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Leukemia Inhibitory Factor (LIF) Modulates Pro-Opiomelanocortin (POMC) Gene Regulation in Stably Transfected AtT-20 Cells Overexpressing LIF

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Leukemia inhibitory factor (LIF) levels are elevated in sepsis and correlate with shock and poor prognosis. We have previously shown that lipopolysaccharide (LPS) administration induces hypothalamic and pituitary LIF expression in vivo, which is associated with the acute rise in circulating adrenocorticotrophic hormone (ACTH) levels. As AtT-20 cells respond to LIF, we established murine LIF (mLIF) stably transfected AtT-20 cell lines to study LIF regulation of pro-opiomelanocortin (POMC) expression and ACTH secretion. Our results show that mLIF transfectants accumulated mLIF (up to 15.6 ± 3.2 ng/mL after 24 h) as well as increased ACTH secretion (up to 2.4-fold above control cells) in conditioned medium. The magnitude of ACTH induction correlated with mLIF concentrations in different transfectants (r = 0.75–0.88, $p < 0.05$). Moreover, mLIF transfectants showed a higher sensitivity to CRH stimulation with an increased ACTH production within 8 h ($p < 0.05$), whereas control cells were responsive to CRH at 24 h. Additionally, mLIF transfectants exhibited a maximum threefold ACTH induction, compared to 1.7-fold in control cells. Furthermore, mLIF transfectants have a blunted dexamethasone-mediated inhibition of ACTH (35% inhibition in control cells vs no inhibition in mLIF-transfected cells at 24 h). These findings support and extend the previous observations of LIF acting at the pituitary level, and indicate that mLIF stably-transfected AtT-20 cells are a useful model for studying mLIF-mediated gene regulation in pituicytes.

Key Words: Leukemia inhibitory factor (LIF); AtT-20 cells; pro-opiomelanocortin (POMC); ACTH.

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Introduction

Leukemia inhibitory factor (LIF), a neuropoietic cytokine, influences the function and/or development of several genes *(1–4)*. LIF exerts its biological action by binding to specific cell-surface receptors, which, in turn, triggers heterodimerization between the LIF receptor and gp130 subunits, and subsequently, induces intracellular phosphorylation cascades *(5–7)*. Moreover, interleukin-6 (IL-6), interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin 1 (CT-1), and LIF share the common gp130 molecule as a component of their receptor complex *(8–12)*.

LIF is expressed at low levels in normal adult tissues, as well as in vitro in many cell types *(13)*. Expression of LIF can be induced in vitro and in vivo in response to physiological and pathological perturbations *(14,15)*. For example, in mouse uterine endometrium, LIF expression peaks at ovulation just prior to the onset of blastocyst implantation *(16,17)*. We have previously shown that LIF was expressed in human fetal and adult murine pituitary cells *(18)*. Both LIF and LIF receptor mRNA are expressed in mouse hypothalamus and pituitary *(19)*, and are upregulated in vivo by lipopolysaccharide (LPS). Induction of LIF and CRH either precedes or parallels the acute rise of circulating ACTH levels *(19)*, indicating that LIF plays a role in regulating the hypothalamic–pituitary–adrenal axis in response to stress.

Murine pituitary AtT-20 cells respond to LIF by exhibiting enhanced rat pro-opiomelanocortin (rPOMC) promoter activity, increasing mouse POMC mRNA levels and the increasing secretion of ACTH. CRH acts synergistically with LIF to induce POMC gene transcription, whereas dexamethasone (Dex) blunts LIF-mediated effects *(20)*. Since wild-type AtT-20 cells express low endogenous levels of LIF, we established stably-transfected AtT-20 cells overexpressing mLIF, and studied the regulation of ACTH secretion by CRH and Dex in this cell model. The results show that accumulation of mLIF in the medium of transfected cells is associated with persistently enhanced ACTH secretion. The magnitude of ACTH induction correlates

