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GLUCOCORTICOIDS AND CYCLIC AMP SYNERGISTICALLY REGULATE THE ABUNDANCE OF PREPROENKEPHALIN MESSENGER RNA IN NEUROBLASTOMA-GLIOMA HYBRID CELLS

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SUMMARY: Regulation of preproenkephalin gene expression was studied in NG108-15 neuroblastoma-glioma hybrid cells. Untreated cells contain 20-120 fg preproenkephalin mRNA per μg cellular RNA. Treatment of cells with a glucocorticoid (e.g. dexamethasone) for 24 hr or 8 days elevated the abundance of this mRNA to 3 or 9 times the control, respectively. Treatment with 8-bromo-cyclic AMP or an adenylate cyclase activator such as prostaglandin E_1 or forskolin elevated preproenkephalin mRNA to twice the control or less. Treatment with both glucocorticoid and forskolin for 24 hr or 8 days markedly increased preproenkephalin mRNA to 5-8 and 30 times the control, respectively. Intracellular Met-enkephalin immunoreactivity was increased in parallel with the mRNA abundance. The results demonstrate that preproenkephalin gene expression is synergistically regulated by glucocorticoids and cAMP.

The opioid peptide precursor proenkephalin (or proenkephalin A) undergoes proteolytic cleavage in specific neurons and endocrine cells to yield Met- and Leu-enkephalin, as well as several larger enkephalin-like opioid peptides (reviewed in ref. 1). The nucleotide sequences of mRNA coding for the gene product preproenkephalin (ppEnk) from bovine adrenal medulla (2,3), human pheochromocytoma (4,5), and rat brain (6,7) have been determined.

Little is known about the regulation of ppEnk gene expression by hormones and neurotransmitters. Elevations of the ppEnk mRNA content in specific brain regions have been observed upon treatment of rats with a dopamine-receptor antagonist (8,9), electroconvulsive shock (10), or reserpine (11). Elevations of ppEnk mRNA levels also have been elicited in bovine adrenal chromaffin cells by treatment with 8-bromo-adenosine-3',5'-monophosphate (8-Br-cAMP), activators of adenylate cyclase, or nicotinic acetylcholine receptor

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Abbreviations: Dex, dexamethasone; ppEnk, preproenkephalin (A); Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PGE_1, prostaglandin E_1; 8-Br-cAMP, 8-bromo-adenosine-3',5'-cyclic monophosphate; poly(A)$^+$ RNA, polyadenylated RNA; bp, base pairs.

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stimulation (12-14). The relevant molecular mechanisms of these effects may be difficult to ascertain in these complex systems, particularly in brain.

Therefore we have sought a clonal neural cell line in which to study the regulation of ppEnk gene expression under defined conditions. NG108-15 mouse neuroblastoma x rat glioma hybrid cells, which assume a neuronal phenotype upon prolonged elevation of cAMP levels (15), were evaluated for this purpose. These cells contain small amounts of enkephalin (16-19) which are increased 3-5-fold by treatment with glucocorticoids for 24 hr or longer (17) or increased 2-fold by treatment with cAMP derivatives for 5 days (19). The mechanism(s) of these increases have not been heretofore studied. In this study we demonstrate a synergistic action by glucocorticoids and cAMP to regulate the ppEnk mRNA abundance in NG108-15 cells. Some of this work has been presented in abstract form (20).

MATERIALS AND METHODS

Materials. Steroids, PGE_1, and 8-bromo cyclic nucleotides were from Sigma. Forskolin was from Calbiochem. Ro20-1724 was from Hoffman-LaRoche, Inc. Plasmid pSP65 and SP6 RNA polymerase were from Promega-Biotec Co.

Cell culture. NG108-15 cells (passage 16-23) were cultured at 36°C in 75 cm^2 flasks containing 25 ml of either of the following media equilibrated with humidified 10% CO_2-90% air: Serum-supplemented medium contained 90% Dulbecco's modified Eagle's medium supplemented with 0.1 mM hypoxanthine, 1 µM aminopterin, 16 µM thymidine, and 10% fetal bovine serum that had been treated at 55°C for 1 hr with charcoal and Dextran T40 to extract endogenous steroids (21). Serum-free medium was described by Wolfe and Sato (22). Experiments were initiated only after cell cultures had a density of at least 4 x 10^6 cells per flask. Stock solutions (X1000) of steroids, forskolin, and PGE_1 were prepared in 95% ethanol. The final ethanol concentration of up to 0.19% was not found to affect significantly the ppEnk mRNA abundance.

