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Induction of Metallothionein by Stress and its Molecular Mechanisms

SAMSON T. JACOB,*[1] KALPANA GHOSHAL,* AND JOHN F. SHERIDAN†

**Department of Medical Biochemistry, The Ohio State University College of Medicine,*
†Section of Oral Biology, The Ohio State University College of Dentistry, Columbus, OH 43210

This article describes the effect of restraint stress or social reorganization stress on the induction of metallothionein (MT) in the liver, heart, lung, and spleen. Both MT-I and MT-II mRNA were elevated as much as 30-fold following just 12 h (one cycle) of restraint stress. The amount of MT protein also increased following stress. The MT induction was the highest in the liver, followed by the lung, heart, and spleen. MT-I induction was also observed in the fore, mid, and hind regions of the brain whereas the brain-specific MT-III gene was not activated by stress. The increase in MT mRNA correlated well with the rise in stress-induced serum corticosterone. The induction occurred at the transcriptional level and was mediated essentially by the activation of glucocorticoid receptor. The MT mRNA returned to the control level after nine cycles of stress. Exposure of these habituated mice to a different type of stress (treatment with heavy metals such as cadmium or zinc sulfate) led to further MT induction. Because heavy metals induced MT via activation of the factor MTF-1, distinct molecular mechanisms should be responsible for the activation of MT promoter by different inducers.

Metallothionein Restraint stress Social reorganization stress Molecular mechanisms

STRESS is generally defined as a state of altered homeostasis caused by an external or internal stimulus, which can be restored to the control level by a variety of adaptive neuroendocrine mechanisms (49). Suppression of several immune response elements as a result of stressful life can lead to major health consequences such as increases in the susceptibility to, and severity of, infectious diseases (9). In mammals, stress is known to induce acute phase response in the liver, which correlates with plasma glucocorticoid levels and with production of proinflammatory cytokines. Although simple physical or psychological stress (e.g., restraint stress) can induce a few proteins (e.g., heat shock proteins), the molecular mechanism of this process and the probable cellular advantages of the increased level of these proteins have not been elucidated.

Metallothioneins (MT) are ubiquitous cysteine-rich, low molecular weight, heavy metal-binding proteins (20,31). Four isoforms of MT have been discovered in mammals that are arranged in tandem, 6 kb apart, in mouse chromosome 8 (44,47). MT-I and MT-II genes are probably the most widely expressed, and are regulated coordinately in all tissues (44,56) whereas MT-III is brain specific (45) and MT-IV is expressed mainly in the stratified squamous epithelium of skin, tongue, etc. (47). Because the latter two isoforms were identified only recently and not much is known about their functions, almost all the studies to date have focused on MT-I and MT-II. These two isoforms have been implicated in the scavenging of toxic metals such as cadmium and mercury, as well as biologically essential metals like zinc and copper by forming trimercaptide linkages (20,31). The recent reports, however, suggest that the detoxification of heavy metals is not the only or even the primary function of MT. Indeed, these studies reported a key role for MT-I and MT-II in copper homeostasis (5,10,34), as a donor of zinc for the zinc-dependent transcription factors (30,53), in maintenance of redox balance (40) and protection of cells against reactive oxygen intermediates (46,50, 51,55) and electrophilic anticancer drugs (33,36). Several lines of evidence indicate that MT prevents cellular damage caused by

[1]Address correspondence to Samson T. Jacob, 333 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210. Tel: (614) 688-5494; Fax: (614) 688-5600.

a variety of agents. For example, overexpression of these proteins appears to protect the cells against DNA-damaging agents (51). A recent study has elegantly demonstrated that selective overexpression of MT in the cardiac tissue can protect these transgenic mice from the cardiotoxic effects of adriamycin, a potent anticancer drug (32). Further, cultured ventricular myocytes from these overexpressor mice are protected from hydrogen peroxide toxicity, which provides a more direct proof for the capacity of MT to prevent damage from oxidative injury (54). Contrary to the results obtained from the cells or transgenic mice that overexpress metallothioneins, targeted disruption of MT genes can drastically enhance the sensitivity of the organisms with the knock-out genes to heavy metals (41), the oxidant *tert*-butyl hydroperoxide, and the herbicide Paraquat (37). In general, the cells or tissues that are resistant to heavy metals or to reactive oxygen species or other DNA-damaging agents appear to tolerate these insults by increased production of MT.

