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Regulation of Pro-opiomelanocortin Gene Transcription in Individual Cell Nuclei

ROBERT T. FREMEAU, JR., JAMES R. LUNDBLAD, DOLAN B. PRITCHETT, JOSIAH N. WILCOX,* JAMES L. ROBERTS

A nonrepetitive complementary RNA probe specific for an intervening sequence of the rat pro-opiomelanocortin (POMC) gene primary transcript was used to analyze the hormonal regulation of POMC gene transcription in individual cell nuclei in the rat pituitary by in situ hybridization. This probe recognized only full-length POMC heterogeneous nuclear RNA, as verified by Northern blots of pituitary RNA. When pituitary sections were hybridized with this ^{3}H-labeled POMC intron A probe, silver grains were predominantly localized over the nuclei of cells that expressed POMC in the anterior and intermediate lobes. Adrenalectomy increased both the average grain density over corticotroph nuclei and the number of cells in the anterior pituitary with significant numbers of silver grains over their nucleus. Dexamethasone administration to intact or adrenalectomized rats results in the rapid (within 30 minutes) disappearance of silver grains over the nuclei of corticotrophs in the anterior lobe, suggesting that POMC gene transcription had been inhibited. However, adrenalectomy or dexamethasone administration did not alter the silver grain density over nuclei of intermediate lobe melanotrophs. Thus, this in situ hybridization assay utilizing an intervening sequence–specific POMC probe can measure rapid physiological changes in POMC heterogeneous nuclear RNA in individual cell nuclei.

THE PRO-OPIOMELANOCORTIN GENE and its peptide products, including adrenocorticotrophic hormone (ACTH), β-endorphin, and the melanotropins, are subject to tissue-specific differential regulation of peptide secretion and gene expression (*1*). For example, in anterior lobe corticotrophs (*2*), but not in intermediate lobe melanotrophs (*2*), ACTH is one of the primary end products of proteolytic process-

Department of Biochemistry and Center for Reproductive Sciences, Columbia University College of Physicians and Surgeons, 630 West 168 Street, New York, NY 10032.

*Present address: Department of Molecular Biology, Genentech Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080.

ing (*2*). In a classic neuroendocrine feedback loop, glucocorticoids, which are synthesized in and secreted from the adrenal cortex in an ACTH-dependent manner, inhibit pro-opiomelanocortin (POMC) peptide secretion and gene transcription in the anterior lobe corticotroph, but have no effect on the intermediate lobe melanotroph (*3*). In contrast, adrenalectomy (ADX) elevates anterior lobe POMC gene transcription and POMC messenger RNA (mRNA); this increase can be reversed by administration of the synthetic glucocorticoid, dexamethasone (DEX) (*3*). The absence of an effect of glucocorticoids on POMC gene expression in the neurointermediate lobe is believed to be due to a lack of functional glucocorticoid receptors (*4*). Thus, for the pituitary POMC system, tissue-specific differences in regulation at the secretory level are paralleled by changes in the level of gene transcription. These studies, as well as others on prolactin (*5*) and growth hormone (*6*), suggest that a metabolic event during receptor-mediated stimulus-secretion coupling stimulates transcription of the gene encoding the secreted peptide to maintain a production rate of the peptide precursor sufficient for the secretion rate.

Studies of the secretory activity of individual cells have indicated that individual cells within a tissue thought to function homogeneously may respond heterogeneously to specific secretagogues (*7*). For example, isolated rat anterior pituitary corticotrophs respond heterogeneously to corticotropin-releasing hormone (CRH) (*7*). Thus, to determine if the correlation between POMC peptide secretion and gene transcription exists at the single-cell level, it would be of value to directly examine the transcriptional and secretory activity of individual POMC-expressing cells. To approach this question we developed an assay capable of analyzing the transcriptional activity of specific genes in individual cells.

In situ hybridization techniques employing coding sequence probes have been used to study the physiological regulation of various neuroendocrine peptide mRNA's in the brain and pituitary (*8, 9*). One shortcoming of these studies is the relatively slow time course of the change in cytoplasmic mRNA levels because of their large pool size. We reasoned that, with a probe from the first intervening sequence of the rat POMC gene, we could detect rapid alterations in the level of the POMC gene primary transcript within individual cell nuclei before we could detect alterations in the corresponding cytoplasmic mRNA.

