Hypermethylation of Metallothionein-I Promoter and Suppression of Its Induction in Cell Lines Overexpressing the Large Subunit of Ku Protein*

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We have shown previously that the heavy metal-induced metallothionein-I (MT-I) gene expression is specifically repressed in a rat fibroblast cell line (Ku-80) overexpressing the 80-kDa subunit of Ku autoantigen but not in cell lines overexpressing the 70-kDa subunit or Ku heterodimer. Here, we explored the molecular mechanism of silencing of MT-I gene in Ku-80 cells. Genomic footprinting analysis revealed both basal and heavy metal-inducible binding at specific cis elements in the parental cell line (Rat-1). By contrast, MT-I promoter in Ku-80 cells was refractory to any transactivating factors, implying alteration of chromatin structure. Treatment of two clonal lines of Ku-80 cells with 5-azacytidine, a potent DNA demethylating agent, rendered MT-I gene inducible by heavy metals, suggesting that the gene is methylated in these cells. Bisulfite genomic sequencing revealed that all 21 CpG dinucleotides in MT-I immediate promoter were methylated in Ku-80 cells, whereas only four CpG dinucleotides were methylated in Rat-1 cells. Almost all methylated CpG dinucleotides were demethylated in Ku-80 cells after 5-azacytidine treatment. To our knowledge, this is the first report that describes hypermethylation of a specific gene promoter and its resultant silencing in response to overexpression of a cellular protein.

Ku is an abundant nuclear protein that consists of two polypeptides of molecular masses 80–86 and 70–72 kDa. It was first identified in the serum of a patient with scleroderma polymysitis overlap syndrome (1), and antibodies against this protein have been detected in the sera of patients with various autoimmune diseases (2). It can bind to the DNA ends (2, 3) as well as the internal DNA sequences (4–7). Ku has been implicated in multiple cellular processes that include DNA replication, recombination, repair, ATPase and helicase activities, transcription, and alteration in chromatin structure (2, 8). It is also involved in cell signaling (9), cell cycle regulation (10), and maintenance of telomere length and telomere silencing (8).

Ku can act both as a transcriptional activator and repressor depending upon the promoter and growth conditions (2, 6, 7, 11–14). Previous study in our laboratory demonstrated that specific antibodies against Ku or peptide fragments of Ku could inhibit RNA polymerase I transcription in vitro, which was overcome by the addition of purified Ku protein to the reaction (6). Further study showed that Ku could physically and functionally interact with another RNA polymerase I transcription activator, CPBF (14), a protein that is structurally and functionally related to USF, a helix-loop-helix-zipper DNA binding factor (15, 16). On the contrary, Ku purified from the growth-arrested cells inhibited polymerase I transcription, which could be restored following addition of purified Ku from the control cells (11).

In the course of our study on the regulation of metallothionein (MT)1 gene expression (for review, see Refs. 17 and 18) by heavy metals and oxidative stress, we observed abolishment of the induction of MT-I and MT-II (19) (the two predominant isoforms of MT) as a result of overexpression of the large subunit (p80) of Ku in rat fibroblast cells, Rat-1. Interestingly, overexpression of the small subunit (p70) of Ku or of the heterodimer (p70 and p80) did not inhibit MT induction by the heavy metals. Nuclear extracts from the p80-overexpressing cells contained a repressor activity that could block transcription from the MT-I promoter (19). The activities of two of the key transcription factors, Sp1 and MTF-1, that modulate MT-I transcription were not modified in the extracts from these cells. The present study was undertaken to determine the molecular mechanism by which MT-I expression is repressed in the p80 overexpressing cells. This study showed that the promoter hypermethylation is primarily responsible for the suppression of MT induction.

MATERIALS AND METHODS

Cell Cultures, Treatment with 5-Azacytidine, Heavy Metals, and Northern Blot Analysis—Rat-1 (parental cell line) as well as cell lines that overexpress p70 subunit (R70-15), p80 subunit (R80-1 and R80-6), and both subunits (R7080-6) were generously provided by Gloria Li, Memorial Sloan Kettering Institute. For convenience, overexpressing cell lines were also designated Ku-70, Ku-80, and Ku-7080, respectively. All experiments except that described in Fig. 2B were performed using the clonal isolate R80-1 (designated Ku-80 throughout the text). In the experiment described in Fig. 2B, R50-1 (Ku-80) and another clonal isolate, designated R80-6, were used. The culture conditions and treatment with the heavy metals were described earlier (19). For 5-AzaC treatment, Ku-80 cells at 25–30% confluency were grown in the presence of increasing concentration of the analog for 72 h. RNA isolation and Northern blot analysis were performed as described (19).