Our laboratory has been interested in the molecular mechanisms of induction of metallothionein. In the course of this investigation, we discovered the importance of an element designated MRE-c′ that spans from −135 to −110 bp of MT-I promoter (12,15) in MT expression. Based on transfection studies using several 5′ deletion mutants, we showed that this element is required for the constitutive expression of MT. Subsequently, we purified two protein factors, designated C′BF-1 and C′BF-2, that interact with MRE-c′ and transactivate the MT-I promoter (1,2). Although MT-I and MT-II promoters can be activated by heavy metals and other inducers, and those that cause oxidative stress, restricted movement of animals such as restraint stress alone can dramatically augment MT expression in the absence of any external agents. Only limited study has been done on the nature of the physiological factors involved in the stress-mediated induction of MT, often with contradictory results (26–29). None of these studies have identified the *trans*-acting factors involved in this process or measured the MT mRNA levels or determined whether enhanced MT induction by physical stress occurs at the transcription level. Further, stress itself was induced by subjecting the animals to complete immobilization for a relatively long period, which can lead to considerable trauma. We took advantage of a simple mouse model system to study the mechanism of MT induction by restraint stress. This article describes (a) the merits of this model system, (b) the role of glucocorticoid and its receptor in MT induction by stress, and (c) habituation of the animals to physical stress with respect to MT-I induction.

MERITS OF THE ANIMAL MODEL SYSTEM USED TO STUDY THE STRESS-MEDIATED ALTERATION IN GENE EXPRESSION

The major disadvantage of the previous models is that the animals were totally immobilized by attaching them to a board or keeping them in a wrapping of metallic net for a relatively long period of time at a stretch, which can lead to significant trauma (4,26,27). To overcome these problems, we have developed a simple animal model for studying stress-induced changes in gene expression (52). Here, C57BL mice (4–6 weeks of age) maintained on 12-h light/dark cycle (with lights on at 0600 h) were transferred to well-ventilated 50-ml conical propylene tubes for 12 h each day (from 2100 to 0900 h). Although the animals were free to move back and forth in the tube, they were unable to turn around, which caused the restraint stress. Each 12-h restricted movement was designated one cycle of stress. Because the animals did not have access to food and water during this period, the control mice were also deprived of food and water. After each cycle of stress, the animals were removed from the tube and were allowed access to food and water ad lib. This model is ideal to study the physiological adaptation after a few cycles of restraint stress, and to investigate whether distinct molecular mechanisms exist for the induction of metallothionein after physical and chemical stress. It has been extensively used to explore the neuroendocrinological changes under stress (13,14,21–24).

The animals were also subjected to another type of psychological stress. This involves a social reorganization paradigm that has been described in detail elsewhere (43). After the initial acclimation period, the "aggressor" mice were identified in each cage by observing their behavioral patterns (3,8). Switching of these mice between cages at the beginning of the 12-h light/dark cycle and social reorganization every second day for four cycles caused severe psychological stress.

INDUCTION OF METALLOTHIONEIN GENE EXPRESSION IN THE TISSUES OF MICE SUBJECTED TO PHYSICAL OR PSYCHOLOGICAL STRESS

To study the effect of restraint stress on MT gene expression, the animals were subjected to nine cycles of stress. For detailed study on MT induction, we chose the liver, as this organ is the major site of MT gene expression (20,31,44). RNA was isolated from the liver after each cycle of stress and subjected to Northern blot analysis either using a random-primed,

[α-^{32}P]dCTP-labeled mouse MT-I minigene or [α-^{32}P]dATP-tailed deoxyoligonucleotides specific to mouse MT-I and MT-II (16). Scanning of the Northern blot by PhosphorImager using ImageQuant program (Molecular Dynamics) and quantitation of ^{32}P signals with Volume Analysis program showed as much 30-fold increase in the level of MT-I and MT-II mRNA levels following just one cycle of stress. The induced level of MT mRNA persisted until nine cycles of stress at which time it was restored to the control level, which suggests habituation or adaptation of the animals to restraint stress with regard to induction of MT. Restraint stress did not induce other genes that are activated after other types of stress, which include the genes for heat shock protein hsp 70 and heme oxygenase. Similarly, MT-III gene (which is largely expressed in the brain) was not induced in the liver following restricted movement of the animals even for 9 days (data not shown). These data thus demonstrate the specificity of MT-I and -II induction in response to restraint stress. MT-I and MT-II were also induced in the lung, heart, and spleen following restraint stress. In general, the highest induction levels were in the liver and lung (Fig. 1A). MT-I gene was also induced in the fore, mid, and hind regions of the brain within 1 day of restraint stress, which declined significantly after nine cycles of stress, especially in the fore and midbrain regions (Fig. 1B). Although MT-III is selectively expressed in the brain, we explored the possibility that the gene for this MT isoform may be induced in the brain after restraint stress. Our study, however, failed to detect any significant change in the expression of this isoform in the three brain segments following restraint stress (K. Ghoshal, unpublished). It would be of considerable interest to study the MT induction in different cell types of the brain following restraint stress. Such investigation is likely to reveal the major target in the brain for the stress-mediated induction of MT-I and MT-II, and may show induction of MT-III in a specific cell type.