The basic premise of this assay is that the primary transcript of eukaryotic genes transcribed by RNA polymerase II is rapidly processed to mature mRNA in the nucleus, appearing in the cytoplasm within 30 minutes after synthesis of the primary transcript (*10*). The excised introns are degraded even more rapidly, as their levels are significantly lower than those of the primary transcript (*10*). Therefore, because of its rapid processing, the nuclear level of the primary transcript should reflect its rate of synthesis, namely, transcription of the gene.

We subcloned a unique-sequence 1.5-kb Bam HI–Kpn I fragment of intervening sequence A (POMC IVS-A) of the rat POMC gene (striped bar in Fig. 1A) into the polylinker of the plasmid pSP6 and synthesized radioactively labeled sense or antisense single-stranded RNA probes for our in situ hybridization studies. The POMC exon III–specific probe used in our study was the I13 POMC complementary DNA (cDNA) (*11*) subcloned into the polylinker of pSP6.

To determine which species of RNA were recognized by the POMC IVS-A probe, a Northern blot analysis of total RNA isolated from rat neurointermediate pituitary was conducted with both POMC IVS-A and exon III probes. The POMC exon III–specific probe hybridized to two RNA's (Fig. 1B, lane 1). The predominant band corresponded to mature 1.1-kb POMC mRNA while the larger molecular weight band had a mobility equivalent to that expected of the rat POMC gene primary transcript, approximately 6.5 kb. When this Northern blot was probed again with ^{32}P-labeled POMC IVS-A complementary RNA (cRNA) (Fig. 1B, lane 2), a single 6.5-kb band was detected. Thus, the POMC IVS-A cRNA probe detected only the primary transcript of the POMC gene and not heterogeneous nuclear RNA (hnRNA) processing intermediates, the excised intron or its degradation products, or the mature mRNA. Furthermore, when a Northern blot of nuclear and cytoplasmic RNA from the neurointermediate pituitary was incubated with ^{32}P-labeled POMC IVS-A cRNA, a single band corresponding to the POMC gene primary transcript was detected in nuclear but not cytoplasmic RNA (Fig. 1B, lanes 3 and 4). Our inability to detect the excised intron by Northern analysis suggests that it is rapidly degraded in the nucleus after splicing, as shown in other systems (*10*). The POMC IVS-A probe can therefore be used as a hybridization probe to detect the POMC gene primary transcript in nuclear RNA.

