Dexamethasone Differentially Affects the Levels of Calcitonin and Calcitonin Gene-related Peptide mRNAs Expressed in a Human Medullary Thyroid Carcinoma Cell Line*

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The TT cells are a continuous line of human C-cells derived from a medullary thyroid carcinoma. These cells produce large quantities of calcitonin and calcitonin gene-related peptide (CGRP) by the differential splicing of a single calcitonin gene transcript. We have used specific cDNA probes for calcitonin and CGRP to study the regulation of the calcitonin gene by dexamethasone, a synthetic glucocorticoid. Northern blot analysis of total cellular RNA isolated from the TT cells showed hybridization of the calcitonin probe to 3600- and 1000-base RNA species. The CGRP probe hybridized to 3600- and 1050-base RNA species. Dexamethasone treatment (10^{-6} to 10^{-8} M) of TT cells (for 6 days) caused a dosage-dependent increase in calcitonin mRNA levels and a decrease in CGRP mRNA levels. These findings were confirmed in time course studies where dexamethasone treatment (10^{-8} M) caused a 2-13-fold increase in calcitonin mRNA and a 40-60% decrease in CGRP mRNA between 4 and 6 days of treatment; the effect was reversible after dexamethasone withdrawal. After excluding an effect of dexamethasone on calcitonin and CGRP mRNA stability, we have concluded that dexamethasone affects the splicing mechanism to favor production of calcitonin mRNA over CGRP mRNA.

The calcitonin gene is novel in that a single gene and RNA primary transcript code for the production of two unique peptides (1-3). Therefore, the regulation of calcitonin gene expression is controlled at multiple levels (1-7). At the level of transcription, expression of the calcitonin gene is enhanced by phorbol esters (4), cAMP analogues (5), and possibly 1,25-dihydroxyvitamin D_3 (6) and dexamethasone (6). The next step at which the calcitonin gene could be regulated is at the level of RNA processing where the primary calcitonin RNA transcript is differentially spliced to produce two unique mRNAs (1-3). Differential splicing and polyadenylation site choice result in the production of either calcitonin mRNA or a different mRNA coding for a peptide called CGRP (1, 7). The two mRNAs are composed of a common 5' region and differ in their specific peptide coding regions and untranslated 3' sequences. The decision to produce calcitonin mRNA or CGRP mRNA appears to be regulated in a tissue-specific manner. In the thyroid C-cell, calcitonin mRNA predominates whereas CGRP mRNA is produced in neural tissue (1, 8). However, this splicing decision is not absolute. The normal thyroid C-cell produces small amounts of CGRP in addition to calcitonin (9). Several tumor types, including carcinoma of the lung and medullary thyroid carcinoma as well as cell lines derived from these tumors, have been shown to produce both peptides (3, 10-12). In this study we have utilized a cell line which produces approximately equal quantities of both calcitonin and CGRP (13). The TT cell line is a continuous human C-cell line which was derived from a medullary thyroid carcinoma (13). We examined the effect of the glucocorticoids on the expression of calcitonin and CGRP mRNA and found dexamethasone to enhance calcitonin mRNA levels while lowering CGRP mRNA, indicating a probable role for glucocorticoids in the regulation of RNA splicing in this cell line.

**Experimental Procedures**

**Cell Culture Methodology**—The TT cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Laboratories), using previously described techniques (14). A fibroblast cell line OB 17 served as a control (15). Cell counts were determined with a Coulter Counter (model ZF), as previously described (14). Dexamethasone was added to the medium as a 25 mM stock prepared in ethanol. Control cells received an equal volume of ethanol. Cell viability was determined by exclusion of 0.04% trypan blue. Greater than 99% viability was observed for all treatment conditions.

**Preparation of RNA**—Total cellular RNA was isolated from various cell types by the guanidine isoothiocyanate procedure (16). The TT cells were plated at an initial density of 3 x 10^5 cells/100-mm dish. The cells were allowed to attach for 24 h prior to treatment. At indicated times medium was removed from the cells and 1.5 ml of 4 M guanidine isothiocyanate, 0.5% sodium isoury sacrose, 25 mM sodium citrate, pH 7.0, and 0.1 M 2-mercaptoethanol were added to lyse cells and denature proteins. Cellular debris was scraped from flasks or dishes and the lysate was passed through a 23-gauge needle to shear the DNA. RNA pellets were obtained by centrifugation through 5.7 M CsCl as previously described (16). The RNA pellets were dissolved in sterile distilled H_2O, and after the A_260 was determined, vanadium-riboside complex (17) was added (final concentration 10 mM), and the sample volume was adjusted accordingly.

