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Metallothionein Gene Expression Is Regulated by Serum Factors and Activators of Protein Kinase C

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The exact physiological role of metallothionein (MT) is not clear. It has been suggested that these low-molecular-weight, highly inducible, heavy-metal-binding proteins serve in the regulation of intracellular Zn metabolism. Among the Zn-requiring systems are several enzymes involved in DNA replication and repair. Therefore, during periods of active DNA synthesis there is likely to be an increased demand for Zn, which could be met by elevated MT synthesis. For that reason, we examined whether stimulation of cellular proliferation leads to increased expression of MT. We report here that treatment of cultured mammalian cells with serum growth factors and activators of protein kinase C, all of which are known to have growth stimulatory activity, led to induction of MT mRNA. One of the required steps in the signal transduction pathways triggered by these agents, ending in MT induction, appears to be the activation of protein kinase C.

Zinc (Zn) is an important component of many enzymes, including alkaline phosphatases, carboxypeptidases, dehydrogenases, RNA and DNA polymerases, and the protein synthesis machinery (see references 28 and 34 for reviews). Zn deficiency leads to severe growth retardation, developmental abnormalities, immunodeficiency, eczematoid dermatitis, and several other disorders (28). As an important ion, the homeostasis of Zn is finely maintained. Metallothioneins (MTs), which are low-molecular-weight heavy-metal-binding proteins (see reference 12 for a review), are likely to occupy a central role in the regulation of zinc metabolism (7, 29). Several Zn-requiring apoenzymes can be reactivated by the transfer of Zn from MT to the apoenzyme (33). Therefore, MTs may constitute a regulatory system whose function is analogous to that of calmodulin in calcium metabolism. In addition, MTs probably serve as an intracellular reservoir of Zn. In support of these roles it has been reported that induction of MT synthesis is responsible for the increased cellular uptake of Zn after treatment with glucocorticoid hormones (13, 15, 16).

As the major Zn-binding proteins in the cell (16), MTs can potentially contribute to many important biological processes that involve Zn-requiring enzymes, including replication, repair, transcription, protein synthesis and turnover, and energy metabolism. It is expected that during periods of active cell growth and proliferation there is an increased demand for Zn that could be met by elevated MT synthesis. In this report we describe the relationship between cellular proliferation and MT synthesis. More specifically, we examined the effect of serum and various growth factors as well as direct activators of protein kinase C, all of which are stimulators of cellular proliferation (25, 26), on the expression of MT mRNA in various mammalian cell cultures. Although MT synthesis is not directly coupled to the cell cycle, it is stimulated in response to treatment with serum, peptide growth factors, and two different activators of protein kinase C.

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MATERIALS AND METHODS

Chemicals. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was from C.C.R., Inc., Eden Prairie, Minn.; 4-α-phorbol and 1,2-dioctanoylglycerol (DiC8) were from Life Sciences Resources, Milwaukee, Wis.; platelet-derived growth factor (PDGF), epidermal growth factor, and the tripeptide glycyl-histidyl-lysine (Gly-His-Lys; also referred to as hepatocyte growth factor or HGF [27]) were from Collaborative Research, Inc., Lexington, Mass.; dexamethasone and calcium ionophore A23187 were from Sigma Chemical Co., St. Louis, Mo.; and insulin, glucagon, insulin-like growth factor-1 (IGF-1) and IGF-2 were kindly provided by Hyam Leffert, University of California, San Diego, who has tested them for biological activity.

Cell culture. All cell lines and primary cultures were grown in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.), supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin (Sigma) per ml, and 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah). For serum starvation, cells (40 to 80% confluent) were incubated for 40 h in DMEM containing 0.5% FBS. At the end of this treatment, all the cells were fully viable as judged by trypan blue exclusion.