Preparation of RNA. Cells were detached from each flask in phosphate-buffered saline. An aliquot (0.1 ml) was withdrawn for protein determination by the BCA (bicinchoninic acid) Protein Assay Reagent of Pierce Chemical Co. with bovine serum albumin as the standard; a cell number of 1.2 x 10^6 contained 1 mg protein by this assay. Cells from each flask were pelleted by centrifugation and lysed by sonication in 2 ml 4 M guanidinium thiocyanate solution (23). Total RNA was purified by differential ethanol precipitations (23) and quantitated by ultraviolet absorbance at 260 nm. Protein and RNA contents are expressed in figure legends as means ± standard errors.

Quantitation of ppEnk mRNA. Because of the low abundance of ppEnk mRNA in NG108-15 cells, Northern blot analysis gave more satisfactory signals than dot-blot analysis and was therefore used routinely. RNA was electrophoresed on 6% formaldehyde-1.2% agarose gels, blotted onto nylon membranes (Nytran, Schleicher and Schuell) and hybridized with the 936 bp Sac I-Sma I fragment of the pRPE2 plasmid, nick-translated with [α-^{32}P]dCTP to 2-5 x 10^8 cpm/µg DNA. The hybridization, washing, and autoradiography of blots were performed according to standard procedures, detailed previously (6). Bands corresponding to ppEnk mRNA were quantitated by a Shimadzu CS-930 scanning densitometer. The relationship between peak areas and RNA added was approximately linear within limits. Only peak area data within this linear range were utilized.

To estimate the amount of ppEnk mRNA bound to a Northern blot, a series of known amounts (1-20 pg) of pYSEC1 transcript RNA (described below) were mixed with carrier rat liver RNA (50 µg), then electrophoresed and blotted adjacent to other samples to be analyzed. The hybridization signals at 950

bases were quantitated by densitometry and used to calibrate ppEnk mRNA signals at 1500 bases after correction for the difference in length.

Preparation of synthetic RNA possessing the partial sequence of rat ppEnk mRNA. The 936 bp Sac I-Sma I fragment of pRPE2 (6) containing the entire coding region was ligated into the polylinker region of plasmid pSP65 (24). One recombinant plasmid, pYSEC1, was linearized by digestion with Sma I and used as a template for SP6 RNA polymerase (24). Analysis by formaldehyde-agarose gel electrophoresis and ethidium-bromide staining revealed that at least 90% of the product migrated as a discrete band of 950 bases (theoretical size 954 bases). The RNA concentration was determined by the absorbance at 260 nm after complete alkaline hydrolysis. The yield was 3.2 μg/μg plasmid.

Met-enkephalin determination. Pelleted cells were homogenized in 2 ml of 1 M acetic acid-0.05 M HCl. The homogenates were heated for 10 min at 100°C to inactivate proteases and centrifuged at 10,000 X g for 20 min. Supernatant fractions were neutralized with NaOH and centrifuged as before. Enkephalins were partially purified by adsorption to Poropak Q beads (Waters Associates) in water followed by elution with 50% ethanol and evaporation in vacuo. The recovery was 75% with [^{125}I]Met-enkephalin. One-third of the eluted material was digested sequentially with trypsin and carboxypeptidase B essentially as described (1,25). Met-enkephalin was determined by radio-immunoassay with [^{125}I]Met-enkephalin (New England Nuclear) and the RB-4 antiserum (25) at 1/40,000 final dilution.

RESULTS

PpEnk mRNA in untreated NG108-15 cells. Total RNA from NG108-15 cells was found to contain a single major species of ppEnk mRNA of chain length approximately 1500 bases (Fig. 1), which is in accord with the size of rat brain ppEnk mRNA (6,9). The abundance of ppEnk mRNA in different preparations of confluent untreated cells varied from 20 to 120 fg/μg total RNA (1.5-9 molecules per cell). For comparison, the abundance of ppEnk mRNA in the striatum of the adult Fischer rat was much higher, 45-60 pg/μg RNA.

