

ARTICLE

## Regulation of c-fos Expression by EGF and GnRH in Specific Anterior Pituitary Cells from Proestrous Female Rats

Jennifer L. Armstrong and Gwen V. Childs

Department of Anatomy and Neurosciences, University of Texas Medical Branch, Galveston, Texas

**SUMMARY** C-fos is an early expression oncogene that can be stimulated by a variety of regulators. It is expressed by subsets of all pituitary cells, with increased expression seen in proestrous rats. However, in freshly dispersed pituitary cells studied during different stages of the cycle, there is limited expression of fos by luteinizing hormone (LH) cells and little basal expression by cells with follicle-stimulating hormone (FSH) antigens. Proestrus is a time during which pituitary gonadotropes express peak levels of receptors for gonadotropin-releasing hormone (GnRH) and epidermal growth factor (EGF). We hypothesized that if GnRH or EGF stimulated fos activity in gonadotropes they would be most effective during the peak expression of their receptors. Anterior pituitaries were removed, cut into small pieces, and stimulated for 30 min. Total RNA was then collected and analyzed by Northern analysis. Both EGF and GnRH caused an increase in c-fos mRNA levels in the anterior pituitary gland compared with unstimulated pituitary glands assayed immediately after removal from the pituitary. However, the stimulatory effects were no greater than those seen with medium alone. This suggested that fos expression could be stimulated by local factors either in the pituitary or the medium itself. The second phase of the study focused on pituitary cells plated for 1 hr and then stimulated with EGF and GnRH for 15 min. Dual immunocytochemistry was done to learn which cell types expressed the fos proteins. After 15 min, EGF and GnRH both increased the percentages of fos-bearing cells above levels seen in medium alone. EGF stimulated fos proteins in subsets of FSH, adrenocorticotropin (ACTH), and growth hormone (GH) cells. GnRH increased fos proteins in subsets of ACTH and GH cells. These results suggest that EGF and GnRH may regulate fos expression, but not necessarily in gonadotropes. They also highlight the need for carefully timed experiments because endogenous factors in the pituitary itself may stimulate immediate early gene expression. (*J Histochem Cytochem* 46:935-943, 1998)

**KEY WORDS**

epidermal growth factor  
gonadotropin-releasing  
hormone  
gonadotrope  
immunocytochemistry

C-fos and c-jun are early expression proto-oncogenes that are expressed in low levels in most cells. They are called primary or immediate early response genes. The fos and jun proteins join to form the AP-1 protein. This protein can then bind to the AP-1 binding site on DNA to induce transcription of various genes (Rauscher et al. 1988). Expression of c-fos mRNA and antigens occurs once the cell has been stimulated. This expression of the c-fos gene occurs rapidly and tran-

siently (Kruijer et al. 1985; Franza et al. 1988; Sagar et al. 1988). Mitosis, synthesis of proteins, and secretion of proteins are all examples of changes in cell function that are associated with the expression of c-fos.

Gonadotropin-releasing hormone (GnRH) is an important regulator of fos activity in both the brain and the pituitary (Lee et al. 1990). GnRH stimulates c-fos expression in the immortalized GnRH neuronal cell line GT1. GT1 cells treated with GnRH increased expression of c-fos, as shown by Northern analysis (Cesnjaj et al. 1993). Cesnjaj et al. (1994) also reported that GnRH stimulated c-fos in primary pituitary cell cultures from randomly cycling female rats, as well as c-fos, c-jun, and jun-B in the  $\alpha$ T3-1 gonadotrope cell line. Recent studies in our laboratory con-

Correspondence to: Gwen V. Childs, PhD, Dept. of Anatomy and Neurosciences, MRB 10-104, 303 University Blvd., U. of Texas Medical Branch, Galveston, TX 77555.

Received for publication December 29, 1997; accepted April 15, 1998 (7A4561).

firm that *c-fos* is expressed by gonadotropes, particularly those bearing luteinizing hormone- $\beta$  (LH $\beta$ ) antigens or mRNA (Armstrong and Childs 1997b).

Another regulatory factor for early gene expression is epidermal growth factor (EGF). Muller et al. (1984) utilized 3T3 fibroblast cells and showed that stimulation with EGF increased the levels of *c-fos* mRNA in the cell cultures within 1 hr. Sagar et al. (1991) treated retinal Muller cells after treatment with EGF or TGF $\alpha$ . This group showed stimulation of *c-fos* mRNA within 30 min, and *fos* protein was induced within 1 hr.

We and other workers have shown that EGF may play an important role in the stimulation of gonadotropes during the estrous cycle (Fan et al. 1995; Armstrong and Childs 1997a,c). However, no one knows the exact role of EGF in gonadotrope function. EGF has been shown to stimulate the release of LH (Miyake et al. 1985). Our recent studies have shown EGF receptors (EGFR) on immature gonadotropes early in the cycle. These cells were identified by their content of LH $\beta$  mRNA and absence of FSH $\beta$  mRNA or gonadotropin antigens. The expression of EGFR by LH and FSH antigen-bearing gonadotropes increased as the gonadotropes increased their gonadotropin stores during diestrus (Armstrong and Childs 1997a). This increase was parallel to the increase seen in GnRH receptors in pituitary gonadotropes (Childs et al. 1994b; Lloyd and Childs 1988).

Furthermore, expression of EGFR could be regulated by EGF itself, which is a product of subsets of pituitary cells including gonadotropes and growth hormone (GH) cells (Armstrong and Childs 1997c). This suggests a local autocrine or paracrine regulatory loop that may facilitate the development of the population as it approaches the proestrous surge.

EGF's functions in the gonadotrope population appear to involve differentiation events (such as production of EGF receptors; Armstrong and Childs 1997c). We hypothesized that EGF may work through *fos* to stimulate one or more differentiation steps. Similarly, GnRH is already known to stimulate *fos* in pituitary cells. However, the exact cell type had not yet been identified in the normal pituitary cell population. The GnRH-stimulated *fos* activity in a gonadotrope cell line reported by Cesnjaj et al. (1993) supports the hypothesis that GnRH stimulates *fos* in normal pituitary gonadotropes as well.