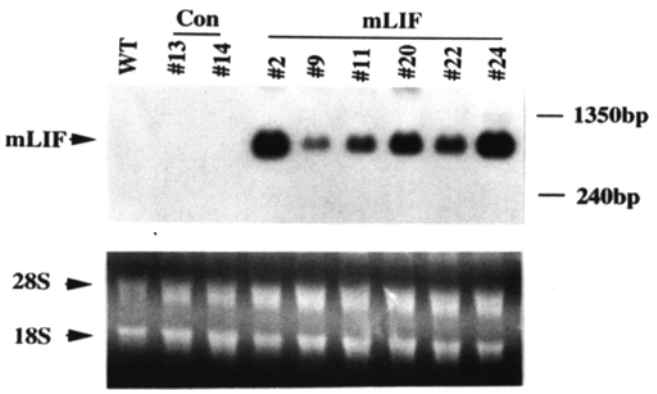

Fig. 1. Northern blot analysis of mLIF mRNA in mock-transfected and mLIF stably transfected AtT-20 cells. Twenty micrograms of total cellular RNA from wild-type and transfected clones were analyzed using a mLIF cDNA probe as described in Materials and Methods. Ethidium bromide staining demonstrated integrity of RNA and comparable loading on the gel.

with mLIF concentrations. Moreover, LIF sensitizes AtT-20 cells to CRH, and blunts Dex-mediated inhibition. These findings further support the in vivo implication of LIF action at the pituitary level and indicate that mLIF stably transfected AtT-20 cells are a useful model to study mLIF-mediated POMC gene regulation.

Results

Expression of mLIF mRNA in Transfected AtT-20 Cells

Low levels of mLIF mRNA expression (weak signal detected only by ribonuclease protection assay) in wild-type AtT-20 cells were previously demonstrated *(18)*. Expression of mLIF mRNA from wild-type, mock, and mLIF-transfected AtT-20 cells were tested by Northern blot (Fig. 1). In wild-type and control AtT-20 cells, the predicted 4.5-kb mLIF mRNAtranscripts were undetectable using 20 μg of total RNA and 18 h of autoradiography (data not shown), consistent with our previous observations. In contrast, all six analyzed mLIF stably transfected clones expressed variable amounts of unique hybridization signals at approx 800 bp.

mLIF and ACTH Secretion in Transfected AtTr-20 Cells

To assess effects of expressed mLIF on ACTH production in these transfectants, levels of mLIF were analyzed, and ACTH secretion was measured for up to 72 h in culture medium. Control cells produced a relatively low amount of mLIF, as shown in Fig. 2 (two representative clones). In contrast, mLIF concentrations in the medium of mLIF transfectants during 3 d of cell culture were consistently elevated ($p < 0.05$), and achieved concentrations of 1 n*M* (20 ng/mL) after 24 h. Therefore, the 24-h time period was chosen to characterize the effects of CRH and Dex treatment. Interestingly, ACTH secretion was enhanced ($p < 0.05$) in mLIF transfectants after 36–72 h (Fig. 3). In parallel experiments, the expression of mPOMC mRNA

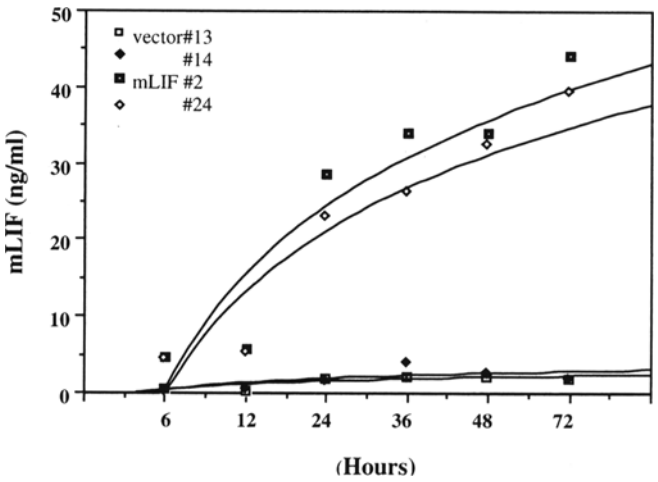

Fig. 2. Time-course of mLIF secretion in two representative clones from both mock-transfected (#13 and #14) and mLIF-transfected (#2 and #24) AtT-20 cells. Cells were seeded in 24-well plates (5×10^4 cells/well) in G418 containing growth medium for 24 h. Medium was replaced by OPTIMEM supplemented with 5% CSS. Cultured supernatants were collected as indicated and murine LIF concentrations were measured. Results are expressed as the mean value of three wells from one representative experiment.