**Hybridizations**—RNA dot blot hybridizations were performed by

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2The abbreviations and trivial names used are: CGRP, calcitonin gene-related peptide; dexamethasone, 8a-fluoro-16a-methyl prednisolone; estradiol, 1,3,5(10)-estratrien-3,17-β-diol; kb, kilobase.

3In the TT cell line, secreted values range from ~100-600 ng/48 h/10^5 cells for calcitonin; ~50-40 ng/48 h/10^5 cells for CGRP, while intracellular values are slightly higher (G. J. Cote, J. A. Gould, and R. F. Gagel, manuscript in preparation).
immobilization of purified total RNA (0.625, 1.25, 2.5, and 5 μg) onto nitrocellulose paper in the presence of 20 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) as described by Thomas (18). Northern blot hybridizations were performed by fractionation of total RNA (15 μg) on formaldehyde agarose gels as described by Lehrach et al. (19), followed by capillary transfer of RNA to nitrocellulose as described by Thomas (18). The calcitonin-specific probe was the BglII/NsiI fragment of the rat pCal plasmid (kindly provided by J. W. Jacobs, Merck) (20). This fragment was labeled by random priming and new DNA was synthesized by the addition of Klenow fragment polymerase in the presence of 0.5μScNPdCTP (21). CGRP mRNA levels were measured using an end-labeled 41-base synthetic oligomer which codes for amino acids 3–16 (2). End-labeling was performed using T4 kinase as previously described (22). Prehybridization, hybridization, and washing procedures were identical to those of Thomas (18). Generally 1–2 × 10⁶ cpm/ml labeled cDNA probe was used in the hybridization solution. The end-labeling of the CGRP oligomer resulted in cDNA probes of lower specific activity compared to calcitonin resulting in a lower hybridization signal strength.

Autoradiographs were quantitated by computer-assisted densitometric scanning with a video camera as described by Mariash et al. (23). The range over which measurements of intensity were linear was determined for our instrument to ensure accurate quantification of our results.

Measurement of RNA Stability—The stability of calcitonin and CGRP mRNA were measured by actinomycin D chase as previously described by Dani et al. (24). The TT cells were plated as above at an initial density of ~3 × 10⁵ cells/100-mm dish. The cells were allowed to attach 24 h prior to treatment and then received either control medium or medium containing 1 × 10⁻⁶ M dexamethasone for 5 days with medium changed at 48-h intervals. On day 4 actinomycin D (4 μg/ml) was added to treated and control dishes. Total RNA was isolated from these dishes at the time of actinomycin D addition and at 1, 3, 6, 12, and 24 h after addition, and RNA dot hybridization was performed to determine mRNA levels as described above.

RESULTS AND DISCUSSION

Calcitonin and CGRP are produced in approximately equal amounts in the TT cell line. The production of both calcitonin and CGRP mRNA by the TT cell line makes it an ideal model system in which to study the regulation of the splicing event because there are easily detectable amounts of both calcitonin and CGRP mRNA. Fig. 1 shows Northern hybridization of TT cell total RNA with our probes. The calcitonin probe recognized a 1-kb band and a 3.6-kb precursor band, whereas the CGRP probe revealed a 1.05-kb band and a precursor band of identical size to the calcitonin RNA precursor. Neither probe hybridized to control RNA isolated from a fibroblast cell line. These findings agree with the previously reported calcitonin and CGRP mRNA sizes for this cell line with the exception that CGRP mRNA has been reported to be only 1 kb (12). The 3.6-kb fragment corresponds in size to a partially processed common precursor (3, 11).

Previous studies in our laboratory show the synthetic glucocorticoid dexamethasone to inhibit the transcription of the somatostatin gene in the TT cell line (25). We wanted to see if dexamethasone had a similar effect on calcitonin and CGRP mRNA production. TT cells were treated with a range of dexamethasone concentrations for a period of 6 days and then calcitonin and CGRP mRNA levels were measured by dot hybridization (Fig. 2). The serum-containing medium used to grow the TT cells contained approximately 10⁻¹⁰ M endogenous cortisol (as determined by radioimmunoassay), and for

**Fig. 1. Northern analysis of calcitonin and CGRP mRNA in TT cells.** Total RNA was isolated from TT cells (TT) and OB 17 fibroblast control cells (OB). The total RNA (15 μg) was fractionated on a formaldehyde-agarose gel and transferred to nitrocellulose for hybridization with either a calcitonin or CGRP cDNA probe (see “Experimental Procedures”). Values given indicate RNA size in bases relative to λ HindIII markers.

