Identification of Glucocorticoid Response Element of the Rat TRH gene

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Objectives: It was suggested that glucocorticoid exerts cell-specific effects on thyrotropin-releasing hormone (TRH) gene expression at the transcriptional level. Althought there is no typical palindromic glucocorticoid response element (GRE) on the rat TRH gene promoter, a perfect GRE half-site is found between -205 bp and -211 bp. We investigated whether the segment between -242 bp and -200 bp of the rat TRH gene promoter is responsible for glucocorticoid response.

Methods: For the 5' deletion study of the TRH gene, four differnt plasmid constructs, pTRH($-554/84$), pTRH($-242/84$), pTRH($-200/6$), pTRH($-113/84$) were used for transient transfection study. The plasmid pMAMneo-LUC was used as a positive control and pRSV-GR expression vector, as a co-transfection study. Transfection was performed by the modified calcium precipitation method with 2 μg of each lasmid on the HeLa cells.

Results: Dexamethasone (DEX) stimulated the transcriptional activity of pTRH($-554/84$)-LUC and pTRH($-242/84$)-LUC by approximately 3.2 fold at 10^{-8} M and 5.4 fold at 10^{-6} M. On the contrary, deletion of the region between -242 to -200 bp reduced the basal transcriptional activity by 90% and also completely abolished the DEX-induced transcriptional activation of the luciferase gene. The DEX-induced transcriptional activation of pTRH($-242/84$)-LUC was in dose-dependent manner. While the co-transfection of glucocorticoid receptor expression vector (pRSV-GR) did not increase the basal transcriptional activity of pMAMneo-LUC, it increased the basal transcription of pTRH($-242/84$)-LUC by 1.8 fold. The pRSV-GR co-transfection and DEX tratment further increased the transcription of pTRH($-242/84$)-LUC by 2-4 fold at the concentration of 10^{-8} M.

Conclusion: These findings suggest that a cis-acting element(s) which is important for the basal transcriptional activation and glucocorticoid response of the rat TRH gene is located between -242 bp and -200 bp. The gene has a weak GRE glucocorticoid response and seems to be mediated by an interaction between glucocorticoid receptor and other transcriptional factor (s).

Key Words: TRH promoter, GRE, Transcription factors, HeLa cells, Transfection.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a tripeptide that was originally characterized in extracts of ovine and porcine hypothalamus[1]. Much of the hypothalamic TRH is localized within the pavocellular subdivision of the paraventricular nucleus. Cells in this region project to median eminence to regulate anterior pituitary thyrotropin secretion. TRH is also present in many extrahypothalamic loci and plays roles as a neurotransmitter and/or a neuromodulator in the central nervous system and in the gastrointestinal tract[2~4]. In 1986, Lechan et al. isolated a nearly full length TRH cDNA from a rat hypothalamic bacteriophage lgt11 expression library[5] which allowed investgations of the regulation of TRH mRNA levels in intact animals.

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Evidence for a direct effect of glucocorticoids on the TRH :gene expression in the paraventricular nucleus (PVN) of the hypothalamus include the presence of glucocorticoid receptors in the PVN[6] and the decreased TRH gene expression by glucocorticoid treatment in the rat PVN shown by in situ hybridization of Lechan and co-workers[7]. In contrast to the rat TRH gene expression in the PVN, glucocorticoid treatment stimulated the expression of extrahypothalamic rat TRH gene. Glucocorticoid treatment increasd the TRH mRNA in the murine neoplastic medullary thyroid carcinoma cell line (CA77)[8] and the fetal diencephalic neuron[9~10]. It also increased a parallel rise of TRH, pre-pro-TRH -(25-50) and pro-TRH mRNA in the somatotrophs of long-term cultured rat anterior pituitary gland[11], indicating that the effect probably did not occur at the post-translational level. Furthermore, Rondeel et al. suggested that dexamethasone (DEX) did not alter pro-TRH mRNA stability in the rat anterior pituitary gland[12]. Therefore, one might speculate that glucocorticoid regulates the TRH gene expression at the transcriptional level.