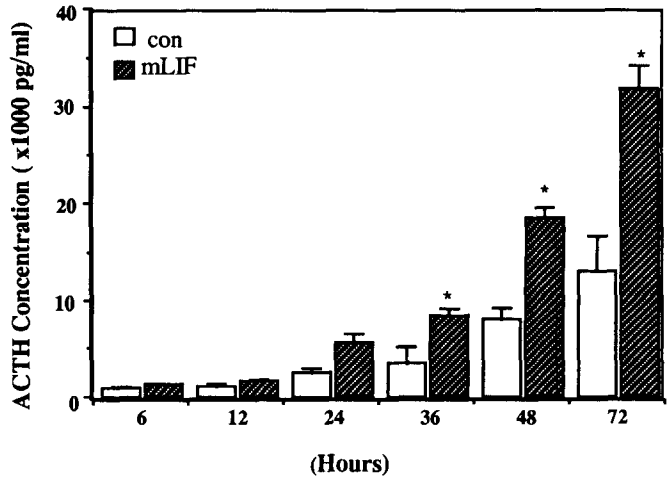

Fig. 3. Time-course of ACTH secretion in both mock-transfected and mLIF-transfected AtT-20 cells. Cells were plated and medium collected as described above. Results are expressed as mean ± SEM of 3 wells from one representative experiment with 2 mock transfectants and 6 mLIF transfectants. *$p < 0.05$ compared to mock transfectants.

was also measured and is depicted in Fig. 4. Mouse POMC mRNA in mLIF transfectants increased markedly within 24 h, but the earliest significant differences in ACTH secretion were only observed after 36 h of incubation.

Correlation of ACTH Secretion and Variable mLIF Concentration in the Medium of mLIF Transfectants

ACTH production in different mLIF transfectants was also tested to determine whether ACTH secretion reflects the observed dose response of exogenous mLIF treatment.

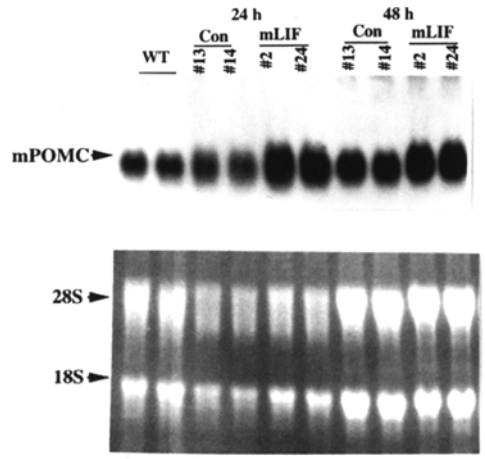

Fig. 4. Northern blot analysis of mPOMC mRNA in mock transfectants and mLIF transfectants. Cells were seeded for 24 h, followed by incubation with OPTI-MEM containing 5% CSS for 24 and 48 h, and RNA extraction. Lanes 1 and 2: mock transfectants (#13 and #14); lanes 3 and 4: mLIF transfectants (#2 and #24).

A series of incubations were performed, and the supernatants were collected for RIA for both ACTH and mLIF. The correlation of ACTH and mLIF secretion is shown in Fig. 5. ACTH and mLIF concentrations correlated significantly after 36 h, and this correlation persisted for up to 72 h (r = 0.767 and 0.752, respectively).

ACTH Secretion in Response to CRH

To compare the potentiation of CRH induction in both lines of transfectants, two clones from control and mLIF transfected AtT-20 cells were tested. Cells were cultured with CRH (10^{-8} M) for up to 24 h. Induction of ACTH varied between these two groups during the time course of CRH treatment, as shown in Fig. 6. Maximal stimulation (1.7-fold) by CRH was observed at 24 h in control cells, whereas significant ($p < 0.05$) stimulation (1.6-fold) in mLIF transfectants was detected after 8 h of exposure. Moreover, significant ($p < 0.05$) ACTH induction (2.0 to 3.0-fold) persisted in mLIF transfectants exposed to CRH for 16–24 h.

