cAMP signaling can antagonize potent glucocorticoid post-transcriptional inhibition of stanniocalcin gene expression

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Abstract

Stanniocalcin (STC) is a glycoprotein hormone first discovered in fish as a homeostatic regulator of calcium and phosphate transport; it has recently been discovered in mammals, in which it appears to have a similar role. It has also been implicated in a number of different physiological processes through correlative studies, but the factors regulating its production have not been elucidated. In this report, we show that steady-state STC mRNA levels in the mouse corticotrope tumor line, AtT-20, were exquisitely sensitive to glucocorticoids. Hydrocortisone and dexamethasone (Dex) induced a dramatic reduction in steady-state STC mRNA levels in AtT-20 cells through a post-transcriptional mechanism. Similarly, glucocorticoids down-regulated STC mRNA levels in the human fibrosarcoma cell line, HT1080. The specificity of the

glucocorticoid-mediated decrease in STC mRNA abundance was shown using the glucocorticoid receptor antagonist, RU-486. Activation of the cAMP-signaling pathway in glucocorticoid-cultured AtT-20 cells transiently restored STC gene expression. Treatment of AtT-20 cells with the transcriptional inhibitor, actinomycin D, rescued steady-state STC mRNA levels from Dex-induced repression, indicating that the Dex-mediated decrease in STC gene expression requires current gene transcription. Taken together, these results describe a unique model system in which cAMP-stimulated events can reverse post-transcriptional repression of gene expression by glucocorticoids.

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Introduction

Stanniocalcin (STC) is a secreted homodimeric glycoprotein originally discovered in bony fishes, in which it has a critical role in regulating serum Ca^{2+} and PO_4^{2-} homeostasis (Wagner 1994). STC protects fishes from increased plasma Ca^{2+} concentrations through inhibition of Ca^{2+} uptake by the gills and gut (Wagner *et al.* 1986, 1988, Butkus *et al.* 1987, Sundell *et al.* 1992), and by increasing PO_4 reabsorption by the kidneys (Lu *et al.* 1994). The production and secretion of STC by the corpuscles of Stannius in fish are, in turn, controlled primarily by serum Ca^{2+} concentrations (Wagner *et al.* 1989, 1991).

Mammalian STC shares approximately 73% amino acid sequence homology with fish STC, implying that its function might be highly conserved across species (Chang *et al.* 1995, 1996, Olsen *et al.* 1996, Varghese *et al.* 1998). Indeed, the hormone appears to regulate the movement of Ca^{2+} and PO_4 in mammalian kidney and intestine (Wagner *et al.* 1997, Madsen *et al.* 1998). There is good evidence indicating that STC regulates mineral

metabolism, but little is understood with regard to its function in tissues not associated with mineral homeostasis. Recently, numerous reports have implicated STC in different biological processes by associating an up-regulation of its steady-state mRNA levels with a particular physiological state or event. An increase in STC mRNA levels was correlated with atherogenic treatment of human umbilical vein endothelial cells (Sato *et al.* 1998), *in vitro* differentiation of endothelial cells into vessels (Kahn *et al.* 2000), fibroblast responsiveness to serum-borne growth factors (Iyer *et al.* 1999), and *in vitro* differentiation of a neuronal cell line (Zhang *et al.* 1998b). Collectively, these reports indicate that STC production is sensitive to a multiplicity of biological stimuli and STC may thus contribute to the control of diverse physiological processes. However, the inter- and intracellular messengers that are responsible for increasing steady-state levels of STC mRNA described above have not been delineated.

We have shown that STC production is widespread in the adult mouse and is highly expressed in a variety of tissues throughout mouse development (Varghese *et al.* 1998, Stasko & Wagner 2001). An understanding of how

the STC gene can be regulated will be critical to our understanding of its function. With regard to hormonal regulation, only one report exists that showed that STC mRNA levels could be up-regulated in whole kidney tissue by vitamin D (Honda *et al.* 1999). To identify suitable cell-line models and discover factors capable of regulating STC transcription and production, we tested more than 20 different cell lines by northern analysis and found that STC was highly expressed by those of pituitary origin. In particular, the mouse corticotrope tumor line, AtT-20, produces the highest levels of STC. We have also shown that the STC gene is active in mouse pituitary and, on the basis of our cell-line survey, STC is probably expressed by all hormone-producing cell types of the intermediate and anterior lobes.

Much of our knowledge regarding the regulation of pro-opiomelanocortin (POMC), in addition to the synthesis and post-translation processing of adrenocorticotropin, has been obtained using AtT-20 cells (Bilezikjian *et al.* 1991, Horiba *et al.* 1993, Therrien & Drouin 1993). To understand the mechanisms controlling production of STC and thereby gain insight into its potential functions, we assessed the effects of various hormonal agents and intracellular secondary messengers on STC production by AtT-20 cells. Our study provides the first direct evidence that steady-state STC mRNA levels can be dramatically down-regulated by glucocorticoids, and that activation of the cAMP-signaling pathway and inhibition of *de novo* gene transcription can abrogate glucocorticoid inhibition of STC mRNA levels. The widespread expression of STC in mammals, coupled with the fact that glucocorticoids are essential physiological regulators, suggests that the results presented here may reflect the regulatory role of glucocorticoids at several sites of STC production.

Materials and Methods

Materials

OptiMEM cell culture medium and fetal bovine serum (FBS) were purchased from Life Technologies (Burlington, Ontario, Canada). UltraMEM and Ultra-Culture media were obtained from BioWhittaker, Inc. (Walkersville, MD, USA). Actinomycin D (Act D), α -amanitin, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), cycloheximide (CHX), dexamethasone (Dex), 2 α -dihydrotestosterone (DHT), 17 β -estradiol, forskolin, human/rat corticotropin releasing factor (CRF), progesterone, and the glucocorticoid receptor (GR) antagonist RU-486 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aprotinin, 8-bromo-cAMP (8Br-cAMP), leupeptin, phenylmethylsulfonyl fluoride (PMSF), and RNase A were obtained from Roche Diagnostics (Laval, Canada). Proteinase K was obtained from Life Technologies and RNase-free DNase I was purchased from Worthington Biochemical Corp.

(Freehold, NJ, USA). Stock solutions of Dex, DHT, β -estradiol, progesterone, and RU-486 were prepared in ethanol, those of Act D and forskolin were made in dimethylsulfoxide, and 8Br-cAMP, CHX and CRF were dissolved in water.

The various cDNAs used for northern analysis included: a 500 bp mouse POMC cDNA fragment in pBKSII from Dr M G Rosenfeld (University of California at San Diego, CA, USA), an 800 bp mouse STC cDNA encompassing the entire coding region, pBKSII containing 18S ribosomal DNA (rDNA), and pGEM-4Z carrying a 380 bp rat cyclophilin cDNA consisting of 320 bp of the coding region and 52 bp of 3' untranslated sequence. The 372 bp mouse metallothionein-1 (MT-1) cDNA plasmid was obtained from Dr James Koropatnick (London Regional Cancer Centre, London, Canada). The plasmid pZL1, containing a 1.6 kb mouse Dexras1 cDNA was the generous gift of Dr Robert J Kempainen (Auburn University College of Veterinary Medicine, Auburn, AL, USA).

