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CCAAT-binding Transcription Factor(CTF) Proteins Required for the Transcriptional Stimulation by Thyroid Hormone in GH_4C_1 Cells

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= Abstract =

CAAT-binding transcription factor(CTF) proteins are implicated in the expression of multiple forms and are probably identical to nuclear facfor-1(NF-1). In herpes simplex virus thymidine kinase gene, CTF is required not only to induce basal transcription of this gene, but also to stimulate the transcription of this gene by thyroid hormone. Gel mobility shift assays and biotin-avidin protein-DNA binding assays were performed to investigate if the synthesis of transcription factors which bind to "CCAAT" sequences of the TK promoter(CTF or CTF-like factors) is stimulated by T_3 and to kdentify the CCAAT-binding proteins in GH_4C_1 cells having endogenous thyroid hormone receptors. There are two different sized(31 and 33 kDa) CCAAT-binding proteins in GH_4C_1 cells having endogenous thyroid hormone receptors. The synthesis of these CTF-binding proteins were not affected by T_3.

Introduction

Viral infection of mammalian cells entails the regulated induction of viral gene expression. The induction of many viral genes, including the herpes simplex virus(HSV) gene encoding thymidine kinase(TK), depends on cellular regulatory proteins that act in *trans*. Because recognition of the TK promoter by cellular transcription factors is well understood, its *trans* induction by regulatory proteins may serve as a useful model for the regulation of eukaryotic gene expression. Jones et al[1] characterized an *in vitro* transcription system derived from uninfected HeLa cells that accurately initiates RNA synthesis at the TK promoter. Analysis of linker-scanning, single-site, and promoter-inversion mutants reveals that the TK upstream elements previously mapped *in vivo* are accurately recognized *in vitro*[2][3]. A protein fraction obtained from uninfected HeLa cells required for TK transcription in reconstitution experiments was found to contain multiple protein species that bind specifically to the TK promoter. DNase I footprint experiments with wild type and mutant promoters revealed that the TK upstream elements contain three distinctive protein binding sites, two of which("GGGCGG" ; −55/−50 in sense strand and −98/−103 in antisense strand) appear to be recognized by the Sp1 transcription factor and one(−82/−86 in antisense strand) interacts with a cellular protein(s), CTF/NF-1 family of factors, that binds to "CCAAT" sequences(Fig. 1). Optimal expression of the TK gene appears to require the coordinate interaction of these two types of transcriptional factors(Sp1 and CTF/NF-1) with the three upstream elements of the promoter(−55/−50, −98/−103, and −82/−86).

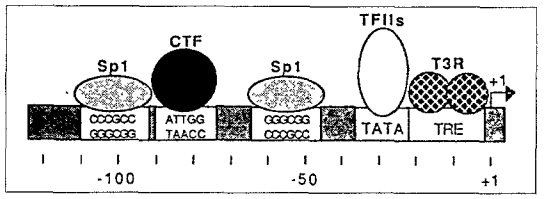

Fig. 1. Schematic illustration of the functional binding sites for nuclear proteins in the TK promoter. Sp1, Sp1 transcription factor ; CTF, CCAAT-binding transcription factor ; TFIIs, transcription factors for RNA polymerase II ; T_3R, thyroid hormone receptors ; TATA, TATA box ; TRE, thyroid hormone response element.

We previously found that the TK gene promoter contains a novel element that can be functionally activated by thyroid hormone(T_3)[4]. T_3 stimulation of the TK gene requires both thyroid hormone responsive element and an CTF site[4]. There are two possibilities to explain these findings. One possibility is that T_3 might increase the synthesis of CTF or other CCAAT box binding factors and these factors would bind to the CCAAT box and stimulate transcription. The other is that a direct protein-protein interaction between the T_3 receptor and a transcription factor could stimulate transcription. CTF proteins implicated in the expression of a variety of genes are composed of multiple forms and are propably identical to nuclear factor-1(NF-1)[5,6].

The present study was undertaken to investigate if the synthesis of those transcription factors which bind to "CCAAT" sequences of the TK promoter(CTF or a CTF-like factor) is stimulated by T_3 and to identify the CCAAT-binding proteins in GH_4C_1 cells having endogenous thyroid hormone receptors. For this purpose, gel mobility shift assays and biotin-avidin protein-DNA binding assays were performed.