In Vivo Genomic Footprinting—In vivo methylation of cellular DNA by dimethyl sulfate (DMS) and the subsequent DNA extraction were done following the protocol of Mueller and Wold (20). The procedure of Ping et al (21) was followed for ligation-mediated (LM)-PCR. MRE and MLTF/ARE sites (adenosine) were subjected to methylation

1 The abbreviations used are: MT, metallothionein; 5-AzaC, 5-azacytidine; DMS, dimethyl sulfate; IVGF, in vivo genomic footprinting; MRE, metal response element, MLTF/ARE, adenosine major late transcription factor/antioxidant response element; LM-PCR, ligation-mediated polymerase chain reaction; bp, base pair(s).

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Fig. 1. Genomic footprinting analysis of MT-I promoter in Rat-1 and Ku-80 cells after induction with ZnSO₄ and CdSO₄. Cells untreated or treated with ZnSO₄ (100 μM) or CdSO₄ (30 μM) were subjected to DMS treatment, followed by DNA extraction and piperidine cleavage (see “Materials and Methods”). The naked DNA from both wild-type and mutant cells were also treated with DMS and piperidine. An identical amount of DNA (2 μg) from each sample was then subjected to LM-PCR with primers specific for the upper and the lower strands. A, schematic diagram of the MT-I promoter depicting cis elements that are relevant to heavy metal-induced expression. LM-PCR of the lower strand (B) and LM-PCR of the upper strand (C) of MT-I promoter in Rat-1 and Ku-80 cells. Lanes 2, 3, and 4 represent Rat-1 cells that are untreated or treated with ZnSO₄ (100 μM) and CdSO₄ (30 μM), respectively; lanes 6, 7, and 8 correspond to similar samples from Ku-80 cells, respectively; lanes 1 and 4 denote naked DNA from Rat-1 and Ku-80 cells, respectively. Arrows (→) indicate G-residues that are protected, whereas asterisks (*) represent hypersensitive G-residues.

RESULTS

The MT-I Promoter in Ku-80 Cells Is Refractory to the Trans-acting Factors, as Revealed by In Vivo Genomic Footprinting—Previous study (19) in our laboratory demonstrated that Ku-80 cells (cells overexpressing the large subunit of the protein Ku) were unable to induce MT-I or MT-II in response to heavy metals such as CdSO₄ or ZnSO₄. Under this condition, the parental Rat-1 cells, a fibroblast cell line, and Ku-70 cells (cells overexpressing the small subunit of Ku) or Ku-7080 cells (cells overproducing both subunits of Ku) could induce the gene by the toxic metals. This study suggested that the noninducibility of MT-I gene in Ku-80 cells was because of the existence of a repressor in the nuclear extract prepared from Ku-80 cells (19).

Alternatively, overexpression of p80 could result in altered chromatin structure that inhibits access of positive factors to the corresponding regulatory elements in the promoter. To address the latter issue, we took advantage of in vivo genomic footprinting (IVGF) to analyze and compare the state of MT-I promoter occupancy in Rat-1 and Ku-80 cells before and after exposure to heavy metals. This would allow us to identify the sequence-specific DNA-protein interactions that are unique to both cell lines, and potential alterations of these interactions specifically in the parental cells after exposure to heavy metals. IVGF analysis of the MT-I promoter was performed by LM-PCR with primers designed to amplify the sequence from –226 to –16 bp, which encompasses all cis-acting elements involved in the basal and metal-induced expression of MT-I. Fig. 1A depicts the metal regulatory elements and other key elements of the rat MT-I promoter. Both Rat-1 and Ku-80 cells were treated with DMS before and after exposure to ZnSO₄ or CdSO₄, and genomic DNA was extracted, cleaved with piperidine, followed by amplification of the promoter fragment by LM-PCR. Naked genomic DNA isolated from both Rat-1 and Ku-80 cells were treated in the same manner as intact cells and amplified to provide the genomic G-ladder. DNA-protein interactions can result in protection of G-residues from DMS reactivity (indicated by arrows in Fig. 1, B and C). Alternatively, such interactions can result in more intense bands of G-residues exhibiting enhanced DMS reactivity (indicated by asterisks in Fig. 1, B and C) when compared with the naked DNA ladder or untreated control. The DMS reactivity and cleavage pattern of both strands of naked genomic DNA from Rat-1 and Ku-80 cells were identical (Fig. 1, B and C).