Cell culture

The AtT-20 cell line was initially obtained from Dr M G Rosenfeld's laboratory (University of California at San Diego). The original AtT-20 cell line (CCL-89) and the adherent variant (D16 v-F2) were purchased from the ATCC (Rockville, MD, USA). Adherent AtT-20 D16 v-F2 cells and HT1080 cells were passaged by trypsinization using 0.25% trypsin and 0.53 mM EDTA and grown in OptiMEM supplemented with 10% FBS (OptiMEM/FBS). The CCL-89 cells grew in suspension and were passaged by dilution in OptiMEM/FBS. For studies evaluating the effects of steroids or other compounds on STC mRNA levels, cells were plated at a density of 1×10^6 cells (or 3×10^6 cells) per T-25 cm² (or T-75 cm²) flask in OptiMEM/charcoal-stripped FBS or in UltraMEM supplemented with 10% FBS (UltraMEM/FBS). After 3 days, fresh medium was added containing the following agents or combinations thereof: 10 μ g/ml Act D, 5 mM 8Br-cAMP, 1 μ g/ml CHX, 10^{-7} M CRF, 10^{-7} M Dex, 10^{-7} M DHT, 10^{-7} M 17 β -estradiol, 10^{-5} M forskolin, 10^{-7} M progesterone, and 10^{-6} M RU-486. At selected times after treatment, cells were harvested for RNA extraction by trypsinization. To assess the effect of different culture media on STC mRNA production, cells were seeded at a density of 3×10^6 cells per T-75 cm² flask in OptiMEM/FBS. After 3 days, cultures were washed and the media replaced with UltraMEM/FBS. Alternatively, cells were grown in UltraMEM/FBS for 3 days, washed three times, and the media replaced with OptiMEM/FBS. In both experiments, cells were harvested at various times after the media switch, for RNA extraction. In experiments assessing the growth potential of AtT-20 cells, triplicate cultures were plated at a density of 1×10^5 cells per T-25 cm² flask in either OptiMEM/FBS or UltraMEM/FBS and grown continuously for 0 to 7 days.

At each time point, cells were harvested and counted using a hemocytometer. In addition, the growth potential of AtT-20 cells in OptiMEM/FBS was simultaneously compared with changes in *STC* mRNA expression. Briefly, AtT-20 cells were seeded at 3×10^5 cells per T-75 cm² flask in OptiMEM/FBS, cultured continuously for 0 to 7 days, and then harvested for RNA isolation.

RNA extraction and Northern analysis

Total RNA was extracted using Trizol (Life Technologies) according to the manufacturer's instructions. For most experiments, 50 µg total RNA was analyzed on 1% agarose containing 20 mM 3-morpholinopropanesulfonic acid, 5 mM sodium acetate and 1 mM EDTA, and 0.37 M formaldehyde, transferred to Amersham Hybond-N nylon membranes (Amersham Pharmacia Biotech, Baie-d'Urfe, Quebec, Canada), and the blots baked for 2 h at 80 °C. Baked blots were hybridized overnight at 65 °C in 5 ml 0.5 M phosphate buffer pH 7.2, containing 7% (w/v) SDS, and 10 mM EDTA with 15×10^6 c.p.m. of the indicated probe. Radiolabelled DNA probes were prepared with [α -³²P]deoxy-CTP (3000 Ci, mmol; Amersham Pharmacia Biotech) using the Ready-to-Go DNA labeling beads (deoxy-CTP) from Amersham Pharmacia Biotech. Labeled DNA was separated from unincorporated radionucleotide using Sephadex G-50 columns (Amersham Pharmacia Biotech). After hybridization, blots were washed twice in $2 \times$ SSC and 0.1% SDS for 15 min at room temperature, and then with $0.1 \times$ SSC and 0.1% SDS for 30 min at 65 °C. Results were visualized by autoradiography, and quantitative analysis was performed using a PhosphorImager and the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA, USA). Blots were stripped with boiling 0.5% SDS for 5 min, and subsequent hybridization was conducted as above with ³²P-labelled cDNA. Northern blots performed with radiolabeled cyclophilin cDNA or 18S rDNA provided RNA loading controls to which *STC*, *MT-1*, *POMC* and *Dexas1* mRNA signals were normalized as a ratio. For the analysis of steady-state *STC* mRNA levels, the signal intensity of the major 4 kb transcript was determined as a measure of *STC* mRNA steady-state levels. All experiments were performed two to five times and data shown are representative of reproducibly obtained results.

Nuclear run-on transcription

The effect of Dex on the rate of gene transcription was determined using a nuclear run-on assay (Greenberg & Bender 1997) in five separate experiments. Briefly, AtT-20 cells were seeded at a density of 7×10^6 cells per 175 cm² flask in OptiMEM/charcoal-stripped FBS. After 3 days, cells were cultured for 24 h in fresh medium with or without 10^{-7} M Dex. After the above treatment, cells

were scraped into ice-cold PBS, pelleted at 4 °C, and lysed in buffer containing 10 mM Tris HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40 (NP-40). After 10 min at 4 °C, nuclei were counted using a hemocytometer, washed twice by centrifugation at 500 *g*, and then resuspended in reaction buffer (5 mM Tris HCl, pH 8.0, 2.5 mM MgCl₂, and 0.15 M KCl). A 400 µl volume of 5×10^7 nuclei was incubated for 30 min at 30 °C in reaction buffer supplemented with 2.5 mM dithiothreitol, 120 µCi [α -³²P]CTP (3000 Ci, mmol; Amersham Pharmacia Biotech), and 0.5 mM each of ATP, GTP and UTP. The reactions were stopped with 600 µl of a solution containing 42 µg/ml RNase-free DNase I in 10 mM Tris HCl, 0.5 M NaCl, 50 mM MgCl₂, and 2 mM CaCl₂. After 5 min at 30 °C, a 200 µl volume of solution containing 10 mg/ml proteinase K in 0.5 M Tris HCl, pH 7.6, 0.25 M EDTA, and 5% SDS was added to the reaction mixture. After 30 min at 42 °C, total RNA was extracted using Trizol as described above, and radio-labeled transcripts were purified from unincorporated nucleotides using Sephadex G-50 columns.

Plasmid DNAs (5 µg), containing cyclophilin, *STC*, *POMC*, and *MT-1* cDNA, 18S rDNA fragments, or pBKSII vector alone were linearized, denatured in 0.25 M NaOH for 5 min at 25 °C, and neutralized in 0.4 M Tris HCl, pH 7.6. Denatured plasmid DNAs were treated with equivalent volumes of $20 \times$ SSC and dot-blotted onto nitrocellulose membrane (Micron Separations, Inc., Westborough, MA, USA). After baking, blots were hybridized with approximately 4×10^6 c.p.m. of α -³²P-labeled AtT-20 RNA at 65 °C overnight in 2.5 ml of hybridization buffer used for northern blotting. Subsequently, blots were washed twice in 40 mM Na₂HPO₄ buffer and 0.1% SDS for 20 min at room temperature, and then with $2 \times$ SSC supplemented with 0.01 mg/ml RNase A at 37 °C, followed by 40 mM Na₂HPO₄ and 0.1% SDS at 37 °C. Results were analyzed as above for northern blotting.