To test these hypotheses, pituitary cells were taken at the time in the cycle when EGF or GnRH receptors were at a peak (proestrous AM). Dual immunocytochemistry was employed to determine the identity of the cells with *fos* proteins and each of the pituitary hormones. Northern analysis was utilized to determine if either EGF or GnRH stimulated an increase in *c-fos* mRNA in pituitaries taken from proestrous rats. This article reports the results of this investigation.

## Materials and Methods

### Animals and Collection of Anterior Pituitary Glands

Female Sprague-Dawley rats (Harlan-Sprague Dawley; Houston, TX) weighing 250–300 g were housed three per cage under artificial illumination between 0600 and 1800 hr. The rats were given food and water ad libitum. They were acclimated for 7–10 days before the start of daily vaginal smearing, which was performed to determine the stage of the cycle. The females underwent at least two complete normal 4- or 5-day estrous cycles before they were used. The animal care and use protocol was approved annually by the Institutional Review Committee. All animals were sacrificed between 0900 and 1000 hr. The animals were removed from their housing room and sacrificed by decapitation in a separate, adjoining room within seconds of removal from the cage. The same animal handler performed these rapid procedures, which were approved by the Animal Care and Use Committee.

Anterior pituitary glands were dissected from the animal and rapidly placed in cold Dulbecco's modified Eagle's medium (DMEM) (JRH Biosciences; Lenexa, KS) containing 0.3% bovine serum albumin (BSA; Sigma, St Louis, MO), 1.8 g/500 ml HEPES (Sigma), and 24.65 ml/500 ml sodium bicarbonate (JRH Biosciences). To prevent bacterial growth, 1  $\mu$ l/100 ml gentamicin (Sigma) was used.

### Collection of Total RNA

For the assays of *c-fos* mRNA, the pituitaries were washed three times with DMEM and then chopped into smaller pieces. The anterior pituitary pieces were then stimulated for 30 min with 10 ng/ml EGF (Upstate Biotechnology; Lake Placid, NY), 100 nM GnRH (Peninsula Laboratories; Belmont, CA), or DMEM alone. The DMEM used at this time contained identical reagents as listed above as well as 1  $\mu$ g/50 ml ascorbic acid (Sigma). In addition, some RNA extractions utilized DMEM with these additives: 0.005 mg/ml insulin, 0.05 mg/ml transferrin, and 0.001 mM sodium selenite. Stimulation occurred at 37C in a CO<sub>2</sub>-controlled environment. Total RNA was isolated by the single-step method (Chomczynski and Sacchi 1987) using TRIzol (Gibco BRL; Gaithersburg, MD). The total RNA pellets were allowed to air-dry and then were stored in the -70C freezer.

### Northern Analysis

Northern blot analysis was carried out as previously described (Fan and Nagle 1996). At the time of use, the total RNA collected from the female anterior pituitary gland was quantified by comparisons of optical density (OD) absorbance at 260 and 280 nm. A value >1.6 for A<sub>260</sub> to A<sub>280</sub> was required for the RNA to be determined as uncontaminated.

Total RNA (20  $\mu$ g) collected from the anterior pituitary was separated on a 1% agarose gel (Gibco). The RNA was transferred from the gel onto a Nytran membrane (Schliecher & Schuell Maximum Strength Nytran Plus; Keene, NH). Once the transfer to the Nytran membrane was complete, the membrane was uv-crosslinked (Ultra Lum UVC 515 Ultraviolet Multilinker), dried for 1 hr, and prepared for prehybridization. The prehybridization and hybridization buffer consisted of 2  $\times$  Denhardt's solution (Sigma), 50%

formamide, 0.5% SDS,  $5 \times$  SSC, salmon sperm DNA, and DEPC-H<sub>2</sub>O.

The DNA probe was labeled using a Prime It-II Random Primer Labeling Kit (Stratagene; La Jolla, CA). Briefly, the c-fos DNA insert (a generous gift from Dr. Tom Curran, St. Jude Children's Research Hospital, Memphis TN) was mixed with DEPC-H<sub>2</sub>O and random primers. Unincorporated nucleotides were removed by chromatography on Nuc-Trap columns (Stratagene). Salmon sperm DNA was mixed into the solution and the radioactivity of the probe was quantified. The probe was added to the prehybridization container and the membrane was hybridized overnight at 42C.

The following day the membrane was washed with  $1 \times$  SSC and 0.1% SDS in DEPC-H<sub>2</sub>O at 42C and then placed in the autoradiography cassette with two intensifying screens and film (Sigma) at -70C. The film was developed the next day.

#### Dissociation of Pituitary Glands

For the plated cultures, pituitary glands were dissociated enzymatically and mechanically as described previously (Childs et al. 1987). This protocol takes about 1 hr. Cells were tested for viability by the trypan blue dye exclusion test. The protocol produced 2-3 million cells/pituitary that were 98% viable. Cells were plated for an additional 1 hr onto glass coverslips (Thomas Scientific; Swedesboro, NJ) that were coated with poly-d-lysine (Sigma) in plastic 24-well trays (Corning; Corning, NY). The cells were plated at a density of approximately 40,000-50,000 cells/well/50  $\mu$ l DMEM. The DMEM contained 0.005 mg/ml insulin (Sigma), 0.05 mg/ml transferrin (Sigma), and 0.001 mM sodium selenite (Johnson Matthey; New York, NY). Cells were allowed to settle down onto the coverslips for 15 min before being placed in a 37C incubator for 45 min. Cells were then stimulated with 10 ng/ml EGF (Upstate Biotechnology), or 10 nM GnRH (Peninsula Laboratories) in DMEM or DMEM alone for 15 min at 37C. After the 15-min stimulation, the cells were fixed with 2% glutaraldehyde for 30 min, followed by four washes with 4.5% sucrose. The cells were then stored in the refrigerator until immunocytochemistry could be performed (no longer than a week).

