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The immediate vicinity of mouse metallothionein-I gene contains two sites conferring glucocorticoid inducibility to the heterologous promoter

Sergey Y. Plisov[a,*], Marina G. Nichiporenko[a], Alla L. Shkapenko[a], Victor P. Kumarev[b], Ludmila V. Baranova[b], Tatyana I. Merkulova[a]

[a]*Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences, Lavrentyev 10, Novosibirsk 6300890, Russian Federation*
[b]*GENSET, 75001 Paris, France*

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Abstract Glucocorticoid responsive elements (GREs) located − 252 to − 209 bp upstream and + 1011 to + 1054 bp downstream of the transcription initiation site of the mouse metallotionein-I (mMT-I) gene were identified in transient transfection eperiments. However, the promoter region of the mMT-I gene (− 330 to + 70 bp) was found to provide low, if any, glucocorticoid induction of the linked CAT gene, while showing strong cadmium regulation, comparable with the in vivo level.

Key words: Metallothionein; Glucocorticoid regulation; Promoter; Regulatory element

1. Introduction

The metallothioneins are small cystein-rich proteins that bind heavy metals [1]. In mice, metallothionein genes are inducible by heavy metals and glucocorticoid hormones. Both inducers act at the transcriptional level [2]. As to glucocorticoids, the hormone–receptor complexes interact with specific DNA sequences, termed glucocorticoid responsive elements (GREs) that are located in the vicinity of responsive genes and affect their transcription [3]. Deletion analysis is the accepted approach to detect GREs. With this approach, for instance, a functional GRE was found at the 5′-flanking region between − 263 and − 248 bp) of the human metallothionein-II_A gene [4]. This element was coincident with the DNA-binding site for the glucocorticoid receptor, and shared strong homology with the GRE consensus sequence (GGTCANNNTGTTCT) [5]. As to the mouse metallothionein genes, however, transfections did not allow identification of any GRE because the transfected genes, although inducible by cadmium, were not inducible by glucocorticoids [6,7].

The proximal 5′-flanking region of the mouse metallothionein I (mMT-I) gene contains a sequence between − 244 to − 229 bp that displays high homology with the GRE consensus sequence. We showed that the DNA fragment corresponding to the gene region between − 252 to − 209 bp and containing this sequence is able to specifically bind the glucocorticoid receptor [8]. This region was also classified as a potential GRE with our computer method [9], which revealed another likely GRE, located at the 3′-flanking region of the mMT-I gene between + 1011 and + 1054 bp, and considerably differing from the consensus sequence. In this study we have examined the ability of these presumed GREs to confer glucocorticoid inducibility on a chloramphenicol acetyltransferase (CAT) reporter gene the transcription of which was driven by the Herpes virus thymidin kinase gene promoter.

*Corresponding author. Fax: (7) (3832) 35 65 58.
E-mail: merk@cgi.nsk.su

Abbreviations: GRE(s), glucocorticoid responsive element(s); mMT-I, mouse metallothionein-I; CAT, chloramphenicol acetyltransferase.

2. Materials and methods

2.1. Plasmid constructions

Oligonucleotides corresponding to both strands of fragments from − 259 to − 209 bp and from + 1011 and + 1054 bp of the mMT-I gene were synthesized by the phosphoramidate method and purified by reverse-phase liquid chromatography [10]. The oligonucleotides were annealed and ligated into the *Sma*I site in pUC18. Their nucleotide sequences were verified by DNA sequencing [11] A series of CAT plasmids shown in Fig. 1A were generated by inserting *Hin*dIII–*Eco*RI fragments of the recombinant pUC clones into the *Bam*HI site of pTKCAT [12] after end-repair with Klenow enzyme.

Mouse genomic clone pKH was a gift of R.D. Palmiter (University of Washington School of Medicine, Seattle) [6]. The *Eco*RI–*Hin*dIII fragment of pKH, containing the mMT-I gene, was cloned into pBR322. The *Bst*EII–*Bgl*II fragment, corresponding to the − 300 to + 70 bp region of the mMT-I gene was subcloned into the *Bam*HI site of pBL6CAT vector kindly provided by G. Schutz (German Cancer Research Center, Heidelberg) [13].