RNA isolation and analysis. RNA was isolated (5) and analyzed by agarose gel electophoresis and transferred to nitrocellulose as previously described (11). The levels of hybridization were visualized by autoradiography and quantitated by densitometry with a Zeineh Soft Lazer scanner. MT mRNA levels were normalized to the amount of α-tubulin mRNA to correct for any differences in the amount of RNA loaded. Transcription in isolated nuclei was performed as described by McKnight and Palmiter (24). Each ^{32}P-labeled RNA sample was hybridized to duplicate filters to which 1 μg of phMT-II$_3$, pKd-1(α-tubulin), and pBR322 DNA was immobilized. After washing, the amount of radioactivity hybridized to each filter was determined by scintillation counting. Hybridization efficiency was determined with an in vitro-synthesized [^{3}H]RNA probe and was approximately 30%.

Probes. The *Bam*HI-*Pvu*II fragment of phMTII$_3$, an hMT-II$_A$ cDNA clone (19), and the *Pst*I fragment of Kα-1, an

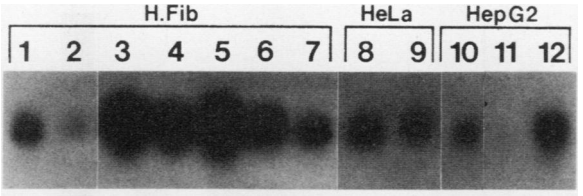

FIG. 1. Effect of serum on MT mRNA in human cell lines. Primary skin fibroblasts (H.fib) (lanes 1 to 7), HeLa cells (lanes 8 and 9), and HepG2 cells (lanes 10 to 12) were grown in DMEM ⊕ 10% FBS. While some of the cultures were kept in that medium (lanes 1, 3, 4, 8, and 10), others were given DMEM ⊕ 0.5% FBS for 36 h and then incubated for another 6 h in fresh DMEM ⊕ 0.5% FBS (lanes 2, 9, and 11), 10% FBS (lanes 7 and 12), 10% FBS with either 5 μM Cd^{2+} (lanes 3) or 10^{-6} M deamethasone (lane 4), or DMEM ⊕ 0.5% FBS with either 5 μM Cd^{2+} (lane 5) or 10^{-6} M dexamethasone (lane 6). At the end of the incubation period total cellular RNA was extracted, separated on agarose gels, hypbridized to an hMT-II_A coding-region probe, and visualized by autoradiography.

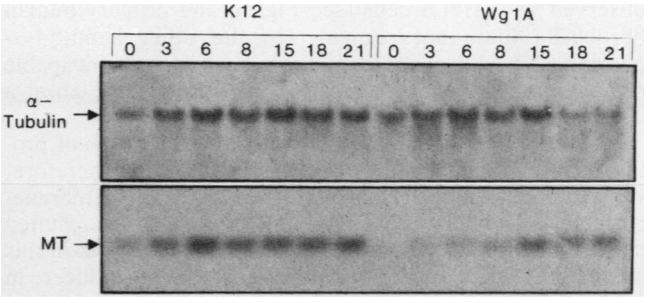

FIG. 2. MT mRNA is not cell cycle regulated but is serum inducible. Cultures of Chinese hamster fibroblasts were arrested either by incubation at the nonpermissive temperature (K12 cells) or by serum starvation (Wg1A cells) and then stimulated to divide by either shifting them back to the growth-permissive temperature (K12) or feeding them with medium containing 10% FBS (Wg1A). RNA was extracted from cells harvested at different times as indicated above each lane (in hours) after restimulation of growth and analyzed by Northern blot hybridization with either an α-tubulin or an MT probe. The same blots also were hybridized to a histone probe which showed S-phase-specific expression of histone mRNA (shown in reference 2).

α-tubulin cDNA clone (8), were isolated from agarose gels, purified on Elutip columns (Schleicher & Schuell, Inc., Keene, N.H.), and radiolabeled by nick translation (23) with [α-^{32}P]dATP and [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.).