We also measured the amounts of the protein induced after stress by immunoblot analysis with antibodies against MT (commercially obtained from Zymed). There was a significant increase in the MT content that was proportional to increase in MT mRNA levels following stress.

The effect of social stress on MT induction was studied as follows. After identifying the aggressor males, these mice were switched between cages at the beginning of the 12-h light/dark cycle, and social reorganization was performed every second day for four cycles. This stress has been shown to activate the hypothalamic–pituitary–adrenal axis, and cause reactivation of latent herpes simplex virus type 1 (43). It was of considerable interest to determine whether social stress can also activate MT-I promoter. Using this model system, we showed that MT-I gene in the liver was activated as much as 20-fold in response to social stress (K. Ghoshal, published data).

CORRELATION OF THE LEVELS OF GLUCOCORTICOID AND METALLOTHIONEIN mRNA IN THE LIVER

Stress is known to activate the hypothalamus–pituitary–adrenal gland axis that leads to increased levels of glucocorticoid and catecholamine (22,23). Potentially, these agents can then modulate expression of different target genes in tissues that contain their receptors. The role of these physiological factors in MT induction following restraint stress has not been clearly established. Although pharmacological doses of glucocorticoid have been shown to induce MT (19,42), the potential induction of MT by physiological doses of this hormone during stress has not been explored. Using other models that involve complete immobilization of animals, another laboratory (26) has studied the role of glucocorticoid on MT induction. Initially, these investigators reported that pretreatment of the animals with RU 486, a type II glucocorticoid (GC) receptor antagonist, increases the constitutive or stress-induced levels of MT-mRNA (26), which suggested a negative regulation of MT induction by this hormone. Subsequent study by the same investigators, however, showed that the increase in the level of GC correlated with the augmented level of MT mRNA following restraint stress (29). To investigate the role of glucocorticoid released under stress on MT induction, we determined the MT-I mRNA and GC levels at different times (0600, 1200, and 1800 h) after releasing the mice from one cycle of stress. First, we measured the serum corticosterone level following stress by radioimmunoassay. For this purpose, sera from four to five animals were pooled for each measurement (16). At 0600 h, the time of releasing the mice from stress, the GC level increased more than threefold, then declined to almost the control level at 1200 h and began to increase by 1800 h. At this time, the GC levels in the control and the stressed groups were identical. Concurrently, the MT-I mRNA level was highest at 0600 h (30-fold greater than the control level), declined at 1200 h, and was further reduced at 1800 h. The induction of MT mRNA correlated well with the stress-induced increase in the GC level, rather than the diurnal fluctuations of the corticosterone.