Materials and Methods

1. Cell culture

GH_4C_1, HeLa, and COS1 cells were grown in Dulbecco's Modified Eagle's Medium(Gibco) containing 15mM HEPES, pH 7.5, 0.1mg/ml pyruvate, 50μg/ml penicillin-G(DHAP medium), and either 5%(v/v) fetal bovine serum(Gibco) for GH_4C_1 and HeLa cells or 10%(v/v) calf serum for COS1 cells.

2. Oligonucleotides

A CCAAT box motif(CTTGTCATTGGCGAATTCGAACA ; CTF-23) for CTF or CTF-like transcription factor binding was derived from the native TK promoter spanning bases −92 to −72.

3. Preparation of nuclear extracts

Nuclear extracts were prepared according to the method of both Digman et al[7] and Ye and Samuels[8] with modifications. In brief, cells were pelleted by centrifugation, washed in phosphate-buffered saline, resuspended in STM-Triton buffer(20mM Tris-HCl, pH 7.8, 1.1mM $MgCl_2$, 250mM sucrose, and 0.2% Triton X-100) and homogenized. Nuclei were pelleted and extracted with buffer containing 400mM KCl in GTME buffer(15% glycerol, 25mM Tris-HCl, pH 7.8, 0.05% Triton X-100, 10mM 2-mercaptoethanol, 0.5mM EDTA, and 0.5% phenylmethylsulfonyl fluoride). Insoluble debris was removed by centrifugation, and the supernatant was dialyzed against 100mM KCl in GTME buffer. The nuclear extracts were stored in small aliquots at −80℃, and freshly thawed material was used in each experiment.

4. Whole cell labeling with L-^{35}S-methionine

At the time of labeling, cells were washed once with L-methionine- and L-leucine-free(met^-leu^-) Ham's F-10 medium(Gibco) and then incubated for 25min at 37℃ in the same medium. The cells were labeled by incubation for 2 h at 37℃ with Ham's F-10(met^-leu^-) supplemented with 10%(v/v) calf serum, 13μg/ml L-leucine, and 0.2μM L-^{35}S-methionine(New England Nuclear ; 700−1000Ci/mmol). The cells were chilled to 4℃ and washed three times with saline. Nuclear extracts were prepared as described on the above. The extracts were stored frozen at −80℃ until use. Trichloroacetic acid precipitate counts per min present in the nuclear extracts were measured.

5. Gel mobility shift assays

^{32}P-labeled oligonucleotides(30,000cpm) were incubated with 10μg of nuclear extracts in the presence of 10μg poly(dIdC) in a final volume of 25μl. Incubations were carried out at room temparature for 30min. All binding reactions contained 25mM Tris-HCl, pH 7.8, 0.5mM EDTA, 100 ng aprotinin, 3μg

ovalbumin, 10mM 2-mercaptoethanol, 0.05% Triton X-100(v/v), 10% glycerol(v/v), and 75mM KCl. The samples were chilled on ice and then loaded onto a 5% nondenaturing polyacrylamide gel in low ionic strength buffer(10mM Tris base, 7.5mM acetic acid, 0.04mM EDTA, pH 7.8). The electrophoresis was performed at 4℃ at voltage gradient of about 15 V/cm with rapid buffer circulation. The gel was dried and autoradiographed at −80℃ for 12−24 h using Kodak X-Omat film.

6. Biotin-avidin protein-DNA binding assays

For the biotin-avidin assay, end-labeled biotinylated oligonucleotides were generated by filling in the ends with the Klenow fragment of E. Coli DNA polymerase. The reaction mix contained, per nmol DNA, 10 nmol bio-11-dUTP(Sigma), 50 nmol each of dATP, dCTP, and dGTP. After biotinylation, unincorporated nucleotides were removed by chromatography on DEAE-cellulose. The biotinylated oligonucleotides were quantitated using a Hoefer TKO Mini-fluorometer(Hoefer), following the directions of the manufacturer. Biotin-avidin protein-DNA binding assay was performed as follows. ^{35}S-labeled nuclear proteins(5×10^5cpm) were incubated with 5 pmol biotinylated oligonucleotide in a 150-μl volume containing 16μg poly(dIdC)(Pharmacia) and 75mM KCl in GTME buffer. After a 30min incubation at 25℃, the samples were incubated on ice for 10min. Protein-biotinylated DNA complexes were isolated by inculation with 20μl of a 50%(v/v) solution of streptavidin-agarose(Bethesda Research Laboratories) at 4℃ for 10min. Pellets were washed three times with GTME buffer-75mM KCl and boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) sample buffer, and the supernatants were run in 10% SDS gels with ^{14}C-labeled molecular mass standards(Sigma). Labeled proteins were visualized using fluorography with EN^{3}HANCE(New England Nuclear) and exposure at −80℃ using Kodak X-Omat film.