RESULTS

Regulation of MT mRNA level by serum. To determine whether MT gene expression is coupled to cell growth and proliferation, we examined the influence of FBS, which supplies cultured cells with growth factors, on MT mRNA levels in various human cell cultures. The level of MT mRNA was greatly reduced after incubation of foreskin fibroblasts or HepG2 cells (hepatoma cell line) in medium containing 0.5% FBS (Fig. 1, lanes 2 and 11 versus lanes 1 and 10). HeLa cells, on the other hand, were not as sensitive to serum starvation, and the level of MT mRNA in these cells decreased marginally (Fig. 1, lane 9 versus lane 8). Increasing the serum concentration from 0.5 to 10% restored MT mRNA expression to its normal basal level within 8 h (Fig. 1, lanes 7 and 12). This effect was specific since the level of α-tubulin mRNA was not greatly affected (data not shown, but see Fig. 2).

While reducing the basal level of MT mRNA expression, incubation of cells in low serum had no effect on induction by either Cd^{2+} or dexamethasone (Fig. 1, lanes 3 to 6), consistent with the early report about induction of MT by these agents in serum-free medium (15). In other experiments not shown here, we examined the time course of the decrease in MT mRNA after shifting the cultures from high to low serum and found that the minimum was reached within 12 to 24 h.

The effect of serum on MT gene expression can be mediated by one of two basic mechanisms. The first possibility arises because serum starvation blocks cell division and leads to accumulation of cells at the G1 phase. If MT gene expression is cell cycle dependent, e.g., specific for the S phase, as progression through the cell cycle is inhibited, a decrease in the level of MT mRNA would be observed. The second possibility is that serum contains factors which affect MT gene expression directly, regardless of the position within the cell cycle, perhaps by modulating the activity of a *trans*-acting factor.

To discriminate between these two possibilities, we examined MT synthesis in synchronized cultures of Chinese hamster fibroblasts. The K12 cell line is a temperature sensitive mutant in cellular proliferation derived from the parental cell line Wg1A. K12 cells can be conveniently synchronized by first blocking their division at the nonpermissive temperature, which arrests cells in mid-G1 (2 h before the onset of DNA synthesis), and then shifting them back to the permissive temperature to release that block (2, 30). There were no significant differences in the level of MT mRNA relative to that of α-tubulin mRNA in synchronized K12 cells harvested at different points throughout the cell cycle (Fig. 2). In contrast, the level of histone mRNA in these same cells exhibited marked cell cycle dependence (data not shown, but see reference 2). Chinese hamster fibroblasts can also be arrested by serum starvation. In this case the trigger for initiation of the cell cycle is the addition of serum. MT mRNA expression in serum-stimulated K12 (data not shown) and Wg1A (Fig. 2) cells increased gradually, remained constant after 15 h, and did not correlate with any particular phase of the cell cycle. In other experiments we examined HeLa cells collected at different phases of the cell cycle by elutriation and again found that the level of MT mRNA was constant (M. Karin and C. Distellhorst, unpublished data). From these results we conclude that although MT expression is induced by serum, it is not coupled to the cell division cycle.

Induction of MT mRNA by activators of protein kinase C. Recent studies indicate that the mitogenic activity of serum is due to the presence of various growth factors. Some of these factors are known to stimulate the turnover of inositol-containing phospholipids, after binding to their cell surface receptors which leads to release of diacylglycerols and inositol-phosphates that act as activators of protein kinase C and Ca^{2+} mobilization, respectively (25, 26). These events are thought to be necessary steps in the pathways leading to the initiation of cell division. To test whether either of these pathways is involved in the induction of MT mRNA by serum, we examined the effects of the phorbol ester TPA, a known activator of protein kinase C (4), and the Ca ionophore A23187 on MT mRNA levels. Incubation of HepG2 cells with increasing concentrations of TPA led to significant induction of MT mRNA (Fig. 3A) that peaked 8 h after the addition of this agent (Fig. 3B). Similar effects were

observed with HeLa cells (see Fig. 6) and primary human fibroblasts (data not shown). On the other hand, 4-α-phorbol-12,13-didecanoate, a phorbol ester that is incapable of activating protein kinase C (4), had no effect even at a concentration of 1 μg/ml (data not shown).