To confirm the role of glucocorticoid in MT in-

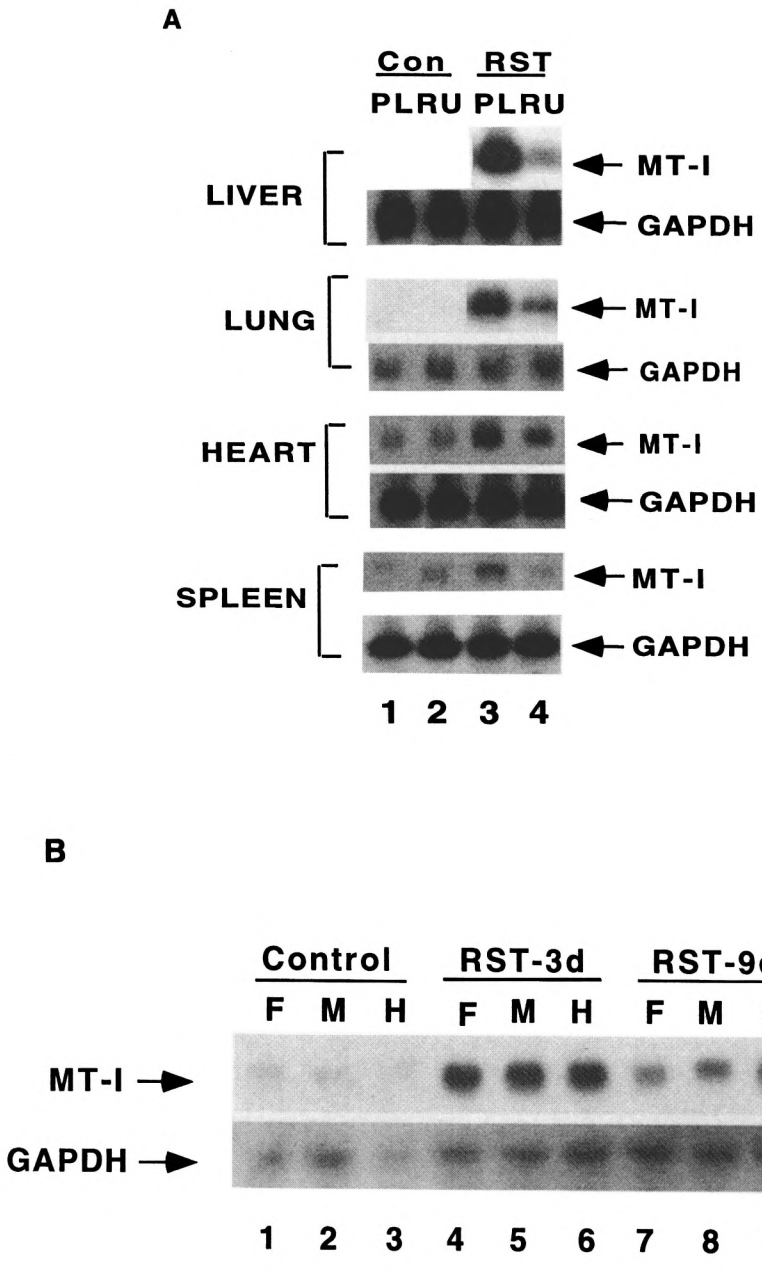

FIG. 1. (A) Northern blot analysis of MT-I mRNA in different tissues of mice restraint stressed for three cycles. C57BL/6 mice were injected daily with RU 486 or RU (a type II glucocorticoid receptor antagonist) at a dose of 25 mg/kg body weight or the solvent polyethylene glycol (PL) 1 day prior to restraining. Mice were then restrained for 12 h (2100 to 0900 h) for 3 consecutive days and total RNA was isolated from the liver, lung, heart, and spleen from the stressed (RST) and control (Con) animals. Total RNA (30 μg) was subjected to Northern blot analysis with ^{32}P-labeled random-primed MT-I mini gene, *Bgl*II-*Hin*dIII fragment of MT-I mini gene (pMT-IΔi), or GAPDH cDNA. Lanes 1 and 2 represent mRNA level in the tissues of control mice injected with PEG (PL) and RU 486 (RU), respectively, whereas lanes 3 and 4 indicate those in the restraint mice treated similarly. (B) Northern blot analysis of MT-I mRNA in the different regions of the brain of C57BL/6 mice restrained for three and nine cycles. Different brain regions (fore, mid, and hind) were dissected out from the control and restraint mice and total RNAs (30 μg) isolated from these regions were subjected to Northern blot analysis as described in (A). Lanes 1–3 represent mRNA level in the fore (F), mid (M), and hind (H) brain of the control mice, whereas lanes 4–6 and 7–9 denote the mRNA level in the same regions of the brain of mice restrained for 3 days (RST-3d) and 9 days (RST-9d), respectively.

duction in response to restraint stress, the mice were pretreated with RU 486, the GC receptor antagonist, prior to stress. The animals were implanted SC with continuous-release pellets containing 0.5 mg/pellet RU 486 or a placebo pellet. The pellets were implanted using 14-gauge trocar 3 days before subjecting the animals to stress. After three cycles of stress, the animals were killed, total RNA was isolated and subjected to Northern blot analysis. The MT-I mRNA level decreased by at least 50% in the mice treated with RU 486 whereas there was no detectable change in the MT-I mRNA level in the livers of mice implanted with the placebo pellet (Fig. 2A). To investigate whether the remaining part of MT induction is due to the effect of catecholoamine released under stress, the experiments were repeated with nadolol, a β-adrenergic receptor antagonist (13). Unlike RU 486, nadolol did not inhibit the stress-mediated increase in MT-I expression (Fig. 2B). This observation does not support a role for the catecholamines in the augmented MT-I expression, at least in the liver. Alternatively, endogenous opioids may be involved in this process. Indeed, opioids are known to be released in response to stress. It has been shown earlier that opioids modulate immune function (6). Further, we have shown that stress-induced suppression of natural killer cell activity during an influenza viral infection is mediated by opioids (J. Sheridan, unpublished data). The effect of naltrexone or naloxone, the opioid receptor antagonists, on the MT induction should be studied in order to elucidate the potential role of opioids on this induction process. All these inducers (glucocorticoids, catecholamines, and opioids) can induce MT when administered exogenously in pharmacological doses (28,31). We have, however, demonstrated that corticosterone induced by stress has a definitive role in the increased expression of MT-I and -II genes.

