Communication

CTG Triplet Repeat in Mouse Growth Inhibitory Factor/Metallothionein III Gene Promoter Represses the Transcriptional Activity of the Heterologous Promoters*

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Growth inhibitory factor/metallothionein III (GIF/MT-III) is expressed specifically in brain, and neither mRNA nor protein is detected in other organs. This tissue-specific expression might be regulated by negative elements as well as positive elements, such as tissue-specific enhancers. To investigate the repression mechanisms of this gene in organs other than the brain, transfection experiments were performed by using various deletion mutants. Interestingly, a 25 × CTG repeat in the promoter region seemed to contribute to the repression activity. Moreover, the repression activity of this 25 × CTG repeat was also observed in various promoters and in a direction and position independent manner, indicating that this element could act as a silencer. However, no binding protein was detected by gel-shift and footprint analyses. These results strongly suggest that the CTG repeat functions as a negative element, and that this effect is caused by unknown mechanisms, rather than by interactions between specific cis-elements and specific trans-acting factors as reported previously. It is also possible that the CTG repeat functions as a general silencer in many genes.

Gene expression is regulated mainly at the transcription level. Many trans-acting factors, such as enhancer-binding proteins, participate in this regulation (see Refs. 1 and 2 for review). For tissue-specific expression, tissue-specific enhancers are known to be involved. Recently, it was proposed that, as well as enhancer elements and enhancer-binding proteins, negative regulators are also involved. Mechanisms include competition, quenching, direct inhibition, and squelching (see Refs. 3 and 4 for review). However, compared with enhancer functions, the mechanisms of negative regulation still remain to be investigated.

Growth inhibitory factor (GIF) has been purified from human brain (5). Both amino acid sequence analysis and cDNA cloning have revealed that GIF is a small protein, 68 amino acids long, and quite similar to metallothionein (MT) with insertions of 1 amino acid and 6 amino acids in the N-terminal and C-terminal portions, respectively (5–8). Therefore, GIF is also termed MT-III, since MT-I and MT-II have been reported (6). In addition, a new MT isoform, MT-IV was reported recently (9). MTs are small, cysteine-rich, and metal-binding proteins, and MT-I and MT-II are thought to contribute to detoxification of heavy metals, such as mercury and cadmium, and homeostasis of essential trace elements, such as zinc and copper (see Refs. 10 and 11 for review). Whereas MT-I and MT-II are expressed widely in almost all organs and are strongly induced by heavy metals, GIF/MT-III is expressed strictly in the brain and not induced by heavy metals (6). GIF/MT-III is also known to be deficient in the brain of Alzheimer’s disease (AD) patients (5). Therefore, in addition to β-amyloid precursor protein and t, GIF/MT-III seems to have an important role in Alzheimer’s disease (5, 12–14).

In this report, we characterized the promoter region of the mouse GIF/MT-III gene and found that a CTG repeat in this region functions as a silencer-like element and contributes to negative regulation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The mGIF/MT-III gene was a kind gift from Dr. R. Palmiter (6). The fragment from base pairs −807 to +57 in the promoter region of the GIF/MT-III gene was made by polymerase chain reaction techniques (PCR) (15) and ligated to the multicloning site in the promoter-less luciferase vector, PGV-P (Nippon Gene) according to standard protocol (16). Various deletion mutants were constructed from this construct by deletion at the 5’ end by exonuclease III and mung bean nuclease digestions. Internal deletions and the 25 × CTG fragment for insertion within various promoters were constructed by PCR. All constructs used here were checked by sequencing with the dideoxy method using denatured plasmid templates (17).

Cell Culture and DNA Transfection—The HepG2 cells, human hepatoma cell line, were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were transfected by the calcium phosphate co-precipitation techniques described by Chen and Okayama (18). All the transfection experiments were performed at least three times by using two or three different preparations of DNA, and the mean values are shown under “Results.” Transfection efficiency was checked by co-transfection with pRSVGal, a eukaryotic expression vector containing the Escherichia coli β-galactosidase structural gene controlled by a Rous sarcoma virus long terminal repeat as an internal or an external standard. Luciferase activity was assayed by Pikka Gene (Toyo Ink Mfg. Co., Ltd.) and a lumiphotometer, and β-galactosidase activity was determined as described (19).

RESULTS

Repression Activity of CTG Repeat in GIF/MT-III Gene Promoter—The GIF/MT-III gene is not expressed in any organs except the brain. To investigate the repression mechanisms of this gene, various deletion mutants with the luciferase gene as a reporter were transfected into human hepatoma cell line HepG2 cells. As shown in Fig. 1, the −257/−172 construct revealed the highest activity among the constructs tested. All other constructs showed lower activity. This result indicates that there is a repressible element between −477 and −257.

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1 The abbreviations used are GIF, growth inhibitory factor; MT, metallothionein; PCR, polymerase chain reaction.
The nucleotide sequence of this region is shown in Fig. 2. A data base search for trans-acting factors revealed some typical cis-elements for LBP-1, LF-A1, and ADR-1 (data not shown). More interestingly, we have found that CTG is repeated 25 times in this region. To elucidate the function of this $25 \times$ CTG repeat on trans-activation activity, we next transfected the internal deletion mutant lacking the CTG repeat. Compared with the native −807GIF construct, the construct with deleted CTG repeats revealed higher luciferase activity (Fig. 3A). When $25 \times$ CTG repeats were joined to the CTG-less −257GIF construct, lower activity was observed (Fig. 3B). These results indicate that the CTG repeat has repressive activity on the GIF/MT-III gene promoter.