#### Immunocytochemical Studies

Anterior pituitary cells were collected and dispersed as mentioned above. Coverslips were single labeled for c-fos protein or dual labeled for c-fos protein and one of the six pituitary hormones. Single and dual immunochemical techniques have been described previously (Childs et al. 1994a,b; Armstrong and Childs 1997a-c). Briefly, cells were incubated at 37C with the primary antibody raised against fos at a dilution of 1:1200. This rabbit polyclonal antibody Ab-2 to c-fos protein was purchased from Oncogene Science (Cambridge, MA). The antibodies were detected using a DAKO (Santa Barbara, CA) rapid detection kit with streptavidin-peroxidase and black nickel-intensified diaminobenzidine (DAB). Coverslips to be utilized for dual labeling protocols were then incubated at 37C in one of the six pituitary hormone antibodies (for dilutions see below). The antibodies were detected using the DAKO rapid detection kit with streptavi-

din-peroxidase and orange DAB. Antisera to pituitary hormones were obtained from the following locations and used at the described dilutions. Anti-bovine LH $\beta$  was a gift from J. G. Pierce and was used at a dilution of 1:40K. Anti-human FSH $\beta$  was used at a dilution of 1:10K and was generously provided by the Pituitary Hormone Distribution Program (NIDDK; Bethesda, MD) as was the anti-rat- $\beta$  thyrotropin which was used at a dilution of 1:45K. Anti- $\beta$ -ACTH was made in this laboratory from the 17-39 C-terminal fragment of ACTH (Moriarty and Halmi 1972) and was used at a dilution of 1:30K. Rabbit anti-rat prolactin, used at a dilution of 1:40K, and rabbit anti-rat GH, used at a dilution of 1:35K, were purchased from Chemicon (Temecula, CA).

Control labeling protocols were performed for both the single label for c-fos protein and the dual label with c-fos protein and pituitary hormones. For both primary antisera, the antibodies were omitted from the diluent buffer and the coverslips were incubated in buffer alone. In addition, the antisera had been preabsorbed with each antigen as previously described (Armstrong and Childs 1997a-c; Childs et al. 1994a,b). All controls showed no labeling for the antigen in question.

#### Statistical Analysis

One or two rats were collected at a time for each experimental group until there were 6-8 rats/group. All rats were taken on the morning of proestrus because of the peak expression of EGF receptors (Armstrong and Childs 1997a) and GnRH receptors (Lloyd and Childs 1988) seen at that time. Each experiment was repeated at least three times. The final averages reported are of data from each of the repeated experiments ( $n = 3-5$ ). Data points for each experiment were collected by counting cells plated on three coverslips. The counts were done at  $\times 40$ . Fields were randomly selected and the first 100-250 cells encountered were counted and analyzed. The raw counts of cells with single or dual labels were inserted into an Excel template which automatically calculated the percentage of cells labeled for each antigen as well as the percentage of dual labeled cells. This enabled us to constantly monitor the dual labeling to ensure that it did not interfere with the detection of either antigen. The percentages from the three coverslips provided an average for that experiment. These values were then averaged to produce the final data point ( $n = 3-5$  experiments). To determine significance in a group of experiments,  $1 \times$  ANOVA or  $2 \times$  ANOVA tests were performed. If the  $f$  value was significant ( $p < 0.05$ ), individual differences were then detected by the Fisher's least significant difference (LSD) post hoc test.

## Results

#### Regulation of c-fos mRNA by EGF and GnRH

The Northern analyses were done on pituitary pieces that were freshly removed from the animals as well as those that had been incubated in DMEM alone or DMEM containing GnRH or EGF for 30 min. C-fos mRNA was undetectable in pituitary pieces immediately after removal. However, a 30-min incubation in defined medium with or without 10 ng/ml EGF or 100

nM GnRH increased c-fos mRNA levels. There were no significant differences when the effects of medium alone, EGF, or GnRH were compared (data not shown). The defined medium contained the following additives: insulin, transferrin, sodium selenite, and bovine serum albumin (0.25%). The BSA was needed because at least two of the pituitary cell types will not respond to secretagogues without it. To test possible effects of insulin, transferrin, or sodium selenite, all additives except the BSA were removed. Incubation in the remaining medium continued to stimulate the same increment in expression of c-fos mRNA. Neither EGF nor GnRH increased expression beyond that of the medium alone (data not shown).

#### Fos Stimulation by EGF and GnRH in Total Pituitary Cell Populations

The dissociation protocol took 1 hr and the resulting pituitary cultures were plated for an additional hour. Therefore, in view of the endogenous increases in fos activity seen in the previous group of experiments, it was not surprising to detect fos proteins in these 2-hr cultures. After 1-hr plating in defined medium, 26.3% of pituitary cells expressed fos proteins.

After an additional 15–30 min in DMEM alone, the values did not change. Figure 1A illustrates a field showing baseline fos expression in several pituitary cells after 1-hr plating and an additional 15 min in medium alone. Note that the density of the labeling varies from cell to cell.

Figure 2 shows the changes seen after EGF or GnRH exposure. After only 15 min of incubation in EGF or GnRH, the percentages of pituitary cells with fos proteins were increased significantly to 34.2% and 31.8%, respectively. No further stimulation was seen after 30 min. These values are significantly higher than those seen for the DMEM controls. Figures 1B and 1C illustrate the increased percentages of cells labeled for fos in EGF- (Figure 1B) or GnRH- (Figure 1C) treated cultures.

#### Fos Stimulation Within Specific Cell Types in the Pituitary Gland

The dual labeling identified the specific cell types affected by EGF or GnRH. EGF treatment significantly increased fos expression in cells bearing the following antigens: FSH (1.2–3.7% of pituitary cells), ACTH (1.7–5.3% of pituitary cells), or GH (6.3–13.6% of pituitary cells) (Figure 3). EGF treatment had no effect on fos-immunolabeled cells that also contained LH, TSH, or prolactin antigens.

