, and two outside the cell to provide measurements of background staining. Staining intensity was measured as gray level units between 0 and 255 and for each square the mean gray level of intensity of staining was calculated from the gray level values of the individual pixels. Groups were compared using an ANOVA followed by Bonferroni's multiple comparison test.
RESULTS AND DISCUSSION

A Loss of MT-1 mRNA Localization Leads to a Loss of MT-1 Protein Localization—In the present study, we have investigated the effects of alteration in mRNA localization on MT-1 protein localization. Clones of cells expressing either the 5'-UTR, coding sequence and 3'-UTR from the MT-1 gene (MTMT cells), or an MT-1 gene construct in which the endogenous 3'-UTR had been replaced by that from cytosolic glutathione peroxidase (MTGSH cells) were selected and screened on the basis of their MT-1 content. Two clones were chosen for further study (one MTMT, one MTGSH) which expressed similar levels of MT-1 protein (150 ng/mg of total protein). The abundance of this protein is approximately 7-fold higher than the very low level found in untransfected CHO cells.

Using the anti-MT monoclonal antibody E9, staining was detected in both clonal cell lines but no staining was observed in the untransfected cells where the level of MT is very low (Fig. 1, panel B3). Furthermore, no staining was observed when either the primary antibody was replaced by normal mouse serum or when the primary antibody was omitted and replaced with PBS (results not shown). These negative controls show that the secondary antibody was not binding to nonspecific sites and that the signal obtained with the E9 anti-MT was specific. Furthermore, since there was no staining with untransfected cells the staining pattern obtained in the transfected clones must reflect the distribution of the transgene product and not the low level of endogenous MT.

Immunostaining showed that in unsynchronized, exponentially growing populations of MTMT cells, MT-1 was essentially localized to the perinuclear cytoplasm (Fig. 1, panel B1). In contrast, in MTGSH cells there was no particular distribution of MT-1 with the protein being found localized throughout the cytoplasm (Fig. 1, panel B2). These two patterns of staining resemble the mRNA distribution detected by in situ hybridization (18). Similar staining patterns were found using a polyclonal antibody (21) known to detect MT-1 (data not shown).

Further analysis of the immunostaining patterns was carried out by confocal microscopy. Such analysis using optical sectioning every 0.5 μm showed comparable distribution of MT-1 to standard microscopy and confirmed that in the MTMT cells the MT-1 had a perinuclear localization (Fig. 2, panel A1).

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but not in the MTGSH cells (Fig. 2, panel A2). Since this analysis was carried out using optical sections the perinuclear distribution in MTMT cells appears to reflect protein distribution rather than increased thickness of the cell in this region.

Image analysis of the staining pattern was carried out on confocal images using Fenestra/Cyclops software. As shown in Fig. 2, the differences in MT-1 distribution between MTMT and MTGSH cells were confirmed by image analysis. A profile of staining across a typical MTMT cell showed an enrichment of staining around the nucleus whereas the staining was very low in the rest of the cytoplasm and the nucleus (Fig. 2, panel B1). In contrast, the profile of the MTGSH cells showed uniform staining in the cytoplasm and less staining in the nucleus (Fig. 2, panel B2). Quantification of the staining in the two cell lines was also carried out by making intensity measurements in discrete regions of the cells. The data for nucleus, peripheral cytoplasm, and perinuclear cytoplasm are shown in Table I as gray level intensities. Since these values are derived from measurements on a standard area of electronic slices of standard thickness the values reflect MT-1 concentration. In MTMT cells, staining was significantly greater (p < 0.001) in the perinuclear region of the cell, compared with the nucleus and the peripheral cytoplasm. Although staining in MTGSH cells was significantly greater in the perinuclear cytoplasm compared with the nucleus (p < 0.001), there was no difference in staining intensity between the perinuclear and peripheral cytoplasmic regions. In conclusion, both forms of image analysis and quantification confirm that in MTMT cells the MT-1 protein is largely perinuclear whereas in MTGSH cells it has a uniform cytoplasmic distribution with no localization.

