Overexpression of the large subunit of the protein Ku suppresses metallothionein-I induction by heavy metals

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ABSTRACT Metallothioneins (MT) are involved in the scavenging of the toxic heavy metals and protection of cells from reactive oxygen intermediates. To investigate the potential role of the protein Ku in the expression of MT, we measured the level of MT-I mRNA in the parental rat fibroblast cell line (Rat 1) and the cell lines that stably and constitutively overexpress the small subunit, the large subunit, and the heterodimer of Ku. Treatment with CdSO₄ or ZnS₅, elevated the MT-I mRNA level 20- to 30-fold in the parental cells and the cells (Ku-70) that overproduce the small subunit or those (Ku-7080) overexpressing the heterodimer. By contrast, the cells (Ku-80) overexpressing the large subunit of Ku failed to induce MT-I. In vitro transcription assay showed that the MT-I promoter activity was suppressed selectively in the nuclear extracts from Ku-80 cells. The specificity of the repressor function was shown by the induction of hsp 70, another Cd-inducible gene, in Ku-80 cells. Addition of the nuclear extract from Ku-80 cells at the start of the transcription reaction abolished the MT-I promoter activity in the Rat 1 cell extract. The transcript once formed in Rat 1 nuclear extract was not degraded by further incubation with the extract from Ku-80 cells. The repressor was sensitive to heat. The DNA-binding activities of at least four transcription factors that control the MT-I promoter activity were not affected in Ku-80 cells. These observations have set the stage for further exploration of the mechanisms by which the Ku subunit mediates suppression of MT induction.

Metallothioneins (MT) are ubiquitous, low molecular weight, cysteine-rich (30 mole %), heavy metal-binding proteins that are induced in cells in response to a variety of stimuli (for reviews, see refs. 1–3). Four isoforms of MT have been discovered in animal cells, which are arranged in tandem on mouse chromosome 8 (4). The expression of MT-I and MT-II genes are regulated coordinately in all tissues (5, 6) whereas MT-III is brain-specific (7) and MT-IV is expressed in the stratified squamous epithelium of skin and tongue (4). Although MT can scavenge toxic metals such as cadmium, mercury, and nickel, detoxification of these metals does not appear to be the only function of these proteins (1–3). MT-I and MT-II have been implicated in the delivery of zinc to the zinc-dependent transcription factors (3) and protection of cells against reactive oxygen intermediates generated under a variety of conditions (8, 9). Overexpression of MT in cells results in a significant decrease in the sensitivity of the cells to the membrane-permeant oxidant tert-butyl hydroperoxide (8), DNA-damaging agents (9), and certain anticancer drugs (10). On the contrary, embryonic cells (11) or transgenic mice (12) targeted disruption of MT-I and -II genes are markedly sensitive to the cytotoxic effects of cadmium, the oxidant tert-butylhydroperoxide, and the herbicide paraquat.

Because the cells that are resistant to heavy metals and to free oxygen or hydroxyl radicals tolerate these insults by producing relatively high levels of MT, there has been significant interest in the elucidation of the molecular mechanisms of MT induction. Considerable efforts have been made in the identification of the factors that can activate the MT promoters. In addition to the well defined transcription factors Sp l and USF/MLTF, which interact with the specific sequences on the MT genes, other protein factors that modulate MT expression also have been characterized. MTF I is a 70- to 80-kDa polypeptide with 6 zinc fingers (13) that is required for the basal as well as induced transcription of MT gene by heavy metals (14) and in response to oxidative stress (15). Embryonic stem cells with targeted disruption of the MTF I gene (14) are very sensitive to heavy metal ions, as there is no constitutive or induced expression of MT gene in these cells. Recently, we characterized two nuclear protein factors, one from rat liver (16) and the other from a rat hepatoma (17), that can trans-activate the mouse MT-I promoter. The liver protein, a dimer of a 33-kDa polypeptide, and the tumor protein, a monomer with a molecular size of 28 kDa, interact with the MRE-c’ sequence located between –108- and –135-bp positions with respect to the +1 site of mouse MT-I gene, an element that is involved in the basal transcription of the gene (18).