The mechanism by which glucocorticoid stimulates gene transcription has been studied in detail. In the absence of hormone, glucocorticoid receptor (GR) is located in the cytoplasmic space in association with a protein called heat-shock protein 90 (hsp90). When the concentration of glucocorticoid in the extracellular medium is increased, the hormone moves across the plasma membrane into the cytoplasmic space where it bidns to the GR and induces dissociation of the GR-hsp90 complex and dimerization of the GR. The resulting complex of hormone and GR moves into the nucleus through nuclear pores. Within the nucleus, the glucocorticoid-GR complex binds to the glucocorticoid response element (GRE) on a target gene[13,14]. The consensus sequence of the GRE has been revealed to be located within a few hundred base pairs of the transcription start site of hormone responsive genes, and has a palindromic structure known as GAACAnnnTGTTC[15].

The discrepancy between glucocorticoid-induced regulation of the TRH gene expression in the PVN and extrahypothalamic sites suggests that the diversity of response might be based on the interaction betwen a cell-specific transcription factor(s) and GRE. However, the GRE of the rat TRH gene has not been defined, to date. As the first step

to understand the molecualr: mechanism for the glucocorticoid regulation of the TRH gene expression, we tried to identity the GRE on the promoter of the rat TRH gene.

MATERIALS AND METHODS

1. Materials

HeLa cell was purchased from American Type Culture Collection. Dulbeco's modified Eagles Medium (DMEM), fetal calf serum (FCS), penicillin and streptomycin and trypsinwere obtained from Gibco-BRL. Dexamethasone, O-nitrophenyl-D-galactopyranoside (ONPG), adenosine triphosphate (ATP) and D-luciferin were obtained from Sigma. Calf intestinal alkaline phosphatase, T4 kinase, T4 ligase, Klenow fragments and all restriction enzymes were purchased from Promega. Reagents for syntheitic oligonucleotides were supplied by Applied Biosystem. All the other chemicals were purchased from Promega and BRL.

2. Plasmids

For the 5′ deletion study of the TRH gene, six different plasmid constructs, pTRH(−554/84)-LUC, pTRH(−242/84)-LUC, pTRH(−200/6)-LUC, pTRH(−113/84)-LUC, pTRH(−77/84)-LUC and pTRH(−47/84)-LUC, were used for transient gene expression. Each plasmid was constructed by subcloning the serially truncated 5′ upstream regulatory region of the rat TRH gene at the position of −554, −242, −200, −113, −77, −47 into the promoterless luciferase expression vector, pA3LUC[16,17], which was generously provided by Dr. W. Wood. All of the plasmid construct were verified by sequencing.

The plasmid pMAMneo-LUC was used as a positive control plasmid for GRE. The plasmid contains the mouse mammary tumor virus promoter, including multiple copies of GRE[18], which was kindly provided by Dr. B. Sandboun.

The plasmid pRSV-GR, provided by Dr. K. Yamamoto, was a plasmid vector that highly expresses GR. It includes a full length GR cDNA which was subcloned downstream to the promiscous Rous Scarcoma Virus (RSV) promoter[19].

3. Transfection

Monolayer culture of HeLa cells was maintained in DMEM supplemented with 10% fetal calf serum

and penicillin–streptomycin at 37℃ under 5% CO2. HeLa cells were cultured in 100 mm dishes for 1–2 dyas until 60% confluent before transfection. Transfection was performed by the calcium phosphate precipitation method[20] with 2 μg of pMAM-LUC (MMTV-LUC) as a positive control or 2 μg of each of the pTRH-LUC vectors described above, 2 μg of the GR expression vector (pRSV

5′-FLANKING SEQUENCE OF THE RAT TRH PROMOTER

-244   -210 -205
ctcgctcagcccccacttgttccctagtcaga*tgttctc*ttagtcaacagacctccggagctgggcgccaacctgcgccagccgtgcctcctc

             -113   -94
gctcagcccccaccagggtttccggaaagcgggcgggtcccccggctctgccgtcagcacccctgtcttcggcctgcgccccctccccg

 -52   +1
ctgacctca caggcgccgcgtctccagctcatataacctcagcccctcctcggaggagcGCGCAGTCGACCCTG–
                                     –>