ACTH Production in Response to Dex Treatment

Cells were treated with either different doses (10^{-6}–10^{-9} M) of Dex for 24 h (Fig. 7A), or 10^{-8} M Dex for 24–72 h (Fig. 7B). In control cells, Dex inhibited ACTH secretion in a dose dependent fashion. Maximal inhibition was achieved (up to 35%) at 10^{-8} M Dex ($p < 0.05$). No further inhibition was observed with increasing Dex concentrations. In contrast, in mLIF transfectants, the inhibitory effect of Dex was only achieved by increasing Dex concentrations to 10^{-7} M or higher at 24 h incubation. However, prolonged exposure for 48 and 72 h restored Dex inhibition (44–50%, $p < 0.05$) in mLIF transfectants (Fig. 7B).

Discussion

LIF gene expression in human fetal and murine pituitary tissues were identified by ribonuclease protection assay and RT-PCR *(18,19)*. LIF binding and immunoreactivity were demonstrated to be associated predominantly with corticotroph cells *(18)*. We have previously shown that mLIF mRNA expression *(18)* and secretion of mLIF in AtT-20 cells are low *(21)*. The existence of a functional LIF receptor complex in murine AtT-20 cells was confirmed by fluorescence-activated cell sorting *(18)*, and the finding of increased POMC mRNA expression and ACTH secretion in response to LIF treatment *(20,22,23)*. In the present study, an mLIF stably transfected AtT-20 cell line was established and used to explore further the ability of LIF to modulate ACTH secretion in the presence or absence of CRH or Dex.

By Northern blot using 20 μg total RNA and 18 h of autoradiography, we were unable to detect 4.5-kb mLIF

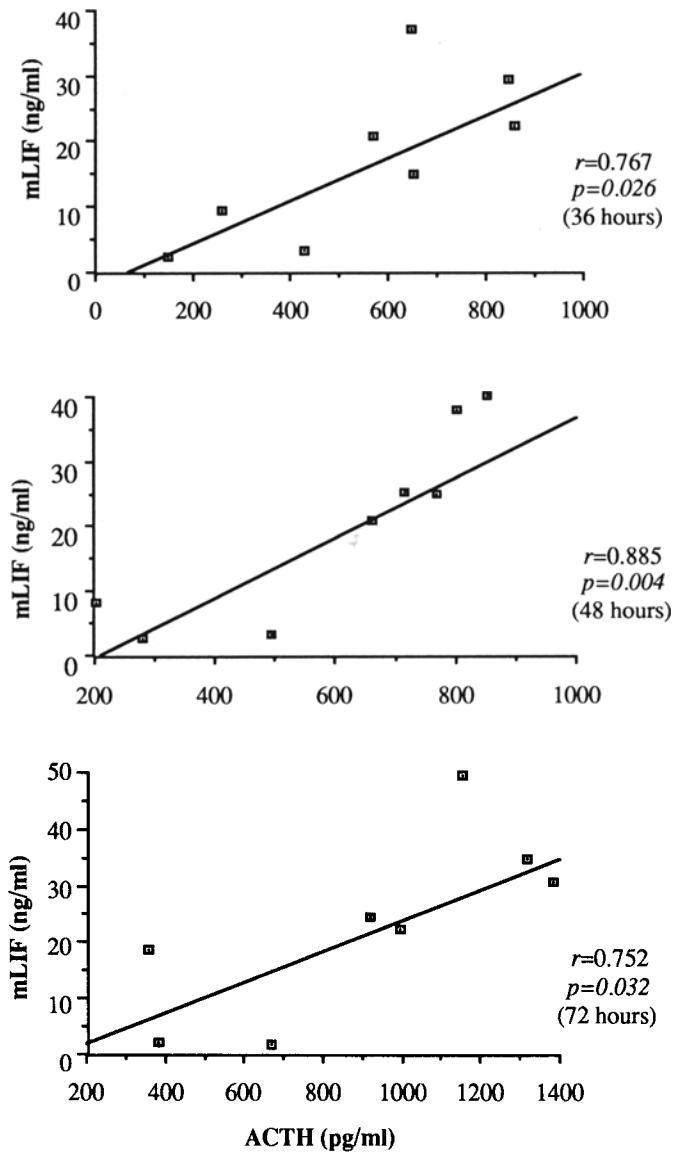

Fig. 5. Correlation of mLIF and ACTH secretion in different transfectants. Cells were seeded and cultured in OPTI-MEM supplemented with 5% CSS. Cultured supernatants were collected after 36, 48, and 72 h of incubation. Murine LIF and ACTH were measured by RIA. Results are expressed as mean value of three wells from a representative experiment.