In addition to the positively acting transcription factors, there is indirect evidence for the existence of a repressor of MT gene transcription (19–21), as cells treated with the protein biosynthesis inhibitor cycloheximide accumulate MT-I mRNA in the absence of metal ion treatment. Our laboratory has embarked upon the identification of the repressor(s) for MT induction and elucidation of its molecular mechanism(s) of action. We have shown that the Ku protein, an autoantigen, or an inhibitor associated with this protein can suppress RNA polymerase I (Pol I) transcription of the rat ribosomal RNA gene when the cells are starved for serum (22), although Ku is a positively acting Pol I transcription factor under normal growth conditions (23, 24). The present study was undertaken to investigate whether Ku plays a role in suppressing MT induction. We show that the cells overexpressing the large subunit (p80) of Ku do not induce MT-I whereas MT-I induction in response to the heavy metals proceeds unabated in the parental rat fibroblast cells or the cells overexpressing either the small subunit (p70) of Ku or Ku heterodimer. Further, we used in vitro transcription assay to demonstrate the existence of a repressor for MT-I expression in the nuclear extracts from the p80-overproducing cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Rat 1 cell lines overexpressing different subunits of Ku and the parental cell line

Abbreviations: MT, metallothionein; EMSA, electrophoretic mobility-shift assay.

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were generous gifts from Gloria Li, Memorial Sloan Kettering Institute. These cell lines were grown in DMEM containing 10% FBS. The cell line overexpressing the 70-kDa subunit of Ku (R70-15) was maintained in the same medium containing hygromycin B (100 µg/ml), and the cell line overproducing the p80 subunit of Ku (R80-1/R80-6) was maintained in G418 (200 µg/ml). The cell line overexpressing both subunits (R7080-6) was grown in the presence of both drugs (25). For the sake of convenience, the overexpressing cell lines are also designated Ku-70, Ku-80, and Ku-7080, respectively.

TREATMENT OF RAT I AND KU OVEREXPRESSING CELLS WITH THE METAL IONS, ISOLATION OF TOTAL RNA, AND NORTHERN BLOTTING.

Analysis.

Metal Ions, Isolation of Total RNA, and Northern Blot Analysis.

When cells were in culture, metal ions were added to the medium in the following concentrations: 100 µM CdSO4 and 100 µM ZnSO4. Because Zn at the concentrations used is not toxic to Rat 1 cells, the presence of ZnSO4 was evaluated in order to study the induction of MT-I. Total RNA was isolated from the cells and an aliquot of RNA from the untreated control and the treated cells was subjected to Northern blot analysis with random-primed cDNA probes for mouse MT-I (27), human hsp 70 (28), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (29).

Preparation of Transcriptionally Active Nuclear Extract. Rat 1 cells were grown in 20 150-mm tissue culture dishes. Cells were harvested by scraping and spinning down at 1,800 × g at 4°C. The cell pellet was washed twice with ice-cold PBS. The transcriptionally active nuclear extract was prepared as described (30).

Electrophoretic Mobility-Shift Assay (EMSA). The whole-cell extracts used for measuring the DNA-binding activities of Sp1 and MTF 1 were prepared as described (31) and incubated with [α-32P]dGMP-labeled MRE-d and MRE-c′ (16) oligonucleotides as the probe.

In Vitro Transcription Assay. Transcription of MT-I gene in vitro was performed with the nuclear extract by using pMTI(C2AT) as the template following the published protocol (32). In this plasmid, the MT-I promoter was cloned directly into the G-fragment cassette plasmid, p(C2AT), which yields a 394-nt-long transcript in vitro. This plasmid yielded consistently cleaner and more reproducible transcript in vitro than that obtained with other recombinant MT-I plasmids (33).

