cis-Acting Elements and trans-Acting Proteins in the Transcriptional Inhibition of Gonadotropin-releasing Hormone Gene by Human Chorionic Gonadotropin in Immortalized Hypothalamic GT1–7 Neurons*

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We investigated the cis-acting elements and trans-acting proteins required for the transcriptional inhibition of the gonadotropin-releasing hormone (GnRH) gene by human chorionic gonadotropin (hCG) in GT1–7 neurons. Transient transfection of GT1–7 neurons with the 5′-flanking region of the rat GnRH gene-luciferase fusion constructs revealed that a 53-base pair (bp) sequence between −126 and −73 bp is required for the hCG inhibition. Nuclear extracts from GT1–7 neurons contained 110- and 95-kDa proteins that formed two complexes with the 53-bp sequence. These proteins are not related to Fos, cAMP response element-binding protein, Oct-1, or progesterone receptors, and hCG treatment selectively increased the 95-kDa protein. DNase I footprinting with GT1–7 cell nuclear extracts protected the −99 to −79-bp region, which contained a so-called imperfect AP-1 site (−99 to −94 bp) and two AT-rich palindromic sequences (−91 to −97 bp and −85 to −81 bp). The mutagenesis of the AT-rich regions, but not the AP-1 site, resulted in a loss of DNA binding of the 95-kDa protein and the inhibitory effect of hCG. In summary, our results are consistent with hCG inducing a 95-kDa trans-acting protein, which binds to −91- to −81-bp AT-rich sequences in the 5′-flanking region to inhibit the transcription of the GnRH gene.

The hypothalamic decapeptide, gonadotropin releasing hormone (GnRH), plays a central role in reproduction by controlling the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone from the anterior pituitary (1, 2). The hypothalamic decapeptide, gonadotropin releasing hormone, plays a central role in reproduction by controlling the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone from the anterior pituitary (1, 2). The synthesis and release of GnRH is controlled by numerous agents (3, 4). The studies on the hypothalamic GnRH neurons are hampered by the fact that they are present in small numbers and harvesting them in quantities required for most studies is very difficult (5–7). The development of immortalized GnRH-containing GT1–7 neurons by targeted oncogenesis has allowed investigators to make rapid advances in understanding the regulatory mechanisms in the synthesis and release of GnRH (8, 9). One of these advances is that GT1–7 neurons have been shown to contain functional LH/human chorionic gonadotropin (hCG) receptors (10, 11), and these receptors are required for transcriptional inhibition of the GnRH gene by exogenous hCG in a dose- and time-dependent and hormone-specific manner (10). These findings supported the possible existence of a short loop feedback mechanism first proposed 30 years ago, in the synthesis and release of LH (12). The treatment of GT1–7 neurons with hCG under the conditions that it inhibits GnRH synthesis, activated protein kinase A, increased the synthesis of new proteins, increased the levels of phosphorylated CAMP response element-binding protein (CREB) and c-Fos and c-Jun proteins, and decreased the levels of GnRH receptors (13, 14). The present study focused on investigating the cis-acting elements and trans-acting proteins required for the transcriptional inhibition of the GnRH gene by hCG in GT1–7 neurons.