Western blot analysis

The amounts of AtT-20 cell intracellular and secreted *STC* proteins were assessed by western blots. Cells were seeded at a density of 1×10^6 cells per 25 cm² flask in OptiMEM/FBS. After 3 days, cells were washed and then cultured in OptiMEM alone with or without 10^{-7} M Dex for various time periods. For secreted protein, conditioned medium was collected and then concentrated approximately fivefold using a centrifugal filter (Amicon, Beverly, CA, USA). For intracellular protein, cells were harvested from flasks using PBS containing 0.53 mM EDTA, pelleted by centrifugation at 500 *g*, and then lysed in buffer containing 50 mM Tris HCl, pH 8.0, 20 mM EDTA, pH 8.0, 50 mM sodium fluoride, 200 µM sodium orthovanadate, 1 mM PMSF, 10 µg/ml each aprotinin and leupeptin, and 1% NP-40. After 15 min at 4 °C, insoluble

material was removed by spinning the lysates at 13 000 *g* for 15 min and the post-nuclear supernatants collected. Protein concentrations of post-nuclear supernatants were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Fifty micrograms of post-nuclear supernatants or 60 µl of concentrated conditioned medium were resolved on 8% non-reducing SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Boehringer Manneheim). As a positive control, recombinant human *STC* was included on the protein blots. Blots were probed with rabbit anti-human *STC* polysera (De Niu *et al.* 1998), followed by horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech), and subsequently developed with Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA, USA).

Cell transfection and reporter gene assay

AtT-20 cells were seeded at a density of 3×10^5 cells per six-well plate in OptiMEM/FBS and cultured for 24 h. After incubation, cells were transfected for 24 h with ExGen 500 (Euromedex, Mundolsheim, France) and various luciferase reporter plasmids built with pGL3-Basic (Promega, Madison, WI, USA). The following luciferase reporter vectors were transfected in triplicate: pCMV-Luc, a positive control vector containing the cytomegalovirus (CMV) enhancer and immediate early promoter fused to the luciferase gene; pGL3-STC669, a pGL3 plasmid containing 669 bp of *STC* immediate 5'-flanking DNA, and pGL3-basic, which contains a promoterless luciferase gene as the negative control. After transfection, cells were washed twice with PBS, and then incubated for 24 h in OptiMEM/charcoal-stripped FBS with or without 10^{-6} M Dex, or UltraMEM. Luciferase activity was then measured in lysates using the Luciferase Assay System (Promega) and Berthold Lumat LB 9507 luminometer.

Results

Steady-state *STC* mRNA levels in AtT-20 cells are exquisitely sensitive to specific media

To determine whether expression of *STC* could be modulated by different growth conditions, we tested a variety of culture media in the absence or presence of FBS. Surprisingly, we observed widely different levels of *STC* mRNA that correlated with AtT-20 cell growth in specific cell culture media. AtT-20 cells demonstrated robust *STC* gene activity when grown in either OptiMEM or UltraCulture media (Fig. 1). In contrast, *STC* mRNA was barely detectable in AtT-20 cells cultured with UltraMEM and was quantified at 3–10% concentrations found in cells grown in either OptiMEM or

Figure 1 Steady-state *STC* mRNA levels in AtT-20 cells is significantly regulated by the culture medium. (A) AtT-20 cell lines from the ATCC (CCL-89 cells grow in suspension, D16 v-F2 are adherent) and from Dr M G Rosenfeld (AtT-20), were cultured to confluency in OptiMEM (OM), UltraMEM (UM) or UltraCulture (UC), each supplemented with 10% FBS. The representative autoradiographs show the mRNA abundance of *STC* and POMC relative to cyclophilin (CP). (B), (C) Graphical representations of the signal intensity for *STC* and POMC mRNA from each cell line in panel (A) after normalization to those for cyclophilin mRNA plotted as the percent change in the *STC*:CP and POMC:CP ratios from the values obtained in OptiMEM-cultured cells. Bars correspond to and are aligned with each lane of panel (A).

UltraCulture, despite the presence of 10% FBS and the fact that UltraMEM is considered to be a nutrient-rich medium for serum-reduced cell culture. These results were confirmed with fresh AtT-20 cells (D16 v-F2) from the ATCC and the original AtT-20 line (CCL-89) from which the adherent variant was derived. In all three cases,

drastically reduced *STC* mRNA levels were observed when the cells were cultured continuously in UltraMEM, indicating that the UltraMEM-mediated repression of *STC* gene expression was not unique to our AtT-20 cell line and was not mediated by FBS. Interestingly, *STC* gene expression in the original non-adherent AtT-20 cells was significantly lower than that seen in the adherent cell line. Moreover, the steady-state *POMC* mRNA levels in all AtT-20 cell lines were reduced two- to fivefold when cells were grown in UltraMEM compared with the other media. One explanation for the reduction in *POMC* gene expression is that UltraMEM contained glucocorticoids, as it is well established that corticosteroids exert negative feedback repression on *POMC* transcription and ACTH release from corticotropes, including AtT-20 cells (Drouin *et al.* 1989, 1993). It should be noted that the composition of these nutrient-rich media was not available.

In addition to the reduction in *STC* and *POMC* mRNA levels, UltraMEM induced other distinct morphological changes in the adherent AtT-20 corticotrope cell line. In OptiMEM, AtT-20 cells consistently appeared as large clusters of rounded cells that grew on top of cells with a stellate morphology, whereas they adopted a singular stellate morphology in UltraMEM (Fig. 2A). In addition to radical alterations in morphology, these nutrient-rich media had a significant impact on AtT-20 cell growth. Analysis of the proliferation rate over a 7-day period indicated that AtT-20 cells grew three to nine times more slowly in UltraMEM compared with parallel cultures in OptiMEM (Fig. 2B). This result was also another indication that glucocorticoids were present in UltraMEM, because they have been demonstrated to attenuate AtT-20 cell growth (van Wijk *et al.* 1995). We also observed that the exponential growth of AtT-20 cells in OptiMEM (Fig. 2B) was accompanied by an increase in steady-state *STC* mRNA levels (Fig. 2C–D). During this period of rapid growth, the abundance of *STC* mRNA gradually increased, to be six- to 19-fold greater at maximum cell density on day 7 compared with day 0 (Fig. 2C–D). In contrast, there were no significant increases in *POMC* mRNA levels during this period of cell growth (Fig. 2C–D). Taken together, these results suggest that AtT-20 cells are significantly altered in their growth potential, morphology and steady-state level of *STC* mRNA when cultured in the presence of different nutrient-rich media.

The apparent loss of STC gene expression in UltraMEM is reversible

Further investigation was performed to determine whether the virtually complete abrogation of *STC* mRNA expression in AtT-20 cells, mediated by UltraMEM, was reversible. To test this, AtT-20 cells were cultured in UltraMEM, washed extensively to remove factors in UltraMEM that were potentially down-regulating *STC*

gene expression, and then switched to OptiMEM. Alternatively, cells were cultured in OptiMEM, washed, and then switched to UltraMEM. In two independent experiments, the switch from UltraMEM to OptiMEM resulted in a profound eight- to 36-fold increase in *STC* mRNA abundance (Fig. 3A), whereas the reverse switch caused a 6- to 13-fold decrease and almost complete loss of *STC* mRNA (Fig. 3B). These results clearly suggested that a component of UltraMEM was responsible for significantly down-regulating *STC* and *POMC* gene expression.