The effect of glucocorticoid hormones alone. Treatment of the cells for 24-48 hr with 1 μM dexamethasone (Dex) elevated the abundance of ppEnk mRNA to 3 times the control level (Fig. 1). Half-maximal and maximal effects were achieved with approximately 10^{-8} and 10^{-6} M Dex, respectively (not shown). Results with other steroids, tested at 1 μM for 24 hr, were as follows: the naturally occurring glucocorticoids hydrocortisone and corticosterone were as effective as Dex, while deoxycorticosterone had a slight effect (158% of control), and β-estradiol, progesterone, and testosterone were ineffective (not shown). This pattern of response to steroids of various classes is consistent with a glucocorticoid-receptor mediated effect.

The effects of cAMP and the combination glucocorticoid + cAMP. NG108-15 cells were treated for 24 hr with 8-Br-cAMP, PGE_1, which strongly stimulates adenylate cyclase of these cells, or forskolin, an activator of the catalytic subunit of adenylate cyclase, each in the presence and absence of Dex. As shown in Fig. 2A, treatment with 8-Br-cAMP, PGE_1, or forskolin, with or without Ro20-1724, an inhibitor of cAMP phosphodiesterase activity, elevated the ppEnk mRNA slightly or not at all (0-70%). Dex alone elicited an 80%

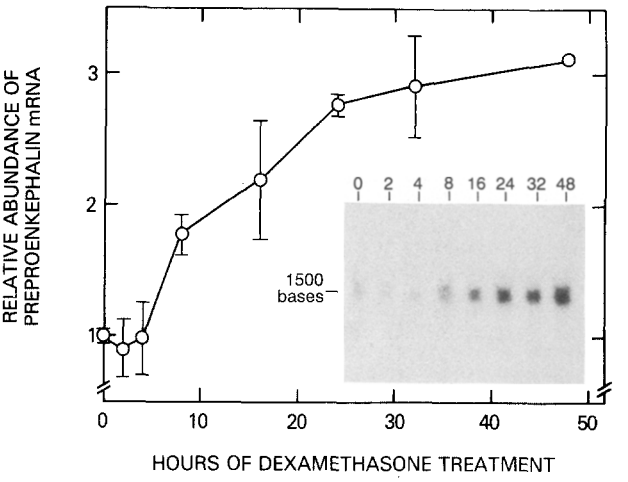

Fig. 1. The relation between the duration of Dex treatment and the abundance of ppEnk mRNA. Cells cultured in serum-supplemented medium received 1 μM Dex at various times prior to harvest. No difference in protein content (4.8 ± 0.1 mg/flask) or RNA yield (308 ± 6 μg/flask) was found. Northern blot analysis of 50 μg RNA from duplicate flasks of cells was performed. Inset: Autoradiogram (2 day exposure) of RNA from one set of cultures. Numbers at top indicate hours of Dex treatment. The number at left is the length (bp) of the hybridized RNA. Graph: PpEnk mRNA abundances relative to the zero-time control, plotted as means ± SE of duplicate flasks. The apparent absolute abundance in the zero-time control was 80 fg/μg total RNA.

