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Cell cycle regulation of metallothionein in human colonic cancer cells

(proliferation/cell synchronization/anti-metallothionein antibody/immunocytochemistry)

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ABSTRACT **Elevated levels of metallothionein (MT) found in rapidly growing tissues such as neonatal liver and various types of human tumors have suggested a role for MT in cell proliferation. To further explore this possibility we investigated the concentration of MT in human colonic cancer (HT-29) cells at different stages of proliferation by means of immunocytochemistry and competitive binding. MT is increased in subconfluent proliferating cells relative to growth-inhibited confluent cells, much as it is in growing tissues. Cycling cells synchronized with compactin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, revealed an oscillation of cytoplasmic MT that reached a maximum in successive late G_1 phases and at the G_1/S transition. Individual phases of the cell cycle were assessed by [^{3}H]thymidine incorporation and by immunofluorescence employing an antibody that detects a nuclear antigen associated with proliferation. An enzyme-linked immunosorbent assay was used to quantify the relative amounts of MT in homogenate supernatants of HT-29 cells. A 2- to 3-fold increase in MT in actively proliferating cells and the regulation of the protein during the mitotic cell cycle point to a physiological role for MT in cellular proliferation and suggest that it may also serve as a proliferation marker.**

Mammalian metallothioneins (MTs) are a family of low molecular weight, cysteine-rich proteins that bind the essential metals Cu and Zn under physiological conditions and the toxic metals Cd and Hg under pathological conditions. Most of the several functional MT genes in higher eukaryotes can be induced by metal ions (1) via metal-responsive elements. MT gene expression is also controlled by glucocorticoids, various cytokines, and growth factors (2). Despite detailed knowledge of the factors that induce MT, it has been difficult to discern a definite biological role(s) of the protein. Detoxification of heavy metals, radicals, and xenobiotics has been inferred from exposing either animals or cells to various types of nonphysiological stress. However, little emphasis has been given to the actual expression of the protein under noninduced conditions, except for studies of its role in metal homeostasis during embryogenesis (3) and in different states of fetal and perinatal development (4, 5). Elevated concentrations of MT in rapidly proliferating cells have been attributed to an increased demand for Cu and Zn (6–8) and increased MT has been noted in various tumors (9–13). If it is a homeostatic molecule, these elevated levels might reflect an increased demand for an active metal regulatory system. This, in turn, might control the activity of Zn-requiring proteins, which influence gene expression (14, 15) at different stages of cell proliferation.

The above findings imply an important role for MT in proliferating cells, but the regulation of MT concentration during the cell cycle remains controversial. Induction of MT biosynthesis in several cell lines by various growth factors was found not to be coupled directly to the cell cycle (16). However, the localization of MT in nuclei of primary hepatocytes in the early S phase after stimulation with epidermal growth factor and insulin suggested that it might be related to special stages of the cell cycle (17). Thus far, regulation of MT during the cell cycle has not been demonstrated directly for several reasons. First, current methods lack the sensitivity to detect the low basal MT levels in uninduced cells. To overcome this problem, investigators switched to Cd-resistant substrains (18) or to metal-induced cell cultures (19). The use of nonphysiological metal concentrations limits the value of such studies. Second, previous studies performed on cells induced to progress from quiescence, G_0, into S phase involved factors that could potentially stimulate MT. Consequently, cell cycle-dependent events could not be separated and differentiated from overall MT induction.

The present study takes a different approach to discern the role of MT in cell proliferation. It uses a sensitive anti-MT monoclonal antibody (mAb) that recognizes an epitope of rat and human MT other than the amino terminus (20). This mAb makes it possible to examine physiological steady-state—i.e., ng—levels of MT at different stages of cell proliferation without the need for pathophysiological metal concentrations. Furthermore, we have employed synchronized, continually dividing cells—i.e., human colonic cancer (HT-29) cells that are progressing from G_1 to S, which differs biochemically from the G_0/S progression (21). Synchronization was achieved with compactin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which provides G_1 synchrony without affecting gene expression or protein biosynthesis (22, 23). We find that the MT concentration oscillates during progression of HT-29 cells through the cell cycle, and this appears to have functional significance.