2.2. DNA transfections and CAT assay

LTK^- cells were grown at 37°C in Eagle's essential minimal medium supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere. Transient transfections of cells were performed by the calcium phosphate-mediated precipitation method [14]. Twenty hours after transfections cells were treated with 3 μM of dexamethasone or 5 μM of $CdSO_4$ for an additional 20 h. Cell extracts, normalized for total protein content, were assayed for CAT activity as previously described [15].

3. Results and discussion

Data shown in Figs. 1 and 2 provide experimental evidence that an isolated binding region of the glucocorticoid receptor from the 5′-flanking region of the mouse MT-I gene between − 252 and − 209 bp (pMT2CAT) increases the CAT gene expression 3.6-fold ($P > 0.95$) following treatment by dexamethasone. The same effect is produced by the + 1011–+ 1054 bp fragment from its 3′-flanking region (pMT43CAT). Interestingly, despite a considerable difference in sequences, corresponding to the GRE consensus, between these fragments (Fig. 3), they do not differ much in ability to function as GREs when inserted upstream of the heterologous promoter.

The regulatory function of the 5′-GRE (located in the 5′-flanking region) of the mMT-I gene remains the same in

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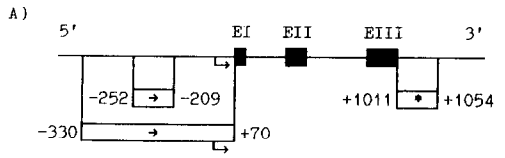
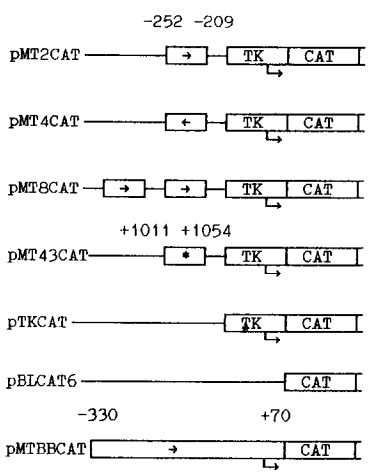


B)



Fold of CAT induction by dexamethasone	Number of experiments
3.6 ± 0.5 *	9
4.1 ± 0.3 *	5
8.1 ± 2.1 **	7
4.0 ± 0.3 *	5
0.8 ± 0.1	8
1.1 ± 0.2	7
1.6 ± 0.2	7

Fig. 1. (A) Map of the mMT-I gene. DNA fragments of the gene used in mMT-I-CAT constructions are indicated below. (B) CAT assay in extracts from LTK$^-$ cells after transient transfection with mMT-I-CAT fusion plasmids. The control plasmids (pTKCAT and pBLCAT6) and mMT-I-containing plasmids (pMT2CAT, pMT4CAT, pMT8CAT, pMT43CAT, pMTBBCAT) are shown schematically on the left. Boxed arrows indicate the orientation and copy number of the mMT-I gene fragment from −252 to −209 bp, and asterisks show the +1011 to +1054 bp fragment of the mMT-I gene inserted upstream of the TK promoter and reporter CAT gene. The plasmid pMTBBCAT contains the intact region of the mMT-I gene from −330 to +70 bp upstream of the reporter CAT gene. The table on the right shows the fold of CAT gene induction by dexamethason and the number of experiments performed with the plasmids. Statistical significance of differences was calculated by Students *t*-test where $*P > 0.95$ vs. pTKCAT, $**P > 0.95$ vs. pMT2CAT.

either orientation relative to the transcription start site (pMT2CAT and pMT4CAT), and a duplication of the fragment leads to a 2-fold increase in the induction of CAT gene expression by dexamethasone (pMT8CAT); properties of the

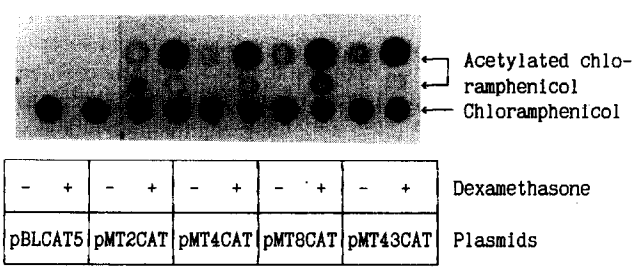


Fig. 2. Dexamethasone-dependent transcriptional enhancer activities of the mMT-I gene DNA fragments. A typical autoradiogram of CAT assays performed with extracts of LTK$^-$ cells transfected with different mMT-I-CAT constructions.

known GREs found in a range of glucocorticoid-dependent genes and viruses [16–18].