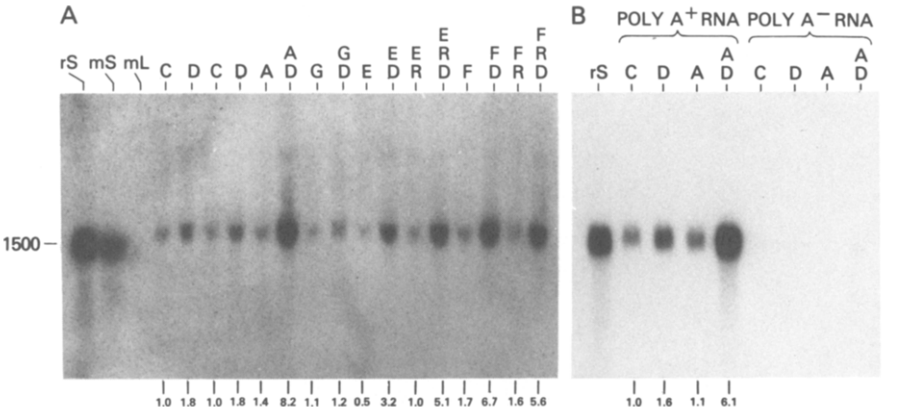

Fig. 2. The effects of Dex and/or cyclic nucleotide elevation on the ppEnk mRNA abundance. NG108-15 cells cultured in serum-supplemented medium were treated with the indicated compounds for 24 hr. At harvest, the protein content was 9.8 ± 0.2 mg/flask. A: Northern blot analysis of total RNA (4-day film exposure). Rat (Fischer strain, young adult) striatum RNA (rS, 0.5 μg), mouse (young adult) striatum RNA (mS, 0.5 μg), mouse liver RNA (mL, 50 μg), and NG108-15 RNA (50 μg) were electrophoresed in indicated lanes. Abbreviations: C, control; D, 1 μM Dex; A, 1 mM 8-Br-cAMP, G, 1 mM 8-Br-cGMP; E, 10 μM PGE_1; F, 10 μM forskolin; R, 0.25 mM Ro20-1724. The number at left is length (bp). The apparent absolute abundance in untreated control cells averaged 99 fg/μg total RNA. B: Northern blot analysis of poly(A)$^+$ and poly(A)$^-$ RNA (2 day exposure). RNA was fractionated by oligo(dT)-cellulose chromatography (2 cycles). The poly(A)$^+$ fractions were 1.7-1.9% of the applied RNA. The following were electrophoresed per lane: 0.5 μg rat striatum total RNA, 6 μg NG108-15 poly(A)$^+$ RNA, 50 μg NG108-15 poly(A)$^-$ RNA. Abbreviations are as in panel A. Numbers at bottom of panels A and B are abundances of ppEnk mRNA in each lane relative to that of untreated cells. The difference in mobilities of striatal and NG108-15 ppEnk mRNA in panel A is apparently due to different amounts of RNA and residual salt applied.

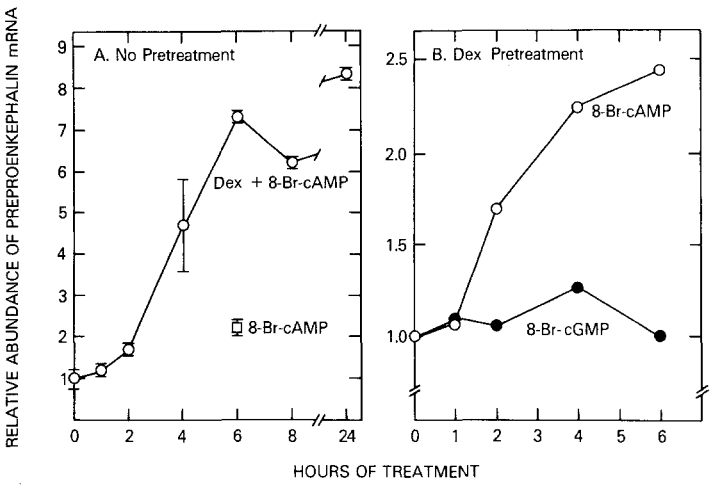

Fig. 3. Effects of short durations of treatment with Dex and/or cyclic nucleotides on the ppEnk mRNA abundance. NG108-15 cells were cultured in serum-supplemented medium. A: Cells were treated with 1 mM 8-Br-cAMP with (○) or without (□) 1 μM Dex. The average protein content (mg/flask ± SE) and average total RNA yield (μg/flask) were 11.7 ± 0.1 and 866 ± 26, respectively. The results (means ± deviations of duplicates flasks) are plotted as the ratios of the level of untreated cells. The apparent absolute abundance in untreated cells was 82 fg/μg total RNA. B: Cells were treated with 1 μM Dex for the 32 hr prior to harvest. At indicated times prior to harvest 0.5 mM 8-Br-cAMP (○) or 8-Br-cGMP (●) was added in the continued presence of Dex. The average protein content and RNA yield were 5.0 ± 0.1 mg/flask and 257 ± 8 μg/flask, respectively. The ppEnk mRNA abundance of the Dex-pretreated zero-time cells was 223 fg/μg total RNA.

elevation. In contrast, the combination of Dex with either 8-Br-cAMP or adenylate cyclase activator elicited a marked and synergistic elevation of ppEnk mRNA to 5.1-8.2 times the level of untreated cells. In contrast to 8-Br-cAMP, almost no stimulation by 8-Br-cGMP in the presence of Dex was found. The same pattern of stimulation was found when equal amounts of poly(A)$^+$ RNA rather than total RNA were compared (Fig. 2B). Essentially all ppEnk mRNA in NG108-15 cells is poly(A)$^+$, as with rat brain (not shown).