THE NATURE OF THE SPECIFIC DNA BINDING FACTOR THAT IS ACTIVATED AFTER RESTRAINT STRESS

Several protein factors are known to interact with specific *cis*-acting elements on the MT-I and -II promoter and the regions proximal to the promoter. Although some of these factors are likely to be activated by inducers of MT gene, a most logical candidate is the glucocorticoid receptor for the following reasons.

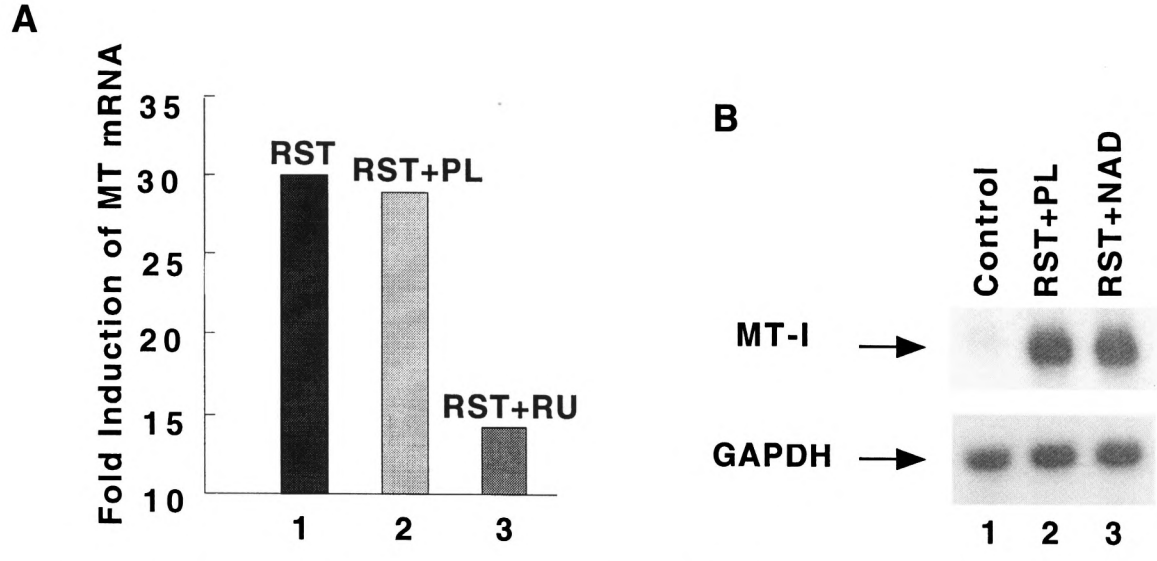

FIG. 2. Effect of RU 486 and nadolol (β-adrenergic receptor antagonist) on MT-I mRNA induction in the livers of restraint mice. (A) Mice were implanted with RU 486 or placebo 3 days before restraint. After three cycles of restraint, the liver RNA was isolated from these mice as well as normal mice. Total RNA (30 μg) was subjected to Northern blot analysis with MT-I mini gene or GAPDH cDNA as probe. For graphical representation of the effect of RU 486 ^{32}P signal for MT-I mRNA in the control liver was normalized to that for GAPDH mRNA, and was arbitrarily assigned the value of 1. The fold induction was calculated by comparing the MT signal in each treatment group normalized to GAPDH signal to that of the control value. Lanes 1–3 represent the fold induction of MT in the livers of restrained (RST), placebo-implanted restrained (RST+PL), and RU486-implanted restrained (RST+RU) mice. The results are the mean of three independent experiments on the pooled liver RNA from each group consisting of 4–5 animals. [These data were derived from (16) with permission from the American Society of Biochemistry & Molecular Biology]. (B) Mice were implanted with a sustained release pellet of nadolol or placebo 3 days prior to restraining and after three cycles of stress, total RNA from the livers was subjected to Northern blot analysis for MT-I or GAPDH. Lanes 1–3 represent the mRNA level in the nadolol-implanted control mice (control), placebo-implanted (RST+PL), and nadolol-implanted (RST+NAD) restrained mice, respectively. [These data were derived from (16) with permission from the American society of Biochemistry and Molecular Biology.]