Heavy metal-induced footprinting was observed in the lower
strand at MRE-d element where varying levels of protection of the G-residues spanning this element were detectable upon Zn$^{2+}$ or Cd$^{2+}$ treatment of the Rat-1 cells (Fig. 1B, lanes 3 and 4). Distinct footprinting of MRE-c element and the overlapping MRE-c' element were clearly detectable in the lower strand of MT-I promoter of Rat-1 cells, which were indistinguishable for Zn$^{2+}$- and Cd$^{2+}$-treated samples (Fig. 1B, lanes 3 and 4). The G-residues encompassing MRE-e, MRE-b, and MRE-a element of the lower strand of MT-I promoter from the heavy metal-treated Rat-1 cells were also protected (Fig. 1B). In addition, Zn$^{2+}$- and Cd$^{2+}$-induced footprinting was observed at the composite element MLTF/ARE in the lower strand of control Rat-1 cells when compared with the naked G-ladder (Fig. 1B). The G-residues in Sp1 binding element were constitutively protected (Fig. 1C, lane 2, double arrow), and some were hypersensitive in the upper strand of control Rat-1 cells (Fig. 1C, asterisk) before and after ZnSO$_4$ or CdSO$_4$ treatment. A very prominent DMS reactivity (hypersensitive residue) was observed at another Sp1 binding site overlapping with the MLTF/ARE element in the upper strand of MT-I promoter from both Zn$^{2+}$- and Cd$^{2+}$-treated Rat-1 cells (Fig. 1C). On the contrary, none of the conspicuous constitutive and/or metal-induced footprinting observed in Rat-1 cells were visible in Ku-80 cells irrespective of metal ion treatment (Fig. 1, A and C). This observation indicates that overexpression of p80 protein results in altered chromatin structure. Such chromatin modification is likely to lead to a conformation of the MT-I promoter refractory to any positive factor binding, which could explain the lack of heavy metal-induced MT-I induction in Ku-80 cells.

**MT-I Gene Is Activated by Various Inducers in Ku-80 Cells after 5-AzaC Treatment**—The failure of the positive factors to footprint on the promoter of other genes silenced because of methylation of CpG dinucleotides has been reported by many laboratories (23). Based on these published reports and our own IVGF data with MT-I gene (Fig. 1), we reasoned that MT-I promoter may be silenced in Ku-80 cells because of promoter methylation. To test this possibility, we determined the effect of 5-AzaC, a potent DNA demethylating agent (24) on MT-I expression. As treatment with 10 $\mu$m 5-AzaC for 24 h did not result in induction of the gene in response to heavy metals (19), we first determined the concentration of the cytosine analog and duration of treatment that can activate MT-I gene in Ku-80 cells. Increase in 5-AzaC concentration from 10 to 20 $\mu$m and time of incubation with the drug from 24 to 72 h resulted in significant expression of MT-I mRNA after CdSO$_4$ treatment (Fig. 2A). These data showed that MT-I gene can indeed be induced in Ku-80 cells (R80-1 clonal line) by heavy metals after prolonged treatment with relatively high concentration of 5-AzaC. The MT-I promoter methylation in the clonal cell line used in the present study may be because of a critical chance event of methylation that contributed to further methylation. To rule out this possibility, we used another randomly selected clonal isolate (R80-6) of p80 overexpressing cells. This study showed that MT-I gene is repressed in these cells as well and that demethylation with 5-AzaC reactivated the promoter (Fig. 2B). The silencing of MT-I gene in the cells overexpressing the 80-kDa subunit is, therefore a specific event. After demethylation with 5-AzaC, MT-I gene could be activated by other inducers as well, e.g. zinc, dexamethasone, cycloheximide (data not shown). We then compared the level of induction of MT-I mRNA in Rat-1 cells following exposure to CdSO$_4$ to that of Ku-80 cells treated with 5-AzaC plus CdSO$_4$ (Fig. 2C). Under this condition, MT-I mRNA level in the 5-AzaC-treated Ku-80 cells reached almost the same level as that in Rat-1 cells. We could not detect any basal expression of MT-I in Ku-80 cells after 5-AzaC treatment. This data suggests that hypermethylation of MT-I gene plays a key role in its silencing in Ku-80 cells.