Dexamethasone induces dramatic down-regulation of STC gene expression in AtT-20 cells

As mentioned above, the down-regulation in *STC* and *POMC* gene expression observed in AtT-20 cells cultured in UltraMEM may have reflected the presence of glucocorticoids (Drouin *et al.* 1989, 1993). To test this, we examined the effect of Dex on the abundance of *STC* and *POMC* mRNA levels in AtT-20 cells cultured in OptiMEM in nine independent experiments (Fig. 4). Dexamethasone dramatically reduced steady-state *STC* mRNA levels within 4 h; this effect was routinely maximal at 24 h, with a five- to 22-fold loss of *STC* mRNA signals in the presence of 10^{-7} M Dex. Consistent with reports demonstrating the sensitivity of *POMC* gene expression to Dex (Drouin *et al.* 1989, 1993), *POMC* mRNA levels were consistently reduced twofold by 48 h (Fig. 4). These results clearly indicated that *STC* gene activity was much more sensitive to glucocorticoid repression than was the *POMC* gene. As a further control for glucocorticoid activity, we evaluated the expression of the *Dexras1* gene, a member of the Ras superfamily of *GTPase* genes, as it has been shown to be rapidly induced in AtT-20 cells upon addition of Dex (Kemppainen & Behrend 1998). Consistent with a previous report (Kemppainen *et al.* 1998), *Dexras1* mRNA accumulation was significantly induced in AtT-20 cells, routinely reaching 200- to 300-fold greater values within 3 h after Dex treatment (Fig. 4). The induction of *Dexras1* gene expression preceded the reduction in *STC* mRNA levels and confirmed the rapid gene-stimulatory effects of glucocorticoids on AtT-20 cells.

Given the dramatic changes induced by the synthetic glucocorticoid on *STC* mRNA abundance, we investigated whether other steroid hormones could induce similar changes in AtT-20 cells. In two independent experiments, the hormones DHT, 17β -estradiol and progesterone produced only modest (less than twofold) changes in *STC* mRNA levels, compared with the five- to eightfold reduction by Dex (Fig. 5).

To determine whether glucocorticoid repression of *STC* mRNA accumulation was specific to AtT-20 cells, two independent experiments were carried out with the human fibrosarcoma cell line HT1080, which maintains high steady-state *STC* mRNA levels (Chang *et al.* 1995).

Figure 2 Culture medium induces dramatic alterations in morphology, growth rate and *STC* gene expression in AtT-20 cells. (A) Phase-contrast micrographs of AtT-20 cells grown in either OptiMEM/FCS or UltraMEM/FCS. AtT-20 cells appear as large clusters of rounded cells that grow on top of cells with a stellate morphology when cultured in OptiMEM/FCS. In contrast, AtT-20 cells adopt a singular stellate morphology in UltraMEM. (B) AtT-20 cells were seeded at 1×10^5 cells per culture dish containing either OptiMEM/FBS or UltraMEM/FBS and then harvested at the indicated times for cell counting. The plot, which was representative of multiple experiments, compares the rate of growth of AtT-20 cells in OptiMEM (●) and UltraCulture (○). The results shown were the mean $\pm$ S.D. of triplicate samples. (C) Approximately 3×10^5 AtT-20 cells were seeded per culture dish containing OptiMEM/FCS and then harvested for RNA extraction at the times indicated. A typical northern blot shows the steady-state level of *STC* and *POMC* mRNA relative to that of cyclophilin. (D) The change in *STC* and *POMC* mRNA levels in panel (C) were quantitated, normalized to cyclophilin mRNA and plotted as the percent change in the *STC*:CP (●) and *POMC*:CP (○) ratios relative to day 0 values.

The HT1080 cells, like AtT-20 cells, routinely exhibited a significant down-regulation in the abundance of *STC* mRNA, with a maximal fivefold reduction at 24 h after treatment (Fig. 6). Thus the strong repressive effects of glucocorticoids on *STC* gene expression did not occur via a mechanism restricted to AtT-20 cells.

Effects of dexamethasone on *STC* protein in AtT-20 cells

We next assessed whether the repressive effects of Dex on *STC* mRNA abundance in AtT-20 cells resulted in similar changes in *STC* protein concentrations. We simultaneously examined intracellular and secreted *STC* in

Figure 3 Loss of *STC* gene expression in AtT-20 cells under UltraMEM (UM) culture conditions is reversible. (A), (B) AtT-20 cells were cultured for 3 days in OptiMEM/charcoal-stripped FCS (or UltraMEM/FCS), washed, the medium switched to UltraMEM/FCS (or OptiMEM/FCS), and the cells harvested at the indicated times for RNA extraction. (A), (B) Typical autoradiographic results indicating the mRNA levels of *STC* relative to cyclophilin (CP). (C) Graphical representation of the data in panels (A) (UltraMEM to OptiMEM; ●) and (B) (OptiMEM to UltraMEM; ○), showing the change in normalized *STC* mRNA at the indicated times as the percent change from time 0, on a log scale.

Dex-treated cells. Dexamethasone did not affect the abundance of intracellular *STC* protein in the post-nuclear fraction of AtT-20 cells (Fig. 7B). Similarly, AtT-20

Figure 4 Dexamethasone represses *STC* gene expression in AtT-20 cells. AtT-20 cells were treated with 10^{-7} M Dex in OptiMEM/charcoal-stripped FCS for the times indicated and then harvested for RNA extraction. A northern blot, representative of several experiments, shows the steady-state mRNA levels of *STC*, *Dexras1*, and *POMC* relative to cyclophilin.

secretion of *STC* into the culture medium was not perturbed by Dex treatment over the 30-h treatment period, as determined by quantitation of the bands on westerns blots (Fig. 7B). However, further investigation using a human *STC* (hSTC) radioimmunoassay (De Niu *et al.* 2000) found that Dex significantly down-regulated the level of *STC* in conditioned medium by 48 h after treatment (Fig. 7C). Thus the Dex-mediated changes in abundance of *STC* mRNA were accompanied by a significantly delayed reduction in secreted *STC*, possibly reflecting the long half-life of the hormone *STC* under tissue culture conditions.

The GR antagonist RU486 abrogates repression of STC by glucocorticoids

To confirm that Dex repressed *STC* gene expression via activation of the GR, we assessed the ability of the glucocorticoid antagonist, RU486, to overcome the glucocorticoid-mediated reduction in AtT-20 steady-state *STC* mRNA levels. The RU486 treatments were carried out in cells cultured in UltraMEM, in which *STC* mRNA levels are suppressed without the addition of Dex. Glucocorticoid radioimmunoassay of UltraMEM indicated that it contained at least 0.8 μ M hydrocortisone, whereas OptiMEM and UltraCulture do not contain glucocorticoids. In five independent experiments, the GR antagonist routinely increased steady-state *STC* mRNA levels in UltraMEM-cultured AtT-20 cells, indicating that it rescued *STC* gene expression in the presence of glucocorticoid-rich media (Fig. 8A). These findings were supported by data from experiments of RU-486 treatment of AtT-20 cells when cultured in OptiMEM