We have previously shown that MT-1 mRNA is localized in the perinuclear cytoplasm and associated with the cytoskeleton (18). Furthermore, the 3′-UTR of MT-1 mRNA was responsible for this localization since no specific localization was observed in the MTGSH cell line, in contrast to the MTMT cells which show perinuclear localization. The present data describing MT-1 protein distribution indicate that the delocalization of MT-1 mRNA leads to MT-1 protein no longer being concentrated in the perinuclear cytoplasm. Thus, MT-1 mRNA localization determines the subsequent distribution of the encoded protein.

MT-1 mRNA Perinuclear Localization and Association with the Cytoskeleton Is Necessary for MT-1 Protein Shuttling into the Nucleus—The majority of cells (>80%), in the exponentially growing MTMT cells, have the distribution of MT-1 protein showed in Fig. 1. However, in a small proportion of the population, some nuclear staining was observed (data not shown). Further experiments were carried out to investigate if this phenomenon was cell cycle related since MT-1 has been found in the nucleus of some cells during the G\textsubscript{1} to S phase transition (15–17). Cells from both clonal CHO lines were synchronized by arrest in G\textsubscript{0} using serum deprivation followed by release of the cells from serum block after readdition of serum. Values shown are means from three different experiments. The S.E. values were too small to be shown clearly on the graph.

The present results indicate that disruption of the normal perinuclear localization of MT-1 mRNA and its association with the cytoskeleton causes a loss in localization of MT-1 protein, most notably that it is no longer concentrated in the nucleus at the G\textsubscript{1} to S phase transition of the cell cycle. Thus, the data show that localization of MT-1 mRNA is required for...
the perinuclear localization of MT-1 protein and its shuttling into the nucleus; furthermore, they provide the first evidence for a physiological role for perinuclear mRNA localization.

c-myC mRNA is localized around the nucleus (6) and mRNAs for a number of proteins which are imported into the nucleus (e.g. c-Fos and cyclin A) are also associated with the cytoskeleton (24). Thus, targeting of mRNAs to the perinuclear cytoskeleton to promote subsequent efficient import of the protein into the nucleus may be relevant to a wide range of proteins including transcription factors and proteins that shuttle into the nucleus in a cell cycle-dependent manner. The detailed mechanism by which mRNA localization promotes subsequent nuclear import is at present not clear but synthesis of the proteins on polysomes associated with the cytoskeleton suggests that a transport process involving cytoskeletal filaments is likely.

Although MT-1 is partly a cytoplasmic protein, it has been found in the cell nucleus during cell proliferation (13). This nuclear localization appears to occur at the time of the G₁ to S phase transition in the cell cycle, suggesting that MT-1 localization changes during the cell cycle such that the protein is shuttled into the nucleus at the beginning of S phase. The nuclear role of the protein is not known but it may have a protective role (17). Indeed, our preliminary data suggests that a loss of MT-1 mRNA and protein localization is accompanied by increased susceptibility to DNA damage and the availability of cell lines differing in the ability to localize MT-1 in the nucleus will provide tools to investigate the nuclear function of MT-1.

**Table II**

Quantification of MT-1 distribution in synchronized transfected CHO cells

| Cell lines | Distribution of MT-1 protein |
|------------|-----------------------------|
|            | Nucleus | Perinuclear cytoplasm | Peripheral cytoplasm |
| MTMT       | 64.5 ± 5.7 (15)^a,b          | 22.1 ± 3.5 (15)^a       | 20.7 ± 3.6 (15)^a     |
| MTGSH      | 9.1 ± 0.4 (15)^c,d           | 24.9 ± 0.9 (15)^c       | 25.9 ± 1.2 (15)^d     |

The perinuclear localization of MT-1 protein and its shuttling into the nucleus; furthermore, they provide the first evidence for a physiological role for perinuclear mRNA localization.

**Fig. 4.** Confocal microscopy of metallothionein distribution in synchronized transfected CHO cells. Cells were synchronized by serum depletion and analyzed by immunocytochemistry 10 h after readdition of serum. Cells were stained with anti-MT-1 antibody (E9) and FITC-labeled anti-mouse IgG. A, MTMT cells showed staining in the nucleus (1) but MTGSH cells showed general cytoplasmatic staining with no MT-1 present in the nucleus (2). Bars represent 10 μm. B, typical staining profile across a MTMT cell (1) and a MTGSH cell (2) after quantification of the staining pattern using Cyclops software. This analysis confirmed that MTMT cells show a distinct nuclear localization of MT-1 (1) but that MTGSH cells show no localization.
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