As mentioned above, the physiological activators of protein kinase C are thought to be diacylglycerols. Therefore, we incubated HepG2 cells with the synthetic membrane-permeable diacylglycerol DiC8. This treatment also resulted in the induction of MT mRNA (Fig. 4). (Even though in this experiment DiC8 appears to be a more potent inducer, in most experiments its effect was equivalent to that of TPA.) The response to DiC8 proceeded with similar kinetics as the response to TPA and was optimal at a concentration of 100 nM (data not shown). Furthermore, TPA and DiC8 are primary inducers of MT mRNA, as their activity is independent of de novo protein synthesis (Fig. 4). In agreement with previous observations (13), inhibition of protein synthesis by cycloheximide led to small but significant induction of MT mRNA. The effect of the protein kinase C activators was additive with that of cycloheximide alone. These results suggest that TPA and DiC8 induce MT expression by increasing the activity of a *trans*-acting factor already present in the cells.

At least 50% of the MT mRNA expressed in human cells is derived from the hMT-II_A gene (20) which is known to

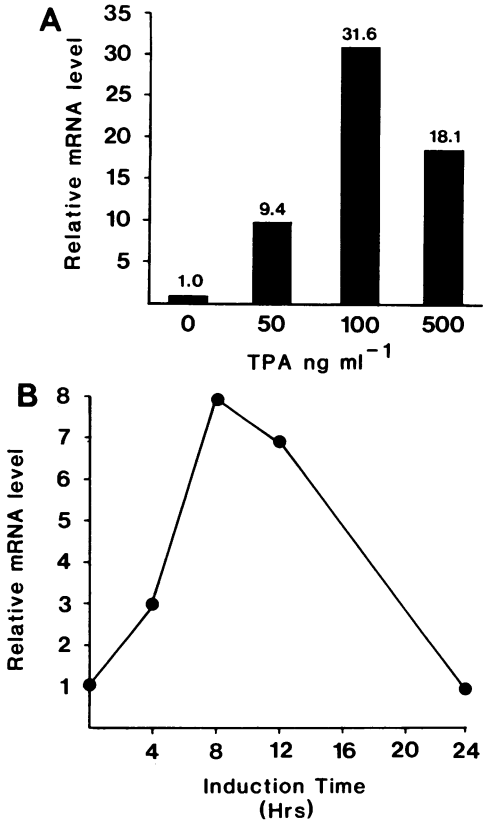

FIG. 3. Induction of MT mRNA by TPA. Serum-starved HepG2 cells were incubated for 8 h in the presence of different amounts of TPA as indicated in panel A or were incubated for various lengths of time, as indicated in panel B, with 100 ng of TPA per ml. The amount of MT mRNA relative to that of α-tubulin mRNA was determined by Northern hybridization, autoradiography, and densitometry. The uninduced basal level was arbitrarily set at 1.

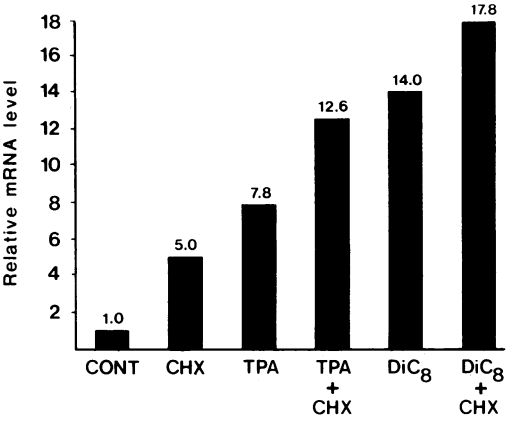

FIG. 4. Induction of MT mRNA by TPA and diacylglycerol does not require on-going protein synthesis. HepG2 cells were incubated for 8 h either in DMEM ⊕ 0.5% FBS (CONT) or in the same medium containing cycloheximide (CHX) at 300 μg/ml, TPA (TPA) at 100 ng/ml, a water-soluble diacylglycerol (DiC8) at 100 nM, or with the indicated combinations of these drugs. The amount of MT mRNA relative to that of α-tubulin mRNA was determined as indicated above.