Figure 3 also shows that GnRH treatment significantly increased fos expression within each of the following antigen-bearing cells: ACTH (1.8–4.4% of pi-

tuinary cells) and GH (6.3–10.4% of pituitary cells). The other four pituitary cell types did not increase fos expression compared with the control levels. Surprisingly, GnRH stimulation had no effect on gonadotropes.

Figure 4 illustrates these data. Figures 4A and 4B show different magnifications of nonstimulated pituitary cells. A cluster of cells from Figure 4A is shown, at higher magnification, in Figure 4B. The cluster includes orange-labeled growth hormone cells (GH). Note that fos expression is seen in the nucleus of one of the GH cells in this cluster. The remaining GH cells do not appear to express fos, although fos is expressed by non-GH cells in the field (Figures 4A and 4B).

Figures 4C–4D show fos expression after stimulation by EGF. The lower magnification shown in Figure 4E illustrates two clusters, each of which shows a GH cell with fos expression. Cluster 1 is magnified in Figure 4C and cluster 2 is magnified in Figure 4D to better visualize the dual labeled GH cells.

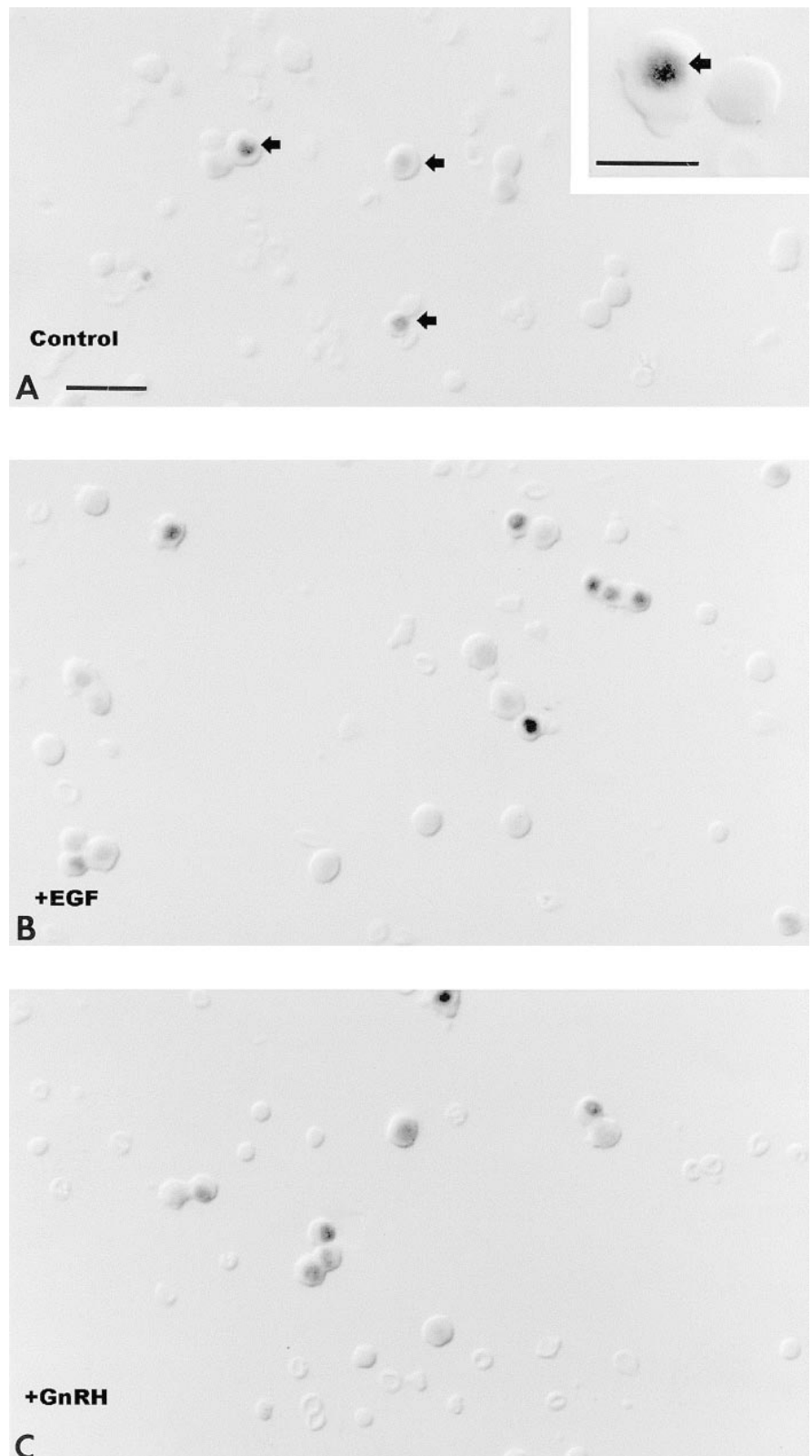
Finally, Figures 4F and 4G show the effects of EGF on FSH gonadotropes. A cluster of cells is shown in Figure 4F which was taken from a control (DMEM only) population. One of the FSH cells expresses fos proteins. After EGF stimulation, Figure 4G illustrates the increased expression of fos by FSH cells. The FSH labeling is also denser as a result of EGF stimulation.