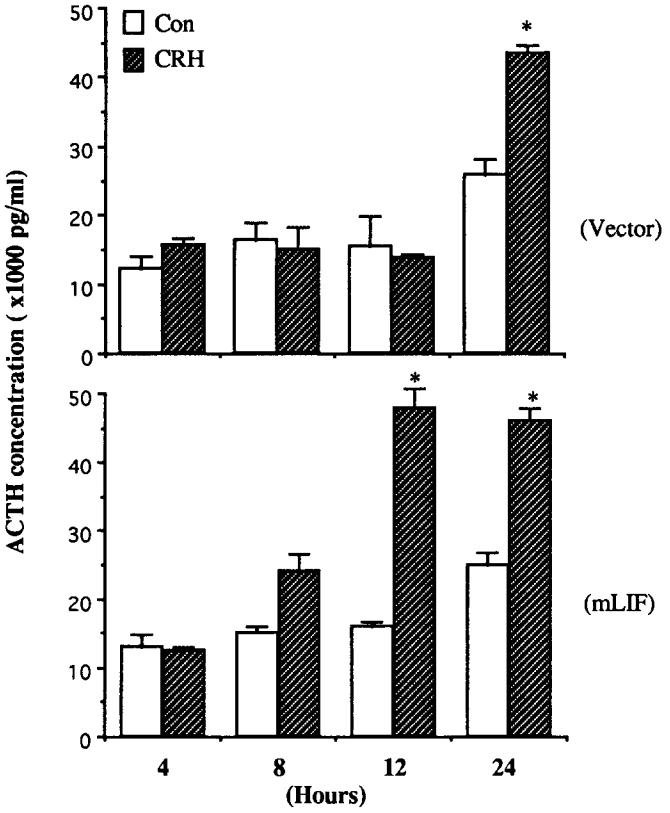

Fig. 6. Time-course of ACTH secretion in response to CRH treatment: Cells were treated with CRH for up to 24 h. ACTH concentrations in the media were measured by RIA. Results are expressed as mean ± SEM of ACTH concentrations from a representative experiment. $*p < 0.05$ compared to the same cells without CRH treatment.

