We have investigated the mechanisms underlying cell-specific glucocorticoid repression of calcitonin/calcitonin gene-related peptide (CGRP) gene expression. Treatment with the synthetic glucocorticoid dexamethasone has been shown to decrease mRNA levels in the 44-2C thyroid C cell line. Nuclear run-on assays showed that dexamethasone repressed transcription 2-3-fold in 44-2C cells. In contrast, dexamethasone stimulated calcitonin/CGRP transcription 4-6-fold in the CA77 thyroid C cell line. Transient transfection assays were used to map repression of reporter gene activity in 44-2C cells to a neuroendocrine cell-specific enhancer located between -920 and -1125 base pairs (bp). Within this region, an 18-bp element was found that conferred both full basal enhancer activity and dexamethasone-dependent repression in 44-2C cells. The 18-bp region contains possible binding sites for AP-1 and helix-loop-helix transcription factors as well as a glucocorticoid receptor half-site. Colocalization of repression and enhancer activity was then investigated in other cell lines. In CA77 cells, while the 820-1125 region strongly enhanced transcription, the 18-bp region conferred only partial activation and dexamethasone had little effect on reporter gene activity. Dexamethasone did not repress the calcitonin/CGRP activity in the heterologous HeLa and Rat1 fibroblast cell lines. These results suggest that glucocorticoids repress transcription of the calcitonin/CGRP gene by inhibiting cell-specific transcription factor activity.

Glucocorticoid and other steroid hormones are able to regulate expression of many genes in both a positive and negative manner (1). Interestingly, in an increasing number of cases, glucocorticoid regulation has been shown to be tissue-specific. This can be manifested as positive regulation by glucocorticoids in some cells and with negative regulation or the absence of regulation in other cell types. Several examples of this have been identified in vivo and in studies with tumor cell lines. For example, expression of the phosphoenolpyruvate carboxykinase gene is stimulated by glucocorticoids in the liver and kidney but repressed in adipose tissue (2). Similarly, dexamethasone treatment stimulates rat angiotensinogen mRNA levels in the brain and liver but does not affect gene expression in the kidney (3). Other genes that have shown cell-specific regulation by glucocorticoids include the pro-opiomelanocortin (4), corticotropin-releasing hormone (5), and thyrotropin-releasing hormone genes (6). While in general the mechanisms underlying cell-specific glucocorticoid regulation are not known, recent work with the proenkephalin gene enhancer has provided some insights (7). These studies indicated that selective interactions between the glucocorticoid receptor and the AP-1 transcription factor at the enhancer can either stimulate or repress transcription of a reporter gene.

The calcitonin/calcitonin gene-related peptide (CGRP) gene is an example of a gene that is regulated by glucocorticoids in a cell-specific manner. The calcitonin/CGRP gene encodes multiple neuroendocrine peptides that are expressed in specific endocrine and neuronal tissues. Alternative splicing yields calcitonin mRNA in thyroid C cells and the neu- ropeptide CGRP in central and peripheral neurons (8). Glucocorticoid treatment increases mRNA levels of the calcitonin/CGRP gene in the human TT and rat CA77 medullary thyroid carcinoma cell lines (9-11) but decreases calcitonin/CGRP expression in rat 44-2C medullary thyroid carcinoma (12) and human NCI-H727 bronchial carcinoma cell lines (13). Studies done in vivo and with primary cultures of thyroid cells have demonstrated that glucocorticoid treatment increases calcitonin expression in the rat thyroid (14). In contrast, glucocorticoid treatment does not increase CGRP mRNA levels in the brainstem, suggesting that regulation of the calcitonin/CGRP gene by glucocorticoids in vivo may also be controlled in a cell-specific manner. As a starting point to understanding the cell-specific glucocorticoid regulation of the calcitonin/CGRP gene, we have investigated the mechanism of repression of the gene in the 44-2C medullary thyroid carcinoma cell line. We first demonstrated that repression occurs at the level of transcriptional initiation. Transient transfection of calcitonin/CGRP gene 5'-sequences coupled to reporter genes was used to map cell-specific repression by dexamethasone. Interestingly, the site of negative glucocorticoid regulation is located within an 18-bp element which also functions as a neuroendocrine cell-specific enhancer of basal transcription. The absence of dexamethasone-treatment stimulated calcitonin/CGRP transcription.
amethasone-dependent repression in cell lines where the cell-specific enhancer was not active suggest that glucocorticoids may repress transcription by inhibiting cell-specific transcription factors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The 44-2C cell line was maintained in DMEM (high glucose) with 1% equine serum (HyClone Laboratories, Logan, UT), and 0.1% L-glutamine; CA77 cells in Ham's F-12 medium/DMEM (low glucose) (1:1) with 10% fetal bovine serum (HyClone Laboratories); HeLa cells in Ham's F-12 medium with 10% fetal bovine serum; and RNA cell in DMEM (high glucose) with 10% calf serum. Also, 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO) were added to each culture medium. For dexamethasone treatments prior to nuclei isolations, CA77 cells were plated in serum-free medium and washed for 2 h in 2 cm electrode gap, Bio-Rad) using a Bio-Rad gene pulser at 960 V, 90 min at 37 °C and washed for 30 min at 37 °C. 6-Actin was included as a loading control. Filters were exposed to x-ray film for 1-7 days and quantitated using an LKB Ultroscan densitometer.