respond to several different inducers including heavy-metal ions, glucocorticoids (14), and interleukin-1 (17). Nuclease S1 protection experiments have confirmed that the hMT-II_A gene also is induced by TPA and that this treatment has no effect on the choice of transcriptional initiation sites (data not shown). The effect of TPA was exerted at the level of hMT-II_A gene transcription as indicated by the results of the nuclear runoff experiments (Table 1). The increased transcription rate can account for the majority of the TPA effect on MT mRNA. It remains to be tested whether other members of the MT gene family are also induced by TPA.

Induction of MT mRNA by growth factors. Since the liver is the major site for MT synthesis in vivo, we chose the HepG2 cell line for determining which of the growth factors present in serum is responsible for MT mRNA induction. First we examined the response of these cells to the tripeptide Gly-His-Lys (or HGF), which is reported to exert growth stimulatory activity on hepatoma cells (27), and to PDGF. In the presence of 0.5% FBS, a dose-dependent increase in MT mRNA by Gly-His-Lys but not by PDGF

TABLE 1. TPA increases the transcription rate of the hMT-II_A gene[a]

Cells	Treatment	hMT-II_A transcription (ppm)	α-Tubulin transcription (ppm)
Primary skin fibroblasts	Control (expt 1)	0.8 ± 0.1	3.3 ± 0.3
	Control (expt 2)	1.6 ± 0.2	3.2 ± 0.2
	TPA (expt 1)	5.4 ± 0.6	2.9 ± 0.2
	TPA (expt 2)	7.2 ± 1.0	2.6 ± 0.2
HepG2	Control	0.57 ± 0.2	1.8 ± 0.3
	TPA	1.76 ± 0.1	1.2 ± 0.3

[a] Nuclei were isolated from either control cells or cells treated with 100 ng of TPA per ml for 1 h. Endogenously preinitiated transcripts were labeled by incubation with [^{32}P]UTP, extracted, and hybridized to duplicate nitrocellulose disks to which pBR3222, phMT-II_3, and pKd-1(α-tubulin DNAs were immobilized, and the average values of radioactivity hybridized to the filters were converted to parts per million (ppm) of total transcription ([^{32}P]UTP incorporation) taking into consideration the hybridization efficiency (approximately 30%). The background hybridization (to pBR322) was between 15 and 25 cpm per filter.

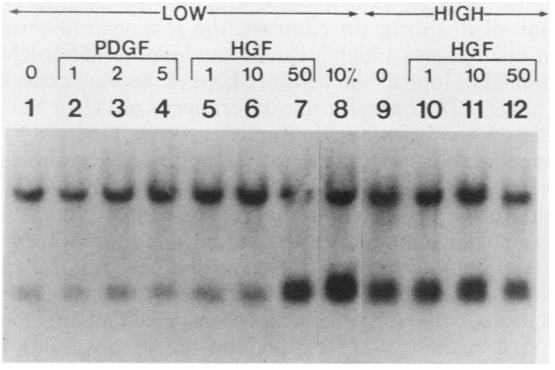

FIG. 5. Induction of MT mRNA by Gly-His-Lys (HGF). HepG2 cells maintained in either 0.5% FBS (LOW, lanes 1 to 8) or 10% FBS (HIGH, lanes 9 to 12) were incubated for 8 h with PDGF (lanes 2 to 4) or Gly-His-Lys (lanes 5 to 7 and 10 to 12) at the indicated concentrations measured in either half-maximal units for PDGF (determined by the manufacturer) or nanograms per milliliter for Gly-His-Lys. Total cellular RNA was extracted and analyzed as described in the legend to Fig. 3. The upper band represents α-tubulin in mRNA, and the lower band is MT mRNA.