First, corticosterone is released into the serum following restraint stress and its level is correlated with the level of MT mRNA. Second, the antagonist of the glucocorticoid receptor can suppress MT induction in response to restraint stress. Third, a recent study (35) has identified the glucocorticoid response element (GRE) in the upstream region of the MT gene. The mouse element consists of two GRE consensus sequences separated by a few base pairs and differs from the conventional GRE element only in two or three positions. It is located as much as 7 kb from the transcription start site of MT-I gene and 3 kb from the +1 site of MT-II gene. This slightly modified GRE, however, could bind to glucocorticoid receptor very efficiently and mediate glucorticoid-mediated activation of the MT genes in transfected cells (35). Our study showed that a specific GR–GRE complex was formed when an oligonucleotide corresponding to one of the GRE elements on the MT gene was used to detect the GR activity in the liver nuclear extracts derived from the control mice in electrophoretic mobility shift assay (EMSA). Interestingly, the extracts from the mice stressed for three cycles showed at least a fourfold increase in the complex formation, which indicates that GR was activated after restraint stress (Fig. 3). The specificity of the complex was shown by (a) lack of alteration in the amount of a nonspecific complex after stress, (b) abolishment of the complex in the presence of 100-fold excess of unlabeled GRE oligo, and (c) the lack of competition with excess of a mutant GRE oligonucleotide in EMSA.

MT induction after social stress should not be a surprising observation, as the coritcosterone level rises following social stress and, consequently, the glucocortioid receptor is likely to be activated in the livers of these animals as well. It is, therefore, logical to conclude that psychological stress (which includes restraint and social stress) induces metallothioneins via a common mechanism whereas induction of the same gene by treatment with heavy metals and other inducers is likely to involve other factors such as MTF-1.

We also explored the potential activation of other factors that are involved in the basal and constitutive expression of MT. MTF-1 is a factor required for basal as well as induced transcription of MT in response to heavy metals (25,48) and oxidative stress (11,18). Its activity was measured in EMSA using the liver nuclear extract and MRE-d oligonucleotide to which MTF-1 is known to bind efficiently (25). Two distinct complexes were formed with this oligonucleotide, as both MTF-1 and Sp1 (a general RNA polymerase II transcription factor) can interact with this oligo (25). Competition EMSA using unlabeled Sp1 oligo will, therefore, form a specific complex corresponding to MTF-1, whereas competition with unlabeled MRE-s oligo (a variant form of MRE-d where Sp1 site is mutated) will yield a specific complex with Sp1. Under these conditions, there was no significant alteration in the DNA binding activities of Sp1 and MTF-1 following restraint stress. MLTF/USF is another protein factor that is involved in the constitutive expression of MT gene (7). This factor