**Fig. 2. Dose-response curve for the effect of dexamethasone on calcitonin (CT) and CGRP mRNA production.** The TT cells were plated at an initial density of ~3 × 10⁵ cells/100-mm dish and allowed to attach in growth medium (24 h), prior to changing to medium containing the indicated concentration of dexamethasone. Growth was continued in the presence of the indicated concentration of dexamethasone for 6 days with medium changed at 48-h intervals. The cell number was determined by use of a Coulter Counter. Each number represents the mean ± S.E. The values for mRNA measurement represent the average of two dishes as determined by dot hybridization and quantitated by a computer-assisted densitometer.
this reason exogenous steroid was limited to concentrations greater than this. Dexamethasone caused a dose-related increase of calcitonin mRNA and decrease of CGRP mRNA; these effects were maximal for calcitonin mRNA at $1 \times 10^{-5}$ M and $1 \times 10^{-6}$ M for CGRP mRNA. In this experiment and in similar experiments, somatostatin mRNA was undetectable at dexamethasone concentrations greater than $1 \times 10^{-5}$ M (data not shown).

Fig. 3 shows the time course of dexamethasone action on calcitonin and CGRP mRNA levels. Within 48 h after initiation of treatment with dexamethasone there was an increase of calcitonin mRNA which reached a maximum 6-8 days after onset of treatment (Fig. 3A). CGRP mRNA levels decreased 50% over the same time course (Fig. 3B). Withdrawal of dexamethasone from treated cells causes mRNA levels to return to the levels of control. In the control cells the levels of both calcitonin and CGRP mRNA rise during the first 4 days of the experiment followed by a decline to original levels. We have observed this pattern in other experiments and it suggests that there may be a cell growth- or density-dependent expression of these genes in this cell line. TT cells treated in a similar manner with estradiol show no difference in calcitonin and CGRP mRNA levels compared to controls (data not shown).

Northern blot analysis was performed on total RNA isolated from cells treated with dexamethasone for 6 days to determine the effect of steroid treatment on precursor and mature RNA levels. Fig. 4 shows the specific enhancement of both mature and precursor RNA for calcitonin and specific inhibition of CGRP mRNA. The densitometric scanning of the precursor band in this and other Northern blots suggests a 2-4-fold enhancement of precursor after dexamethasone treatment. Unfortunately, we have been unable to quantitate the effect of dexamethasone on the precursor using the CGRP cDNA because of the lower specific activity of this probe. The densitometric scanning of calcitonin and CGRP mRNA bands yielded the same results obtained by dot hybridization.

In this paper we have demonstrated that dexamethasone

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**Fig. 3.** The time course and reversibility of dexamethasone treatment on calcitonin (CT) and CGRP mRNA production in the TT cells. TT cells were grown in the presence (●) or absence (○) (control) of $1 \times 10^{-6}$ M dexamethasone using the experimental protocol described in Fig. 2. On day 4, dexamethasone was withdrawn (▲) from one group of treated cells. Medium was changed at 48-h intervals. RNA measurements were made at indicated time points after initiation of treatment as described in Fig. 2. The hybridization value obtained for day 0 served as point of reference for the determination of all other values. **Dex,** dexamethasone.

**Fig. 4.** Northern analysis of RNA isolated from dexamethasone-treated TT cells. Northern blot analysis was performed on total RNA (15 μg) isolated from TT treated for 6 days with $10^{-6}$ M dexamethasone (D) or a paired control (C). Molecular weights were determined relative to λ HindIII markers.
enhances calcitonin while simultaneously depressing CGRP mRNA levels. These results might be explained in a number of different ways. First, the effect of glucocorticoids on gene transcription is well documented. Indeed the increased level of calcitonin precursor RNA during treatment suggests dexamethasone may in fact be acting to stimulate transcription of the calcitonin gene, a mechanism previously shown in the rat C-cell (6). However, if the effect were entirely transcriptional we would expect to find a parallel increase in both populations of cells to occur we would expect calcitonin and CGRP to remain unchanged by the removal of dexamethasone. Therefore, after evaluating all these possibilities we have concluded it is likely that dexamethasone acts both to increase the transcription of the calcitonin gene and alter the splicing of the RNA precursor to favor the production of calcitonin mRNA.

We can envision at least three mechanisms by which dexamethasone could act to affect the RNA splicing machinery. The first is by direct transcriptional regulation of factors involved in splicing. RNA splicing requires several proteins and small nuclear RNAs any of which might be targets for glucocorticoid regulation (27). Second, a cell cycle-dependent effect on RNA splicing is also possible. We have found that the treatment of TT cells with dexamethasone causes growth arrest and the cell cycle-dependent expression of genes has been previously shown (28). Finally, it is possible that dexamethasone, a known differentiating agent (29), is stimulating a state which more closely resembles that of the normal thyroid C-cell in which calcitonin production predominates. For each of these possibilities, however, the final point of regulation must somehow involve the actual RNA splicing process. Future studies will require a more direct examination of the effects of dexamethasone on RNA splicing.

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REFERENCES

Differential Expression of Calcitonin and CGRP mRNA