was observed (Fig. 5). The lack of response to PDGF is consistent with the absence of its receptors on liver cells (3). The response to 50 ng of Gly-His-Lys per ml (Fig. 5, lane 7) was equivalent in magnitude to the induction observed with 10% FBS (lane 8). No additional response to Gly-His-Lys was observed in cells continuously maintained in 10% FBS (lanes 10 to 12), suggesting that the two agents act through a similiar pathway. We also tested the ability of a number of other growth factors that are known to stimulate DNA synthesis in liver cells (22) to induce MT mRNA. While no significant response was observed with physiological concentrations of IGF-2 (Fig. 6A, lane 14) moderate stimulation was observed after incubation with IGF-1 (lane 13), epidermal growth factor (lane 12), and glucagon (lane 16). A large induction was observed after treatment with insulin (lanes 6 and 15).

Binding of insulin to its cell surface receptor stimulates the turnover of inositol-containing phospholipids generating diacylglycerol, which consequently leads to activation of protein kinase C (31). To test whether the effect of insulin on MT mRNA could be mediated by activation of protein kinase C, we examined the sensitivity of this response to 1-(5-isoquinolinsulfonyl)-2-methylpiperazine dihydrochloride (H-7), a protein kinase inhibitor with preferance for protein kinase C (21). While induction of MT mRNA by TPA was completely blocked by treatment with H-7 (Fig. 6A, lanes 3 to 5), the response to insulin was only partially inhibited (Fig. 6A, lanes 6 and 7). These experiments suggest that additional pathways stimulated by insulin not involving protein kinase C also lead to MT mRNA induction. This alternative pathway does not seem to involve protein kinase A, since dibutyryl cyclic AMP and isomethylbutylxanthine, a phosphodiesterase inhibitor, had no significant effect on MT mRNA in HepG2 cells (R. Chiu and R. J. Imbra,