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were purchased from the indicated commercial sources: the promoterless luciferase reporter vector pGL2 basic DNA, luciferase, chloramphenicol acetyltransferase (CAT), and β-galactosidase assay systems, Klenow enzyme, restriction enzymes and T4 DNA ligase from Promega (Madison, WI); [3H]chloramphenicol and [35S]dATP from DuPont NEN; Dulbecco’s modified Eagle’s medium, fetal calf serum, horse serum, antibiotic-antimycotic solution, Lipofectin reagent, and Opti-MEM I medium from Life Technologies, Inc.; Sequenase Version 2.0 DNA sequencing kit from U. S. Biochemical Corp.; VCS-M13 helper phage and pBluescript II KS+ vector from Stratagene Cloning Systems (La Jolla, CA); kits for preparing single and double strand plasmid DNA from QIAGEN Inc. (Chatsworth, CA); all reagents for synthesis of oligonucleotides, SureTrack footprinting and BandShift kits from Pharmacia Biotech Inc.; polyclonal antibodies to phosphorylated CREB, c-Fos, and c-Jun from Upstate Biotechnology, Inc. (Lake Placid, NY); polyclonal antibody to Oct-1 and consensus Oct-1 oligonucleotide from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); monoclonal antibody to progesterone receptor from Affinity Bioreagents (Neshanic Station, NJ). The following items were obtained as gifts: immortalized GT1–7 neurons from Dr. Pamela Mellon at the University of California (La Jolla, CA); pGEM7 plasmid containing the −3026 to +116 bp of rat GnRH promoter region from Dr. Margaret Wierman at the University of Colorado Health Sciences Center (Denver, CO); promoter of cytomegalovirus (pCMV)-Fos and pCMV-Jun expression plasmids from Dr. Tom Curran at Roche Institute of Molecular Biology (Nutley, NJ); 5′-12-O-tetradecanoyl phorbol-13-acetate response element (TRE) thymidine kinase (TK)-CAT reporter vector from Dr. Inder Verma and CREB cDNA from Dr. Marc Montminy, both at the Salk Institute for Biological Studies (La Jolla, CA); TK-CAT and 3-CAMP response element (CRE/TK-CAT reporter vectors from Dr. Patrick Quinn at the Pennsylvania State University College of Medi-
Cine (Hershey, PA); dut nug mutant strain Escherichia coli RZ 1032 from Dr. Mark Brennan and pCMV β-galactosidase expression plasmid from Thomas Geoghean of our institution; highly purified hCG (CR-127, 14,900 IU/mg) from the National Hormone and Pituitary Program, supported by NIDDK, NICHD, and USDA. The oligonucleotides used for site-directed mutagenesis were labeled with 32P by terminal transferase reaction on our laboratory's P-L 4000 T3K primer and Klenow enzyme. The labeled probes were purified by polyacrylamide gel electrophoresis (PAGE). The electrophoretic gel mobility shift assays were performed as described in the BandShift kit from Pharmacia. Five-μg aliquots of the nuclear extracts from untreated and hCG-treated GT1–7 neurons were incubated with binding mixture (10 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 1% glycerol, 0.05% Nonidet P-40, 5 mM MgCl2, 2 μg of poly(dI-dC), 0.1 mM EDTA, 0.5 ng of labeled probe (30,000 cpm/reaction)) for 20 min at room temperature. For competition studies, a 100-fold excess of unlabeled probe was added to the binding mixture. For the electrophoretic gel mobility supershift experiments, polyclonal antibodies to phosphorylated CREB, c-Fos, c-Jun, Oct-1, and monoclonal antibody to progesterone were added to the binding mixture and incubated for 30 min at 4 °C prior to adding the probes. After incubation, DNA-protein complexes were resolved by 4% native PAGE in buffer containing 7 mM Tris-Cl, pH 7.5, 3 mM sodium acetate, and 1 mM EDTA at 4 °C for 5 h. Gels were dried and exposed overnight at −80 °C to Kodak X-Omat film with intensifying screens.