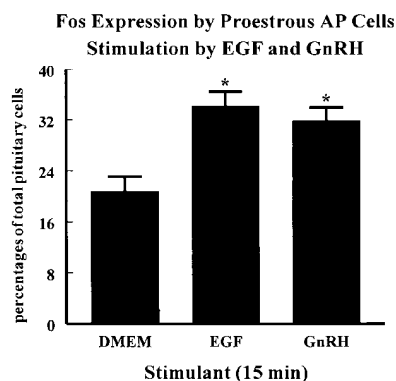
#### Discussion

Our recent cytochemical studies showed that fos protein activity is highest in cells from proestrous rats. However, the dual labeling evidence indicated that most of the fos-bearing cells in this population are not gonadotropes (Armstrong and Childs 1997b). This initially raised a question about fos as a third messenger in the activation of the gonadotrope during proestrus and led to subsequent studies designed to learn if GnRH can stimulate fos expression by gonadotropes from proestrous rats. Furthermore, because the maturing gonadotrope also expresses EGF receptors (Armstrong and Childs 1997a,c), we studied EGF as a possible activator of fos in gonadotropes. We hypothesized that both EGF and GnRH would stimulate fos expression in gonadotropes during a period when their receptors were at a peak. Therefore, proestrous cells were used in this phase of the study because expression of receptors for GnRH or EGF is at a peak (Lloyd and Childs 1988; Childs et al. 1994a,b; Armstrong and Childs 1997a,c).

Activation of c-fos occurs in a very short time frame, as shown by these and other experiments (Autelitano and Sheppard 1993; Cesnjaj et al. 1993). The Northern analysis experiments showed that c-fos mRNA levels were upregulated by medium alone within 30 min after the pituitary glands were collected. These results

**Figure 1** Single labeling for fos proteins in population of cells from pro-estrous rats. (A) Some pituitary cells express fos proteins after 1 hr in culture and 15 min in medium alone (arrows). The labeling is confined to the nucleus (inset), but it varies from a light gray to dense black. Both EGF (B) and GnRH (C) stimulated additional cells to produce fos proteins after only 15 min. Note also that there are more cells that exhibit the darkest label for fos proteins in their nucleus. Bar = 20  $\mu$ m; inset = 10  $\mu$ m.





**Figure 2** Bar graph illustrating the counts of fos-labeled cells. Cells incubated for 15 min in medium alone showed 21–26% cells with fos proteins in the nucleus. Exposure to EGF or GnRH produced a 31–38% increase in expression. \*, significantly different from control values.

agree with those of Sheng and Greenberg (1990), who reported that c-fos expression can only be seen for a short time before it is degraded. We modeled our initial studies after those of Cesnjaj et al. (1994), who looked at cell cultures from randomly cycling female rats. They stimulated the pituitary cell cultures with GnRH for 30 min and found significant increases in c-fos expression. The present studies correlate with those of Cesnjaj et al. in that they show that GnRH does increase expression of fos proteins in primary cultures of pituitary cells in 15–30 min.

#### Endogenous Regulation of c-fos mRNA Expression

The present studies raised questions about the events that occur as the pituitaries are removed from the animal. This affects interpretation of data from assays of fos mRNA or proteins. When we assayed pituitary pieces immediately after removal, no c-fos mRNA was detected. However, after 30 min of incubation in medium alone, c-fos mRNA was activated. Neither GnRH nor EGF stimulated c-fos mRNA levels above the background levels seen with this defined medium. Similarly, additional experiments in medium without insulin, sodium selenite, and transferrin also showed that medium alone continued to be a stimulant for c-fos expression. Therefore, under these experimental conditions we were unable to separate possible stimulatory effects of GnRH and EGF on c-fos mRNA from effects seen after removal from the pituitary and incubation in a defined medium.

These data suggest that c-fos mRNA is stimulated by local factors, possibly in response to the stress associated with pituitary removal from the body. Locally produced EGF or other growth factors by pituitary cells themselves are candidates for regulation of this response. Therefore, to show fos stimulation by any

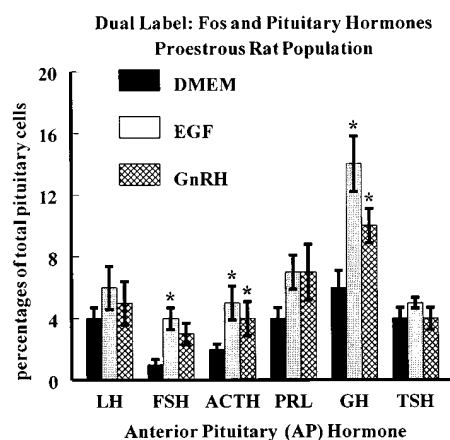
growth factor, one must define the basal conditions carefully and recognize that exogenous stimulation may be superimposed on endogenous stimulation by resident growth factors. The pituitary will undoubtedly respond to its removal from the influence of the hypothalamus, and early gene expression may mediate that response.

#### Identity of the fos-producing Cells

The findings from the Northern analysis suggested that one must be cautious about interpreting data from any in vitro studies. Nevertheless, the studies by Cesnjaj et al. (1994) and the present study show that information about factors that regulate fos expression could be obtained once baseline values were low enough. It is possible that the endogenous stimulation is self-limiting, which may allow tests of regulatory factors at later time points.