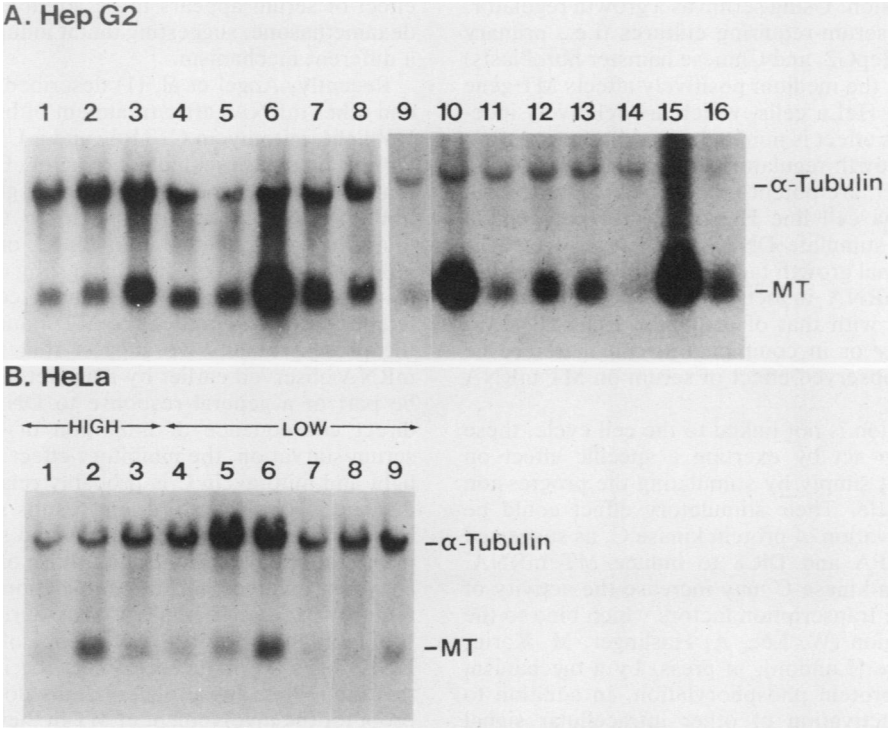

FIG. 6. Effect of peptide hormones, protein kinase C activator, and inhibitor on MT mRNA. (A) HepG2 cells were incubated for 8 h in DMEM containing 0.5% FBS with no additions (lane 1), 10% FBS (lane 2), 100 ng of TPA per ml (lane 3), 20 μM H-7 (lane 4), 100 ng of TPA ⊕ 20 μM H-7 (lane 5), 20 ng of insulin per ml (lane 6), 20 ng of insulin per ml plus 20 μM H-7 (lane 7), 7 μM A23187 (lane 8), control medium (lane 9), 50 ng of Gly-His-Lys per ml (lane 10), 2.5 half-maximal units of PDGF per ml (lane 11), 20 ng of epidermal growth factor per ml (lane 12), 20 ng of IGF-1 per ml (lane 13), 20 ng of IGF-2 per ml (lane 14), 20 ng of insulin per ml (lane 15), or 20 ng of glucagon per ml (lane 16). (B) HeLa cells maintained in DMEM containing either 10% FBS (lanes 1 to 3) or 0.5% FBS (lanes 4 to 9) were incubated for 8 h with control medium (lane 1), 100 ng of TPA per ml (lane 2), 20 ng of insulin per ml (lane 3), control medium (lane 4), 10% FBS (lane 5), 100 ng of TPA per ml (lane 6), 20 ng of insulin per ml (lane 7), 7 μM A23187 (lane 8), or 50 ng of Gly-His-Lys per ml (lane 9). RNA was extracted and analyzed as described in the legend to Fig. 3.

unpublished data). However, an increase in intracellular Ca^{2+} may have some role in the action of insulin and other growth factors, as treatment with A23187, a Ca^{2+} ionophore, caused a moderate induction of MT mRNA (Fig. 6A, lane 8). It remains to be tested whether the activation of cyclic AMP phosphodiesterase by insulin (31) is possibly involved in regulation of MT gene expression.

Insulin and Gly-His-Lys had no significant effect on MT mRNA expression in HeLa cells (Fig. 6B, lanes 3, 7, and 9), ruling out the possibility that the Zn and Cu ions which are known to be complexed by these peptides, respectively, could be responsible for their effects, because HeLa cells are at least as responsive to metal induction as HepG2 cells (11). In contrast to its moderate effect on HepG2 cells, treatment with A23187 did not lead to MT induction in HeLa cells (Fig. 6B, lane 8). TPA on the other hand was an effective inducer in HeLa cells (Fig. 6B, lanes 2 and 6).

DISCUSSION

The results presented in this paper indicate that various agents known to have mitogenic effects, such as serum growth factors and TPA, stimulate MT gene expression in cultured cells. Therefore, these agents can be added to the long list of MT inducers that already includes heavy-metal ions, glucocorticoid hormones, interferon, and interleukin-1 (see reference 12 for a review).

The present study was undertaken to determine whether there is a relationship between stimulation of proliferation, which is likely to increase the intracellular demand for Zn, and MT gene expression. Using serum as a growth regulator, we showed that in serum-requiring cultures (i.e., primary human fibroblasts, HepG2, and Chinese hamster fibroblasts) the level of serum in the medium positively affects MT gene expression, while in HeLa cells, which are relatively independent of serum, its effect is minimal. In addition to serum, at least two other growth regulators of hepatic cells, insulin and Gly-His-Lys (27), are potent inducers of MT mRNA in the human hepatoma cell line HepG2. Other polypeptide hormones that also stimulate DNA synthesis in liver cells (22), such as epidermal growth factor, IGF-1, and glucagon, also lead to MT mRNA induction, but their effects are moderate compared with that of insulin and Gly-His-Lys. These factors, singly or in combination, can therefore be responsible for the observed effect of serum on MT mRNA synthesis.