Figure 5 STC gene expression demonstrates differential responsiveness to steroid hormones. (A) AtT-20 cells cultured in OptiMEM/charcoal-stripped FCS were treated with 10^{-7} M Dex, 10^{-7} M DHT, 10^{-7} M β -estradiol (E), or 10^{-7} M progesterone (P) for 4 and 24 h. The representative autoradiograph shows the abundance of STC mRNA relative to 18S rRNA. (B) Numerical depiction of the normalized mRNA signals in panel (A), indicating the percent change in steady-state STC mRNA levels compared with untreated cells at each time point. Bars correspond to and are aligned with each lane of panel (A). DHT, β -estradiol and progesterone have a minor effect on STC expression, in contrast to the strong inhibition caused by Dex.

supplemented with Dex. The GR antagonist consistently abrogated the Dex-mediated down-regulation of STC mRNA levels in AtT-20 cells (Fig. 8B). Steady-state STC mRNA levels in AtT-20 cells consistently reached 80% of control levels when the cells were treated with both RU486 and Dex for 48 h, compared with the six- to 12-fold decrease in STC mRNA abundance seen in cells treated with Dex alone. RU486 had no significant effect on the accumulation of STC mRNA compared with that in untreated cells. These data showed that Dex repression of the abundance of STC mRNA in AtT-20 cells was mediated by the GR.

Figure 6 Dexamethasone repression of STC expression is not limited to AtT-20 cells. (A) HT1080 cells were treated with 10^{-6} M Dex in OptiMEM/charcoal-stripped FCS for the times indicated and then harvested for RNA isolation. Typical northern blot, indicating the steady-state mRNA levels of STC relative to 18S rRNA. (B) Results for STC mRNA from panel (A), quantitated and normalized to the 18S rRNA signal and plotted as the percent change in STC:18S ratios relative to untreated cells at 0, 24, and 48 h. The bars correspond to each lane of the northern blot in panel (A). Dex decreased HT1080 STC mRNA levels by 80% after 24 h treatment.

Activation of cAMP-signaling pathway antagonizes the repressive effects of hydrocortisone on STC mRNA levels in UltraMEM-cultured AtT-20 cells

In previous studies, activation of the cAMP signaling pathway was reported to alter *POMC* gene activity in AtT-20 cells (Knight *et al.* 1987, Lundblad *et al.* 1988, Autelitano *et al.* 1989). Given that gene expression of both *STC* and *POMC* demonstrated sensitivity to glucocorticoids, we investigated whether induction of the protein kinase A (PKA) pathway could alter the response of the *STC* gene to glucocorticoids. Briefly, intracellular cAMP levels were increased in UltraMEM-cultured AtT-20 cells by treatment with the adenylate cyclase activators: forskolin or CRF, or the cAMP analog 8Br-cAMP. The drug forskolin stimulates the adenylate cyclase system by

constitutive activation of adenylate cyclase (Tang & Hurley 1998), whereas CRF induces the adenylate cyclase pathway via binding to CRF receptors expressed on corticotrope cells (Luini *et al.* 1985, Reisine *et al.* 1985). In two independent experiments, northern blot analysis indicated that the cAMP-inducing agents dramatically increased *STC* mRNA levels in UltraMEM-cultured

AtT-20 cells, routinely reaching maximum values 4 h after the addition of these agents and returning to basal values at 24 h after treatment (Fig. 9). The level of *STC* mRNA induction in UltraMEM-cultured AtT-20 cells was three- to eightfold with CRF, five- to 15-fold with forskolin, and seven- to 30-fold with 8Br-cAMP. In contrast, stimulation of UltraMEM-cultured AtT-20 cells with phorbol 12-myristate 13-acetate, a protein kinase C (PKC) activator, failed to induce *STC* mRNA expression (data not shown), suggesting that stimulation of the PKC pathway and the subsequent increase in intracellular Ca^{2+} concentrations cannot rescue *STC* gene expression. Consistent with this finding, a fourfold increase in CaCl_2 concentration in UltraMEM was insufficient to induce *STC* mRNA levels in AtT-20 cells (data not shown), implying that extracellular Ca^{2+} could not antagonize the repressive effects of Dex on *STC* gene expression. In summary, these findings strongly suggest that induction of the intracellular cAMP pathway can specifically overcome the glucocorticoid-mediated inhibition of *STC* gene expression in AtT-20 cells.

Dexamethasone does not alter *STC* reporter gene expression

The Dex-mediated repression in steady-state *STC* mRNA levels may be secondary to a reduction in *STC* gene transcription. To examine this, AtT-20 cells were transiently transfected with pGL3 mSTC669, a pGL3 plasmid with the luciferase gene under the control of a 669 bp region of the mSTC proximal promoter. After transfection, cells were cultured in either UltraMEM or OptiMEM, with or without Dex, to determine the effect of glucocorticoids on *STC* promoter activity in three independent experiments. The presence of glucocorticoids in the culture medium routinely did not modulate the CMV promoter activity of the pCMV-luciferase positive control reporter gene, indicating that glucocorticoids were

Figure 7 Dex repression of *STC* is not apparent at the protein level until 48 h after treatment. (A) Cells were cultured in OptiMEM/charcoal-stripped FBS in the presence or absence of 10^{-7} M Dex for the indicated periods of time. The autoradiograph illustrates *STC* steady-state mRNA levels relative to cyclophilin mRNA. (B) Post-nuclear supernatants (PNS) and conditioned media (CM) were prepared from AtT-20 cells cultured in OptiMEM with or without 10^{-7} M Dex treatment for 30 h (cells from which RNA was isolated and used to generate panel (A)). Representative western blots loaded with 50 µg post-nuclear supernatants protein per sample and 60 µl conditioned medium protein per sample indicated *STC* protein concentrations. A lane of recombinant human *STC* (hSTC) was loaded as a size control. (C) Cells were cultured in OptiMEM/charcoal-stripped FBS for 3 days and then treated with (■) or without (□) 10^{-7} M Dex for the times indicated. The concentration of *STC* in conditioned medium was measured using an hSTC radioimmunoassay. The results shown are the means $\pm$ S.D. of triplicate samples; they reveal a significant loss of *STC* from the media after 48 h in the presence of Dex.

not globally suppressing transcription (Fig. 10A). In cells transfected with pGL3 mSTC669, glucocorticoids consistently had no effect on *STC* proximal promoter activity. These results clearly indicated that GR did not directly

down-regulate the strong *STC* proximal promoter activity in AtT-20 cells, suggesting that transcriptional inhibition may not be responsible for the Dex-mediated down-regulation of steady-state *STC* mRNA levels.

*Dexamethasone does not alter the rate of *STC* gene transcription*

The lack of glucocorticoid effects on mSTC proximal promoter activity did not preclude the possibility of a negative effect on transcription mediated by other parts of the *STC* gene. To address this possibility, we performed nuclear run-on assays. Briefly, the rate of nascent *STC* transcript formation was assessed in nuclei isolated from AtT-20 cells cultured in the presence or absence of Dex for 24 h in OptiMEM. In untreated cells, the rate of *STC* gene transcription was barely detectable relative to 18S rRNA, and this routinely remained unaltered, as determined by quantitative PhosphorImager analysis, after Dex treatment in four independent experiments (Fig. 11B1). Similar results were observed for cyclophilin (*CP*) gene transcription. As observed by others (Mayo *et al.* 1981, Drouin *et al.* 1989, 1993), Dex dramatically reduced *POMC* transcription and enhanced *MT-1* gene transcription by two- to 12-fold and three- to 13-fold, respectively, indicating that Dex treatment was effective and that the nuclear run-on assays were functional. Although we cannot conclude definitively that glucocorticoids down-regulate *STC* gene transcription, it seems unlikely that the large decrease in *STC* mRNA accumulation caused by glucocorticoids is due solely to direct transcriptional inhibition.