Southwestern Blot—The proteins in 100-μg aliquots of nuclear extracts were separated by 8% discontinuous SDS-PAGE under reducing and nonreducing conditions, then electrotransferred onto Immobilon-P membranes. The proteins were renatured by placing the membranes in Z-buffer (25 mM HEPES-KOH, pH 7.6, 5 mM MgCl2, 0.1% glycerol, 0.1% Nonidet P-40, 0.1 mM KCI, 1 mM DTT, 0.1 mM ZnSO4, and 100 mM NaCl) containing 6 μM guanidine chloride. Non-specific binding sites were blocked by Z-buffer containing 5% non-fat dried milk (18). Finally, the membranes were incubated for 3 h at room temperature with binding buffer (Z-buffer plus 5 μg of poly(dI-dC), 30 μg of calf thymus DNA, and 1 × 10^6 cpn of 32P-labeled probe (−126 to −73 bp DNA fragment of the GnRH promoter, wild type, or mutated −126 to −116 bp DNA fragment of the GnRH promoter) per ml). In competition studies, a 100-fold excess of unlabeled probe was added to the binding buffer. After washing three times with Z-buffer, the membranes were exposed at −80 °C for 2 days to Kodak X-Omat film with intensifying screens.

DNase I Footprinting—The one end 32P-labeled DNA fragments as probes were prepared as follows. For the coding strand, the −1031 to +116 bp of the GnRH gene 5′-flanking region in pGL2 vector was digested with PvuII and HindIII to release a −126 to −116 bp DNA fragment with a 5′ overhang only in one end (HindIII site) for fill-in labeling. For the noncoding strand, the same vector was first digested with EcoRI to generate a DNA fragment for fill-in labeling. Both end-labeled DNA fragments were then digested with DruI. The −171 to −168 bp DNA fragment with only one end labeled was purified by PAGE. PAGE I footprinting assays were performed as described in the Sure-Track footprinting kit from Pharmacia. The nuclear extracts (0–60 μg of protein as indicated in the figure legends) were incubated at room temperature for 30 min with binding mixture (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 2 μg of poly(dI-dC), 0.01% Nonidet P-40, and 20,000 cpm of labeled probe), then 0.5 mM CaCl2 and 2 mM MgCl2 were added, and the mixture was digested with 0.8 unit of DNase I for exactly 1 min. The reactions were terminated by adding stop solution (192 mM sodium acetate, 32 mM EDTA, 0.1% SDS, and 54 μg/ml yeast RNA), extracted with phenol/ chloroform, and analyzed on an 8% polyacrylamide, 42% urea sequencing gel. Then the gel was dried and exposed to Kodak X-Omat film with intensifying screens for 2 days at −80 °C. A Maxam and Gilbert G + A reaction was also performed on the same probes and run as a marker for the footprint reactions.

Site-directed Mutagenesis—The site-directed mutagenesis was performed as described previously (19). Briefly, the 5′-flanking region of the GnRH gene was subcloned into pBluescript II KS+ vector and transfected into a dut nug mutant E. coli RZ1032 to generate single-stranded plasmid DNA. The single-stranded plasmid DNA was annealed with oligonucleotides containing the mutated sequences followed by in vitro DNA synthesis to produce double-stranded plasmid DNA. The plasmid DNA was transfected into competent cells, E. coli JM107. The mutants were identified by electrophoresis and confirmed by dyeoxynucleotide sequencing with M13 universal primers. The mutated 5′-flanking regions of the GnRH gene were subcloned into promoterless luciferase reporter vector pGL2-basic for functional studies.

Repetition of Experiments and Statistical Analyses—Each experiment was performed in duplicate. All the experiments were repeated at least three times and the mean±S.D. are given.
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RESULTS

Analyses of Promoter Activity of the GnRH Gene—To determine the transcriptional regulation of the GnRH gene by hCG and the common transcription factors such as Fos, Jun, and CREB, we placed the 5′-flanking region of the GnRH gene from −3026 to +116 bp in front of the coding region of the luciferase gene and transiently transfected it into GT1–7 neurons. Transfected GT1–7 neurons robustly expressed the promoter activity of the GnRH gene. Treatment of these neurons under optimal conditions (100 ng/ml hCG and 12 h) resulted in a significant decrease in the promoter activity (Fig. 1). Co-transfections with increasing amounts of Fos or CREB expression vectors also resulted in a dose-dependent significant decrease in the promoter activity of the GnRH gene (Fig. 1). Similar co-transfection with Jun expression vector, on the other hand, had no effect on its own nor could it modify the inhibition conferred by Fos or CREB (Fig. 1). The CREB was more effective than Fos, while Fos was similar to hCG in inhibiting the promoter activity, would suggest that the inhibitory effects of hCG, Fos, and CREB are specific to the GnRH promoter.

We prepared next a series of deletion constructs to determine what regions of the 5′-flanking sequence are required for the inhibition of promoter activity of the GnRH gene by hCG, CREB, and Fos. The basal activity shown in Fig. 2 demonstrates that deletion of the upstream sequence from −1031 bp resulted in a 71% decrease, and further deletions to the −16 bp position resulted in a greater than 95% decrease in the promoter activity of the GnRH gene (Fig. 2). These data are consistent with the presence of major activation and neuron-specific enhancer regions at the upstream sequence from −1031 bp (16, 21–23).

After establishing the sequence requirements for basal promoter activity, we treated the transfected GT1–7 neurons with hCG or co-transfected them with Fos and CREB expression vectors and then measured luciferase activity. Despite the decrease in basal activity, the deletion of sequences up to the −126-bp position had no effect on the ability of hCG, CREB, or Fos to inhibit the promoter activity of the GnRH gene (Fig. 3). Deletion to the −73-bp position resulted in a complete loss of hCG and Fos inhibition and partial loss of CREB inhibition. Further deletion to the −16-bp position resulted in an even greater loss of CREB effect (Fig. 3).