MATERIALS AND METHODS

Chemicals. Compactin (Mevastatin) and D,L-mevalonic acid were purchased from Fluka; the inactive lactone form of compactin was converted to its active form as described (22); [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Cell culture products were from Bio-Whittaker. All other chemicals were purchased from Sigma.

Cell Culture, Synchronization, and Assay of DNA Synthesis. HT-29 cells (HTB 38; American Type Culture Collection) were grown in 162-cm^2 culture flasks as described (24) with human transferrin (10 μg/ml) (GIBCO/BRL) and using gentamycin sulfate (25 μg/ml) as antibiotic. For synchronization, confluent cells were trypsinized, dispersed by multiple pipetting, and inoculated into six-well plates at 1.5×10^4 cells per cm^2. The medium was changed twice and after 48–56 h replaced with fresh medium containing 50 μM compactin. Cells were maintained under these conditions for 28 h. The medium was then removed, the cells were washed with Hanks'

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Abbreviations: mAb, monoclonal antibody; MT, metallothionein; FITC, fluorescein isothiocyanate.

balanced salt medium, and the G_1 block was released by addition of fresh medium containing 5 mM mevalonic acid. The medium was changed every 24 h. DNA synthesis was measured by the incorporation of [^{3}H]thymidine into HT-29 nuclei as described (23).

Evaluation of MT. All immunoassays were performed with the mAb II-10a, raised against monomeric rat liver MT-I (20). For crossreactivity assays, rat liver MT-I and human liver MT-1 were isolated as described (25, 26). Rabbit MT was purchased from Sigma; different isoforms were separated on a Mono Q column (Pharmacia), with a gradient of 3–300 mM Tris·HCl (pH 8.6).

Immunocytochemistry. Cells grown on microscope slides in Petri dishes were rinsed three times with phosphate-buffered saline (PBS), fixed in methanol [100% (vol/vol), −20°C, 15 min], incubated with primary antibody for 12 h at 4°C in a humidified chamber and, after rinsing, treated with fluorescein isothiocyanate (FITC)-labeled goat F(ab')$_2$ anti-mouse IgG (Caltag, South San Francisco, CA) for 1 h at room temperature. Fluorescence microscopy was performed with a Labophot fluorescence microscope (Nikon). The mAb Ki-67 (Dako) diluted 1:75 was used for visualization of cell cycle stages (27).

ELISA. For quantitation of MT in homogenate supernatants, cells were washed with PBS, scraped with a rubber policeman, collected by centrifugation at 2000 × *g*, and stored in 1 ml of PBS at −20°C. The cells were thawed, and 0.5 μg of leupeptin per ml, 0.5 mM phenylmethylsulfonyl fluoride, and 65 nM dithiothreitol were added to a final volume of 1 ml. The cell suspension was sonicated on ice using an ultrasonic disintegrator (Branson). After centrifugation at 10,000 × *g* for 30 min the homogenate supernatants were assayed for protein with bicinchoninic acid (28) and adjusted to 2 mg of protein per ml. A standard curve was developed with 100 ng of rat MT-I per well as coating antigen (20). Background correction used the homogenate supernatant of confluent HT-29 cells as diluent at 500 μg/ml. Serial dilutions (1:2) of rat liver MT-I standards and HT-29 samples were made in duplicate. The mAb II-10a (0.08 μg per well) was delivered in 0.15% (wt/vol) bovine serum albumin in PBS. The plates were kept overnight at 4°C. The amount of antibody bound was determined by measuring the enzymatic activity of alkaline phosphatase-conjugated second antibody at 405 nm as described (29). Absorbances were evaluated by a four-parameter logistic equation (30).

RESULTS

HT-29 Cells as a Model for Cell Proliferation. We chose the colonic cancer cell line HT-29 (31) to investigate MT in the cell cycle since it is an established model of cell polarity and undergoes terminal differentiation when glucose is depleted in the culture medium (32, 33). Cells grown in medium containing glucose and fetal calf serum do not manifest any of the functional properties characteristic of intestinal epithelial cells (34). Consequently, they should be suitable to study cell features in general and to address the possible physiological role of MT in cellular proliferation in particular.