RESULTS

Overexpression of the 80-kDa Subunit of Ku Inhibits the Heavy Metal-Induced Expression of Metallothionein-I Gene. Rat 1 cells stably transfected with the cDNA for p70 subunit (R70-15 or Ku-70), p80 subunit (R80-1 or Ku-80), or Ku heterodimer (R7080-6 or Ku-7080) expressed the foreign gene(s) constitutively, as determined by immunoblot analysis (Fig. 1). Ku-70 cells expressed 70-kDa polypeptide at significantly higher level than the parental Rat 1 cells whereas Ku-80 cells, which is consistent with the greater stability of Ku heterodimer relative to that of the individual subunits (25). The above four cell lines under normal conditions did not express MT-I mRNA at any detectable level as analyzed by Northern blot analysis (Fig. 2, lanes 1, 5, 9, and 13, respectively). After treatment with 30 µM CdSO4, the level of MT-I mRNA increased 20- to 30-fold in Rat 1 (lanes 2–4) as well as Ku-70 (lanes 6–8) and Ku-7080 (lanes 14–16) cells. Surprisingly, the heavy metal did not induce MT-I expression in Ku-80 cells (lanes 10–12). As cadmium is toxic to the cells, the metal-exposed cells were transferred to cadmium-free medium, and the MT-I mRNA level was monitored up to 4 hr after removal of the metal. In Rat 1, Ku-70, and Ku-7080 cells, the MT-I mRNA level increased after 2 hr (lanes 3, 7, and 15, respectively), and it remained high even after 4 hr of cadmium withdrawal (lanes 4, 8, and 16, respectively), whereas Ku-80 cells did not activate MT-I gene under these conditions (lanes 11 and 12). Because the induction of MT-I gene by heavy metals is known to occur primarily at the level of transcription (5), the inhibition of MT-I mRNA accumulation in response to cadmium is probably a result of transcriptional repression. The induction of MT-I continued for a significant period even after withdrawal of the metal in all cell lines except Ku-80. These results demonstrate that overexpression of p80 subunit of Ku inhibits cadmium-induced accumulation of MT-I message. We also repeated this experiment with another cell line overexpressing p80 (R80-6) and obtained identical results (data not shown). Since cDNA used as the probe detects both MT-I and MT-II, these data show that both genes are not induced in Ku-80 cells, which is consistent with the coordinated regulation of MT-I and MT-II (6).

To determine the specificity of this response, the mRNA level of another stress-response protein, namely hsp 70, was measured by rehybridizing the same blot to human hsp 70 cDNA (Fig. 2 Middle). Hsp 70 can be induced in cells in response to heat shock and cadmium treatment (34). Unlike MT-I expression, hsp 70 mRNA could not be detected in the parental cells or the overproducing cells after 2 hr of exposure to Cd (lanes 2, 6, 10, and 14). The hsp 70 message, however, appeared in the parental cells after 2 hr of exposure to Cd followed by incubation in Cd-free medium for 2 hr (lane 3) or 4 hr (lane 4). Under these conditions, the hsp 70 mRNA also accumulated in the Ku-80 cells (lanes 11 and 12, respectively). On the contrary, the cadmium-induced expression of hsp 70 was inhibited completely in Ku-70 and Ku-7080 cells (lanes 7, 8, 15, and 16, respectively), which is consistent with the inhibition of hsp 70 induction in these cell lines after heat shock (25, 35). This experiment clearly shows that overexpression of the p80 subunit of Ku does not inhibit expression of all cadmium-inducible genes.