*Dexamethasone-mediated decrease in AtT-20 *STC* mRNA levels requires current gene transcription*

Because glucocorticoids failed to have an effect on *STC* gene transcription, we investigated whether glucocorticoids were acting at the post-transcriptional level to reduce steady-state mRNA levels. In an effort to determine whether Dex repression was due to a change in *STC* mRNA stability, cells were treated with Dex, Act D, or both, and changes in *STC* mRNA accumulation were assessed. As a control, the level of *MT-1* mRNA was

Figure 8 RU486 antagonizes the Dex-mediated down-regulation of *STC* mRNA levels in AtT-20 cells. (A) AtT-20 cells cultured in UltraMEM/FCS were treated with 10^{-7} M RU486 at the times indicated and then harvested for RNA analysis. RU486 rescued *STC* mRNA after just 1 h of treatment. (B) AtT-20 cells cultured in OptiMEM/charcoal-stripped FCS were treated with 10^{-7} M Dex, 10^{-7} M RU486, or both, and then harvested at 24 and 48 h after treatment, for northern analysis. (C) The results in panel (B) were quantitated as described in Materials and Methods and presented as the percent change in the *STC*:cyclophilin (*CP*) ratio relative to untreated cells for corresponding time periods. The bars are aligned with corresponding lanes of panel (B).

Figure 9 Activation of the cAMP-signaling pathway transiently antagonizes repression of *STC* gene expression in UltraMEM-cultured AtT-20 cells. (A) AtT-20 cells cultured in UltraMEM/FCS were treated with 5 mM 8Br-cAMP (●), 10^{-7} M CRF (▲), or 10^{-5} M forskolin (○) and then harvested at the times indicated, for northern blot analysis. The autoradiographic signals in panel (A) were quantitated, normalized as described previously, and plotted as the percent change in the *STC*:cyclophilin (CP) ratio from time 0 h.

measured in parallel. The transcriptional inhibitor, Act D, did not appreciably alter *STC* transcript accumulation, because mRNA levels were at least 65% of controls 24 h after Dex treatment (Fig. 11), indicating that *STC* mRNA was remarkably stable. In contrast, the MT-1 mRNA level was diminished by 72% and 93% at 14 h and 24 h, respectively, demonstrating that the Act D-treated cells had undergone an arrest in gene transcription.

The Dex-mediated repression of AtT-20 *STC* mRNA levels was unexpectedly attenuated at 14 h and 24 h after addition of Act D (Fig. 11). Co-incubation of Act D and Dex abrogated the repressive effect of Dex, by maintaining *STC* mRNA abundance at nearly 70% of control levels. Dexamethasone alone reproducibly diminished steady-state *STC* mRNA to virtually undetectable levels within

Figure 10 Dexamethasone does not affect *STC* gene transcription in AtT-20 cells. (A) Cells in OptiMEM were transfected with luciferase reporter genes, washed, and incubated for 24 h in OptiMEM (light-shaded bars), OptiMEM with 10^{-7} M Dex (open bars), or UltraMEM (dark-filled bars). Luciferase activity was determined as described in Materials and Methods and expressed as the mean $\pm$ S.D. of triplicate cultures. pGL3-STC669 carried 669 bp of the mouse *STC* proximal promoter region and produced equivalent luciferase activity under all culture conditions, which was significantly greater than the promoterless Control luciferase reporter, pGL3-Basic and less than the positive control plasmid, pCMV-Luc. (B) Cells were cultured in OptiMEM with or without 10^{-7} M Dex for 24 h. Nuclei were isolated from cells and nuclear run-on assays were performed as described in Materials and Methods. The representative autoradiogram indicates hybridization of radiolabeled RNA to membranes containing duplicate dots of cDNAs for *STC*, *POMC*, *MT-1*, cyclophilin (CP), and 18S rRNA. The intensity of the signal was indicative of the level of transcription compared with non-specific hybridization to pBKSII.

24 h. Consistent with the findings of previous studies (Mayo & Palmiter 1981), expression of MT-1 increased approximately twofold in response to Dex treatment. After 48 h of Act D treatment, the *STC* mRNA signal was almost lost (85%), presumably because of normal mRNA metabolism. Therefore, the ability of Act D to rescue Dex-mediated repression of *STC* mRNA levels was eventually overcome by the normal mRNA degradative pathways leading to loss of all mRNAs. Similar experiments were carried out with other transcriptional

Figure 11 Dexamethasone-mediated decrease in *STC* mRNA levels in AtT-20 cells requires current gene transcription. (A) AtT-20 cells cultured in OptiMEM/FCS were treated with 10^{-7} M Dex, 10 μ g/ml Act D, 50 μ g/ml CHX or combinations of these, and then harvested at the times indicated, for northern blotting. The northern blots (30 μ g total RNA/lane) show the steady-state mRNA levels of *STC* and *MT-1* relative to 18S rRNA in AtT-20 cells at 14, 24, and 48 h after the indicated treatments. (B), (C) The results for *STC* and *MT-1* mRNA in panel (A) were normalized against the 18S rRNA signal and plotted as the percent change in the *STC*:18S and *MT-1*:18S ratios compared with untreated cells at corresponding time periods. Each bar is aligned with the corresponding lane in panel (A).

inhibitors, α -amanitin and DRB, and we obtained essentially identical results with respect to *STC* mRNA accumulation.

In addition to Act D, we evaluated the effect of the protein synthesis inhibitor, CHX, on Dex-mediated repression of *STC* mRNA accumulation. In contrast to Act D, but similar to Dex, CHX alone reduced *STC* mRNA to undetectable levels after 14 h, which suggested that CHX blocked the translation of proteins required for *STC* gene transcription or those required for *STC* mRNA stability. CHX obviously did not rescue *STC* mRNA from Dex down-regulation even though it has been shown to enhance the stability of several different mRNAs (Philippe & Missotten 1990, Yoon *et al.* 1998, Rahgeb *et al.* 1999, Soloff *et al.* 2000, Wang *et al.* 2000). Because of the unexpected stabilizing effect of transcriptional inhibitors on *STC* mRNA, it was not possible to determine its half-life accurately in the presence of glucocorticoids. There was no significant change in *STC* mRNA abundance over the 24-h period in Act D; however, cell viability was significantly compromised and, consequently, it was not possible to obtain an accurate indication of the *STC* mRNA half-life. Nevertheless, in the absence of glucocorticoids, it was clear that *STC* mRNA was long-lived. The results are consistent with a mechanism in which Dex destabilization of *STC* mRNA is dependent upon transcription of another gene(s).