Most differentiation studies with HT-29 cells have been performed on confluent or even postconfluent cells. Since we were interested in proliferation we therefore used subconfluent cells under conditions established for this purpose. Dispersed cells were seeded at 1.5×10^4 cells per cm^2 and cultured in the presence of 5% (vol/vol) fetal calf serum and 25 mM glucose. After attachment to the plastic surface, the cells grow exponentially in small islands characteristic of adenocarcinomata. They distribute asynchronously throughout the four stages of the cell cycle, as shown below and are herein referred to as *subconfluent, proliferating* cells. As the cells approach confluency, [^{3}H]thymidine incorporation in continuously labeled nuclei reaches a plateau (data not shown), indicative of a stationary density (35, 36). The four stages can be visualized and identified by immunofluorescence employing mAb Ki-67, which recognizes a proliferation-associated human nuclear antigen expressed exclusively by cycling and but not by resting cells (27, 37, 38).

Staining for Ki-67 is seen in ≥95% of subconfluent, proliferating HT-29 cells but is almost absent in confluent cells (data not shown). Thus, the cells undergo density-dependent growth inhibition and are growth arrested at confluency (quiescence, G_0), an additional feature that makes them useful for studying cellular proliferation in cell culture, because large numbers of G_0 cells can be obtained without serum starvation.

HT-29 Cell Synchronization. Synchronization of HT-29 cells, to our knowledge not heretofore reported, was achieved by the use of compactin (39), an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase that presumably stops DNA replication and cell growth by blocking mevalonate production (40).

Synchronization of HT-29 cells by compactin could be followed by [^{3}H]thymidine incorporation. A typical time course is shown in Fig. 1. Exposure of subconfluent, proliferating HT-29 cells to 50 μM compactin over 28 h resulted in ≥80% inhibition of DNA synthesis. Inhibition was reversed by addition of 5 mM mevalonate and DNA synthesis resumed after an 8- to 12-h lag period. Maximal [^{3}H]thymidine incorporation (100%) was reached at 28 h, decreased to 45% at the onset of M phase at 44 h, and increased again during the next S phase to peak at around 60 h (Fig. 1). Synchrony could be followed for at least two cycles after release of the G_1 block. One cycle for logarithmic-phase cells took 24–26 h.

Indirect Immunofluorescence. The specificity of mAb II-10a, raised against monomeric rat liver MT-I is demonstrated in Fig. 2. It recognizes residues 20–25, which are conserved among the human MT isoforms, but not the acetylated amino terminus (41), and crossreacts with rat and human liver MT-1, but not rabbit liver MT-1. Individual, asynchronous, subconfluent HT-29 cells exhibit differences in cytoplasmic MT as measured by mAb II-10a staining intensity. This varies from dense to faint and appears to correlate with the size and shape of each cell. Smaller, postmitotic cells stain more intensely than larger cells (Fig. 3*A*), indicating a change in MT concentration during the cell cycle. Cells progressing beyond mid-S to late S and G_2 phase exhibit an intense staining of nucleoli and nuclear matrix with mAb Ki-67 (37).

Synchronized HT-29 cells were used to further investigate these observations. Treatment of subconfluent growing cells with increasing concentrations of compactin (up to 50 μM) over a period of 28 h increases the number of postmitotic, G_1-synchronized cells, which exhibit intense cytoplasmic stain-

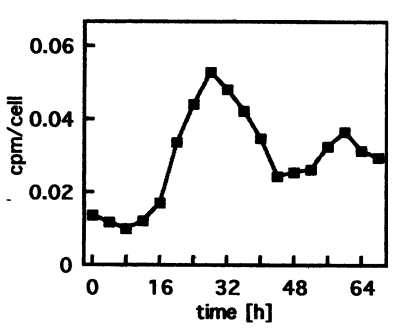

FIG. 1. Synchronized [^{3}H]thymidine uptake by HT-29 cells. For each determination, HT-29 cells were pulsed with 5 μCi of [^{3}H]thymidine per well for 0.5 h at 37°C and harvested at the times indicated. Cells in parallel wells were used for determination of cell number using a Coulter Counter (model Z_f). DNA synthesis is expressed as cpm per cell with each point obtained in duplicate. The standard deviation of the amount of incorporated radioactivity per sample was ≤7.5%.