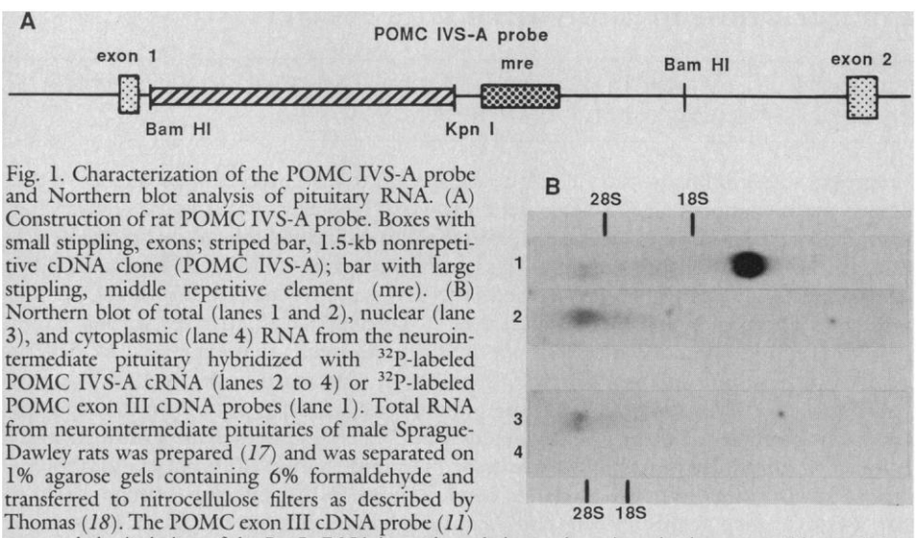

Fig. 1. Characterization of the POMC IVS-A probe and Northern blot analysis of pituitary RNA. (A) Construction of rat POMC IVS-A probe. Boxes with small stippling, exons; striped bar, 1.5-kb nonrepetitive cDNA clone (POMC IVS-A); bar with large stippling, middle repetitive element (mre). (B) Northern blot of total (lanes 1 and 2), nuclear (lane 3), and cytoplasmic (lane 4) RNA from the neurointermediate pituitary hybridized with ^{32}P-labeled POMC IVS-A cRNA (lanes 2 to 4) or ^{32}P-labeled POMC exon III cDNA probes (lane 1). Total RNA from neurointermediate pituitaries of male Sprague-Dawley rats was prepared (*17*) and was separated on 1% agarose gels containing 6% formaldehyde and transferred to nitrocellulose filters as described by Thomas (*18*). The POMC exon III cDNA probe (*11*) was made by isolation of the Pst I cDNA insert by gel electrophoresis and subsequent nick translation with ^{32}P-labeled nucleotides (specific activity, 5×10^8 cpm/μg). The blotted nitrocellulose filters were hybridized with 25×10^6 cpm overnight at 42°C and washed at high stringency. The final wash was 0.2× standard saline citrate (SSC) and 0.1% SDS at 50°C. (1× SSC is 150 m*M* NaCl, 15 m*M* sodium citrate, *p*H 7.0). Filters were exposed to Kodak XAR-5 x-ray film with a Cronex screen at −70°C for 24 hours. The filters were then boiled in water to remove bound probe and probed again with 100×10^6 cpm of ^{32}P-labeled POMC IVS-A cRNA at 50°C overnight. The filters were then washed at high stringency (final wash, 0.1× SSC and 0.1% SDS at 55°C), dried, and exposed as above for 1 week. To isolate nuclear and cytoplasmic RNA, neurointermediate pituitary lobes were homogenized in 1 ml of AT buffer (10 m*M* tris-HCl, *p*H 8.0, 3 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 0.3*M* sucrose, 0.5 m*M* dithiothreitol (DTT), and 0.15% Triton X-100) in a Dounce homogenizer with a loose-fitting pestle (ten strokes). The homogenate was layered over 0.4 ml of AT buffer containing 0.4*M* sucrose and centrifuged at 2500*g* for 10 minutes at 4°C to pellet the nuclei. The supernatant was retained for isolation of cytoplasmic RNA as described above. The nuclear pellet was resuspended in 100 μl of 5*M* guanidine thiocyanate, 10 m*M* EDTA, 50 m*M* tris-HCl, *p*H 7.5, and 8% β-mercaptoethanol and vortexed. The nuclear homogenate was transferred to a Dounce homogenizer and homogenized with ten strokes of a loose-fitting pestle. Nuclear RNA was then isolated from the nuclear homogenate as described above.

Previously, we determined the distribution of cells that expressed POMC in the rat pituitary by in situ hybridization with a POMC exon III cDNA probe (*8*). Five percent of the cells in the anterior lobe and more than 95% of the cells of the intermediate lobe of the rat pituitary expressed the POMC gene; no labeling of cells in the posterior lobe was observed. This pattern of gene expression paralleled the distribution of POMC cells determined by immunohistochemistry on serial sections. When pituitary sections were hybridized with antisense ^{32}P-labeled POMC IVS-A cRNA, x-ray exposures showed a distribution of radioactivity similar to that of POMC (Fig. 2A): heavy labeling over the intermediate lobe (the horseshoe-like structure), light labeling over the anterior lobe (outside the horseshoe), and no labeling over the posterior lobe (inside the horseshoe). Consistent with the fact that there was approximately 20 times less nuclear POMC hnRNA than cytoplasmic POMC mRNA (Fig. 1), several days rather than several hours of exposure were required for a comparable autoradiographic signal.

To characterize the specificity of the signal, we examined the effects of ribonuclease A (RNase A) and deoxribonuclease I (DNase I) treatment of the pituitary sections on subsequent hybridization with ^{32}P-labeled POMC IVS-A. Treatment of the tissue sections with RNase A (20 μg/ml) at 22°C for 30 minutes before hybridization with ^{32}P-labeled POMC IVS-A cDNA dramatically reduced the autoradiographic signal (Fig. 2B). In contrast, when the tissue sections were pretreated with DNase I (200 μg/ml) (Cooper Biomedical) at 22°C for 15 minutes before hybridization with ^{32}P-labeled POMC IVS-A cRNA, no significant reduction in the signal was observed (Fig. 2C). Furthermore, when pituitary sections were hybridized with the POMC IVS-A probe in the sense orientation, no specific labeling was observed (Fig. 2D). Hence, the autoradiographic signal was derived primarily from ^{32}P-labeled POMC IVS-A cRNA-hnRNA hybrids and not from hybridization to the two copies of the POMC gene in the diploid genome.