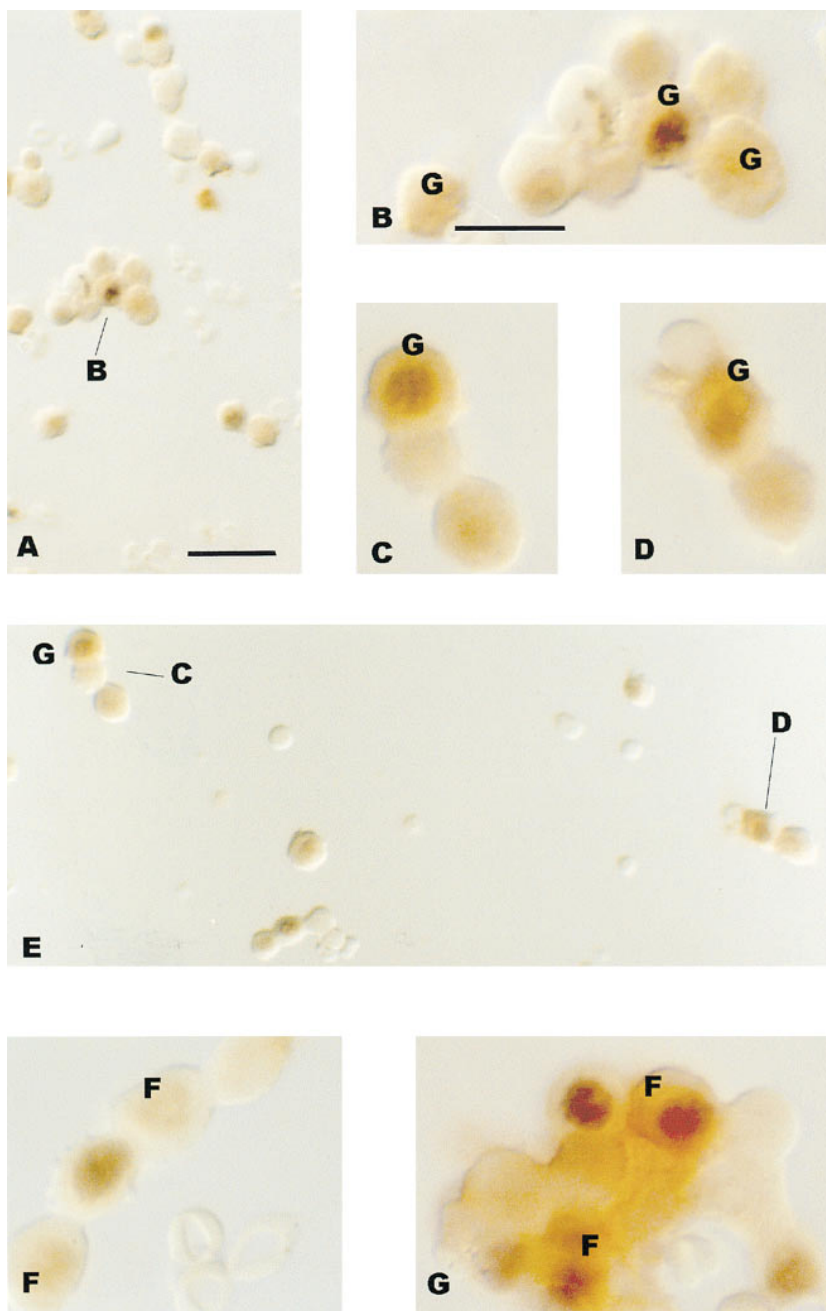
In the studies by Cesnjaj et al. (1994) the pituitary cells had been grown for several days in serum-containing medium. The serum was then removed and the cells were incubated in serum-free medium with or without GnRH for 30 min. In their studies, c-fos mRNA was detected in the control cultures and GnRH stimulated increases in c-fos mRNA beyond that of the control values.

Therefore, we knew that GnRH could stimulate fos. The finding that it was stimulatory in populations of a gonadotrope cell line led to the hypothesis that it stimulated fos production in normal gonadotropes as well. Therefore, one objective of our in vitro studies was to test this hypothesis and determine which cell types might be responding to GnRH with fos produc-



**Figure 3** Dual labeling showed changes in fos proteins in subsets of some of the pituitary cell types. After 15 min, 10 nM GnRH stimulated significant increases in cells with fos and GH or ACTH antigens. EGF stimulated significant increases in cells with fos and FSH, GH, or ACTH antigens. \*, significant difference between control and experimental groups.

**Figure 4** Dual labeled fields allowed the identification of the cells expressing fos and pituitary hormones. **A–E** were dual labeled for fos proteins and growth hormone (GH). **A** and **B** were incubated in DMEM alone. Clusters of cells are seen with only fos or GH immunoreactivity alone. **(B)** One cluster shown at higher magnification contains a GH cell that also expresses fos proteins. **(C–E)** Cells after 15 min in EGF; **C** is low-magnification view. **(C,D)** Two cell clusters are shown in higher-magnification views. Both clusters contain a GH cell with fos immunoreactivity. **(F,G)** FSH cells and fos proteins. Only one of the FSH cells contains fos proteins in **F**, which is from a control population. **(G)** A larger colony of more densely labeled FSH cells. Several of the FSH cells show labeling for fos proteins in their nuclei. Bars: **A** = 20  $\mu\text{m}$ ; **B** = 10  $\mu\text{m}$ .



tion. Furthermore, since GnRH and EGF receptors peak at the same time (proestrous AM), we hypothesized that the gonadotropes may be acutely sensitive to effects of EGF or GnRH early in proestrus. This would be an ideal time to test EGF's effects on fos proteins.

In these experiments, we also wanted to reduce the time between removal from the animal and fixation to that needed for dissociation (1 hr) and plating (1 hr), to reduce the time during which endogenous factors may be stimulating fos. When we compared cells fixed at the end of the plating period with those fixed after

the 15 min needed for the stimulation, medium alone did not produce increases in fos proteins as detected in immunocytochemical tests. Therefore, a baseline was established above which the effects of stimulatory factors could be detected. Both GnRH- and EGF-treated cultures showed 30–40% increases in the percentages of cells expressing fos proteins overall in the pituitary (over those produced by medium alone) (Figure 1).

However, our studies showed some surprising results about the identity of the fos-producing cells. GnRH stimulated fos expression in ACTH, GH, and prolactin cells but did not stimulate fos production in

proestrous gonadotropes. EGF stimulated fos expression in FSH cells, ACTH cells, and GH cells, cells known to possess EGF receptors (Fan and Childs 1995).

#### Effects of GnRH on fos Proteins

There is no doubt that gonadotropes express the *c-fos* early response gene and that GnRH stimulates expression of *c-fos* mRNA or fos proteins in the anterior pituitary (Cesnjaj et al. 1994; Padmanabhan et al. 1995). The present studies and recent publications (Cesnjaj et al. 1994; Armstrong and Childs 1997b) provide proof that normal gonadotropes express fos. In our recent study, the highest expression of fos by pituitary gonadotropes (metestrus) did not coincide with the highest expression of GnRH receptors (Lloyd and Childs 1988) (proestrus). It is possible that any GnRH effect on gonadotrope fos occurs in the cells that retain receptors early in the cycle (about 40% of the gonadotropes). This is a subject for future studies.