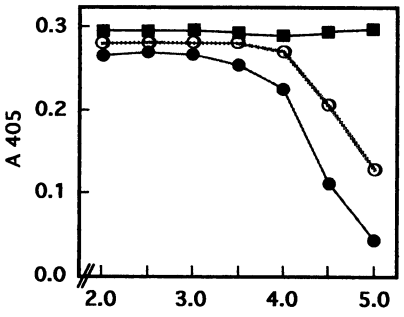

FIG. 2. Crossreactivity of various isolated MTs with mAb II-10a using rat liver MT-I as coating antigen. Serial dilutions of rat liver MT-I (●), human liver MT-1 (○), and rabbit MT-1 (■) were made in triplicate. Reproducibility was <±5%.

ing for MT. Clearly, cytoplasmic accumulation of MT seems to be a characteristic feature of G_1-arrested HT-29 cells (Fig. 3*B*). To determine whether or not this staining pattern is due to a cell cycle-specific induction of MT rather than to a unique induction by compactin, growth arrest was released by mevalonic acid. As the cells progress through a lag phase, where the lowest DNA synthesis rate was observed (Fig. 1), the staining for MT decreased. An increase in staining was obtained initially at 24 h (Fig. 3*C*) in the first cycle and thereafter in successive cycles close to the G_1/S transition. Double staining with mAbs II-10a and Ki-67 identified the maximum of the MT staining as the time of faint nucleolar or undetectable Ki-67 staining. The MT maximum observed in compactin-treated HT-29 cells arrested in early G_1 (23) appears to be shifted toward late G_1 and G_1/S transition in cycling cells (Fig. 3*D*). These findings strongly suggest that the recurring cytoplasmic buildup of MT is indeed cell cycle regulated.

In contrast to the bright cytoplasmic staining for MT in G_1 growth-arrested cells, only weak staining indicating a low basal level of MT is observed for cells in G_0 (Fig. 3*E*). This difference

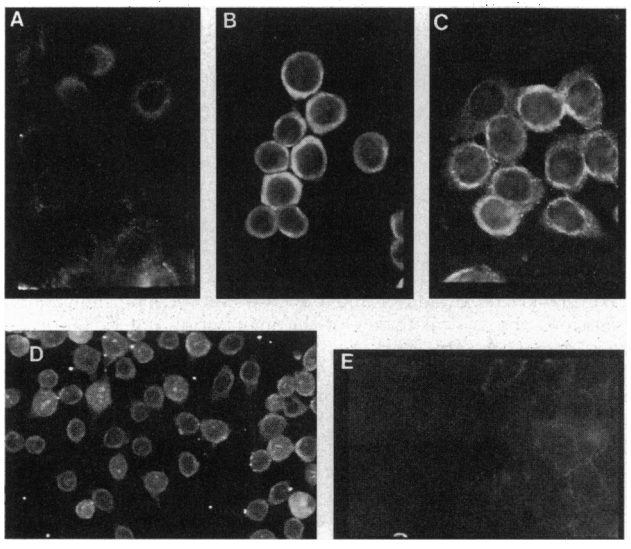

FIG. 3. Immunohistochemical staining of HT-29 cells at different stages of proliferation by mAb II-10a (*A–C* and *E*) or by double staining with mAbs II-10a and Ki-67 (*D*). (*A* and *B*) Subconfluent, proliferating (*A*) and G_1-arrested (*B*) cells. (*C* and *D*) Synchronized cells after release of the compactin-generated block by mevalonate at 24 h (*C*) and at the G_1/S transition in the third cycle (*D*). (*E*) Contact-inhibited HT-29 cells. [*A–C* and *E*, ×130 (original magnification ×400); *D*, ×65 (original magnification ×200).] Controls for the specificity of the MT immunofluorescence signal were (*i*) MOPC 31C (American Type Culture Collection), an unspecific murine mAb of the same IgG subtype as mAb II-10a, and (*ii*) mAb II-10a preincubated with either isolated human MT-1 or rat liver MT-I (not shown).

in staining intensity for MT in cells committed to proliferation versus those at quiescence points to an involvement of MT in cellular proliferation.

ELISA Quantitation of MT in HT-29 Homogenate Supernatants. Homogenate supernatants of HT-29 cells were analyzed by ELISA to quantitatively assess the cytoplasmic accumulation of MT observed by immunofluorescence. Since total protein content varies during cell cycle progression, samples were normalized for protein content rather than cell number (42) to quantitate steady-state levels of MT.