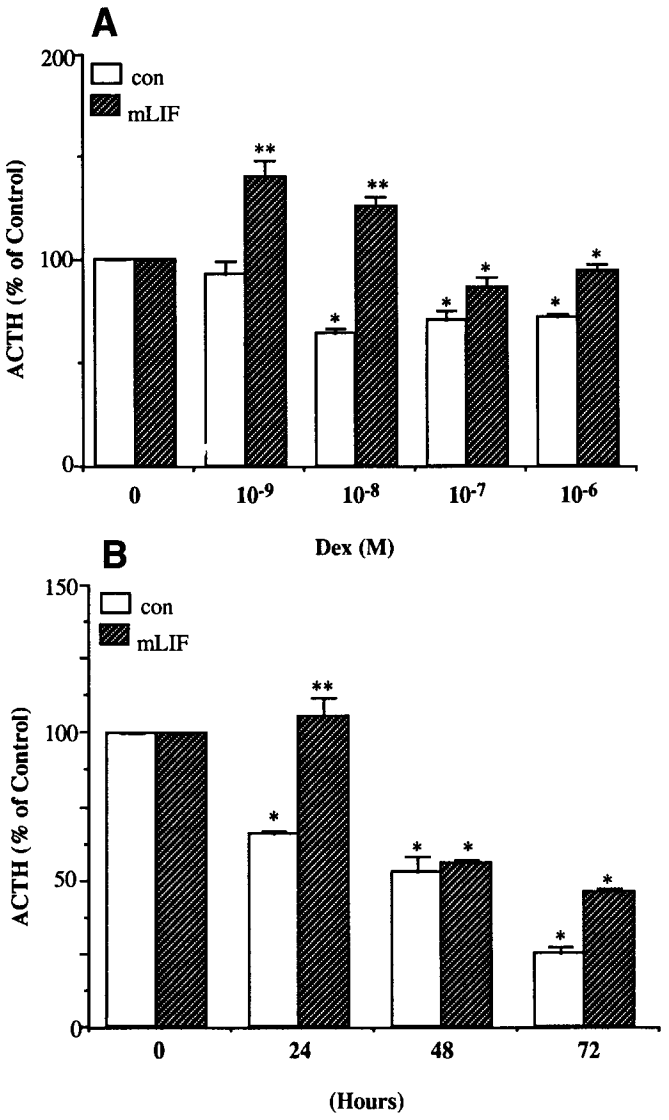

Fig. 7. Time-course and dose–response of Dex treatment: Cells were cultured with different concentrations of Dex for 24 h (**A**) or with Dex (10^{-8} *M*) for various times (**B**). ACTH concentrations in media were measured by RIA. Results are expressed as mean ± SEM percent ACTH in Dex-treated cells compared with untreated cells from a representative experiment. $*p < 0.05$ compared to same cells without Dex treatment. $**p < 0.05$ compared to mock transfectants.

signals in either wild-type or transfected AtT-20 cells (data not shown). In contrast, mLIF transfectants expressing variable amounts of mLIF mRNA transcripts after 2 h of autoradiography (Fig. 1). Using this model, we showed that ACTH secretion was enhanced significantly at 36 h. However, induction of mPOMC mRNA expression occurred earlier, suggesting that LIF-mediated transcriptional regulation of mPCMC gene occurs prior to its effects on ACTH secretion *(18,20–22)*.

CRH exerts synergistic effects with LIF to induce POMC gene transcription and ACTH secretion in wild-type AtT-20 cells *(20,22,23)*. Dex not only inhibits CRH induction of ACTH secretion, but also LIF action *(20)*. The results here further show that mLIF transfectants exhibit both earlier and enhanced ACTH production in response to CRH, as compared to control cells. In contrast to synergistic stimulation with CRH, LIF overcomes the inhibitory effect of physiological concentrations of Dex. The glucocorticoid receptor (GR) has been shown to form a complex with $STAT_5$ to mediate gene transactivation *(24)*, and LIF can reverse Dex-induced growth arrest in human myeloma cell lines *(25)*. This evidence suggests that crosstalking may exist between two unrelated pathways. However, the molecular mechanism for LIF interference with Dex in ACTH production remains unclear. Prolonged Dex treatment of LIF transfectants restored the Dex-mediated inhibitory effect. We hypothesize that LIF may function acutely to overcome feedback of elevated glucocorticoids and continued activation of the hypothalamic–pituitary–adrenal axis in response to stress. Although LIF mRNA expression in rat primary pituitary cultures has been demonstrated to be downregulated by Dex *(25)*, the phenomenon observed in the present model is unlikely because of inhibitory effect of Dex on LIF transcription, since transcription of LIF was driven by a CMV promoter.

Increasing CRH and mLIF mRNA expression preceded or paralleled that of ACTH induction in vivo after treating

mice with LPS *(19)*. Transfected AtT-20 cells are therefore a useful model for studying corticotroph cells in response to stress stimuli, since regulation of ACTH secretion by CRH and Dex was examined with increasing mLIF concentration in mLIF transfectants. Several studies have emphasized the importance of LIF in the neuroendocrine system. LIF can cause specific neuron phenotype switching and induce expression of multiple neuropeptides in sympathetic neurons *(26,27)*, which subsequently modulates hormonal secretion. LIF expression may also have important pathophysiological implications, such as increasing human plasma LIF levels in sepsis correlated with shock and poor prognosis *(28,29)*. However, enhanced LIF synthesis and release from multiple sources likely contribute to elevated circulating LIF. In this article, mLIF stably transfected AtT-20 cells provide a cell-culture system to study altered response to secretagogs owing to prolonged exposure to LIF.

Materials and Methods

Cell Line and Stable Transfection

AtT-20 cells (ATCC, Rockville, MD) were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, streptomycin, and penicillin (Gibco, Gaithersburg, MD). Murine LIF expression plasmid, $pCDNA_3$-mLIF, was generated by cloning the coding region of mLIF cDNA (kindly provided by Tracy Wilson, the Walter Eliza Hall Institute of Medical Research, Melbourne, Australia) into the *Eco*R1 and *Xba*1 sites of the $pCDNA_3$ vector (Stratagene, La Jolla, CA), which contains a neomycin resistance gene. The plasmid $pCDNA_3$- mLIF contains the complete open reading frame of the mLIF (623 bp) under the control of a CMV promoter.