To determine the intracellular localization of the POMC IVS-A probe, pituitary sections were hybridized with ^{3}H-labeled POMC IVS-A cRNA and the hybrids were detected by nuclear track emulsion autoradiography (Kodak NTB2). Silver grains were specifically localized over the nuclei of POMC-expressing cells in both the anterior and intermediate lobes of the pituitary (Fig. 3, A and B). No localization of silver grains occurred over nuclei in the posterior lobe. Similar observations were made on 30 different sections derived from 13 animals. In contrast, as expected from the larger size of the pool of cytoplasmic POMC mRNA compared to nuclear POMC hnRNA or mRNA, when pituitary sections were hybridized with ^{3}H-labeled POMC exon III cRNA, the majority of silver grains were localized over the cytoplasm of POMC-expressing cells (Fig. 3C). Thus, consistent with the Northern analysis (Fig. 1B), the autoradiographic signal obtained with the POMC IVS-A probes appears to result from specific hybridization to primary transcripts of the POMC gene localized in the nuclei of POMC-expressing cells.

Adrenalectomy rapidly increases (within 1 hour) POMC gene transcription in the anterior lobe, which then remains elevated after 1 week (*3*). In contrast to the rapid increase in transcription, POMC mRNA levels do not increase significantly until approximately 8 hours after ADX (*3*). Similarly, when glucocorticoids are administered to control rats or to rats subject to long-term ADX, there is a rapid inhibition of anterior pituitary POMC gene transcription with maximal inhibition occurring within 30 minutes (*3*). However, consistent with a half-life of 18 to 24 hours (*3*), a detectable reduction in anterior pituitary POMC mRNA levels occurs after a lag period of 6 to 8 hours. In contrast, ADX or DEX treatment has little effect on POMC gene transcription or mRNA levels in the neurointermediate lobe.

One week after ADX, we observed a threefold increase in the percentage of cells in the anterior pituitary with significant numbers (fivefold over background) of silver grains over their nuclei. Grain count analysis (*12*) revealed that 3.3 ± 0.9% (mean ± SD; two sections; two animals; 1091 cells analyzed) of the cells in the anterior lobe of pituitary sections from control animals contained significant numbers of silver grains over their nuclei (Fig. 4A). In contrast, 9.9 ± 2.0% (three sections; one animal; 1822 cells analyzed) of the cells in the anterior lobe of ADX (1 week) rats contained significant numbers of silver grains (Fig. 4B) ($P < 0.001$). In addition to increasing the percentage of detectable cells actively transcribing the POMC gene, ADX also increased the silver grain density over the corticotroph nuclei. Anterior lobe corticotrophs from control pituitary sections contained 14 ± 5 grains per nucleus (mean ± SD; two sections; two animals; 110 cells analyzed), whereas corticotrophs from ADX pituitary sections contained 24 ± 7 grains per nucleus (three sections; one animal, 157 cells analyzed) ($P < 0.001$). Thus the increase in POMC gene transcription in the rat anterior pituitary after ADX is the result of both an increased number of cells actively