**Nuclear Run-on Assays**—Approximately 1 × 10^6 cells were incubated in the presence or absence of 500 nM dexamethasone for 2 h, and nuclei were prepared as described previously (16). Cells were scraped from cold Dulbecco's phosphate-buffered saline (PBS) and then collected by centrifugation and gently lysed in detergent (0.5% Nonidet P-40, 300 mM NaCl). Nuclei were frozen in 200 µl of glycerol storage buffer (40% glycerol, 50 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 0.1 mM EDTA) in liquid nitrogen until assay was performed. Assays were done as described (15, 16) with some modifications. Nuclei (200 µl in storage buffer) were thawed and then incubated for 30 min at 30 °C in a 250-µl volume containing 0.5 mM dithiothreitol, 80 mM KCl, 4 mM ATP, 50, and 200 µM of [α-32P]UTP (ICN, 800 Ci/mmol). The reaction mix was then incubated with 100 µg of RNAase-free DNA (Biochemical Research Laboratories), and 10 µM of 20 mM CaCl₂ for 5 min at 30 °C, followed by digestion for 30 min at 37 °C with 20 µg of proteinase K with 50 µg of tRNA in 0.5% sodium dodecyl sulfate, 12.5 mM EDTA, 50 mM Tris- HCl, pH 7.4. Labeled RNA was extracted with an acid/ guanidinium thiocyanate/phenol procedure (17) and resuspended in 2 ml of hybridization buffer (10 mM Tris, pH 7.4, 0.5 M NaCl, 0.1% sodium dodecyl sulfate, 300 mM NaCl). Nitrocellulose filters containing immobilized plasmid DNA were prepared as described (15). Equal counts (10^5 copies) of labeled RNA were incubated with each filter for 36 h at 65 °C. Filters were washed for 2 h in 2 × SSC (300 mM NaCl, 30 mM sodium citrate) at 65 °C and then incubated with 10 µg/ml RNAse A in 2 × SSC for 30 min at 37 °C and washed for 30 min at 37 °C. Filters were included as a hybridization control and pBSK plasmid DNA as a control for background hybridization. Filters were exposed to x-ray film for 1-7 days and quantitated using an LKB Ultrascan densitometer.

**Cell Transfections**—Cells were grown to approximately 80% confluence, harvested by brief trypsin/EDTA treatment, and resuspended in Ca²⁺/Mg²⁺-free PBS. Approximately 10⁶ cells in 0.8 ml of PBS were electrophoreted with 20 µg of supercoiled DNA/cuvette (0.4 cm electrode gap, Bio-Rad) using a Bio-Rad gene pulser at 960 V, 90 min at 37 °C and 900 V, 90 min at 37 °C. After centrifugation, 20-50 µl of supernatant cell extract was assayed for luciferase activity as previously described (18) using a luminometer (Analytical Luminescence Laboratory Monolight 2001). The assay was linear between 500 (background) and 50,000 cpm. Both luciferase and CAT assays were normalized to 50 µg of protein as determined by the Bio-Rad protein assay (Bio-Rad Laboratories). γ-glutamyltranspeptidase was added to each culture medium as an internal standard. CAT assays were performed as described previously (19). After heating to 65 °C for 10 min, 5-40 µl of cell extract was incubated with 300 µM n-butyryl coenzyme A and 100 µM chloramphenicol (Sigma) with 10 µCi of [14C]n-butyryl coenzyme A (New England Nuclear) for 30-60 min in a volume of 100 µl of 250 mM Tris-HCl, pH 8.0. After 1-24 h at 37 °C, reaction products were isolated by extraction with 300 µl of xylene, back extracted twice, and counted in 5 ml of 4x20 scintillation fluid (Research Products International, Mount Prospect, IL) in a Beckman scintillation counter. The CAT assay was linear between 500 (background) and 50,000 cpm. Both luciferase and CAT assays were normalized to 50 µg of protein as determined by the Bio-Rad protein assay (Bio-Rad Laboratories). γ-glutamyltranspeptidase was added to each culture medium as an internal standard. CAT assays were performed as described previously (20).