The increase elicited by Dex + 8-Br-cAMP became evident at 2 hr of treatment, attained an apparently half-maximal response at 4 hr, and appeared to maximize at 6-24 hr (Fig. 3A). In cells pretreated with Dex for 32 hr (Fig. 3B), the increase elicited by 8-Br-cAMP began after a shorter lag of 1 hr and subsequently followed a course similar to that with Dex + 8-Br-cAMP. In the analysis of longer treatments (Fig. 4), the apparent maximal elevation of the ppEnk mRNA abundance by 24 hr of combination treatment was in fact an early phase preceding a more slowly developing and more pronounced elevation. In serum-supplemented medium (Fig. 4A), the rapid rise elicited by treatment with Dex + forskolin for 24 hr was followed by a decline, or in some experiments a plateau, between day 1 and 2, then a slow rise between days 3 and 8 to a level 30 times the control. The effects of Dex and/or forskolin were studied also in cells cultured in a serum-free medium (22) that contains insulin and trans-

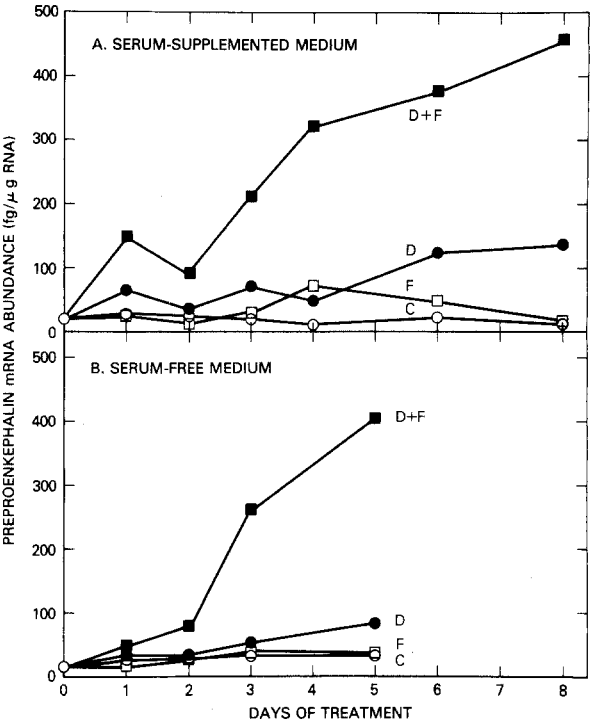

Fig. 4. The effects of prolonged treatment with Dex and/or forskolin on the ppEnk mRNA abundance in NG108-15 cells. A: Cultures in serum-supplemented medium received no drug (C, ○), 1 μM Dex (D, ●), 8 μM forskolin (F, □), or 1 μM Dex + 8 μM forskolin (D + F, ■) and received new medium and drugs daily. Cultures processed on day 8 had 9.5 mg protein/flask and 451 ± 28 μg RNA per flask. B: Cultures adapted to serum-free medium received drugs as described for panel A. Cultures on day 5 contained 4 mg protein/flask.

ferrin but no other hormones or protein growth factors (Fig. 4B). The magnitude of the stimulation by Dex + forskolin was similar to that obtained in serum-supplemented medium, suggesting that other hormones or factors in serum are not required to attain the maximum effect of glucocorticoid + cAMP.

Upon the withdrawal of Dex + forskolin from cells treated for 24 hr, 55-75% of the elevated ppEnk mRNA abundance was lost over the subsequent 24 hr, while 25-45% of the increase persisted for at least 48 hr (not shown).

In the presence of 10 μg/ml actinomycin D, an inhibitor of transcription, the elevations of ppEnk mRNA elicited by 6-hr treatments with Dex, 8-Br-cAMP, and Dex + 8-Br-cAMP were reduced to 20%, 28%, and 11%, respectively, of the elevations in cultures lacking actinomycin D. This suggests that the elevations are not due to merely selective stabilization of ppEnk mRNA. Inclusion of 71 μM cycloheximide to inhibit protein synthesis enhanced by 74% and 104% the increases in ppEnk mRNA elicited by 6-hr treatments with Dex alone and Dex + 8-Br-cAMP, respectively (data not shown). This suggests that the synthesis of an intermediate protein factor is not required, and that the compounds induce protein(s) that attenuate the increase in ppEnk mRNA.