Fig. 1. Candidate GRE site in the rat TRH promoter. Numbers above the sequences depict the position relative to the transcription start site. The candidate half-site for GRE is shown in bold type letters.
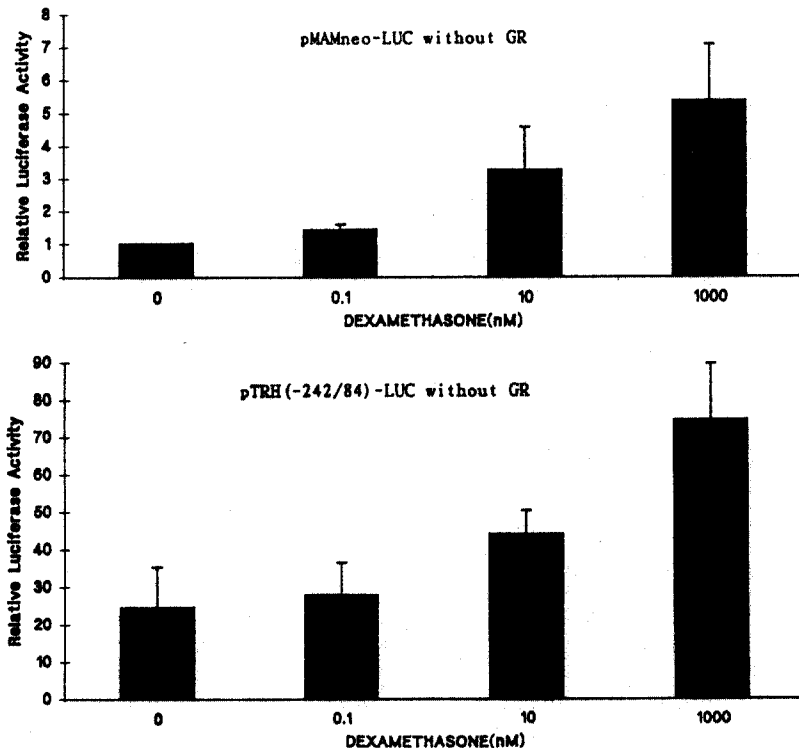

Fig. 2. Dexamethasone increased luciferase activity of the pTRH(−242/84)-LUC and pMAMneo-LUC in dose-dependant manner without pRSV-GR co-transfection in transient transfection assays. Relative luciferase activity shown represents the multiplies of the pMAMneo-IUC without dexamethasone as a control, and mean of the duplicate transfection obtained in 6 separate experiments.
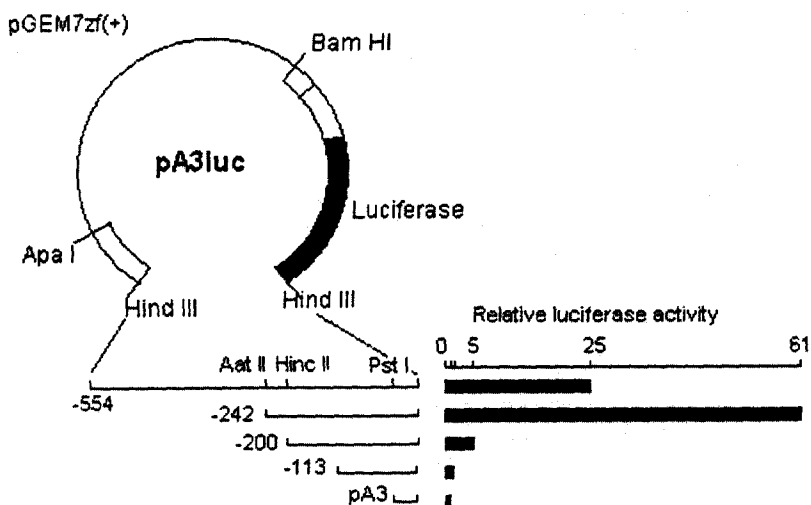
Fig. 3. Structure of pA3LUC and TRH 5'-region constructs and the basal transcriptional activity of the various 5' upstream regions of the rat TRH gene. The transcriptional activity is expressed as the luciferase activity relative to that of pA3LUC as a negative control.
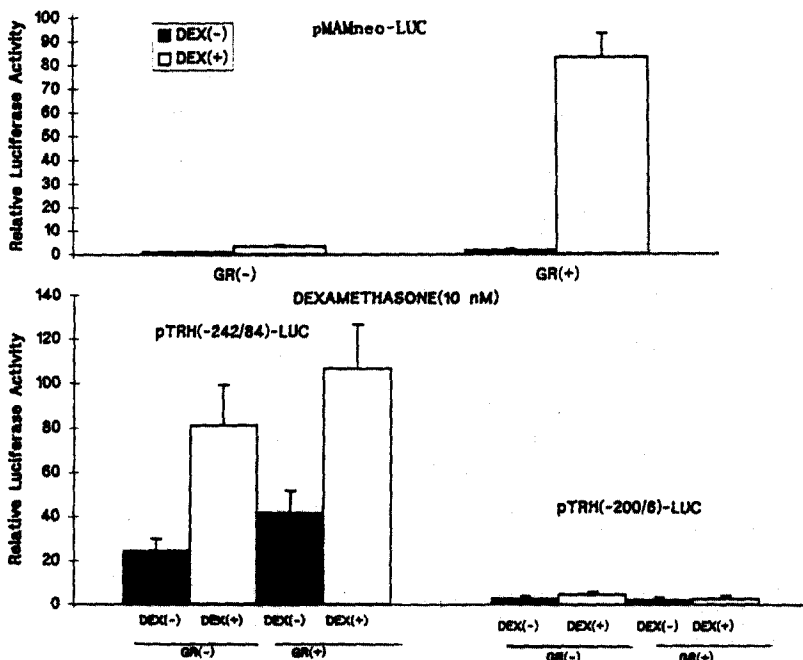