Although β-galactosidase assays showed that transcription efficiencies were approximately the same within each experiment, absolute luciferase activity varied severalfold between experiments due to variation in cell growth rate and transfection survival. To compare results from different experiments, activities were normalized to an internal standard as indicated in the figure legends.

**Construction of Reporter Fusion Constructs**—The luciferase fusion genes containing the calcitonin/CGRP gene 5' sequences in this study have been described previously (21). A series of deletions were made by restriction digestion of the rat calcitonin/CGRP promoter plasmid pBSpAP using PstI (−1362), BglII (−920), Ddel (−717), and DraI (−163). Construction of the −1900 luciferase reporter plasmid (LphRI) and the −1125 luciferase reporter plasmid (Mu3) has been described (21). All fragments share a common 5' terminus at position +21 in exon 1. The PstI (−1362) fragment was also inserted in front of the CAT reporter gene. The internal deletion of the −1362 CAT reporter gene was made by digestion and religation of the BglII sites at −1125 and −920 bp. To construct the −920-1125-TK-LUC reporter gene, the −1125 to −650 BglII fragment was inserted at a BamHI site 5 to a TK-LUC reporter gene (22). The −920-1038- and 1038-1125-TK-LUC fusions contain blunt BglII fragments subcloned into a blunt end-TK-LUC BamHI site. The 1091-1017 insert was constructed using the polymerase chain reaction with flanking primers followed by ligation of BamHI linkers. The 1043-1025-TK-LUC insert was constructed by linking complimentary oligonucleotides containing terminal BamHI sites.

The glucocorticoid receptor expression vector RSV-DexR was provided by S. Adler (Washington University), the MMTV-CAT plasmid by R. Evans (Salk Institute), and cytomegalovirus-βGal by R. Maurer (University of Iowa).

**RESULTS**

Dexamethasone Repression of Calcitonin/CGRP Transcription Rate in 44-2C Cells—Steroid hormones can regulate mRNA levels by directly affecting transcription rate or by acting at a posttranscriptional step such as RNA stability. Previous studies have shown that dexamethasone stimulates transcription of both calcitonin and CGRP-specific exons in CA77 cells (11). However, the mechanism responsible for repression of the gene in 44-2Cs has not been characterized. Therefore, nuclear run-on assays were performed to determine whether glucocorticoids also repress calcitonin/CGRP gene transcription. Cells were treated with dexamethasone for 2 h, after which nuclei were isolated. Labeled nascent RNA transcripts were prepared and incubated with filters containing DNA fragments, including the plasmid vector as background control.

We found that dexamethasone treatment of 44-2C cells decreased the calcitonin/CGRP gene transcription rate to a level consistent with the 3-4-fold decrease seen in steady state mRNA levels (Fig. 1) (12). Over three separate experiments, we saw that transcription initiation following dexamethasone treatment decreased 2-3-fold. To demonstrate that transcriptional repression occurs across both the calcitonin and CGRP exons in 44-2C cells, DNA fragments specific for calcitonin (exon 4) and for CGRP (exons 5 and 6) were used. This was important because in CA77 and TT cells, dexamethasone treatment causes a bias in alternative splicing resulting in a greater increase in calcitonin than in CGRP transcripts (9, 11, 23). Similar 2-3-fold decreases in hybridization occur with both calcitonin and CGRP probes. This indicates that decreased transcription can account for the decrease of both calcitonin and CGRP mRNA levels. In contrast, transcription...
of both the calcitonin and CGRP exons in CA77 cells was shown to increase 4–6-fold, as previously observed (11). In addition, transcription of the human calcitonin/CGRP gene in the TT cell line also increased 4–6-fold (data not shown), which is consistent with the previously reported increase in steady state calcitonin mRNA levels (9). As a control, dexamethasone did not significantly affect transcription of β-actin.