Results

Three different cell lines used in this study were GH_4C_1 cells having endogenous T_3 recpetors and HeLa and COS1 cells having no endogenous T_3 receptors. To study if the synthesis of CTF transcription factors which bind to the TK promoter is stimulated by T_3, gel mobility shift assays were performed using a ^{32}P-CTF binding motif(CCAAT box ; CTF-23). The intensity of the specific bands of CTF-like transcription factors from GH_4C_1 nuclear extracts cultured in either hormone-depleted(CONT) or T_3-supplimented(T_3) media were the same(Fig. 2). CTF or CTF-like factors from HeLa and COS1 nuclear extracts are also shown in Fig. 2, and they migrated to a similar position as the upper specific band from GH_4C_1 nuclear extracts. Even though the mobility of the protein-CCAAT box complex from the three different

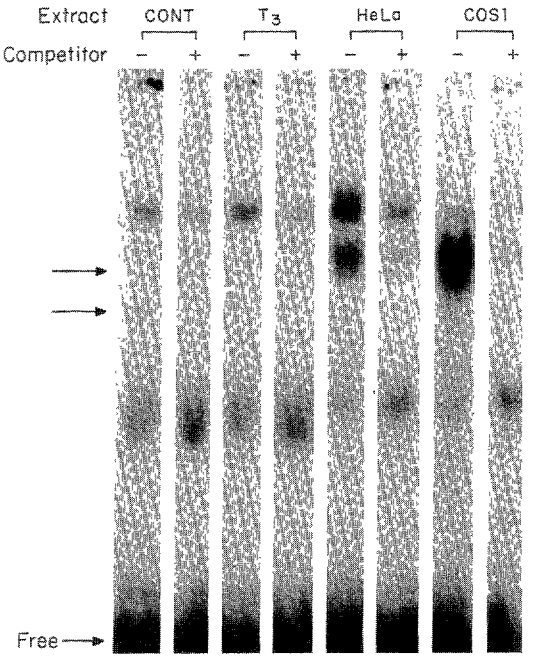

Fig. 2. Binding of nuclear proteins to the CTF-binding motif.
A gel mobility shift assay was performed as described in Materials and Methods. All reactions contained 30,000cpm(4.5 fmol) of ^{32}P-labeled CTF-23 oligonucleotide. The specific complexes are indicated by arrows. Free oligonucleotide is seen near the bottom of the gel. CONT, GH_4C_1 nuclear extract prepared from cells cultured in hormone-depleted media doe 48 h before extraction ; T_3, GH_4C_1 neclear extract prepared from cells cultured in 5nM T_3-supplimented media for 48 h before extraction ; −, No competitor ; +, Addition of a 100-fold molar excess of unlabeled CTF-23 oligonucleotide.

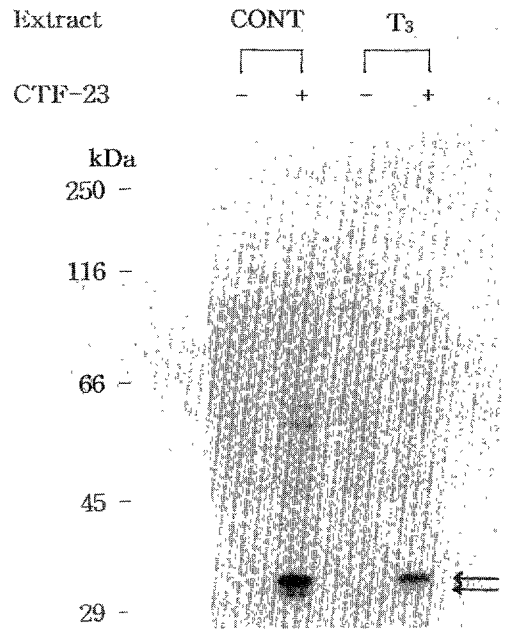

Fig. 3. CCAAT box binding proteins in GH_4C_1 cells.
A biotin-avidin protein-DNA binding assays was performed as described in Materials and Methods. ^{35}S-labeled GH_4C_1 nuclear proteins incubated with no DNA or with the CTF-23 oligonucleotide and separated in a SDS-polyacrylamide gel, and fluorographed. Molecular mass standards are shown on the left. CONT, GH_4C_1 nuclear extract prepared from cells cultured in hormone-depleted media doe 48 h before extraction ; T_3, GH_4C_1 neclear extract prepared from cells cultured in 5nM T_3-supplimented media for 48 h before extraction ; -, No DNA ; +, CTF-23 oligonucleotide.