Fig. 4. Effect of dexamethasone and GR co-transfection under transcription activity of the pTRH(−242/84)-LUC and pMAMneo-LUC. 5'-deletion of the rat TRH promoter to −200 bp abolished dexamethasone stimulation of pTRH-LUC. Data represent the mean±SEM calculated from duplicated values obtained in 6 separate experiments. Results are normalized to the luciferase activity of pMAMneo-LUC without pRSV-GR co-transfection and dexamethasone stimulation as a control.

-GR) and 1 μg of b-galactosidase gene. Fourhovrs after transfection, the cells were washed once with phosphate buffered saline (PBS) and treated with 15% glycerol for 1 min. Afterwards, cells were washed twice with PBS and briefly treated with trypsin before replating in 65 mm dishes. After 24 hrs of incubation, the medium was replaced with fresh medium varing concentrations of dexamethasons (DEX) and the cells were cultured for another 24 hrs. The cellular extracts were subjected to the luciferase assay and β-galactosidase assay for control of transfection efficiency.

4. Statistical Analysis

Data were expressed as mean±standard error from the results of four separate experiments. Statistical analysis was performed by paried t-tests.

RESULT

A single perfect GRE half-site, TGTTCT, was found between 205 and 211 bp upstream of the transcription start site of the rat TRH gene[21] (Fig. 1). To confirm whether the GRE half-site confers the glucocorticoid responsiveness, we examined the transcriptional stimulation of the luciferase gene by the various 5' upstream regions of TRH gene.

The basal transcription of pMAMneo-LUC, a positive control for GRE, was only about 3.2 fold stimulated by DEX at the physiologic concentration of 10^{-8} M and 5.4 fold at 10^{-6} M (Fig. 2). However, co-transfection of GR expression vector (pRSV-GR) enhanced the DEX-stimulated transcriptional activity of pMAMneo-LUC by approximately 83 fold (Fig. 4).

DEX also stimulated the transcriptional activity of pTRH(−554/84)-LUC and pTRH(−242/84)-LUC by apprximately 1.8 fold (p<0.01) at 10^{-8} M and 3.1 fold (p<0.01) at 10^{-6} M. On the contrary, deletion of the region between −242 to −200 bp reduced the basal transcriptional activity by 90% and also completely abolished the DEX-induced transcriptional activation of the luciferase gene (Fig. 3, 4). The DEX-induced transcriptional activation of pTRH(−242/84)-LUC was in dose-dependent manner (Fig. 2). These findings suggest that a cis-acting element(s) which is important for the basal transcriptional activation and glucocorticoid response of the rat TRH gene is located between −242 bp and −200 bp.

While the co-transfection fo pRSV-GR did not increase the basal transcriptional activity of pMAMneo-LUC, it increased the basal transcription of pTRH(−242/84)-LUC by 1.8 fold (p<0.05). The pRSV-GR cotransfection and DEX treatment further increased the transcription of pTRH(−242/84)-LUC by 2-4 fold at the concentration of 10^{-8}M.

DISCUSSION

It was suggested that glucocorticoid exerts cell-specific effects on TRH gene expression at the transcriptional level[12].