Steady-state STC mRNA levels in AtT-20 cells are down-regulated by Dex via induction of early gene(s)

In an effort to examine the rapidity of the Dex effect on *STC* mRNA, we followed the temporal onset of this inhibition after treatment with Act D as a countermeasure. As shown above, Act D antagonized the inhibitory effect of Dex on *STC* mRNA abundance and prolonged its T_{1/2}. Cells were pretreated for 0.5, 1, 2, 5 and 8 h with Dex, followed by 24 h in Act D-containing media, after which *STC* mRNA levels were assessed (Fig. 12A). As controls, parallel cultures were exposed to Dex or Act D alone, or Dex plus Act D simultaneously. The temporal pattern of *STC* mRNA loss after different times of exposure to Dex followed by 24 h with Act D closely mirrored the changes seen in cells given Dex alone (Fig. 12A,B). In AtT-20 cells treated for 1 h with Dex followed by Act D, steady-state *STC* mRNA levels were reduced by 50%, with almost identical results obtained in the presence of Dex alone for 1 h. Therefore, Act D could not rescue Dex-mediated repression of *STC* mRNA accumulation, even after only a 1-h pretreatment, consistent with the notion that GR had induced an early gene product(s) that destabilized *STC* mRNA. In contrast, Act D significantly reduced *MT-1* mRNA accumulation (by 74%), despite a 0.5 h pretreatment with Dex, indicating that transcription was essential for Dex up-regulation of *MT-1* mRNA levels. However, the repressive effects of Act D on

MT-1 gene expression gradually diminished with longer times of exposure to Dex. By 24 h, Dex treatment alone induced *MT-1* gene expression by fivefold. As expected, Act D alone did not significantly affect *STC* mRNA abundance over the 24-h treatment period (Fig. 12C), but it abrogated the Dex-mediated reduction in *STC* gene expression when added simultaneously with Dex (Fig. 12D). An arrest in gene transcription was demonstrated by an 86% loss of *MT-1* mRNA 24 h after addition of Act D in the presence or absence of Dex (Fig. 12C). In summary, these results showed that Dex could rapidly initiate the production of factor(s) that would increase the turnover of *STC* mRNA in AtT-20 cells and that these factor(s) were long-lived and functional even 24 h after cessation of gene transcription. The fact that the repressive action of Dex on *STC* mRNA appeared to be quicker and more potent than that observed for *POMC* mRNA, implied distinct repressive mechanisms.

Discussion

Since the discovery of mammalian *STC* expression in 1995 (Chang *et al.* 1995, 1996, Wagner *et al.* 1995, Olsen *et al.* 1996), various workers have proposed different roles for *STC*, primarily on the basis of correlative data and expression patterns (Sato *et al.* 1998, Zhang *et al.* 1998b, 2000, Iyer *et al.* 1999, Yoshiko *et al.* 1999, Jiang *et al.* 2000, Kahn *et al.* 2000). These studies provided some indication as to which regulatory pathways can induce *STC* production, but the specific factors responsible for the induction were not elucidated. Moreover, factors that can repress *STC* production have not been reported. To understand better the biology and function of *STC* in mammals, it is important to identify molecules capable of modulating its production and thereby provide a molecular basis for changes in *STC* production associated with specific physiological states. To this end, we chose the well-characterized mouse corticotrope tumor cell line, AtT-20, as an *in vitro* model to examine *STC* gene regulation by a variety of intercellular messengers. Unexpectedly, we found that glucocorticoids cause AtT-20 cells to undergo a rapid and significant reduction in steady-state *STC* mRNA levels that was accompanied by a distinct change in cell morphology. The glucocorticoid effect on *STC* mRNA was not restricted to AtT-20 cells, as similar results were evident in the human fibrosarcoma cell line, HT1080. Moreover, studies with the GR antagonist, RU-486, indicated that glucocorticoid-activated GR was responsible for controlling the abundance of *STC* mRNA. The fact that RU486 treatment counteracted the loss of *STC* mRNA in the presence of Dex implies that the underlying mechanism involves DNA binding and inhibition of transcription as expected for a type II nuclear receptor antagonist (Moguilewsky & Philibert 1984, Gass *et al.* 1998, Zhang *et al.* 1998a).

Figure 12 Steady-state STC mRNA levels in AtT-20 cells are down-regulated by Dex via induction of early gene(s). (A) AtT-20 cells were pretreated with 10^{-7} M Dex for 0.5, 1, 2, 5, and 8 h, followed by Act D for another 24 h. The right-hand 24-h time point contains RNA from cells in Dex alone for the duration of the experiment. Lanes labeled 0 and 24 under Co refer to untreated, parallel, control AtT-20 cultures at 0 and 24 h time points of the experiment. Act D could not rescue Dex repression of STC mRNA levels after only a 2-h pretreatment with Dex. AtT-20 cells cultured in OptiMEM/FCS were treated with 10^{-7} M Dex (B), 10 μ g/ml Act D (C), or with 10^{-7} M Dex and 10 μ g/ml Act D together (D) for the indicated times. Comparison of the mRNA signals in panels (B), (C) and (D) indicates that Act D stabilized STC mRNA for at least 24 h in the presence of Dex, whereas MT-1 mRNA was gradually lost.

There are several molecular mechanisms by which activated GR represses gene expression. They focus almost exclusively on the presence of negative glucocorticoid response elements within a gene promoter (Drouin *et al.* 1993, Subramaniam *et al.* 1997, Malkoski & Dorin 1999), and on *trans*-repression models in which GR interacts directly with other transcription factors to alter their activity (Meyer *et al.* 1997, De Bosscher *et al.* 2000). This is particularly relevant to the expression of POMC in

AtT-20 cells, in which a *cis*-active negative glucocorticoid response element was localized and shown to mediate transcriptional inhibition of *POMC* gene expression (Drouin *et al.* 1989). As expected, we found that Dex treatment of AtT-20 cells caused a reduction in POMC mRNA, but the time-course was much slower and the degree of inhibition much less than for STC mRNA. The GR also regulates gene expression post-transcriptionally, by either enhancing the stability of mRNAs (surfactant

protein C (Boggaram & Margana 1994) and fatty-acid synthase mRNAs (Xu & Rooney 1997)) or increasing mRNA decay (somatostatin (Liu & Patel 1995), cyclin D3 (Reisman & Thompson 1995), and insulin mRNAs (Philippe & Missotten 1990)). We examined the mechanism by which glucocorticoids down-regulated the steady-state level of *STC* mRNA in AtT-20 cells. The results from the nuclear run-on studies were not conclusive; however, transient transfection analysis with the *STC* proximal promoter suggested that glucocorticoid-mediated repression of *STC* is not at the transcriptional level. When AtT-20 cells were treated with the transcriptional inhibitor Act D, *STC* mRNA was protected from Dex-mediated repression. These results thus imply that glucocorticoids destabilize *STC* transcripts through *de novo* transcription of genes encoding proteins responsible for degrading *STC* mRNA.