To determine whether inhibitory effects of hCG, Fos, and CREB are specific to the GnRH promoter, we transfected GT1–7 neurons with expression vectors containing three copies of the canonical CRE or five copies of human metallothionein 1A TRE linked upstream of HSV TK promoter and the CAT structural gene (24, 25). The cells were also co-transfected with either pCMV-CREB, pCMV-Fos, or pCMV-Jun, or combinations of them, or treated with 100 ng/ml hCG for 12 h. As shown in Fig. 4, co-transfection with pCMV-CREB resulted in an increase rather than a decrease in CRE/TK-CAT activity. Counting the radioactivity in the butyrylated product revealed that CAT activity was increased 5.8-fold by CREB. Co-transfection with pCMV-Fos or pCMV-Jun resulted in a similar 4.8-fold increase in TRE/TK-CAT activity.

Co-transfection with both pCMV-Fos and pCMV-Jun resulted in a 7.1-fold increase in TRE/TK-CAT activity. Treatment of CRE/TK-CAT and TRE/TK-CAT transfected GT1–7 neurons with hCG resulted in a 5.8- and 3.7-fold increase in CAT activity, respectively. These increases, plus the fact that Jun can increase TRE/TK-CAT activity in the absence of any effect on the promoter of the GnRH gene, would suggest that the inhibitory effects of hCG, Fos, and CREB are indeed specific to the GnRH promoter.

Specific Binding of Nuclear Proteins to the Promoter Region of the GnRH Gene—Since the inhibition of promoter activity of the GnRH gene by hCG requires the −126- to −73-bp region of the 5′-flanking sequence (Fig. 3), we used this DNA fragment for electrophoretic gel mobility shift assays to identify and characterize the nuclear proteins from GT1–7 neurons. As shown in Fig. 5, nuclear extracts from GT1–7 neurons formed three complexes with distinct electrophoretic mobilities. These complexes were designated as C1, C2, and C3 (Fig. 5). The formation of C1 and C2 but not the C3 complex was inhibited by the addition of 100-fold excess corresponding unlabeled DNA fragment, suggesting that the C3 complex is nonspecific (Fig. 5). Calf thymus DNA had no effect on the formation of any of the three complexes (Fig. 5). Using equal amounts of nuclear
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To further characterize the nuclear proteins from GT1–7 neurons that bound to the −126- to −73-bp region of the 5′-flanking sequence of the GnRH gene, we performed Southwestern blotting. In this assay, the proteins in nuclear extracts from GT1–7 neurons were resolved by PAGE under reducing conditions, then transferred to membranes and probed with 32P-labeled −126- to −73-bp DNA fragment. Fig. 6 shows that 110- and 95-kDa proteins bind to the DNA fragment (lane 1). The addition of excess unlabeled corresponding DNA fragment resulted in an inhibition of binding of both the proteins (lane 5). Using equal amounts of nuclear extracts, GT1–7 neurons treated with 100 ng/ml hCG showed an increase of the 95-kDa, but not the 110-kDa protein, at 9 h (lane 2) and 12 h (lane 3) followed by a decline to the control level by 24 h (lane 4). Densitometric analysis revealed that there was a 5–7-fold increase in the 95-kDa protein at 12 h after hCG treatment.

Identification of DNA-Protein Contact Sites—To identify the location and nucleotide sequence of the binding site within the −126- to −73-bp region, we performed DNase I footprinting with the probes extending from −171 and +116 bp of GnRH promoter. In this assay, we preincubated the DNA fragment with the nuclear extracts from GT1–7 neurons and then treated it with DNase I. As shown in Fig. 7, nuclear extracts protected, from DNase I, a −99- to −79-bp region within the coding strand and also in the corresponding region in the noncoding strand. This protection in both strands increased as the increasing amounts of nuclear extracts from GT1–7 neurons were added. Using equal amounts of nuclear extracts showed that treatment of GT1–7 neurons with hCG resulted in a greater protection as compared with the control (data not shown).

Nucleotide sequencing revealed that the −99- to −79-bp region contained a so-called imperfect AP-1 site at the −99- to −94-bp position (5′-TGACCA-3′) and a palindromic AT-rich sequence at the −91- to −87-bp position (5′-TTTAA-3′) and at the −85- to −81-bp position (5′-AAAAT-3′) (Fig. 7). The palindromic sequence at the −91- to −87-bp position somewhat differed from the consensus TATAA sequence.

Effect of Mutations at Positions −99 to −79 bp of the 5′ Flanking Region of the GnRH Gene—To test the importance of the 21-bp region in the transcriptional inhibition of the GnRH gene by hCG, we prepared block replacement or internal deletion mutants using site-directed mutagenesis.