Since MT expression is not linked to the cell cycle, these factors are likely to act by exerting a specific effect on transcription and not simply by stimulating the progression through the cell cycle. Their stimulatory effect could be mediated by the activation of protein kinase C, as suggested by the ability of TPA and DiC8 to induce MT mRNA. Activation of protein kinase C may increase the activity of one or several of the transcription factors which bind to the hMT-II_A control region (W. Lee, A. Haslinger, M. Karin, and R. Tjian, Nature [London], in press) by a mechanism that could involve protein phosphorylation. In addition to protein kinase C, activation of other intracellular signal transduction pathways such as those involving Ca^{2+} can lead to increased MT expression. However, in these experiments the effect of increased Ca^{2+} was not as general as the effect of TPA, since treatment with a Ca ionophore led to induction in HepG2 but not in HeLa cells.

While both serum and TPA activate protein kinase C (25, 26), the time courses of their effects on MT expression are quite different. The induction by TPA is transient, and MT mRNA returns to its low basal level within 24 h after the addition of this drug. In contrast, the response to serum is longer lasting, and a high steady-state level of MT mRNA is maintained as long as the cells are kept in medium containing fresh serum. The basis for this difference is not clear but may be related to down regulation of protein kinase C after prolonged exposure to TPA (6). In addition, serum growth factors may also operate via other mechanisms not involving protein kinase C, such as an increase in intracellular Ca^{2+}, which also has a moderate stimulatory effect on MT expression. Apparently, activation of several different intracellular signaling systems can lead to increased expression of MT genes by an as yet uncharacterized mechanism. In this context it is interesting to note the recent observation by Schmidt and Hamer (32) that cells transformed with the Ha-*ras* oncogene contain elevated levels of MT mRNA. This effect could be mediated by the stimulation of phosphoinositol turnover by the activated Ha-*ras* oncogene (9).

The response of MT genes to serum and TPA is slower than the response of the c-*fos* and c-*myc* genes which are also induced by these agents (10). This difference is due to the longer half-life of MT mRNA (2.5 h [18]) compared with that of c-*fos* and c-*myc* (10).

Previous work in our laboratory has delineated the *cis*-acting elements responsible for induction of the hMT-II_A gene by heavy-metal ions and glucocorticoid hormones (14). The presence of distinct and separable response elements indicates that these inducers do not operate by a common mechanism. At this time, the sequences involved in the response to the various serum factors are not known, but the effect of serum appears to be additive to that of Cd^{2+} and dexamethasone, suggesting that it induces MT expression by a different mechanism.

Recently, Angel et al. (1) described the induction of MT and other mRNAs after treatment of human fibroblasts with UV light, mitomycin C, TPA, and a UV-induced extracellular protein synthesis-inducing factor, EPIF. They suggested that the induction of this group of genes, which includes hMT-II_A, occurs as a response to the inhibition of cell division by UV light, mitomycin C, or possibly even TPA. However, as we show here, arresting cell division by serum starvation or incubation of mutant cells at nonpermissive temperature does not lead to MT induction. On the basis of the present results we suggest that the induction of MT mRNA observed earlier by Angel et al. (1) is more likely to be part of a general response to DNA damage and not a direct consequence of inhibition of cell division. Unlike serum starvation, the inhibitory effect of agents such as UV light and mitomycin C is probably related to their ability to damage DNA. Therefore, our results showing a correlation between increased MT synthesis and stimulation of cellular proliferation do not contradict those of Angel et al. (1). The enzymes involved in both replication and repair of DNA require Zn, and it is likely that during increased cellular proliferation or after the occurrence of extensive damage to DNA, MTs are induced to meet the increased demand for this ion. While these observations do not provide a direct proof for the involvement of MT in these processes, they are in agreement with the original proposal that MTs serve in supplying Zn to various enzyme systems (7, 11).

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