cell lines are similar, it is not clear that they are the same protein or different members of a protein family because of the multiplicity of CCAAT box binding proteins[5,6]. To confirm the result from the gel mobility shift assays and to identify the size of CCAAT-box binding protein(s) present in GH_4C_1 cells, biotin-avidin protein-DNA binding assay was performed. A biotin-avidin protein-DNA binding assay was used in which ^{35}S-methionine-labeled nuclear proteins were incubated with an excess of biotinylated CTF-23. This technique identified highly labeled proteins, extracted from the nucleus in a high salt buffer, which bound to the CTF-23 without additional promoter elements. Fig. 3 shows that two nuclear proteins(31 and 33 kDa) were bound to CTF-23 and the intensity of the two proteins from GH_4C_1 nuclear extracts cultured in either hormone-deplited(CONT) or T_3-supplimented (T_3) media were the same(Fig. 3). Thus, it appears that synthesis of the two CTF-binding proteins(31 and 33 kDa) are not affected by T_3.

Discussion

The herpes simplex virus TK promoter has been extensively analyzed as a model for gene regulation by transcriptional regulators. The functional importance of the binding motifs for CTF/NF-1 are generally found in mammalian cells with multiple forms, the TK promoter has been used as a heterologous promoter to analyze the functional activity of a variety of cis-acting elements, including hormone receptor response elements[9,10]. The findings that both thyroid hormone response element and an CTF site are required for hormone stimulation and that thyroid hormone receptor are reminiscent of the findings that glucocorticoid hormone stimulation of the MTV-long terminal repeat requires both glucocorticoid response element and an NF-1 site[11]. There are two different sized(31 and 33 kDa) CCAAT-binding proteins in GH $_4C_1$ cells having endogenous thyroid hormone receptors(Fig. 3). Since the synthesis of transcription factors bound to the CCAAT box motif in GH_4C_1 cells was not increased by T_3 incubation(Fig. 2, 3), it is possible that direct protein-protein interactions between a transcription factor and the receptor is involved in stimulation of transcription. Another possibilities reported by Martinez et al[13] was that synergistic transcriptional activation by CTF/NF-1 and the estrogen receptor was proposed to result from the enhanced binding of a common target transcription factor by the activation domains of estrogen receptor and CTF/NF-1, rather than by a direct interaction by the two proteins. Further studies are required to identify the mechanisms involved in the synergistic activation of transcription by two activators, the thyroid hormone receptor and CTF/NF-1 family of proteins factor.

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GH_4C_1 세포주에서 갑상선 호르몬에 의한 전사 촉진에 필요한 CCAAT-결합 전사인자(CTF) 단백질에 관한 연구

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CTF 단백질은 유전자의 CCAAT 염기서열 구조에 결합하는 전사 단백질로, 여러 유전자에 광범위하게 존재하며, 하나의 단백질이 아니라 세포주에 따라 여러가지 서로 다른 단백질들로 구성되고, NF-1과도 동일한 단백질로 인식된다. 단순 포진 바이러스의 thymidine kinase 유전자에서 CTF는 이 유전자 전사의 기초 유도에 필요하며, 또한 갑상선 호르몬이 이 유전자의 전사를 촉진시키는 데에도 갑상선 호르몬 수용체 뿐 아니라 이 CTF 단백질이 필요하다. 세포 내에 내인성 갑상선 호르몬 수용체가 존재하는 GH_4C_1 세포주에서 유전자의 CCAAT 구조에 결합하는 CTF 단백질의 크기와 갑상선 호르몬에 의한 합성 증가 여부를 조사하기 위하여 biotin-avidin protein-DNA 결합 방법과 gel mobility shift 방법을 사용하여 실험하였다. GH_4C_1 세포주에 존재하는 CTF 단백질은 그 크기가 31과 33 kDa인 두 개의 단백질로 구성되며, 이 두 단백질의 합성은 갑상선 호르몬에 의하여 증가되지는 않는다.