Because the mechanism of induction of MT-I by Cd and Zn appears to be different (36), the MT-I mRNA level was measured in the overexpressing cells after treatment with ZnSO4. Because Zn at the concentrations used is not toxic to the cells, all cell lines were incubated in the presence of Zn-containing medium to study the induction of MT-I. Total RNA was isolated from the cells after 2, 4, and 8 hr of treatment with the metal ion. There was a time-dependent increase in MT-I mRNA in Rat 1 (Fig. 3, lanes 1–3), Ku-70 (lanes 4–6), and Ku-7080 cells (lanes 10–12) after ZnSO4.
Fig. 2. Level of MT-I mRNA in response to cadmium in the parental cells and cell lines overexpressing Ku heterodimer or different subunits of Ku. Rat 1, R70-15, R80-1, and R7080-6 cells were treated with 30 μM CdSO₄ for 2 hr. The cells then were washed with cadmium-free medium and reincubated for 2 or 4 hr. The same samples in the absence of cadmium treatment were used as the controls. Total RNA was isolated from each batch of cells and subjected to Northern blot analysis with the random-primed, 32P-labeled mouse MT-I cDNA as the probe (Top). The same blot was reprobed with 32P-labeled mouse hsp 70 cDNA (Middle) or rat GAPDH cDNA (Bottom) as the probe. Lanes 1–4 correspond to MT-I mRNA from the control Rat 1 cells (lane 1), Rat 1 cells treated with CdSO₄ for 2 hr (lane 2), or incubated in Cd-free medium for 2 hr (lane 3) and 4 hr (lane 4) after exposure to CdSO₄ for 2 hr. Similarly, lanes 5–8, 9–12, and 13–16 represent mRNA from R70-15, R80-1, and R7080-6 cells, respectively, following treatments described for Rat 1 in lanes 1–4.

treatment. MT-I mRNA was not detected in Rat 1 cells after 2, 4, or even 8 hr of exposure to Zn (lanes 7, 8, and 9, respectively). These results clearly show that overexpression of the p80 homodimer inhibits expression of MT-I gene by the heavy metal whereas heterodimerization of p80 subunit with p70 subunit blocks this repression. Unlike cadmium treatment, the level of MT-I mRNA induced by zinc after 2 hr of exposure was significantly less.

Transcriptional Activity of Mouse MT-I Promoter Is Repressed in Vitro in the Nuclear Extract Prepared from Ku-80 Cells. The inhibition of MT-I mRNA expression by heavy metal ions in Ku-80 cells may be a result of transcriptional repression or enhanced degradation of the message. Because MT-I regulation by various agents occurs mainly at the level of transcription (5, 6), we first explored the MT-I promoter activity in the nuclear extract prepared from the parental Rat 1 and Ku-80 cells. The nuclear extract from Rat 1 cells could transcribe pMT(CTAT) plasmid, generating a transcript of 394 nt (see Materials and Methods), the correct transcript expected from this plasmid (Fig. 4A, lane 1). To show that the transcript is directed specifically by the MT-I promoter, we depleted the extract for a specific transcription factor, MTF 1, that is essential for both basal- and metal-induced transcription of the MT-I gene (14). This was achieved by preincubating the extract with the oligonucleotide (MRE-s) corresponding to the MRE element to which MTF 1 specifically binds (13). As a control, a 37 bp oligonucleotide (E₁) representing the rat rDNA enhancer (23) was used. Preincubation of Rat 1 nuclear extract with increasing amounts of MRE-s reduced the level of RNA transcript in a dose-dependent manner (compare lanes 2 and 3 with lane 1), whereas E₁ oligo had a minimal effect even at the highest concentration (compare lanes 4 and 5 with lane 1).

This result clearly showed that Rat 1 cells extract could transcribe MT-I promoter in vitro. The ability of Rat 1 nuclear extracts to transcribe pMT(CTAT), as compared with relative absence of MT-I mRNA in these cells in the absence of inducers (determined by Northern blot analysis), most probably is a result of the optimal conditions, particularly the naked DNA, used in the transcription assay. We then compared the transcriptional activity of the nuclear extracts prepared from Rat 1 and Ku-80 cells. In this exper-

Fig. 3. Level of MT-I mRNA in cell lines overexpressing different subunits of Ku after ZnSO₄ treatment. Rat 1, R70-15, R80-1, and R7080-15 cells were treated with ZnSO₄ (100 μM) for different time periods. Total RNA was isolated from the cells treated with the metal ion for 2, 4, and 8 hr, respectively. Similarly, lanes 4–6, 7–9, and 10–12 represent MT-I mRNA levels in R70-15, R80-1, and R7080-6 cells, respectively, that were treated for 2, 4, and 8 hr with zinc. (Lower) Level of GAPDH mRNA in each lane.