**Mapping of a Negative Glucocorticoid Regulatory Element—** Reporter fusion genes were used to localize cis-active elements responsible for glucocorticoid repression of the calcitonin/CGRP gene. 44-2C cells were transiently transfected with various lengths of rat calcitonin/CGRP gene 5’-flanking sequence coupled to the luciferase or chloramphenicol acetyltransferase (CAT) reporter genes. To ensure that DNA transfection conditions between control and dexamethasone-treated cells were equivalent, the cells were electroporated in a single cuvette and then split between two dishes before treatment. After 24 h, the cells were harvested and reporter activity was measured. A luciferase fusion gene containing the non-glucocorticoid-responsive thymidine kinase (TK) promoter was included as a control for nonspecific dexamethasone effects on reporter activity. As a positive control for dexamethasone responsiveness, the glucocorticoid-stimulated mouse mammary tumor virus (MMTV) promoter was coupled to CAT and transfected. In some experiments, a cytomegalovirus-βGal reporter plasmid was co-transfected along with the luciferase fusion genes. β-Galactosidase assays confirmed that DNA transfection efficiencies were essentially the same within each experiment. Multiple plasmid preparations were used to show that results were not DNA preparation-dependent.

Mapping experiments in 44-2C cells show that sequences between −920 and −1125 bp from the start site are responsible for negative regulation by dexamethasone (Fig. 2A). Promoter activity of fusion genes containing at least 1125 bp of upstream sequences is repressed 2.5–4.5-fold by dexamethasone. In contrast, shorter constructs are slightly stimulated by dexamethasone treatment. In addition to conferring dexamethasone-mediated repression, the 920–1125 region also enhanced basal transcription of the −1125-CAL-LUC fusion gene approximately 6-fold over that of shorter constructs. This observation is consistent with previous reports that an enhancer of basal transcription in CA77 cells is located between −827 and −1362 bp (21).

We demonstrated that these results were independent of the luciferase reporter gene by also using bacterial CAT as a reporter gene (Fig. 2B). Cells were transfected with CAT fusion genes containing 1350 bp of calcitonin/CGRP 5’ sequence with or without an internal deletion of the 920–1125 region. We saw that fusion genes lacking the 920–1125 enhancer region had 2–3-fold lower basal activity than fusion genes that contained the enhancer. Also, the absence of the 920–1125 region led to loss of repression by dexamethasone treatment.

**Transfer of Cell-specific Glucocorticoid Regulation to a Heterologous Promoter—** To test whether sequences within the 920–1125 region are sufficient for cell-specific negative regulation by glucocorticoids, the region was inserted upstream of the glucocorticoid-nonresponsive thymidine kinase promoter. When the fusion gene was transfected into 44-2C cells (Fig. 3A), basal activity of the promoter was stimulated 70–90-fold over that of the thymidine kinase promoter alone. Treatment of the cells with 500 nM dexamethasone repressed the 920–1125-TK-LUC fusion gene 2.5–3-fold (Fig. 3A), although in a few experiments repression was as much as 10-fold (see Fig. 4). These data demonstrate that cell-specific enhancement and repression are mediated by sequences within the 920–1125 element and can be transferred to a heterologous promoter.

**Localization of Repression by Glucocorticoids to an 18-Base Pair Basal Enhancer Element—** To further map sites of glucocorticoid regulation and basal enhancer activity, smaller regions within the 920–1125-bp element were investigated (Fig. 3A). A sequence either proximal (920–1038) or distal...
while the distal region contains a putative AP-2 binding site along with two consensus helix-loop-helix (HLH) factor binding sites (21). The PuuII site itself is yet another consensus HLH binding site (CAGCTG). In comparison with the 70–90-fold enhancer activity of the entire 920–1125-bp element, neither the proximal nor distal element alone had much enhancer activity (less than 4-fold) and neither mediated repression by glucocorticoids. This suggests that either the binding site was disrupted or that factors binding both upstream and downstream of the PuuII site are necessary for activity.