In fish corpuscles of Stannius cells, Ca^{2+} is critically involved in up-regulating *STC* gene expression (Wagner 1994, Wagner & Jaworski 1994). The stimulatory effect of Ca^{2+} on steady-state *STC* mRNA levels is due, in part, to a post-transcriptional regulatory process involving increased mRNA stabilization (Ellis & Wagner 1995). The mechanism of fish *STC* mRNA stabilization is suggested to involve protein-mRNA interactions in the cytoplasm, whereby the polysomal complex protects mRNA from degradation. Thus post-transcriptional regulation of *STC* gene expression has been conserved from fish to mammalian cells but, in contrast to the findings in fish, we have demonstrated its susceptibility to negative regulation. Based on the fish *STC* data we also assessed the responsiveness of the AtT-20 *STC* gene to increases in extracellular Ca^{2+} (data not shown), and did not detect changes in the steady-state level of *STC* mRNA. In addition, hypercalcemic culture conditions failed to overcome the Dex-mediated repression of *STC* steady-state mRNA levels in AtT-20 cells. Consistent with these results, PKC stimulation by phorbol 12-myristate 13-acetate did not rescue *STC* gene expression when AtT-20 cells were grown in UltraMEM, indicating that intracellular Ca^{2+} mobilization could not rescue glucocorticoid inhibition of *STC* mRNA levels. These results are in contrast to those from a recent study that showed positive changes in steady-state *STC* mRNA levels by increasing extracellular Ca^{2+} in the culture media of MDCK cells, which are of kidney origin (Sheikh-Hamad *et al.* 2000). The fact that AtT-20 *STC* gene expression was not susceptible to Ca^{2+} regulation is not surprising, and implies that regulation by extracellular Ca^{2+} may require factors specific to renal cells.

The molecular basis for the control of mRNA degradation is not completely understood, and it is also not clear how glucocorticoids modulate this process. The mechanism for mRNA turnover is suggested to involve adenosine-uridine-rich sequences or polypyrimidine tracts located in the 3'-untranslated region (UTR) (Lagnado *et al.* 1994, Jacobson & Peltz 1996). The embryonic lethal

abnormal vision (ELAV)-like family of RNA-binding proteins is also implicated in regulating the stability of mRNAs for p21 (Joseph *et al.* 1998) and N-myc (Chagnovich *et al.* 1996). How glucocorticoids can specifically affect mRNA stability through *cis*-active mRNA sequences has not been elucidated; however, it has been reported that Dex can accelerate mRNA breakdown via 3' UTR sequences of the surfactant protein-A and cyclooxygenase II mRNA (Hoover & Floros 1999, Lasa *et al.* 2001). Moreover, glucocorticoids can alter the level of proteins that can bind to the β_2 -adrenergic receptor message while also increasing the level of the β_2 -receptor mRNA (Port *et al.* 1992).

We propose a model for glucocorticoid regulation of *STC* gene expression in which glucocorticoids induce the synthesis of protein(s) that, in turn, directly or indirectly interact with *STC* mRNA to decrease its stability. Given that our results regarding glucocorticoid regulation of *STC* were similar to those for cyclin D3, insulin and somatostatin mRNAs, it is possible that they constitute a class of mRNAs down-regulated by glucocorticoids through a common post-transcriptional mechanism. A recent study observed that glucocorticoid-induced repression of *cyclin D3* gene expression involves two RNA-binding proteins that interact with two binding sites of 26 and 37 nucleotides, in the 3' UTR of cyclin D3 mRNA (Garcia-Gras *et al.* 2000). These proteins were reported to form a constitutive complex with cyclin D3 mRNA, which recruits an unknown protein induced by glucocorticoids, resulting in an unstable complex targeted for degradation. Whether this mechanism also applies to *STC* is not immediately apparent. However, alignment of nucleotide sequences in mouse cyclin D3 3' UTR with mouse *STC* 3' UTR revealed that the 26-nucleotide binding site in cyclin D3 3' UTR had the greatest (68%) identity with the sequence between 3263 and 3286 of *STC* 3' UTR. It remains to be determined whether this homologous nucleotide sequence in the *STC* 3' UTR has a similar role in glucocorticoid-mediated destabilization of *STC* mRNA, as observed for cyclin D3 3' UTR. Alternatively, Dex-mediated destabilization of *STC* mRNA could occur at the 5' end, as observed for rat monocyte chemoattractant protein 1 mRNA (Poon *et al.* 1999).

Our studies also found that the cAMP-signaling pathway, like general transcriptional inhibitors, antagonized the glucocorticoid-mediated down-regulation of *STC* mRNA. An increase in intracellular cAMP concentrations in AtT-20 cells induced *STC* gene expression when cells were cultured in UltraMEM. The results implied that *STC* gene expression was under the control of PKA-mediated phosphorylation events. Although increased concentrations of cAMP did not globally repress transcription, it is entirely possible that cAMP inhibited the activity of glucocorticoid-induced protein(s) that destabilize *STC* mRNA. Increased cAMP could have de-repressed *STC* mRNA levels through PKA-mediated phosphorylation

events at the transcriptional or post-transcriptional level. It could be argued that increased cAMP increased *STC* gene transcription, but the resultant mRNA would, presumably, have been subjected to Dex-mediated destabilization. Furthermore, subsequent experiments indicated that forskolin and CRF, in the absence of glucocorticoid, had no significant effect on steady-state *STC* mRNA levels (data not shown). Moreover, analysis of the m*STC* promoter sequence did not reveal canonical cAMP response element binding protein sites through which cAMP could increase *STC* gene transcription. Alternatively, cAMP may activate transcription through other factors, such as the CCAAT/enhancer binding protein (Roesler 2000).

Interaction between the glucocorticoid and cAMP-activated signaling pathways has been documented at the transcriptional level and probably involves co-regulatory scaffolding proteins, such as cAMP binding protein (CBP) (Collingwood *et al.* 1999, Goldman *et al.* 1997). Glucocorticoids and cAMP generally function in a synergistic or additive manner at the transcriptional level (Liu *et al.* 1994, Pennie *et al.* 1995, Richardson *et al.* 1999), but examples of glucocorticoid antagonism of cAMP-activated transcription have also been reported for several genes, including corticotropin-releasing hormone (Guardiola-Diaz *et al.* 1996), α -glycoprotein hormone subunit (Chatterjee *et al.* 1991), and arginase I (Morris *et al.* 1998). The mechanism by which these antagonistic effects occur has focused on events at the transcriptional level, with GR modulating the binding of transcription factors to the aforementioned gene promoters. To our knowledge, the results described here are the first demonstrating that activation of the cAMP-signaling pathway can alleviate glucocorticoid-mediated inhibition of gene expression at the post-transcriptional level. cAMP has been demonstrated to alter the stability of a variety of mRNAs. The Na⁺/glucose cotransporter, SGLT1 (Lee *et al.* 2000) and lactate dehydrogenase A subunit (Tian *et al.* 1998) mRNAs are stabilized in the presence of cAMP-increasing agents, whereas type-1 plasminogen activator-inhibitor (Tillmann-Bogush *et al.* 1999, Heaton *et al.* 2000) and angiotensin receptor (Xu & Murphy 2000) mRNAs are destabilized by cAMP analogues and cAMP-increasing agents. Therefore, it is likely that cAMP-mediated rescue of *STC* mRNA from glucocorticoid-induced turnover occurred at the post-transcriptional level, possibly through post-translational modification of RNA binding proteins specifically targeted to *STC* mRNA, as described for other genes (Tian *et al.* 1998, Lee *et al.* 2000).

In summary, our findings show for the first time that *STC* gene expression is susceptible to negative regulation, and provide an experimental paradigm with which to study the mechanism by which negative post-transcriptional regulation by glucocorticoids can be overcome by PKA-controlled factors. Further elucidation of this mechanism will be fundamental to our understanding of how glucocorticoids function and will also provide

insights into how *STC* production can be modulated by glucocorticoids and the cAMP signaling pathway.

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