Fig. 4. Transcription of MT-I gene in vitro in the nuclear extract from Rat 1 and R80-1 cells. (A) MT-I transcription in Rat 1 nuclear extract. Nuclear extract (10 μg) from Rat 1 cells was incubated with the template pMT(CTAT) along with ATP, CTP, 3′-0-methyl GTP, and [α-32P]UTP under optimum conditions at 30°C for 45 min (for details, see ref. 32). The [32P]UMP-incorporated RNA was isolated, dissolved in RNA loading buffer, and separated by urea-PAGE and analyzed by autoradiography. Lane 1 indicates the level of 32P-labeled MT-I transcript in Rat 1 nuclear extract, whereas lanes 2 and 3 denote the amount of RNA transcribed when the extract was preincubated with 200 and 400 ng of MRE-s (34 bp) oligo, respectively, and lanes 4 and 5 represent the MT-I transcript level in the presence of 200 and 400 ng of 37 bp rat rDNA enhancer (E₁), respectively, as the competitor. Arrow indicates the transcript. (B) Transcription from MT-I promoter in the nuclear extracts from Rat 1 and R80-1 cells. Identical amounts of the nuclear extract from these cells were analyzed in in vitro transcription reaction as described in A. Lanes 1 and 2 represent MT-I transcript level in 20 and 30 μg of the nuclear extract from Rat 1 cells, whereas lanes 3–5 denote MT-I transcript level in 20, 30, and 40 μg of the nuclear extract from R80-1 cells, respectively.
To eliminate the possibility of activation of a potent nuclease in Ku-80 cells, we added back the nuclear extract from these cells to that of Rat 1 cells at the start and end of transcription reaction (Fig. 5B). The transcription from MT-I promoter was inhibited in Rat 1 extract in a dose-dependent manner by the Ku-80 extract if added at the beginning of the reaction (compare lanes 4 and 5 with lane 1). On the contrary, the transcript level in Rat 1 extract remained unaffected when the extract was added after 45 min of transcription reaction and incubated for an additional 15 min at 30°C (compare lanes 2 and 3 with lane 1). These data suggest that the repressor is not a nuclease.

Overexpression of Ku-80 Polypeptide Does Not Inhibit the DNA-Binding Activities of Four Protein Factors Involved in MT-I Transcription. To elucidate the probable molecular mechanisms for the inhibition of MT-I expression in the cells overexpressing the p80 subunit of Ku, we investigated the activity of four key transcription factors involved in the transcription of MT-I gene, e.g., MTF 1, Sp 1, MLTF/ARE, C'BP-1, and C'BP-2. Among these factors, MTF 1 is required both for basal- and heavy metal-induced transcription of MT-I gene (14). Since the transcription of MT-I gene is inhibited in Ku-80 cells, it seemed likely that p80 polypeptide may interact with MTF 1, resulting in its sequestration or inactivation. Existence of such a repressor has been proposed for MTF 1 (21), as the basal level of MT-I mRNA is very low in many cell lines including Rat 1 cells (Fig. 1, lane 1). Sp 1 is another ubiquitous transcription factor that is required for the basal transcription of MT-I gene (1). To determine whether the repressor in Ku-80 cells inhibits the DNA-binding activity of a transactivator, we performed EMSA with 32P-labeled MRE-d oligo, to which both Sp 1 and MTF 1 bind. The results are shown in Fig. 6. The whole-cell extract from Rat 1 cells had strong Sp 1 activity (Fig. 6A, lane 1) but negligible MTF 1 activity that was stimulated significantly (5-fold) after zinc treatment (compare lane 4 with lane 1). The identities of the complexes were determined by competition with specific oligonucleotides in the extract from zinc-treated cells. The faster-migrating complex appears to be MTF 1, as its formation could be competed with 100-fold excess of cold MRE-s oligo (lane 2), a mutant of MRE-d to which Sp 1 cannot bind (13). The slower-migrating complex probably results from interaction between Sp 1 and the oligo probe, as its formation could be competed with Sp 1 consensus oligo (lane 3). Treatment of these cells with cadmium did not stimulate MTF 1 activity, and Sp 1 activity was decreased.