Because the intact PuuII site was found to be required for enhancer function, smaller regions that span the PuuII site were tested for activity. 74- (1016–1090) and 18-bp (1025–1043) fragments were inserted into the TK-LUC vector. Both of these regions conferred the same 2–4-fold repression as seen with the entire 920–1125 element and acted as strong enhancers of basal transcription. While the 74-bp fragment stimulated TK promoter transcription 40–50-fold, the 18-bp sequence between −1025 and −1043 bp was sufficient to enhance TK transcription 70–90-fold, to the same levels as the entire 920–1125-bp region. The basis for differences between the enhancer activity of the 74- and 18-bp elements was not pursued, but the results suggest that multiple elements may influence enhancer activity. More importantly, these results indicate that elements required for both full basal activity and repression by glucocorticoids are contained within a relatively small 18-bp region of the calcitonin/CGRP promoter.

The sequence of the 18-bp element reveals a number of potential transcription factor binding sites based on sequence homologies (Fig. 3B). In particular, a site homologous to the right half of the palindromic GRE (TGTGCA) was found. In the inverse orientation, this sequence is contained within a previously described site of glucocorticoid repression in the interleukin-6 gene (TTGACA) (24). Also notable is a non-consensus AP-1 binding site (GATTGG) identified as part of an element mediating glucocorticoid repression of the proliferin gene (7, 25). The 18-bp element also contains a possible sequence  between -1025 and -1043 bp was sufficient to stimulate TK promoter transcription 40–50-fold, the 18-bp sequence between −1025 and −1043 bp was sufficient to enhance TK transcription 70–90-fold, to the same levels as the entire 920–1125-bp region. The basis for differences between the enhancer activity of the 74- and 18-bp elements was not pursued, but the results suggest that multiple elements may influence enhancer activity. More importantly, these results indicate that elements required for both full basal activity and repression by glucocorticoids are contained within a relatively small 18-bp region of the calcitonin/CGRP promoter.

The sequence of the 18-bp element reveals a number of potential transcription factor binding sites based on sequence homologies (Fig. 3B). In particular, a site homologous to the right half of the palindromic GRE (TGTGCA) was found. In the inverse orientation, this sequence is contained within a previously described site of glucocorticoid repression in the interleukin-6 gene (TTGACA) (24). Also notable is a non-consensus AP-1 binding site (GATTGG) identified as part of an element mediating glucocorticoid repression of the proliferin gene (7, 25). The 18-bp element also contains a possible site for HLH transcription factor binding (CAGCTG) identical to the AP-4 HLH factor site found in the SV40 promoter (26, 27). The importance of this site is underlined by the fact that when the site is disrupted, almost all transcription enhancement is abolished.

Repression by Dexamethasone Is Mediated by the Glucocorticoid Receptor—The dexamethasone dose dependence of promoter repression was determined to characterize the type of receptor involved (Fig. 4). 44-2C cells were transfected with the 920–1125-TK-LUC plasmid and incubated with dexamethasone concentrations between 10^{-11} and 10^{-6} M for 24 h. An equivalent amount of the ethanol vehicle was added to each control plate. We saw that promoter activity was repressed approximately 10-fold at dexamethasone concentrations above 50 nM. Inhibition was half-maximal with a dexamethasone concentration of 2.5 nM, which is very close to the previously reported dexamethasone Ki range of 3–10 nM for the glucocorticoid receptor (28, 29). This correlation supports the involvement of the glucocorticoid receptor in repression of calcitonin/hCGRP transcription.

Regulation of the Calcitonin/CGRP Promoter in CA77 Cells—We then investigated whether the 920–1125 enhancer is regulated differently in the CA77 thyroid C cell line, where the endogenous calcitonin/CGRP gene is stimulated by glucocorticoids. Reporter genes containing up to 1900 bp of the calcitonin/CGRP promoter sequence were assayed in this cell line (Fig. 5, A and B). As with 44-2C cells, the 920–1125-bp...
region in CA77 cells stimulates basal transcription of the calcitonin/CGRP promoter 8-12-fold. Interestingly, in the CA77 cell line, dexamethasone treatment did not repress activity of calcitonin/CGRP promoter fusion genes containing the 920-1125-bp element. This indicates that, as with the endogenous gene, glucocorticoids differentially regulate reporter gene activity in these cell lines. Overexpression of glucocorticoid receptor in CA77 cells did not cause repression of either -1006-CAL-LUC or 920-1125-TK-LUC reporter activity (data not shown). We conclude, therefore, that lack of repression in the CA77 cell line is not caused by lower glucocorticoid receptor levels.