The GnRH-mediated increase in fos expression appears to be in either GH cells (which may become transitional gonadotropes; Childs et al. 1994a) or corticotropes. The data on GH cells correlate with previous studies showing that 32% of GH cells express GnRH receptors during early proestrus (Childs et al. 1994b). Perhaps GnRH regulates changes in this population to support the estrous cycle activity and fos is a third messenger in the regulatory pathway. For example, GH cells are known to express LH $\beta$  mRNA and FSH $\beta$  mRNA during peak periods of expression in proestrus (Childs et al. 1994a). GnRH regulates transcription of gonadotropin mRNAs (Ramey et al. 1987; Lalloz et al. 1988; Mercer 1990).

GnRH receptors are not found on corticotropes. However, GnRH actions could also be mediated indirectly by paracrine interactions. Further evidence for paracrine interactions modulated by GnRH can be seen in studies by Tilemans et al. (1992). These workers have shown that treatment with GnRH in immature rat pituitary cell aggregates, increased [ $^3$ H]-thymidine incorporation into corticotropes and lactotropes while inhibiting DNA replication in somatotropes. The effects of GnRH on these cells were clearly dependent on gonadotropes. If gonadotropes were removed from the aggregates, GnRH produced no increases in tritiated thymidine.

Finally, one possible paracrine factor that might mediate the GnRH action on these cells is angiotensin II. Gonadotropes have been shown to release angiotensin II (Houben et al. 1990; Schwartz and Cherny 1992). Angiotensin II can act in a paracrine manner to stimulate release of prolactin (Houben et al. 1990) and the release of ACTH (Schwartz and Cherny 1992). Because of these paracrine interactions, it can

be postulated that GnRH binds to its receptor on the gonadotropes to stimulate the release of angiotensin II, which in turn stimulates the release of hormones from target pituitary cell types. This stimulation could involve fos as a third messenger.

#### Effects of EGF on fos Proteins

As stated above, corticotropes do not have GnRH receptors. However, they do have receptors for EGF (Fan and Childs 1995; Armstrong and Childs 1997a). Therefore, they may be stimulated by EGF produced by other cell types, such as GH cells or gonadotropes themselves.

Mouihate and Lestage (1995) have shown that EGF can be released from gonadotropes, and EGF is a known secretagogue for ACTH (Childs et al. 1991; reviewed in Schwartz and Cherny 1992). EGF also stimulates ACTH cell differentiation (Childs et al. 1991) and proliferation in the corticotrope population (Childs et al. 1995). Therefore, the increased fos activity mediated by GnRH may reflect a paracrine interaction with EGF as the mediator. This hypothesis is further supported by our findings in the present study that show direct effects of EGF on expression of fos by corticotropes.

In addition to its effects on corticotropes, EGF increases fos expression in GH cells and FSH cells. The increase in percentages of FSH cells with fos brings the final values to levels similar to those expressed by LH cells. Therefore, it is possible that EGF is selectively stimulating fos expression by LH cells, leading to translation of FSH (monohormonal LH cells are becoming bihormonal). This hypothesis is supported by the cytochemical data showing increased labeling for FSH in EGF-treated cells (Figure 4G). Alternatively, EGF could be acting on a population of monohormonal FSH cells to prepare them for the proestrous or estrous surge secretory activity.

#### Summary and Conclusions

To summarize, both EGF and GnRH may regulate immediate early gene expression in key pituitary cells, either directly or indirectly via paracrine factors. The dual cytochemical evidence showed the identity of the target cells and the fact that multiple cell types may be stimulated. Whereas this study showed that the *in vitro* studies are valuable in that they enable one to test individual secretagogues or growth factors in a system that removes the cells from other regulators (such as steroids or other serum influences), they must also be interpreted with caution. The Northern blot analyses of fos mRNA in the anterior pituitary gland assayed within 30 min of removal from the gland showed significant increases in expression of *c-fos* in medium containing virtually no exogenous growth

factors. Therefore, local paracrine or autocrine regulators may also play a role in regulating early gene expression. This background regulation must be considered in designing the *in vitro* experiments.

#### Acknowledgments

Supported by NSF IBN 9724066 and NIH R01 HD 33915.

We thank Ms Diana Rougeau and Geda Unabia for excellent technical assistance with this work. We also thank Drs Gregg Nagle and Xuemo Fan for help with the Northern Analysis.