FIG. 5. (A) MT-I transcription in Rat 1 nuclear extract preincubated with the nuclear extract from R80-1 cells either untreated or heat-inactivated at 60°C for 10 min. Lane 1 represents transcript level in Rat 1 extract (10 μg), lanes 2 and 3 denote that in Rat 1 extract containing 10 and 20 μg of heat-inactivated R80-1 (HI-R80) extract, respectively. Lanes 4 and 5 indicate MT-I transcript in Rat 1 extract incubated with 10 and 20 μg of untreated R80-1 (R80) extract, respectively. (B) MT-I transcription in Rat 1 nuclear extract after addition of R80-1 nuclear extract before or after transcription reaction. Rat 1 nuclear extract (10 μg) was incubated with 10 and 20 μg of R80-1 extract, respectively, before (lanes 4 and 5) or after (lanes 2 and 3) the transcription reaction. Lane 1 represents transcription in Rat 1 nuclear extract.

FIG. 6. DNA-binding activities of two key transcription factors, MTF 1 and Sp 1, involved in MT-I transcription. (A) DNA-binding activities of MTF 1 and Sp 1 in the cell extract from Rat 1 cells. Ten micrograms of the whole-cell extract from control (lane 1), ZnSO4-treated (lane 4), or CdSO4-treated (lane 5) cells was incubated with 0.1–0.2 ng of 32P-labeled MRE-d oligo at 4°C under optimum binding conditions. To identify the complexes, the extract from ZnSO4-treated cells was preincubated with 100-fold molar excess of unlabeled MRE-s (lane 2) or Sp 1 (lane 3) oligo along with 0.5 μg of poly(dI-dC) as the nonspecific competitor. The DNA–protein complexes formed were separated by polyacrylamide (4% acrylamide) gel electrophoresis and analyzed by autoradiography of the dried gel. Sp 1 indicates the complex characteristic of Sp 1 protein, and MTF 1 corresponds to the complex formed by binding of MTF 1 to MRE-d. (B) DNA-binding activities of MTF 1 and Sp 1 in R80-1 cell. Ten micrograms of the whole-cell extract prepared from the control (lane 1), ZnSO4-treated (lane 2), or CdSO4-treated (lane 3) cells was subjected to EMSA by using MRE-d as the probe. Lanes 4 and 5 represent the complexes formed in the extract from zinc-treated cells preincubated with 100-fold molar excess of MRE-s and MRE-d oligos, respectively.
2-fold (lane 5). Surprisingly, the activities of these two factors, particularly MTF 1, were higher in Ku-80 cells (Fig. 6B). In these cells, both MTF 1 and Sp 1 were activated significantly in response to zinc treatment (compare lane 2 with lane 1). Even exposure to cadmium that did not increase the DNA-binding activity of MTF 1 in Rat 1 cells (Fig. 6A) or other cells (36) led to activation of MTF 1 (lane 3) in Ku-80 cells. It is, therefore, unlikely that the inhibition of MT-I promoter activity in these cells is a result of inactivation of these two positive factors. The higher activities of these factors in Ku-80 cells might be due to their stabilization in the extract prepared from these cells and/or to their activation.

In another experiment, we also tested the activity of the factor C’-BP-1(16) that interacts with the MRE-c’ element and trans-activates the MT-I promoter. The complexes formed with MRE-c’ oligo and the whole-cell extracts from Rat 1 and Ku-80 cells were identical (data not present). Similarly, the activity of MLTF/USF that binds to MT/ARE element of MT-I promoter and that is required for the basal expression of MT-I (37) also remained unaltered in Ku-80 cells (data not presented). These results clearly show that overexpression of Ku-80 subunit of Ku does not suppress induction of MT-I gene by inhibiting the binding of at least four protein factors to specific promoter regions on the MT-I gene.