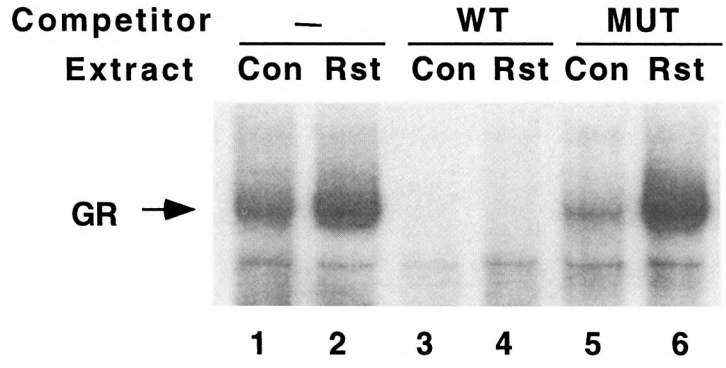

FIG. 3. Electrophoretic mobility shift assay (EMSA) using the liver nuclear extract from normal and restrained mice and ^{32}P-labeled GRE oligonucleotide as probe. Identical amounts (10 μg of protein) of liver nuclear extract from the control and restrained mice were incubated with the ^{32}P-labeled oligonucleotides under optimal binding conditions and the DNA–protein complexes were separated by polyacrylamide (4% acrylamide) gel electrophoresis with 0.5 × TBE as running buffer. The gel was transferred to blotting paper, dried, and subjected to autoradiography and PhosphorImager analysis for quantitation. Lanes 1 and 2 indicate the complexes formed with 10 μg of the extracts from the control (Con) and restrained (Rst) mice, respectively. Lanes 3 and 4 represent the complexes formed in the presence of 100-fold excess of unlabeled wild-type GRE (WT) in the nuclear extracts from the control (Con) and restrained (Rst) animals, respectively. Similarly, lanes 5 and 6 denote complexes formed in the (Con) and (Rst) extracts preincubated with excess of mutant GRE (MUT), respectively. GR indicates the specific complex. [These data, similar to those presented in (16), were obtained from a different experiment.] Note that the nonspecific complex formed (the band below GR) remained unaltered in the presence of vast-excess of GRE (WT) (lanes 3 and 4).

binds E-box element of composite MLTF/ARE (antioxidant response element) and is known to be activated following chemical stress such as treatment with cadmium sulfate (39). Restraint stress did not affect the activity of this factor as well (16). Finally, a recent study (38) has reported that IL-6, a proinflammatory cytokine, is involved in the upregulation of mouse MT-I gene expression in response to treatment with bacterial endotoxin, lipopolysaccharide (LPS). The elevated level of IL-6 in the serum results in the increased binding of STAT (**S**ignal **T**ransducer and **A**ctivator of **T**ranscription) at IL-6 response element on the MT gene. The STAT activity remained unaltered following restraint stress (K. Ghoshal, unpublished data). This is not an unexpected finding, as the level of IL-6 is not significantly elevated after restraint stress (J. Sheridan, unpublished data). These data conclude that the major mechanism of MT induction by restraint or social stress is mediated by augmented activity of the glucocorticoid receptor.

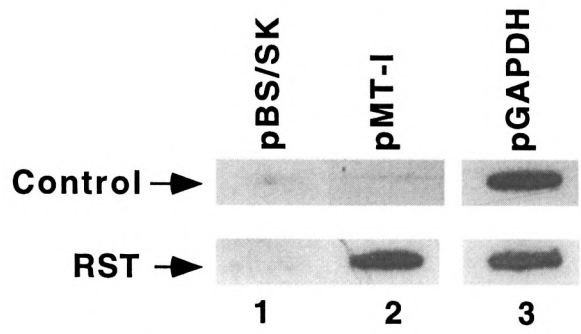

FIG. 4. Transcription in isolated nuclei (run-on transcription) from the livers of the control and restrained mice. Nuclei (5×10^7) isolated from the control mice and the mice restrained for one cycle were incubated with [α-^{32}P]UTP and total RNA containing the same cpm/ml was used as probe to hybridize to different plasmid DNA. Lanes 1–3 represent the ^{32}P signal in pBS/SK (vector plasmid), the plasmids containing mouse MT-I genomic DNA, and the rat GAPDH cDNA, respectively. Each lane contains 10 µg of plasmid DNA. The signal in each lane was scanned in PhosphorImager and quantitated by Volume Analysis program (Molecular Dynamics). [These data were derived from (16) with permission.]

TRANSCRIPTIONAL ACTIVATION OF MT-I PROMOTER IN RESPONSE TO RESTRAINT STRESS

Although MT induction by all the agents appears to occur at the transcriptional level, the dramatic elevation in the levels of MT-I and MT-II mRNA, as shown by Northern blot analysis, may be due to post-transcriptional control mechanisms such as stabilization of mRNAs. To rule out this possibility, a nuclear run-on assay was performed (16). These experiments consisted of incubation of liver nuclei from the control and stressed mice (subjected to one cycle of stress) with [α-^{32}P]UTP and hybridization of identical amounts of radioactivity (cpm/ml) to different plasmid DNAs that correspond to vector, mouse MT-I geneomic DNA, and rat GADPH cDNA. The amount of MT-I transcript formed after stress was at least 20-fold greater than that formed in the control samples (Fig. 4). The levels of GAPDH mRNA remained unaltered, indicating the specificity of the increased MT mRNA levels after stress. Because the MT mRNA level measured by this assay was consistent with the Northern blot analysis, one can conclude that the increased MT-I induction is largely due to increased transcriptional control.