**All CpG Elements within MT-I Promoter Are Methylated in Ku-80 Cells**—To ensure that the activation of MT-I gene by various inducers after 5-AzaC treatment was indeed because of demethylation of the promoter, we performed bisulfite genomic sequencing before and after the drug treatment. Sequence analysis of MT-I promoter (~225 to +1 bp with respect to transcription initiation site) that harbors the cis elements for most of the transacting factors revealed high density of potential methylatable CpG dinucleotides (twenty-one) spanning this region (Fig. 3A). Therefore, it was essential to compare the methylation status of MT-I promoter in Ku-80 cells with that in Ku-70 and Ku-7080 cell lines, which express the smaller subunit of Ku and both subunits together, respectively. The latter two cell lines can express MT-I mRNA at the same level as the parental cell line (Rat-1) in response to heavy metals (19). For this purpose, we performed bisulfite genomic sequencing of DNA isolated from (a) Rat-1, (b) Ku-80, (c) Ku-80 treated with 5-AzaC, (d) Ku-70, and (e) Ku-7080 cells. The bisulfite treatment of the genomic DNA resulted in complete conversion of unmethylated cytosines to uracils without affecting methylated cytosines. Subsequent PCR amplification and sequencing provided an accurate and consistent representation of mCpG sequences.

Completion of bisulfite conversion of the genomic DNA isolated from the five different cell samples were confirmed by Tsp509I digestion. The restriction site for Tsp509I (\(\Delta AATT\)) does not exist in MT-I promoter and is generated only after bisulfite conversion of unmethylated cytosine residues to uracils, which are amplified as thymine during PCR. The Tsp509I digestion analysis of PCR-amplified bisulfite-treated DNA from Ku-7080, Ku-70, Rat-1, and Ku-80 cells treated with 5-AzaC
and Ku-80 cells showed total cleavage of amplified DNA from all five samples (Fig. 3B), indicating completion of bisulfite reaction. To assess the existence of CpG methylation, we digested all the DNA samples with BstUI (CG \( \rightarrow \) CG). The MT-I promoter contains the restriction site for this enzyme, which should be retained after bisulfite conversion if the cytosine residues were methylated. Only the amplified DNA from Ku-80 cells showed cleavage with BstUI, indicating the presence of methyl CpG dinucleotide in this cell line. The bisulfite conversion and restriction enzyme digestion of the amplified DNA were performed at least three times with different batches of chromosomal DNA.

To study the status of all 21 CpG base pairs, we sequenced the amplified DNA from all five cell cultures (see Fig. 4 for schematic representation of the sequencing data). Interestingly, all 21 CpG base pairs (between -225 to +1 site) in Ku-80 cells were methylated, whereas in Rat-1 cells only four methylated CpG dinucleotides were present in the same promoter stretch. It was logical to conceive that the additional CpG methylation of MT-I promoter in Ku-80 cells was a direct or indirect outcome of p80 overexpression in these cells. Interestingly, none of the CpG elements were methylated in Ku-70 or Ku-7080 cells, implying that methylation of MT-I promoter occurred in the cell line that overexpressed specifically p80 but not in cell lines overexpressing p70 homodimer or the p70/p80 heterodimer.

Sequencing of bisulfite-converted DNA from Ku-80 cells treated with 5-AzaC showed extensive demethylation of the MT-I promoter. This explains the ability of 5-AzaC-treated Ku-80 cells to express MT-I upon heavy metal treatment as observed in Northern blot analysis (Fig. 2). This particular batch of 5-AzaC-treated Ku-80 cells retained one methylated CpG at -112 site of the MT-I promoter, which did not interfere with the expression of the gene.

**DISCUSSION**

Although the role of Ku and associated DNA-PK has been well established in V-D-J recombination and double strand DNA break repair, the mechanism by which Ku regulates expression of various genes at the transcriptional level is still an enigma. Earlier studies showed that overexpression of the p70 subunit of Ku or the heterodimer represses induction of Hsp-70 protein (13, 25). The p80 subunit of Ku can function as a receptor for somatostatin (9), whereas the small polypeptide (p70) can directly interact with the proto-oncogene p95\(^{\text{vav}}\) (26) and GCN5, a transcriptional adapter that has chromatin-modifying activity (27). The present study using different clonal isolates of Rat-1 cells overexpressing the large Ku subunit, has clearly demonstrated for the first time that overproduction of a single subunit of a cellular protein can hypermethylate and consequently silence the promoter of a specific gene. These results implicate that the individual subunits of Ku can play important regulatory roles in cellular functions. Although Ku exists predominantly as heterodimer, a certain population of this protein may also exist as homodimers which could exert their specific functions. The ratio of homodimer to heterodimer may vary with the cell type and under certain physiological conditions and disease states.