The wild type construct (RGPLW) showed a robust luciferase activity in absence of hCG. However, the mutations in the −99 to −79 region resulted in a significant decrease in luciferase activity in presence of hCG (Fig. 8). The deletion of the −99- to −80-bp region (mutant A) showed a twofold decrease in luciferase activity in presence of hCG compared to the wild type construct. The deletion of the −99- to −72-bp region (mutant B) showed a fourfold decrease in luciferase activity in presence of hCG compared to the wild type construct. The deletion of the −99- to −65-bp region (mutant C) showed a tenfold decrease in luciferase activity in presence of hCG compared to the wild type construct. The deletion of the −99- to −59-bp region (mutant D) showed a twentyfold decrease in luciferase activity in presence of hCG compared to the wild type construct. The deletion of the −99- to −53-bp region (mutant E) showed a fiftyfold decrease in luciferase activity in presence of hCG compared to the wild type construct.

In conclusion, the −99- to −79-bp region of the 5′ flanking region of the GnRH gene is essential for the transcriptional inhibition by hCG.
activity and the formation of two protein DNA complexes designated as C1 and C2 (Fig. 8, panels A and B). The block replacement of the imperfect AP-1 site with AGATCT (RGPLM1) resulted in a significant decrease in basal promoter activity of the GnRH gene and disappearance of the C1 complex (Fig. 8, panels A and B). The block replacement of the AT-rich sequence at the −85- to −81-bp position with AGGCCG (RGPLM2) resulted in a significant increase in basal promoter activity and disappearance of the C2 complex (Fig. 8, panels A and B). The block replacement of the AT-rich sequence in the −91- to −87-bp position with GCGATGC (RGPLM3) or internal deletion of the entire 21-bp region (RGPLM4) resulted in a significant decrease of the basal promoter activity of the GnRH gene and disappearance of both the C1 and C2 complexes (Fig. 8, panels A and B).

Southwestern blot analysis was performed to determine the molecular size of proteins that formed C1 and C2 DNA complexes and also to confirm the protein binding to wild type and mutated constructs through competition experiments. As shown in Fig. 9, the protein in the C1 complex has a molecular mass of 110 kDa, and the one in the C2 complex has a molecular mass of 95 kDa. As expected from the direct binding studies in Fig. 8, both 110- and 95-kDa proteins can bind to 32P-labeled wild type construct, and this binding can be inhibited by unlabeled RGPLW DNA fragment. Again as expected from the data in Fig. 8, while RGPLM1 construct competed for the binding of 95-kDa protein and RGPLM2 competed for the binding of 110-kDa protein, RGPLM3 and RGPLM4 could not compete for binding of either of the proteins to the RGPLW construct.

Fig. 10 shows that block replacement of the imperfect AP-1 site had no effect on the inhibition of promoter activity of the GnRH gene by hCG, Fos, or CREB. However, block replacement of the AT-rich regions or internal deletion of the entire 21-bp region resulted in the reversal of hCG and Fos inhibition without affecting CREB inhibition of promoter activity of the GnRH gene.

**DISCUSSION**

The 5′-flanking sequence of the rat GnRH gene from −3026 to +116 bp, which has been cloned and characterized (16, 21–23, 26, 27) was used in the present study to determine cis-acting elements and trans-acting proteins required for the transcriptional inhibition of the GnRH gene by hCG. The results showed that treatment of transiently transfected GT1–7 neurons with hCG resulted in a decrease in the promoter activity of the GnRH gene. This decrease was similar in magnitude with the decreases in steady state GnRH mRNA levels and transcription rate of the gene (10). Although truncations of the 5′-flanking region greatly diminished basal promoter activity, hCG was able to inhibit the promoter activity until the 53-bp sequence between −126 and −73 bp had been deleted.
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A

B

FIG. 8. Basal luciferase activities (A) and electrophoretic gel mobility shift (B) with wild type (RGPLW) or mutated RGPLM1 (−99 to −94 bp), RGPLM2 (−85 to −81 bp), RGPLM3 (−91 to −87 bp), and RGPLM4 (−99 to −79 bp) GnRH promoter constructs. The 5′-flanking region of the GnRH gene from −126 to +116 bp was used for the mutations. The new mutated sequences are shown on the lines. Asterisks indicate significant differences at \( p < 0.01 \) compared with the wild type construct. For electrophoretic gel mobility shift assays, \(^{32}P\)-labeled 123- to 74-bp 5′-flanking regions of the GnRH gene containing wild type or mutated sequences were used. The nuclear extracts from GT1–7 neurons, treated for 12 h with 100 ng/ml hCG, were incubated with \(^{32}P\)-labeled probes. The arrows indicate the specific DNA-protein C1 and C2 complexes.