Relative MT contents of HT-29 cells from different stages of the cell cycle are depicted in Fig. 4, which shows the amount of MT in (*i*) confluent and (*ii*) subconfluent, proliferating HT-29 cells (Fig. 4*A*). These results are consistent with and confirm immunofluorescence observations presented in Fig. 3. The low basal level of MT in confluent cells is increased 2- to 3-fold in actively proliferating cells.

To define when in the cell cycle cytoplasmic MT reaches a maximum, homogenate supernatants of synchronized cells, seeded and cultured at the same cell density as in the [^{3}H]thymidine incorporation experiments (Fig. 1), were analyzed by ELISA. Cells were harvested at time 0 and at 20, 24, and 28 h after release of the compactin block. As shown in Fig. 4*B*, the highest amount of MT was found in compactin-arrested cells, which are in early G_1, whereas the maximum in synchronized cycling cells was found at 24 h, which according to [^{3}H]thymidine incorporation is about the G_1/S transition (Fig. 1). This value decreases to about one-third, at 28 h, suggesting an intracellular degradation of the protein in S phase.

DISCUSSION

Discontinuous gene expression is a general feature of the eukaryotic cell cycle (43) and can be followed by measuring either mRNA or protein. Owing to the diverse means of MT mRNA induction, monitoring MT protein levels has several advantages. First, since regulation of genes encoding individual MT isoforms is cell type specific (1, 44), it would be necessary to carry out a detailed examination of the expression of each of the MT genes for HT-29 cells. Second, because of posttranscriptional events, the rate of MT mRNA synthesis is not always directly proportional to that of MT synthesis (45). Third, it is the steady-state level of MT that should correlate with its biochemical function, and hence the investigation of MT protein levels has to be the method of choice.

MT is synthesized rapidly and degraded during progression of the cell cycle (Fig. 4*B*). The turnover rate of MT in other systems is known to be determined by the availability of metal and is influenced by the metal content and the metal species (46). Little is known about intracellular degradation of MT, but it is likely that MT is more resistant to proteases than is thionein. For example, MT induced by dexamethasone was

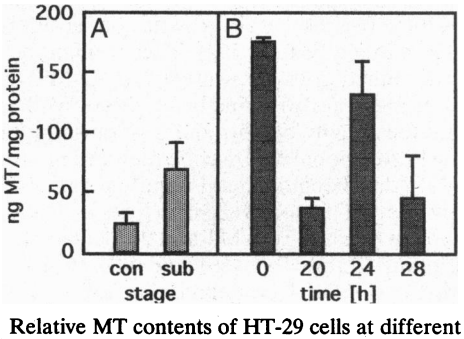

FIG. 4. Relative MT contents of HT-29 cells at different stages of proliferation. (*A*) Confluent grown (con) and subconfluent, proliferating (sub) cells. (*B*) G_1-arrested cells (time 0) and cells after release of the G_1 block at 20, 24, and 28 h. Relative MT contents are expressed as mean ± SEM of two different experiments, each in triplicate.

found to be degraded much more rapidly than that induced by Zn (47), and it has been suggested that hepatic MT degradation is regulated primarily by cellular Zn content (47, 48). Thus, the decrease of MT implies the degradation of MT, perhaps due to the loss of Zn to proteins such as transcription factors at this stage of the cell cycle. Half-times of intracellular degradation for Zn MT are reported to range from 2 to 6 h (26, 49–51), in good agreement with the decrease of MT observed here.

Earlier reports suggesting that MT is involved in proliferation monitored the progression of quiescent, G_0 cells into S phase after induction by a stimulus. For such cells to reenter the cell cycle they must carry out extra metabolism during G_1 and therefore take considerably longer to reach S phase than do cells that have just finished mitosis. This is indicated by dramatic differences in gene expression (21, 52, 53). For example, after partial hepatectomy MT was observed to accumulate in regenerating liver cells, the classic *in vivo* prototype of G_0 cells that are reentering S phase and mitosis (54, 55). Similar findings have been reported upon stimulation of serum-starved cells in tissue culture (16, 56). No direct link between MT biosynthesis and the cell cycle was found, however. In contrast, nuclear localization of MT during early S phase in primary hepatocytes after stimulation with epidermal growth factor and insulin was suggested to be a cell cycle-specific event (17). However, the validity of these findings (regarding nuclear localization of MT) may depend on the specificity of the antibodies employed for immunocytochemistry (unpublished data). It should be noted that all of these studies were with cells undergoing a G_0 to S phase progression, which differs fundamentally from the G_1 to S progression of continually dividing cells. Therefore, it is not clear whether the induction of MT in these experiments is cell cycle specific.