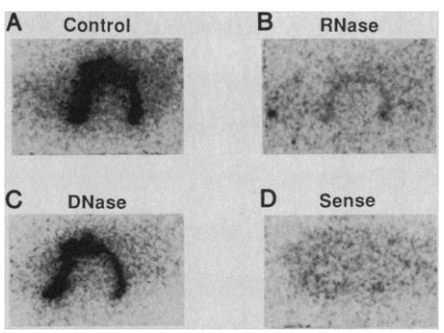

Fig. 2. Characterization of ^{32}P-labeled POMC IVS-A autoradiographic signal in rat pituitary. Adult male Sprague-Dawley rats were anesthetized by injection of sodium pentobarbital (250 mg per 200 g of body weight) and the systemic circulation was rinsed by cardiac perfusion with 20 ml of 0.9% saline at room temperature. Animals were then perfused with approximately 200 ml of ice-cold 4% paraformaldehyde in 0.1*M* phosphate buffer (*p*H 7.4) through the aorta at a pressure of 100 to 140 mmHg for 20 minutes. After perfusion the rats were decapitated, and the pituitaries were removed and immersed in 15% sucrose in phosphate-buffered saline (PBS) for 60 minutes at 4°C. Pituitaries were embedded in M-1 embedding matrix (Lipshaw Manufacturing Co.) and frozen in liquid nitrogen. Ten-micrometer sections were cut with a Hacker Cryostat microtome, thaw-mounted onto gelatin and chrom-alum–coated glass microscope slides, and stored at −70°C until use. Tissue sections were thawed and treated with (A) control buffer, 0.5*M* NaCl, 10 m*M* tris HCl, *p*H 7.5, for 30 minutes at 22°C; (B) RNase A (20 μg/ml) (Sigma) at 22°C for 30 minutes in 05*M* NaCl, 10 m*M* tris-HCl, *p*H 7.5; or (C) RNase-free DNase I (200 μg/ml) (Worthington) at 22°C for 15 minutes in 50 m*M* tris-HCl, *p*H 7.5, 10 m*M* $MgSO_4$, 1 m*M* DTT, and then thoroughly washed in 2× SSC (three changes, 5 minutes each). Tissue sections were then covered with hybridization solution [50% formamide, 0.3*M* NaCl, 20 m*M* tris-HCl, *p*H 8.0, 5 m*M* EDTA, 1× Denhardt's solution, yeast transfer RNA (500 μg/ml), 10% dextran sulfate, and 0.1% SDS] and incubated for 60 minutes at 50°C. After this "prehybridization," the buffer was removed, the tissue sections were washed for 10 minutes in 2× SSC, and the sections were covered with 50 μl of hybridization buffer containing either (i) 2×10^5 cpm of heat denatured (15 minutes at 65°C) ^{32}P-labeled POMC IVS-A antisense RNA (A and C) or message sense RNA (D), or (ii) 2×10^5 cpm of heat-denatured (5 minutes at 100°C) ^{32}P-labeled POMC IVS-A cDNA (B), a cover slip was applied, and the sections were hybridized for 16 hours at 50°C. After hybridization, the slides were washed twice for 10 minutes each in 2× SSC at room temperature. The slides were then treated with RNase A (20 μg/ml) in 0.5*M* NaCl, 10 m*M* tris-HCl, *p*H 8.0, for 30 minutes at 37°C, and washed twice for 10 minutes each in 2× SSC at 45°C. The slides were then washed in 3 liters of 0.1× SSC at 50°C for 60 minutes followed by an overnight wash in 3 liters of 0.1× SSC at room temperature. The slides were then dehydrated in graded ethanol (50%, 70%, 95%; 2 minutes each) containing 0.3*M* ammonium acetate, dried in a vacuum desiccator, and exposed to x-ray film.

transcribing the POMC gene, as well as an increased rate of transcription per cell. In contrast, the silver grain density and hence POMC gene transcription in the intermediate lobe was not significantly altered by ADX, in agreement with previous transcription studies (*3*).

Administration of DEX (100 μg, intraperitoneally) to rats that had been adrenalectomized for 1 week resulted in the rapid (within 30 minutes) disappearance of silver grains over the nuclei of cells in the anterior lobe (Fig. 4C), but not the intermediate lobe. These results are consistent with the rapid inhibition of POMC gene transcription by glucocorticoids and indicate that, like other eukaryotic RNA polymerase II–transcribed genes (*10*), the turnover of POMC hnRNA is rapid, with a half-life significantly less than 30 minutes.