In contrast to the endogenous calcitonin/CGRP gene, however, the reporter genes in CA77 cells were not stimulated by dexamethasone treatment. Consistent with this observation, sequence analysis of 1362 bp of the 5' flanking region did not reveal any sequence with strong homology to both halves of the consensus-positive GRE sequence. As a control to show that glucocorticoids are able to stimulate reporter gene expression in both CA77 and 44-2C cell lines, a CAT fusion gene containing the glucocorticoid-responsive MMTV promoter was transfected into both cell types (Fig. 2B and 5B). In both CA77 and 44-2C cell lines, CAT activity was stimulated to similar levels with greater than 100-fold increases. CA77 and 44-2C cells thus appear capable of similar transactivation by glucocorticoids. We conclude, therefore, that positive regulatory sites may be located further upstream of within calcium/CGRP gene introns. There are precedents for genes with positive GREs further than 2000 bp upstream of the start site (30, 31) and within introns (32).

The 920-1125-bp element and fragments within it were then examined in CA77 cells for enhancer activity as well as regulation by dexamethasone (Fig. 6). As in 44-2C cells, the entire 920-1125 region strongly enhances TK promoter transcription, stimulating activity 30-40-fold. Sequences proximal to the PvuII site at -1038 bp had only 7% of full enhancer activity while distal sequences had no effect on TK-LUC transcription. This indicates that in CA77 cells as well as in 44-2C cells, the intact PvuII site is necessary for enhancer function. The 74- and 18-bp elements conferred only 3 and 9%, respectively, of full activity of the 920-1125 region. This is in contrast to the 44-2C cells in which the 18-bp element alone enhances transcription equally as well as the entire 920-1125 region. Presumably, other transcription factors that bind outside these regions contribute to full enhancer activity in the CA77 cell line. Dexamethasone repressed activity of the 920-1125-bp region by a small but statistically significant amount (1.2-fold, p < 0.05). The repression was more pronounced (1.6-fold, p < 0.01) with smaller elements that include the 18-bp region. Factors necessary for repression by glucocorticoids may therefore be present in CA77 cells but have only a very weak effect on overall transcriptional regulation.

Neuroendocrine Cell-specific Repression of the Basal Enhancer by Dexamethasone—The 920-1125 calcitonin/CGRP enhancer, which stimulates transcription in the thyroid C cell-derived cell lines, had previously been reported to be neuroendocrine cell type-specific (21). The enhancer was reportedly inactive in cell lines that do not express the endogenous calcitonin/CGRP gene, including HeLa and fibroblast cell lines. Because both cell-specific enhancement and dexamethasone repression were localized to the -920 to -1125 region, we wanted to test the hypothesis that negative regulation by dexamethasone may be dependent upon cell-specific enhancer factors that may be binding within the 18-bp element. To do this, we transfected the calcitonin/CGRP reporter fusion genes into the Rat1 fibroblast and epithelial derived HeLa cell lines and then examined dexamethasone regulation of reporter gene activity.

In HeLa cells, we saw that the neuroendocrine-specific 920-1125 enhancer did not increase calcitonin promoter activity and that dexamethasone did not repress activity of the enhancer in this cell line. We observed that calcitonin-luciferase fusion genes without the 920-1125 region were equally as active as those with the region (Fig. 7A). When the 920-1125 region was placed upstream of the TK-LUC gene, it increased transcription approximately 7-fold. This is in contrast to the 40-80-fold enhancement seen in CA77 or 44-2C cells. In HeLa cells, the 18-bp element alone stimulated TK promoter activity only 2-fold. Dexamethasone did not repress activity of either calcitonin/CGRP or TK promoter fusion genes but uniformly stimulated reporter activity of all plasmids 1.5-2-fold, including the TK-LUC reporter gene, which does not

**Fig. 5.** Absence of repression by dexamethasone of calcitonin/CGRP promoter activity in CA77 cells. Luciferase (A) or CAT reporter fusion genes (B) were transfected into CA77 cells and assayed and normalized as in Fig. 2. Activities of 50 μg of extract are shown, except MMTV-CAT for which the activity of 10 pg of extract is shown. Means ± S.E. for at least six independent experiments for -1362-CAL-LUC, -1125-CAL-LUC, and -920-CAL-LUC plasmids, and three experiments with -1900-CAL-LUC, -717-CAL-LUC, -163-CAL-LUC, and CAT plasmids are shown. The cell-specific enhancer region is indicated as a shaded box between -920 and -1125.