In contrast, the present study finds a clear association between MT and the cell cycle. In continually dividing synchronized cells, cytoplasmic accretion of MT was observed in each successive late G_1 phase and G_1/S transition, results that substantiate our original observations with subconfluent, proliferating cells (Fig. 3*A*). Importantly, they suggest that in HT-29 cells MT is regulated during the standard cell cycle under noninduced conditions.

Maximal cytoplasmic accretion of MT coincides with the expression of delayed early genes (57, 58). Induction of the MT-IIa gene in the human fibroblast cell line WI-38 depends on protein synthesis (59), which classifies at least one functional human MT gene as a delayed early gene. The MT maximum in compactin-treated cells (Figs. 3*B* and 4*B*) may therefore be interpreted as an accumulation of a delayed early gene product caused by arresting cells in early G_1.

The fact that MT is regulated during progression of the cell cycle raises questions about its biological function in this process. It accumulates near the G_1/S transition, a period during which cells prepare for DNA synthesis. Treatment of cells with chelating agents has shown that Zn is required at the G_1/S transition (60, 61). Cell growth and proliferation are closely related to Zn content (62). Thus it might be hypothesized that the function of Zn is mediated via MT to regulate the supply of metal for proteins being newly synthesized and to modulate the activity of Zn-requiring transcription factors. Fine tuning of functional MT may be required in order to best accommodate the changing metal requirements of various Zn finger transcription factors (63, 64). The activation of Zn-requiring apoenzymes (65) by MT and the chelate deactivation of transcription factor IIIA (66) and SP1 (67) by thionein underscore the potential modulation of Zn-dependent, biological processes in the cell.

At present the factors that regulate MT during progression of the cell cycle are unknown, but there is some indirect evidence that the regulation of MT involves more than one signaling pathway. Induction of MT mRNA occurs via distinct and separable response elements that do not operate by a common mechanism. In mouse cells, transcription of the MT-1 gene is induced by one transduction pathway triggered by glucocorticoids and another by heavy metals (68). Consequently, $CdCl_2$ can effectively induce MT-1 mRNA in asynchronous and G_2-synchronized L cells. In contrast, the induction of MT-1 mRNA with dexamethasone in G_2-synchronized cells was impaired (69), indicating cell cycle regulation of the glucocorticoid receptor function as well as cell cycle-dependent induction of MT via glucocorticoid-responsive elements (70).

The necessity of MT for cell function has been examined recently by expression of antisense RNA and by gene knockout experiments. The antisense down-regulation of MT in a human monocytic cell line altered three physiological parameters associated with resting and activated monocytes—namely, adherence, invasion, and respiratory burst. It also increased Cd sensitivity (71). Since these parameters have relatively little in common, the authors concluded that MT must have a fundamental physiological function in leukocytes, and they suggested that it is a modulator of basic homeostatic functions rather than an exclusive antidote associated with toxic events.

In contrast, mice carrying null mutations in the MT-I and MT-II loci (72, 73) were examined in terms of embryonic development (4, 74, 75). Disruption of these constitutively expressed genes did not lead to fetal malformations or interfere with early development. These mice exhibited only an increased susceptibility toward Cd intoxication; it was suggested that these widely expressed MTs are not essential for life but are restricted solely to Cd detoxification.

It can be difficult to interpret approaches that depend on loss of function if they do not result in essential alterations. The lack of an effect may be compensated by the expression of genes that are not disrupted, such as MT-III and -IV, or others that are still unknown (72). Furthermore, the minimal amount of functional MT that is essential for cells may be lower than that detectable by the Cd saturation assay, which is probably only useful to measure MT in tissues with high MT concentrations (76).

Overall, the oscillation of MT during the mitotic cell cycle of HT-29 cells with its maximum near the G_1/S transition of the cell cycle, at the onset of DNA synthesis, points to a physiological role for MT in cellular proliferation. Moreover, it may well be that the elevated levels of MT that occur in actively proliferating cells can serve as a marker for proliferation.

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