INDUCTION OF METALLOTHIONEIN BY A DIFFERENT STRESS (HEAVY METAL TREATMENT) IN THE LIVERS OF MICE HABITUATED TO RESTRAINT STRESS

We have shown (16) that each cycle of restraint stress helped maintain a very high level of MT mRNA in the tissues of C57BL/6 mice, particularly in the liver (20–30-fold). After seven cycles of stress, the mRNA level began to decline despite continued exposure to stress, and reached the control level by the 9th day of restraint stress (16). The level of corticosterone in the serum, the primary glucocorticoid in the mouse, does not rise after prolonged restraint stress (22), which further suggests that the animal can habituate to a particular type of stress. It is logical to assume that the glucocorticoid receptor will not be activated under this condition. Although the animals are adapted to one type of stress, they are likely to respond to a different type of stress. To test this possibility, the mice subjected to nine cycles of stress were treated with $CdSO_4$ or $ZnSO_4$, which are known to induce MT. Indeed, the level of MT-I mRNA was elevated as much as 25-fold in response to a different type of stress, namely heavy metal treatment (Fig. 5). An interesting aspect of this induction is that a different *trans*-activating factor is involved in the heavy metal-mediated induction of MT. Restraint stress primarily induces MT as a result of activation of glucocorticoid receptor whereas the heavy metals mediate this process via activation of other factors such as MTF-1 or MLTF/USF (25,39). Thus, different machineries operate in response to different inducers that lead to activation of the same gene promoter.

CONCLUSIONS AND PERSPECTIVES

This study has demonstrated that simple restraint stress or stress caused by social reorganization can result in a robust induction of MT-I and MT-II in

many tissues, particularly in the liver, heart, and lung. The dramatic increase in the mRNA level without any external stimuli is noteworthy. Metallothioneins have been implicated in the protection of cellular damage from a variety of toxic agents and compounds that produce oxidative stress. It is, therefore, logical to conclude that the augmented expression of MT-I and MT-II genes in response to restraint or psychological stress may be a major mechanism for the defense of tissues against the deleterious effects of stress. The different kinds of stress that humans face in daily life are also likely to induce MT that could function as a protective shield. In this respect, human peripheral lymphocytes could afford a useful tool to study MT induction in a human population under a variety of stress conditions. The level of MT in human lymphocytes may indeed be a useful marker to assess the tolerance of an individual to stress. In this context, it is noteworthy that certain strains of mice (e.g., C3Hen) do not induce MT to the same level as C57BL/6 mice in response to restraint stress (our unpublished data). Elucidation of the potential mechanisms of resistance to stress in different strains of the same species and certain human population would be of utmost importance. Although glucocorticoid plays a key role in MT induction, another physiological factor(s), probably opioids, is also involved in this process. Identification of such a factor would be essential to understand fully the relationship between the stress-induced biochemical alterations in the cell and the role of neuroendocrine factors. Another area that is worthy of further investigation is to identify the exact cell type(s) in the brain where MT is induced maximally or suppressed in response to stress, and elucidate the molecular mechanism(s) for the selective expression or repression of an MT isoform in these specific cells. In this context, the role of a potential repressor(s) (17) in the suppression of an MT isoform in some cells should be explored. Finally, the potential stress-induced biochemical, physiological, and behavioral changes in the transgenic mice with disrupted genes for specific MT isoforms should be studied.

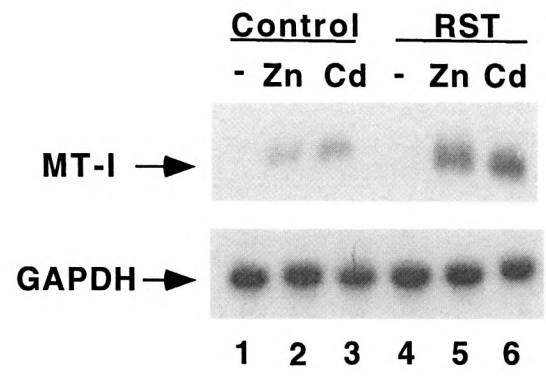

FIG. 5. Level of MT-I mRNA in the livers of mice restrained for 7 days and then challenged with $CdSO_4$ or $ZnSO_4$. Normal mice and restrained mice (7 days) were injected with physiological saline, or $CdSO_4$ (15 μmol/kg body weight) or $ZnSO_4$ in (50 μmol/kg body weight) dissolved in saline. After 4 h, total RNA was isolated from the livers of these mice and 30 μg of RNA was subjected to Northern blot analysis with MT-I cDNA (upper panel) or GAPDH cDNA (lower panel). Lanes 1–3 represent MT-I mRNA level in the livers of mice injected with saline, $ZnSO_4$, and $CdSO_4$, respectively. Lanes 4–6 indicate mRNA level in the livers of restrained mice with saline, $ZnSO_4$, and $CdSO_4$, respectively. [These data, similar to those presented in (16), were obtained from a different experiment.]

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