Effect of CTG Repeat on Transcriptional Activity of Various Promoters—Since the CTG repeat has repressive activity on the GIF/MT-III gene promoter, we next examined whether this effect would occur in various gene promoters. When the CTG repeat was joined to their upstream regions, this repeat showed repressive activity in all promoters tested here (Fig. 4A). Moreover, when the CTG repeat was inserted in opposite orientation, the same results were obtained (data not shown). Even when the CTG repeat was joined downstream of the promoters of three genes, a similar tendency was observed (Fig. 4B). When transfected into 3Y1 cells, a rat fibroblast cell line, and NIH3T3 cells, a mouse embryo cell line, similar results were obtained (data not shown). These results strongly suggest that the CTG repeat acts as a silencer.

Binding Analysis of Nuclear Proteins to CTG Repeat—We next performed gel-shift analysis by using $25 \times$ CTG repeats as a probe and nuclear extracts from HepG2 cells and rat liver. However, no binding protein was detected, although several other trans-acting factors including C/EBPα and Nuclear Factor 1 were detected by each specific probe using the same extract (data not shown). No protected region was detected by DNase footprint analysis by using the GIF promoter as a probe (data not shown). It seems that specific proteins do not bind to the CTG repeat, or that binding affinity is too weak to detect.

**DISCUSSION**

In this report, we characterized the promoter region of the mouse GIF/MT-III gene and identified its CTG repeat as a silencer. Since this gene is not expressed in organs other than the brain (5, 6), this function is quite important for negative regulation in the mouse. Recently, we cloned the human GIF/MT-III gene, including the promoter region up to −22 kilobases, using the coding region as a probe (kind gift from Dr. R. D. Palmiter). Unfortunately, we have not yet identified a CTG repeat in this region by Southern blotting. Therefore, it is possible that the CTG repeat in the GIF/MT-III gene is species-specific. Indeed, only 200-base pair region from the cap site showed high similarity between the mouse and human genes (6). It is unclear whether the CTG repeat contributes to brain-specific transcription of the GIF/MT-III gene, since suitable cell lines for the brain are not available now. Transfection experiments using primary culture and/or in vitro transcription assay may dissolve this question in the near future.

Amplification of CTG or CAG repeats is known in several inherited neurodegenerative diseases including Huntington's disease, spinal and bulbar muscular atrophy, spinocerebellar ataxia, dentatorubral-pallidoluysian atrophy, and myotonic dystrophy (20, 21). In the first four diseases, CAG repeats in the N-terminal portions of their related genes encode polyglutamine residues, and the expansion of a glutamine-rich segment is related to the diseases. Although there is no evidence that an expanded CAG repeat has negative activity on tran-
Transcriptional Repression Activity of CTG Triplet Repeat

Figure 4. Effect of CTG repeat on transcriptional activity of various gene promoters. A. effect of the CTG repeat on various promoters, mgIF, C/EBPβ, SV40, glutathione-S-transferase (GST) P, and human metallothionein IIα (hMT-IIα), in which the CTG repeat was joined to the upstream region of the promoter. B. effect of the CTG repeat on various promoters, mgIF, C/EBPβ, and SV40, in which the CTG repeat was joined to the downstream region of the promoter. Values are represented as relative luciferase activity compared with each CTG-less construct.

transcription, except that the presence of the polyglutamine tract is inhibitory to trans-activation on the androgen receptor gene (22), it is possible that the CAG repeat functions as a silencer under normal conditions. In the case of the myotonic dystrophy gene, in which the CTG repeat is found in the 3′-untranslated region, decreased expression is observed (23). However, an opposite effect has also been reported (24). In Fragile X syndrome, hypermethylation of the CGG triplet repeat in the 5′-untranslated region represses the expression of the Fragile X mental retardation-1 gene (25). Gtf/Mt-III is the first example of a CTG triplet repeat located in the 5′ promoter region, and that has repression activity.

We have tried to detect whether nuclear proteins bind to the CTG repeat. However, we could not observe binding, although the conditions used here could detect other transcription factors. These results strongly suggest that a specific binding protein is not necessary to this function. First, we thought that a conformational change caused by the CTG triplet repeat might cause an inhibition effect on protein-protein interaction between the promoter and basal machinery. However, using the method for the detection of DNA bending by native electrophoresis (26) in the absence of the protein, we failed to detect the conformational change. Moreover, even when the CTG repeat was in the downstream region of the luciferase gene, that is, very far from the promoter, the negative effect was also observed. Therefore, we prefer to consider the following mechanism; while a specific DNA-binding protein would not bind to the CTG repeat, the CTG repeat itself could interact with some proteins in the complexes of basal and specific transcription factors with weak affinity. If this is the case, it is also possible that the CTG repeat may function as an activator. So far as we tested, we observed only a negative effect. We also failed to detect CTG repeat binding activity in the brain nuclear extract, although we could detect the binding activity in other regions in the promoter. Therefore, it seems that the CTG repeat functions as a general silencer which is active in all organs including the brain, and some activator proteins overcome the silencer function in the brain. Further studies are required to clarify the effect of the CTG triplet repeat on gene expression.

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