#### Literature Cited

- Armstrong J, Childs GV (1997a) Changes in expression of epidermal growth factor receptors by anterior pituitary cells during the estrous cycle: cyclic expression by gonadotropes. *Endocrinology* 138:1903-1908
- Armstrong J, Childs GV (1997b) Differential expression of c-fos *in vitro* by all anterior pituitary cell types during the estrous cycle: enhanced expression by luteinizing hormone but not follicle-stimulating hormone cells. *J Histochem Cytochem* 45:785-794
- Armstrong JL, Childs GV (1997c) Regulation of expression of epidermal growth factor receptors in gonadotropes by epidermal growth factor and estradiol: studies in cycling female rats. *Endocrinology* 138:5434-5441
- Autelitano D, Sheppard K (1993) Corticotrope responsiveness to glucocorticoids is modulated via rapid CRF-mediated induction of the proto-oncogene c-fos. *Mol Cell Endocrinol* 94:111-119
- Cesnjar M, Catt KJ, Stojilkovic SS (1994) Coordinate actions of calcium and protein kinase-C in the expression of primary response genes in pituitary gonadotrophs. *Endocrinology* 135:692-701
- Cesnjar M, Krsmanovic L, Catt K, Stojilkovic S (1993) Autocrine induction of c-fos expression in GT1 neuronal cells by gonadotropin-releasing hormone. *Endocrinology* 133:3042-3045
- Childs G, Patterson J, Unabia G, Rougeau D, Wu P (1991) Epidermal growth factor enhances ACTH secretion and expression of POMC mRNA by corticotropes in mixed and enriched cultures. *Mol Cell Neurosci* 2:2885-2895
- Childs GV, Rougeau D, Unabia G (1995) Corticotropin-releasing hormone and epidermal growth factor: mitogens for anterior pituitary corticotropes. *Endocrinology* 136:1595-1602
- Childs GV, Unabia G, Miller B (1994b) Cytochemical detection of gonadotropin-releasing hormone-binding sites on rat pituitary cells with luteinizing hormone, follicle-stimulating hormone, and growth hormone antigens during diestrous up-regulation. *Endocrinology* 134:1943-1951
- Childs GV, Unabia G, Rougeau D (1994a) Cells that express luteinizing hormone (LH) and follicle stimulating hormone (FSH) beta ( $\beta$ ) subunit mRNAs during the estrous cycle: the major contributors contain LH $\beta$ , FSH $\beta$  and/or growth hormone. *Endocrinology* 134:990-997
- Childs G, Unabia G, Tibolt R, Lloyd J (1987) Cytological factors that support nonparallel secretion of luteinizing hormone and follicle-stimulating hormone during the estrous cycle. *Endocrinology* 121:1801-1813
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159
- Fan X, Childs GV (1995) EGF and TGF $\alpha$  mRNA and their receptors in the rat anterior pituitary: localization and regulation. *Endocrinology* 136:2284-2324
- Fan X, Nagle GT, Collins TJ, Childs, GV (1995) Differential regulation of EGF and TGF $\alpha$  in the rat anterior pituitary and hypothalamus induced by stresses. *Endocrinology* 136:873-880
- Fan X, and Nagle GT (1996) Molecular cloning of Aplasia neuronal cDNAs that encode carboxypeptidases related to mammalian prohormone processing enzymes. *DNA Cell Biol* 15:937-945
- Franza BR Jr, Rauscher FJ III, Josephs SF, Curran T (1988) The fos complex and fos-related antigens recognize sequence elements that contain AP-1 binding sites. *Science* 239:1150-1153
- Houben H, Tilemans D, Deneef C (1990) Towards a functional significance of peptides and biogenic amines produced by the anterior pituitary. *J Endocrinol Invest* 13:855-863
- Kruijer W, Schubert D, Verma I (1985) Induction of the proto-oncogene *fos* by nerve growth factor. *Cell Biol* 82:7330-7334
- Lalloz MRA, Detta A, Clayton RN (1988) Gonadotropin-releasing hormone is required for enhanced luteinizing hormone subunit gene expression *in vivo*. *Endocrinology* 122:1681-1688
- Lee W, Smith S, Hoffman G (1990) Luteinizing hormone-releasing hormone neurons express fos protein during the proestrus surge of luteinizing hormone. *Proc Natl Acad Sci USA* 87:5163-5167
- Lloyd J, and Childs G (1988) Changes in the number of GnRH-receptive cells during the rat estrous cycle: biphasic effects of estradiol. *Neuroendocrinology* 48:138-146
- Mercer J (1990) Pituitary gonadotropin gene regulation. *Mol Cell Endocrinol* 73:C63-67
- Miyake A, Tasaka K, Otsuka S, Kohmura H, Wakimoto H, Aono T (1985) Epidermal growth factor stimulates secretion of rat pituitary luteinizing hormone *in vitro*. *Acta Endocrinol* 108:175-178
- Moriarty GV, Halmi N (1972) Electron microscopic study of the adrenocorticotropin-producing cell with the use of unlabeled antibodies and the soluble peroxidase-antiperoxidase complex. *J Histochem Cytochem* 20:590-603
- Mouihate A, and Lestage J (1995) Epidermal growth factor: a potential paracrine and autocrine system within the pituitary. *Neuroreport* 6:1273-1276
- Muller R, Bravo R, Burckhardt J, Curran T (1984) Induction of c-fos gene and protein by growth factors precedes activation of c-myc. *Nature* 312:716-720
- Padmanabhan V, Dalkin A, Yasin M, Haisenleder DJ, Marshall J, Landfeld T (1995) Are immediate early genes involved in gonadotropin-releasing hormone receptor gene regulation? Characterization of changes in GnRH receptor (GnRH-R), c-fos, and c-jun messenger ribonucleic acids during the ovine estrous cycle. *Biol Reprod* 53:263-269
- Ramey JW, Highsmith RF, Wilfinger WW, Baldwin DM (1987) The effects of gonadotropin-releasing hormone and estradiol on luteinizing hormone biosynthesis in cultured rat anterior pituitary cells. *Endocrinology* 120:1503-1513
- Rauscher F, Cohen D, Curran T, Bos T, Vogt P, Bohmann D, Tjian R, Franza B (1988) Fos-associated protein p39 is the product of the *jun* proto-oncogene. *Science* 240:1010-1016
- Sagar S, Edwards R, Sharp F (1991) Epidermal growth factor and transforming growth factor  $\alpha$  induce c-fos gene expression in retinal Muller cells *in vivo*. *J Neurosci Res* 29:549-559
- Sagar S, Sharp F, Curran T (1988) Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* 240:1328-1331
- Schwartz J, Cherny R (1992) Intercellular communication within the anterior pituitary influencing the secretion of hypophysial hormones. *Endocrine Rev* 13:453-475
- Sheng M, Greenberg ME (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* 4:477-485
- Tilemans D, Andries M, Deneef C (1992) Luteinizing hormone-releasing hormone and neuropeptide Y influence deoxyribonucleic acid replication in three anterior pituitary cell types. Evidence for mediation by growth factors released from gonadotropes. *Endocrinology* 130:882-894