Because the Ku homodimers are generally unstable, they are degraded rapidly (2). Consequently, the amount of p80 in Ku-80 cells is considerably less than that in Ku-7080 cells where both p70 and p80 are overexpressed (19, 25). The overproduction of any individual subunit under certain physiolog-
ical conditions or in pathological states may, therefore, have
gone undetected. We have recently demonstrated that MT-I
promoter is hypermethylated in a rat hepatoma and a mouse
lymphosarcoma cell line (relative to the parental cells) which
resulted in the repression of MT-I expression in these cancer
cells.\(^2\) Chromatographic fractionation of the nuclear extracts
from the hepatoma has frequently yielded fractions that con-
tain variable amounts of Ku homodimers.\(^3\) This observation
is consistent with the potential role of Ku subunits in the methyl-
ation of some promoters, which results in their inactivation.

Previous study in our laboratory showed that only the cells
that overproduce the large subunit of Ku fail to express met-
allotheonanein gene and that the suppression of MT-I transcrip-
tion is at least, in part, because of the production of a repressor
in these cells (19). This conclusion was reached by studying the
in vitro transcription of MT-I promoter in nuclear extracts
derived from the wild-type and mutant cells. This study did not
suggest the role of promoter methylation along with the pro-
duction of an active repressor in silencing the MT-I gene. The
present data have clearly demonstrated that long term treat-
ment of the cells with higher concentration of 5-AzaC was
essential to remove the inhibitory methyl groups from methy-
lated CpG residues in MT-I promoter.

The methylation of four CpG elements of twenty one such
dinucleotides in the MT-I promoter of Rat-1 cells did not affect
the ability to express MT-I in response to heavy metals. The
permanence of DNA binding activity of MTF-1, the key tran-
scription factor for MT-I induction, in vitro (gel shift assay) or
ability to transactivate in vivo (transient transaction assay)
(28) following methylation of some MRE sites may probably
explain the capability of Rat-1 cells to express MT-I despite
methylation of a few key cis elements. Interestingly, the DNA
binding and transactivation property of the zinc finger protein
Sp1 are also not inhibited upon methylation of its binding site
(28). The differential methylation pattern of MT-I promoter in
Rat-1 and Ku-80 cells leads us to postulate that the methyla-
tion of just a few key cis elements in Ku-80 cells alone may not
cause MT-I gene silencing. Rather, extensive methylation of
the promoter is likely to recruit the methyl CpG binding pro-
teins along with repressors of transcription, like histone H1 or
Sir3A (29) that leads to alteration in the chromatin structure
and consequently gene silencing. The repressor detected in our
earlier study in Ku-80 cell extract (19) is probably a methyl C
binding protein (MeCP), as it has been shown earlier that
MeCP can inhibit transcription in vitro from unmethylated as
well as methylated promoters depending upon the assay con-
ditions (30). Further, overexpression of MeCP2 is known to
inhibit Sp1-activated transcription of human leukosialin gene,
although methylation of Sp1 binding site does not interfere
with its binding (31). Preliminary UV cross-linking studies
have shown that the activity of a MeCP is significantly higher
in nuclear extract from the Ku-80 cells compared with that
from Rat-1 cells. Alternatively, it is conceivable that a repres-
sor different from MeCP may function in concert with the
methylated promoter to achieve repression of MT induction in
Ku-80 cells.

It is unlikely that a repressor protein binds directly to the
promoter (between -226 to +1 bp) and alters the chromatin
conformation, as no unique or new footprinting appeared in the
MT-I promoter of Ku-80 cells relative to that of Rat-1 cells. We
extended our footprinting analysis up to -350 bp of the pro-
moter using another set of primers, which did not indicate any
DNA/repressor interaction in Ku-80 cells (data not shown). The

\(^2\) K. Ghoshal, unpublished data.

\(^3\) A. Ghosh and S. Jacob, unpublished data.

\(^4\) X. Dong, unpublished data.

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