**DISCUSSION**

The present study has shown that overexpression of the large subunit of the Ku protein can lead to suppression of MT-I gene induction by the heavy metals. The specificity of this inhibitory effect of the Ku subunit was demonstrated by the continued induction of another stress-responsive protein, namely, hsp 70. Interestingly, the thermal induction of hsp 70 was blocked in the cells that overexpress either p70, the small subunit of Ku (35), or Ku heterodimer (25). Therefore, it was not surprising that the heavy metal-induced expression of hsp 70 also was suppressed in these cells (present study). Unlike the repression of hyperthermia-mediated induction of hsp 70 in the cells overexpressing p70 and the heterodimer, the heavy metal-mediated induction of MT is not inhibited in these cells. The nuclear run-on experiment has shown that this repression of MT-I expression in Ku-80 cells is at the level of transcription, and it is specific for MT as expression of ribosomal RNA and GAPDH remained unaffected (data not shown). The lack of activation of MT-I promoter in Ku-80 cells is not a result of quenching or inactivation of the factors MTF 1, Sp 1, MLTF, and C’-BP-1. It is possible that the repressor in Ku-80 cells directly interacts with the basal transcription machinery.

It is not known whether the p80 subunit of Ku can directly inhibit the transcription of MT-I gene. To test this possibility we expressed histidine-tagged p80 and p70 subunits of Ku (generous gifts of Michael Lieber, University of Southern California, Los Angeles) separately or together in reticulocyte lysate (TNT system, Promega) and purified through Ni-NTA resin (Qiagen) after the protocol of Wu and Lieber (38). The recombinant polypeptides could bind to DNA in EMSA with 32P-labeled DNA when cotranslated or mixed after purification but could not bind on their own (ref. 38; data not shown). When we added back these recombinant proteins (p80, p70, or p7080) to Rat 1 or HeLa nuclear extract, none of them could inhibit MT-I transcription (data not shown). This result indicates that p80 cannot directly inhibit MT-I expression; rather, its overexpression activates an intracellular factor(s) that inhibits MT-I gene transcription in vivo. It would be of considerable interest to identify the signal transduction pathway that leads to p80-mediated suppression of MT-I induction.

Because the stably transfected cell lines overexpressing different subunits of Ku were generated by a retroviral vector-mediated gene transfer technique, we tested the possible integration of the recombinant vector within the MT-I gene, resulting in its disruption. To confirm it, we performed Southern blot analysis of the MT-I gene in Rat 1 and Ku-80 DNA digested with different restriction enzymes. The pattern of Southern blot is identical among these two cell lines (data not shown). As MT-I gene is silent in some cell lines because of methylation of the gene (39, 40), we also analyzed the methylation profile of MT-I gene in Rat 1 and Ku-80 cells by digesting the genomic DNA with methylation-sensitive enzymes, MspI and HpaII, and hybridizing to MT-I probe. Identical Southern blot profiles were obtained with these two cell lines (data not shown). Moreover, treatment of Ku-80 cells with azacytidine (an agent that inhibits methylation of DNA) did not facilitate induction of MT-I by heavy metals (data not shown). These results confirm that the MT-I gene is unaltered in Ku-80 cells and the noninducibility is not a result of deletion, insertion, or hypermethylation of the gene.

Finally, although in vitro transcription assay demonstrated the effect of the repressor in the context of naked DNA, we cannot rule out alteration in the chromatin structure as an additional mechanism of repression of MT induction in Ku-80 cells. Such modifications in the chromatin structure are known to affect transcription of other genes (for review, see refs. 41–44), which is manifested by alteration in the DNase I-hypersensitive site or positioning of inhibitory nucleosomes on the promoter regions (42). Under this condition, the positive factors or the basal transcription machinery may not gain access to the promoter, resulting in transcriptional repression. In vivo genomic footprinting and DNase I-hypersensitive site mapping of MT-I and MT-II promoters can address these possibilities. If a diffusible repressor exists in these cells, as our in vitro transcription results indicate, we should be able to purify it further by fractionating the extracts of these cells and assaying the different chromatographic fractions by the in vitro transcription assay standardized in our laboratory (31). Studies along these lines are now in progress.

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