Although there is no typical palindromic GRE on the rat TRH gene promoter, a perfect GRE half-site was found between −205 and −210 bp. Our result shows that deletion of the segment between −242 and −200 bp of the rat TRH promoter abolishes DEX-induced transcriptional activation as well as the basal transcriptional activation. DEX increased the transcriptional activation of pTRH(−242/84)-LUC in dose-dependent manner. It suggests that the segment contains on important element(s) for the glucocorticoid response as well as basal transcriptional activation. In fact, Stevenin et al. recently demonstrated that the DNA binding domain of GR binds tightly to the region between −220 and −200 bp of the rat TRH promoter by using DNase I footprinting[22], which supprts the present result.

Although the transcription of pMAMneo-LUC as a GRE positive control vector was supposed to be stimulated more than 10 fold by DEX, it was, in fact, stimulated only about 5.3 fold by DEX without GR co-transfection in the present study. GR co-transfection, however, dramatically increased the response by 83 fold. It suggests that HeLa cell is deficient of GR. Thus, the possibility can not be excluded that the DEX-induced transcriptional activation of pMAMneo-LUC was mediated by other transcriptional factor(s) induced by DEX. Without GR co-transfection, the transcription of pTRH(−242/84)-LUC was also stimulated to a similar degree by DEX, as was pMAMneo-LUC. Accordingly, the DEX-induced transcriptional activation of pTRH(−242/84)-LUC also could be mediated by other DEX-inducible transcription factor(s).

The basal transcription of pTRH(−242/84)-LUC was approximately 1.8 fold increased by co-transfection of GR, wherease that of pMAMneo-LUC was

not stimulated at all. It suggest that the increased basal transcriptional activity may result from an interaction between GR and other transcription factors that are involved in the basal and hormonal regulation of transcription. In fact, earlier reports demonstrated that there are elements for the binding of cAMP response element binding protein (CREB), c-Jun and thyroid hormone receptor between −47 and −113 bp[23,24,25]. HeLa cell is known to express CREB and c-Jun/c-Fos[26]. Thus, it is possible that GR may interact with other transcription factor(s) including CREB and c-Jun/c -Fos, and which, in turn, enhance basal transcriptional activity.

With DEX stimulation, the increased basal transcriptional activity was further enhanced by 2.6 fold. The magnitude was remarkably smaller than that of pMAMneo-LUC and similar to that without DEX stimulation. It suggests two possibilities. One is that the GRE of the rat TRH gene is much weaker than that of pMAMneo-LUC. As pMAMneo-LUC has multiple copies of GRE, it is known to show very strong response to glucocorticoid. On the contrary, there is only a half-site of GRE on the TRH gene. This could make the difference in the degree of the glucocorticoid response.

The other possibility is that the glucocorticoid effect on the transcription is mediated through the activation of other transcriptional factor(s). Synergistic effect of glucocorticoid and activators of the protein kinase A pathway on the enkephalin gene transcription have been previosly demonstrated[27]. The potentiating effects of two regulators of transcriptional activity may derive from multiple mechanisms, most likely involving the action and/or interaction of transcriptional modulators of the proto-oncogene families[28]. TRH and c-Jun/c-Fos are co-expressed in cultured rat anterior pituitary cells and their expressions are enhanced by DEX in a similar fashion at the transcriptional level[29,30]. Also, the TRH genes are expressed in somatotroph of anterior pituitary gland[31]. Jackson et al. proposed three possible mechanism. Firstly, DEX stimulates TRH gene expression by enhancing c-fos/c -Jun gene expression. Secondly, DEX initially enhances the expression of c-Fos/c-Jun which induces other transacting factors, and which, in turn, increase TRH gene expression. Finally, the gene expressions of TRH and c-fos/c-Jun are stimulated separately by DEX and then c-Fos/c-Jun

is responsible for prolonging the expression of the TRH gene rather than initiation TRH gene expression. We, therefore, hypothesize that the region between −242 and −200 bp of TRH promoter is important for potentiation of transcriptional activity in association with other proto-oncogene families. In conclusion, the sequences between −242 bp and −200 bp of the rat TRH gene promoter contain a weak glucocorticoid response element. The glucocorticoid response seems to be mediated by an interaction between glucocrticoid receptor and other transcriptional factor(s).

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