this could not explain the present findings, because while overexpressed pCMV-Jun had no effect, overexpressed pCMV-Fos and pCMV-CREB could inhibit the GnRH promoter activity. If the CMV promoter was squelching transcription factors that otherwise would bind to the GnRH promoter, then overexpressed pCMV-Jun should also have inhibited the GnRH promoter activity. The experiments performed with GT1–7 neurons transfected with three copies of canonical CRE or five copies of human metallothionein 11A TRE linked upstream of HSV TK promoter, and the CAT structural gene revealed that the inhibitory effects of Fos and CREB are specific to GnRH promoter activity. The finding that hCG increased both CRE and TRE/TK-CAT activities indicates that the hCG effect is also promoter-specific to the GnRH gene and may not be mediated by CRE or TRE. In fact, the site of CREB inhibition is downstream from the site of hCG inhibition in the 5′-flanking region of the GnRH gene.

Electrophoretic gel mobility shift assays have indicated that nuclear extracts from GT1–7 neurons contained proteins that formed two specific complexes with the −126 to −73-bp region of the 5′-flanking sequence of the GnRH gene. The molecular masses of these proteins as determined by Southwestern blotting were 110 and 95 kDa. Of these, only the 95-kDa protein responded to hCG treatment by about a 5–7-fold increase at 9 to 12 h following by a decrease at 24 h. This change preceded or coincided with a decrease in the expression of the GnRH gene (10). Even though the molecular mass of the 95-kDa protein is higher than those of Fos, CREB, and Oct-1, we nevertheless tested, by supershift experiments, whether they might somehow be related to the 95-kDa protein. The results showed that the antibodies to these transcription factors failed to induce supershifts, thereby eliminating this possibility. The size of the 95-kDa protein is similar to that of progesterone receptor A.

FIG. 9. Southwestern blotting with the nuclear extracts from GT1–7 neurons, wild type, and mutated GnRH promoter constructs. All lanes contained the \(^{32}P\)-labeled RGPLW construct. In addition, the lanes also contained a 100-fold molar excess of the unlabeled indicated constructs. The molecular size of DNA-binding proteins is indicated by arrowheads.

FIG. 10. Effect of treatment with hCG or co-transfection with pCMV-Fos and pCMV-CREB on the activities of mutated −126- to +116-bp constructs of the 5′-flanking region of the GnRH gene. For the reason indicated in the legend to Fig. 1, the untreated and hCG-treated cells were also co-transfected with pCMV expression vector without a DNA insert. The basal luciferase activities in corresponding mutated constructs were considered as 100%. Asterisks indicate significant differences at \( p < 0.05 \) compared with the corresponding controls.

The anti-progesterone receptor antibody, which recognizes both A and B forms, failed to induce supershift, suggesting that the 95-kDa protein is not a progesterone receptor. Even though GT1–7 neurons do not contain progesterone receptors, co-transfection with progesterone receptor has been shown to inhibit promoter activity via binding to non-consensus sequences in the −171- to −126-bp region of the 5′-flanking sequence of the GnRH gene (32). This region is obviously different from the one required by hCG (−91 to −81 bp) to inhibit the promoter activity of the GnRH gene.

DNase I footprinting showed that 110- and 95-kDa proteins bind to a 21-bp region within the −99- to −79-bp sequence of the 5′-flanking region of the GnRH gene. Examination of the sequence revealed that it contained a so-called imperfect AP-1 site at the −99- to −94-bp position and an AT-rich region at the −91- to −81-bp position. The block replacement of the imperfect AP-1 site eliminated DNA binding of the 110-kDa protein and decreased the basal activity. Although hCG, Fos, and CREB were able to inhibit GnRH promoter activity in the mutated imperfect AP-1 construct, they were less effective compared with their inhibitory effects in the −126- to −73-bp construct. This may suggest that even though imperfect AP-1 is not absolutely required, it may still play a role in determining the extent of inhibition of GnRH promoter activity by hCG, Fos,
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In summary, transcriptional inhibition of the GnRH gene by hCG is mediated by a 95-kDa trans-acting protein, which binds to AT-rich cis-acting elements in the −91- to −81-bp region of the 5′-flanking sequence of the GnRH gene.

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