However, we ensured that, when located in its natural promoter region of the mouse MT-I gene between −330 and +70 bp, the GRE that we detected is almost unable to exert glucocorticoid regulation over the expression of the CAT gene linked to this region (Figs. 1 and 4). At the same time, this region, containing the metal responsive elements found earlier [19], provided induction of CAT (15-fold) by cadmium ions in our experiments (Fig. 4). These results agree well with those reported by other authors. As was shown, neither an intact mMT-I gene cloned together with its flanking regions [6], nor its promoter region of 1700 bp linked to the coding region of the thymidine kinase gene [7], selectively responded to treatment by dexamethasone following transfection into LTK$^-$ cells, whereas an endogenous MT-I gene of these cells displayed a 4-to 6-fold enhancement of transcription in response to dexamethasone. Amplification of this gene in mouse and hamster sarcoma cells led to a decrease in its sensitivity to dexamethasone, although amplified genes remained transcriptionally active and inducible by heavy metal ions [20]. However, two novel mouse cell mutants (L100 and L80), with amplified metallothionein genes that retained their glucocorticoid inducibility, have been isolated recently [21].

Why regulation of mouse MT-I gene expression by glucocorticoids is lost upon amplification and transfection of the gene is yet to become clear. A possible explanation was that DNA regions required for glucocorticoid regulation of mMT-I gene expression are located far from the gene (over 25 kbp) [20,21]. However, our results show that the mMT-I gene contains at least two potential GREs in its immediate vicinity. At the same time, the 5′-GRE that we have detected is able to function when isolated from its natural environment and not so within its promoter region in transient transfection experiments.

As it is known, a single GRE functions if it is situated only in the immediate vicinity of the TATA box [22], otherwise either several copies of it, or its combination with another regulatory element are required [16,22]. Since the GRE in question and the transcription start site are separated by 240 bp, it can be assumed that the interaction with other regulatory elements is required for its successful function. Elements like these may lie at considerable distances from the GRE, but find themselves in its vicinity following the specific arrangement of DNA within the chromatin. We suggest that the 'assisting' elemement like this may be, in particular, the GRE that we have found at the 3′-flanking region of the mMT-I gene.

Palmiter and associates have recently reported that 10 and 7 kb DNA regions flanking the tandem of mouse metallothionein genes have the properties of locus control regions [23] earlier described for a range of other genes [24,25]. Their study suggests that the chromatin domain structure of the MT

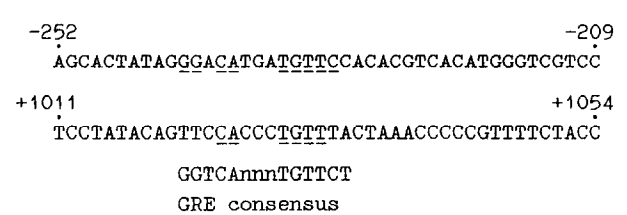


Fig. 3. Nucleotide sequences of the mMT-I GREs revealed. Regions of homology with the GRE consensus sequence are underlined.

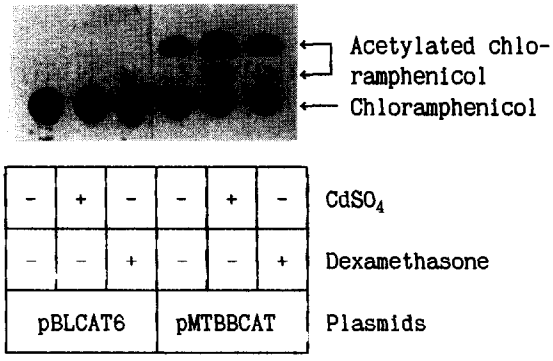


Fig. 4. Dexamethasone and Cd responsiveness of a mMT-I promoter (−330 to +70 bp) ligated to the CAT gene. CAT assays performed with extracts from LTK$^-$ cells transfected with the pMTBBCAT construct and control pBLCAT6 plasmid.

locus is important for glucocorticoid regulation, and fit the idea that distal and proximal elements cooperate in the glucocorticoid induction of the mMT-I gene.

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