To examine the effects of ADX and subse-

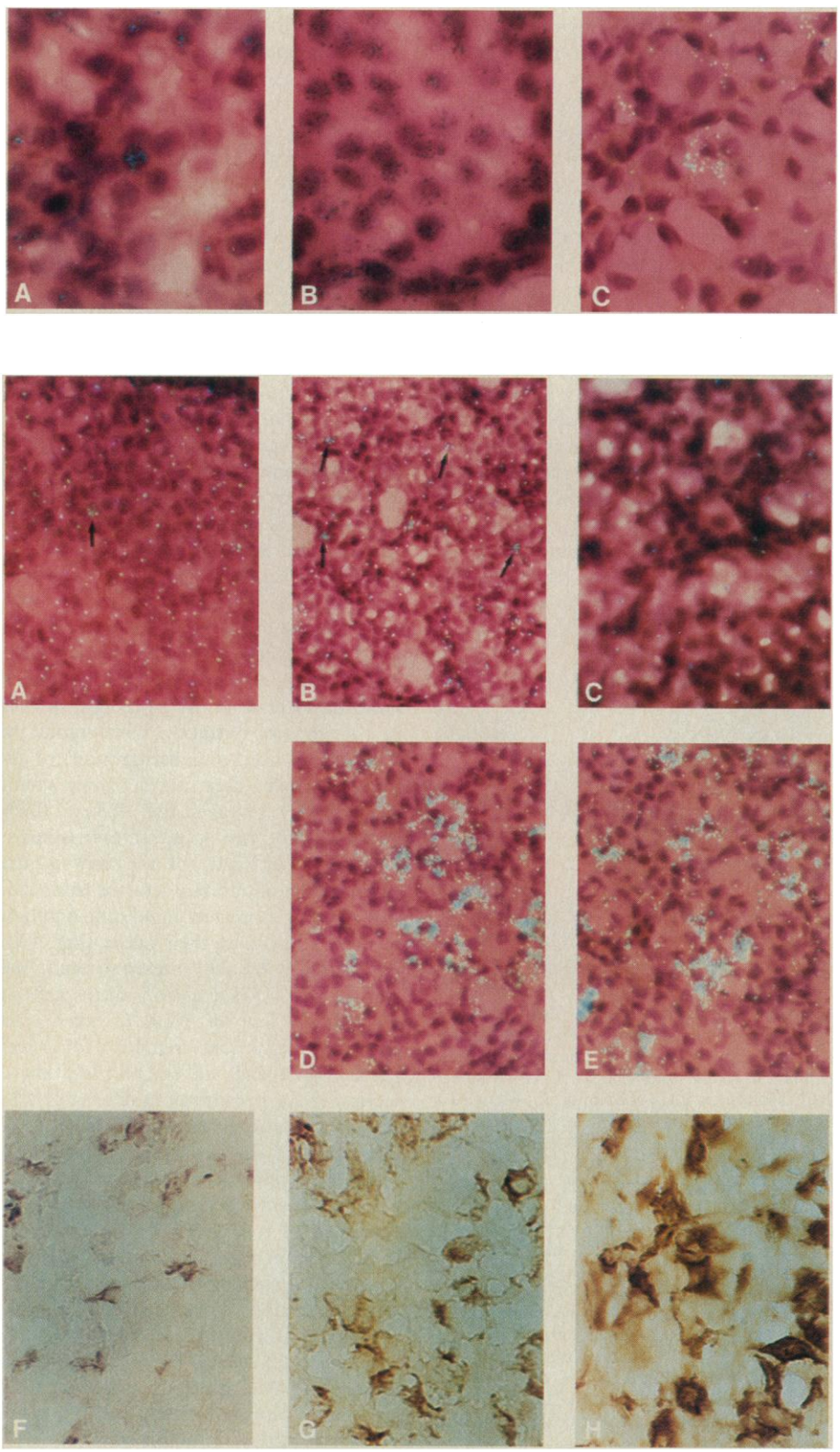

Fig. 3. In situ hybridization of the anterior and neurointermediate lobes of the rat pituitary with ^{3}H-labeled POMC IVS-A cRNA or ^{3}H-labeled POMC exon III cRNA. Nuclear localization of silver grains over (A) anterior lobe corticotrophs and (B) intermediate lob melanotrophs hybridized with ^{3}H-labeled POMC IVS-A cRNA. (C) Cytoplasmic localization of silver grains over an anterior lobe corticotroph hybridized with ^{3}H-labeled POMC exon III cRNA. Ten-micrometer sections of the rat pituitary were prepared and hybridized (as described in Fig. 2) with either 5×10^4 to 10×10^4 cpm of heat-denatured (10 minutes at 65°C) ^{3}H-labeled POMC IVS-A cRNA (A and B) or 2.5×10^4 cpm of heat-denatured (10 minutes at 65°C) ^{32}H-labeled POMC exon III cRNA (C). After hybridization, the slides were washed twice for 10 minutes each in 2× SSC at room temperature. The tissue sections were then treated with RNase A (30 μg/ml) in 0.5*M* NaCl, 10 m*M* tris-HCl, *p*H 8.0, for 45 minutes at 37°C, and washed as described in Fig. 2. The slides were dehydrated in graded ethanols (50%, 70%, 95%; 2 minutes each) containing 0.3*M* ammonium acetate, and dried in a vacuum desiccator for at least 3 hours. They were then dipped in Kodak NTB2 nuclear emulsion diluted 1:1 with distilled deionized H_2O, air-dried for 2 hours in the dark, and exposed for either 21 days (A and B) or 12 days (C) in lightproof boxes, with desiccant. The emulsion was developed and the pituitary sectrions were stained with hematoxylin and eosin (*19*).