**Fig. 6.** Regulation of the 920-1125 region in CA77 cells. The same TK-LUC reporter fusion genes assayed in 44-2C cells in Fig. 3 were transfected into CA77 cells. Luciferase activity of 50 μg of cell extract normalized to that of the 920-1125-TK-LUC plasmid was determined as in Fig. 2. The means ± S.E. of at least three experiments are shown. An asterisk denoted statistically significant repression by dexamethasone (DEX) (p < 0.05 using a two-sided Student’s t test).
Fig. 7. Absence of dexamethasone regulation in heterologous cell lines. Reporter fusion genes were transfected into HeLa (A) or Rat1 fibroblast (B) cells, treated with dexamethasone (DEX), and assayed as described in Fig. 2. Luciferase activity is shown normalized to TK-LUC. The means ± S.E. for at least four independent experiments are shown. C, MMTV-CAT transfection into Rat1 and HeLa cells. Cells were transfected and assayed as in Fig. 2. Extracts were incubated for 24 h in the CAT assay and resulting cpm/50 µg of protein are shown. Activity of vehicle-treated MMTV-CAT extracts was at or below background, approximately 200 cpm.

contain a glucocorticoid-response element. This suggests that dexamethasone may have a general effect on transcription of reporter genes in that cell line.

In the Rat1 fibroblast cell line, dexamethasone treatment did not affect promoter activity of the calcitonin/CGRP or TK promoter fusion genes, whether or not the 920–1125 calcitonin/CGRP enhancer was present (Fig. 7B). As in HeLa, the presence of the 920–1125 did not increase calcitonin promoter activity. The same region increased TK transcription 3–4-fold but was not repressed by treatment with dexamethasone. The relatively modest enhancement of the TK promoter by the 920–1125 element in the Rat1 and HeLa cell lines may be due to binding of non-cell-specific transcription factors in close proximity to the TK start site. In any case, promoter activity in both of these cell lines was not repressed by dexamethasone, suggesting that cell-specific factors required for full enhancer activity are necessary for dexamethasone-dependent repression.

To establish that both cell lines expressed glucocorticoid receptors and were capable of responding to dexamethasone, the MMTV-CAT” gene was transfected into the cells. In the absence of dexamethasone, promoter activity was below the limits of detection, but upon dexamethasone treatment, activity was stimulated at least 100-fold in both Rat1 and HeLa cells (Fig. 7C). These results demonstrate that the lack of dexamethasone repression of genes that include the 920–1125 element stimulates transcription about 3–4-fold but was not repressed by treatment with dexamethasone. The relatively modest enhancement of the TK promoter by the 920–1125 element in the Rat1 and HeLa cell lines may be due to binding of non-cell-specific transcription factors in close proximity to the TK start site. In any case, promoter activity in both of these cell lines was not repressed by dexamethasone, suggesting that cell-specific factors required for full enhancer activity are necessary for dexamethasone-dependent repression.

To establish that both cell lines expressed glucocorticoid receptors and were capable of responding to dexamethasone, the MMTV-CAT” gene was transfected into the cells. In the absence of dexamethasone, promoter activity was below the limits of detection, but upon dexamethasone treatment, activity was stimulated at least 100-fold in both Rat1 and HeLa cells (Fig. 7C). These results demonstrate that the lack of dexamethasone repression of genes that include the 920–1125 enhancer element is not due to a lack of functional glucocorticoid receptors. Multiple DNA preparations and co-transfection controls were included to show that results were not DNA- or transfection-dependent.