Transfections were performed as described *(20)*. Briefly, cells were plated in growth medium 24 h before transfection. Cells were then washed in OPTI-MEM (Gibco) and overlaid with 15 μg of $pCDNA_3$-mLIF plasmid and lipofectamrine (Gibco) mixture for 24 h. Control cells were transfected with pCDNA vector alone (mock transfection). G418-resistant clones were selected in 1 mg/mL of G418 (Gibco) containing growth medium. After 2 wk of culture, several single clones were selected from control and mLIF-transfected cells, and individual clones were further expanded. The mLIF-positive clones were identified by Northern blot using a specific mLIF cDNA probe as described below. Murine LIF concentrations were measured in the culture medium by RIA. Two clones derived from both control and mLIF-transfected cells were used for further study.

RNA Isolation and Northern Blot Analysis

G418-resistant AtT-20 cells were collected for total RNA isolation using Trizol reagent (Gibco), as described *(18)*. Total RNA (20 μg) was fractionated in 1.5% agarose formaldehyde denaturing gel and transferred to a nylon membrane. The mLIF cDNA probe was a 623-bp fragment cleaved from pCDNAs-mLIF expression vector by *Eco*R1 and *Xba*1. The murine POMC cDNA probe, kindly provided by Malcolm J. Low (Portland, OR), was a 600-bp fragment generated by cutting with *Nco*1 and *Xba*1. Both probes were labeled using the random-primed cDNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Membranes were prehybridized and hybridized with α-^{32}P-dCTP-labeled cDNA probe (1×10^6 cpm/mL) in QuikHyb Rapid Hybridization Solution (Stratagene) at 68°C. Membranes were subjected to autoradiography for 2–6 h with a double-intensifying screen after post-hybridization washes.

Hormonal Studies

For time course of mLIF and ACTH secretion, approx 5×10^4 cells/well of mock and mLIF transfectants were seeded into 24-well plates in G418 containing growth medium. After 24 h, cells were washed with 1X PBS and subsequently maintained in OPTI-MEM supplemented with 5% charcoal-stripped FBS (5% CSS), which contains no T_3, Dex, or growth factors, for 6–72 h. Conditioned supernatants were collected for mLIF and ACTH measurement by RIA. Final ACTH concentrations were normalized by cell number in each condition. Experiments for assessment of human CRH (American Peptide Company, Sunnyvale, CA) and Dex (SIGMA, St. Louis, MO) treatment were carried out with the same numbers of cells seeded in 24-well plates for 24 h, and then replaced by 5% CSS OPTI-MEM for 24 h. Cells were then treated with either vehicle alone, CRH (10^{-8} *M*) or Dex (10^{-8}–10^{-8} *M*). To measure mouse POMC mRNA expression at different times, cells were seeded and grown in 2×10^6 cells/dish in 100-mm culture dishes in G418 containing growth medium for 24 h and then replaced by 5% CSS OPTI-MEM. Cells were then lysed by Trizol reagent at the indicated times for total RNA extraction.

Hormone RIAs

ACTH levels were measured using a double-antibody radioimmunoassay kit (Diagnostic Products Company, Los Angeles, CA). All samples were diluted 5- to -20 fold according to the duration of cell culture. Murine LIF levels were analyzed by competition of binding to ^{125}I-anti-mLIF antibody (R&D Systems, Minneapolis, MN) with recombinant mLIF (R&D Systems). The intra-assay coefficient of variation at the mean of 10 ng/mL was 3.29%, and the innerassay coefficient of variation at the mean of 10 ng/mL was 1.58%. The sensitivity was 1 ng/mL *(21)*.

Statistical Analysis

Statistical analyses were performed using one way analysis of variance (ANOVA) for multiple groups and Student's *t*-test for two groups. Correlation of ACTH and mLIF concentration in culture medium was analyzed by linear regression using IBM "PRIMER OF BIOSTATISTICS." Results are expressed as mean ± SEM.

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