Fig. 4. Effect of adrenalectomy and subsequent dexamethasone administration on anterior lobe POMC hnRNA, mRNA, and β-endorphin immunostaining. (A to C) ^{3}H-labeled POMC IVS-A cRNA was hybridized to 10-μm pituitary sections from intact rats (A) or from ADX rats (1 week) after an intraperitoneal injection of saline (B) or DEX (100μg) (C) 30 minutes before killing. (C) Note the complete loss of silver grains over the nuclei of cells in the anterior lobe after 30 minutes of DEX treatment. (D and E) ^{3}H-labeled POMC exon III cRNA was hybridized to 10-μm pituitary sections from ADX rats (1 week) after an intraperitoneal injection of saline (D) or DEX (100 μg) (E) 2 hours before killing. Note that no detectable alteration in cytoplasmic silver grains could be detected 2 hours after DEX administration, a time when POMC gene transcription was maximally inhibited by this dose of DEX. (F to H) Immunohistochemistry with a β-endorphin antiserum (1:500) was conducted on pituitary sections from intact rats (F) or ADX rats (1 week) after an intraperitoneal injection of saline (G) or DEX (100 μg) (H) 2 hours before killing. Note that after ADX the corticotrophs increased both in number and size. Additionally, 2 hours after DEX treatment there was a slight increase in β-endorphin–like immunostaining. Immunocytochemistry was conducted according to the Vectastain ABC method (*20*). The specificity of the β-endorphin antiserum was as described by Liotta *et al.* (*21*).

quent DEX administration on POMC mRNA levels, we hybridized the ^{3}H-labeled POMC exon III cRNA probe to pituitary sections from ADX rats (1 week) that had been injected intraperitoneally with saline or DEX (100 μg) 2 hours before killing. In contrast to the rapid disappearance (within 30 minutes) of nuclear POMC hnRNA's in corticotrophs of DEX-treated ADX rats (Fig. 4, B and C), no detectable change in cytoplasmic silver grain density, and hence POMC mRNA levels, was observed even after 2 hours of DEX treatment (Fig. 4, D and E). Thus, the delay in the decline of POMC mRNA levels relative to transcription rates following DEX treatment can also be observed at the single-cell level.

To examine the effects of ADX and subsequent DEX administration on POMC peptide content, we used an antiserum to β-endorphin in immunohistochemical analysis. One week after ADX, the corticotrophs of the anterior pituitary increased in both size and number, consistent with previous studies (Fig. 4, F and G) (*13*). Two hours after administration of DEX (100 μg, intraperitoneally) to rats adrenalectomized for 1 week, a slight enhancement of β-endorphin immunostaining of corticotrophs in the anterior pituitary was observed when they were compared to untreated ADX pituitary sections (Fig. 4, G and H), consistent with the well-characterized DEX inhibition of POMC peptide secretion (*14*).

We have shown that it is possible to use a nonrepetitive intervening-sequence–specific probe and in situ hybridization techniques to measure rapid physiological changes in POMC gene transcriptional activity in individual cell nuclei in the pituitary. By combining the POMC IVS-A in situ hybridizaton assay with single-cell secretion assays such as the reverse hemolytic plaque assay (*15*), it should now be possible to directly examine the transcriptional and secretory activity of individual POMC-expressing cells. We have also found it feasible to use this assay to study the regulation of POMC gene transcription in individual nuclei of the POMC-expressing neurons in the periarcuate region of the hypothalamus.

Although several recent examples of hormonally mediated alterations in hnRNA processing or stability have been documented (*16*), it is unlikely that the changes in the POMC gene primary transcript we observed in this study could result entirely from alterations in hnRNA processing or stability. Evidence supporting the concept of regulation of gene expression at the posttranscriptional level has arisen from studies that found large, hormonally mediated alterations in hnRNA levels of a specific gene associated with relatively minor alterations in that gene's transcription rate. As previously described however, the corticotrophs respond to ADX or subsequent glucocorticoid replacement with rapid, large changes (greater than fivefold) in the transcription rate of the POMC gene (*3*). Thus, although we cannot exclude posttranscriptional stabilization effects, we believe that the POMC IVS-A autoradiographic signal in our study accurately reflects the transcriptional activity of the POMC gene.

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