DISCUSSION

To begin to understand the mechanisms behind cell-specific glucocorticoid regulation of the calcitonin/CGRP gene in different cell lines, we have mapped negative regulation of the gene to an 18-bp region within a neuroendocrine cell-specific enhancer. In the 44-2C thyroid C cell line, the 18-bp element conferred both negative regulation by dexamethasone and high basal enhancer activity. In the CA77 thyroid C cell line, the 18-bp element contributes a small degree of enhancer activity and a correspondingly small amount of dexamethasone-dependent repression. Furthermore, no repression by dexamethasone was seen in cell lines where the calcitonin/CGRP promoter enhancer was not active. These results suggest that the glucocorticoid receptor may repress transcription by inhibiting stimulatory cell-specific transcription factors binding within the 18-bp element.

A closer examination of the 18-bp element suggests a possible mechanism of repression by dexamethasone (Fig. 3B). Within this region are binding sites for both AP-4 and AP-1 transcription factors. Between the two sites lies a half-GRE, identical to a site where the glucocorticoid receptor was shown to bind DNA to repress interleukin-6 transcription (24). Interestingly, AP-4 and AP-1 sites are found close together in the basal enhancers of other gene promoters (26, 33, 34), and more importantly, AP-4 and AP-1 have been shown to transactivate SV40 transcription in a synergistic manner (26). Negative interactions between the glucocorticoid receptor and either an AP-4-like HLH factor or AP-1 may therefore prevent synergistic activation of the calcitonin/CGRP promoter. Supporting this hypothesis is the fact that the glucocorticoid receptor can repress AP-1 activity by negatively interacting with c-Jun at a composite GRE/AP-1 site in the proliferin enhancer (7, 25). It has also been demonstrated that the glucocorticoid receptor can bind directly to c-Jun and c-Fos proteins to prevent DNA binding and thereby inhibit AP-1 enhancement of the collagen gene (35–37).

It is likely that the transcription factor responsible for cell-specific enhancement may bind to the AP-4 HLH site. Many HLH transcription factors are tissue-specific, such as MyoD, which activates muscle cell genes (38), and achaete-scute, which is neural specific in Drosophila (39). It is especially intriguing that HLH transcription factors such as Pan-1 and Pan-2 have been shown to bind to AP-4 sites in pancreatic gene promoters that are identical to that found in the calcitonin/CGRP 18-bp region (40, 41). It is possible that a thyroid C cell-specific member of the AP-4/Pan family may transactivate the calcitonin/CGRP promoter. Alternatively, the HLH site present in the 18-bp enhancer region may be activated by the MASH (mammalian achaete-scute homolog) HLH transcription factor, which is expressed in some neural crest cells (42). Preliminary studies have shown that MASH1 is expressed in CA77 cells and can transactivate the 18-bp element.³

³ L. A. Tverberg, J. E. Johnson, D. J. Anderson, and A. F. Russo, unpublished observations.
should be noted that the 18-bp element has also recently been shown to mediate retinoic acid dependence of the calcitonin/CGRP gene in CA77 cells. In addition, vitamin D inhibits calcitonin/CGRP expression in vivo (43). Since receptors for these three hormones are all members of the steroid receptor superfamily, it should prove interesting to determine whether these hormones repress transcription by a common mechanism involving negative interactions with AP-1 or with a neuroendocrine-specific transcription factor.

It is tempting to speculate that cell-specific regulation of the gene in tumor cell lines may reflect tissue-specific regulation of the gene in vivo. We and others have seen that dexamethasone or corticosterone treatment increases calcitonin expression in the rat thyroid (14) while not increasing CGRP mRNA levels in the brainstem. While the physiological significance of glucocorticoid regulation of the calcitonin/CGRP is not clear, it may be important in the regulation of bone and mineral metabolism. Calcitonin is used therapeutically to treat osteoporosis, which can be caused by excess these three hormones are all members of the steroid receptor superfamily. It should prove interesting to determine whether these hormones repress transcription by a common mechanism involving negative interactions with AP-1 or with a neuroendocrine-specific transcription factor.

Acknowledgments—We thank Dr. F. Zeytin for generously providing the 44-2C cell line and Drs. C. Glass, S. Adler, R. Evans, R. Maurer, and M. G. Rosenfeld for plasmids. We also thank Tom Lanigan and Dr. R. Maurer for helpful discussions and Dan Wehr for his technical assistance.

References


* T. M. Lanigan and A. F. Russo, submitted for publication.