Table 1. Effects of glucocorticoid and cAMP elevation on the content of Met-enkephalin immunoreactivity

Experiment	Treatment		Average Protein	Met-enkephalin immunoreactivity (fmol/mg protein)	
	Dex	Forskolin	(mg/flask)	Before digestion	After digestion
A.	-	-	6.9	19.7 ± 7.1	48.3 ± 19.8
	+	-	5.8	37.3 ± 12.5	117 ± 26
	-	+	5.3	14.4 ± 2.0	52.7 ± 4.1
	+	+	5.9	214 ± 19	828 ± 105
B.	-	-	12.1	1.8 ± 0.2	2.8 ± 0.2
	+	-	11.6	6.4 ± 1.8	14.3 ± 0.8
	-	+	12.3	1.4 ± 0.5	5.4 ± 0.4
	+	+	9.0	38.5 ± 5.1	118 ± 8

Cells were cultured in serum-supplemented medium (Expt. A) or serum-free medium (Expt. B) in the presence or absence of Dex (1 μM) and/or forskolin (3.3 μM) for 5 days with daily changes of medium. Cells were extracted; an aliquot of the extract was digested with trypsin and carboxypeptidase B. Values are means ± SE of triplicate cultures corrected for 75% recovery.

Effect of glucocorticoids and cAMP on intracellular Met-enkephalin immunoreactivity. Treatment of NG108-15 cells with Dex alone for 5 days increased the Met-enkephalin immunoreactivity to 1.9-3.5 times the control (Table I), as previously reported (17). Treatment with forskolin alone had no effect. Combined treatment with Dex + forskolin resulted in a pronounced increase to 11-19 times the content of the untreated cells. Similar increases were found after digestion of the extracts with trypsin and carboxypeptidase B to liberate enkephalin residues from larger proenkephalin fragments (1). These results indicate that the ppEnk mRNA sequences elevated by Dex and cAMP are translated into proenkephalin molecules, which are post-translationally processed to yield elevated levels of stored enkephalin peptides.

The parental source of the expressed ppEnk gene. NG108-15 cells, which contain an average of 41 rat and 98 mouse chromosomes (26), possess both mouse

Table 2. Species of the ppEnk gene expressed in NG108-15 cells

RNA source	μg RNA applied to gel	Relative intensities of ppEnk mRNA bands (% of rat striatum density)	
		Probe of coding region	Probe of 3'-untranslated region
Rat striatum	0.5	100	100
Mouse striatum	0.5	45	12
NG108-15, untreated	50	7.2	9.7
NG108-15 cells, treated with Dex + 8-Br-cAMP	50	61	80

A Northern blot of indicated total RNA samples was first hybridized with the nick-translated Sac I-Sma I fragment of pRPE2 containing the coding region of ppEnk mRNA. After autoradiography the blot was stripped of probe and rehybridized with the Sma I-Pst I fragment of pRPE2 (insert residues 986-1283, 3' untranslated region), and stringently washed.

and rat ppEnk genes, as revealed by Southern blot analysis. The locations of all examined restriction sites (Bgl II, Eco RI, Hind III, Bam HI, Sac I, and Xba I) within the rat and mouse genes and flanking regions were identical to those of the NG108-15 genes, suggesting no gross rearrangement of these genes in the hybrid cells (not shown).

To determine the species of the gene(s) expressed in the hybrid cells, a probe of the poorly conserved 3' untranslated region was utilized to hybridize preferentially to rat rather than mouse ppEnk mRNA, in contrast to the probe of the coding region. The ratios of hybridization intensities of NG108-15 ppEnk mRNA to hybridization intensities of rat striatum ppEnk mRNA were similar for both probes (Table 2). This result suggests that most of the NG108-15 ppEnk mRNA is transcribed from the rat rather than the mouse gene. This conclusion is consistent with the presence of ppEnk mRNA in the C6BU-1 rat parent but not in the N18TG-2 mouse parent (27).

DISCUSSION

This study demonstrates that the intracellular contents of rat ppEnk mRNA and Met-enkephalin are positively and synergistically regulated by glucocorticoid hormones and compounds that elevate intracellular cAMP. Dual regulation by glucocorticoids and cAMP has been reported for the abundances of several other mRNA species (28-30). In the case of ppEnk mRNA, the mechanism of action of these effectors probably involves the stimulation of transcription, as indicated by nuclear run-off experiments (J. Joshi and S. L. Sabol, preliminary results); however, stabilization of ppEnk mRNA is not ruled out.

It is believed that specific nucleotide sequences near or within genes are involved in the actions of glucocorticoids and cAMP on transcription (e.g. 31,32). The sequenced portions of the rat ppEnk gene (33) do not appear to contain a perfect core consensus sequence (31) for glucocorticoid regulation (GGTACANNNTGT(T/C)CT), although imperfect matches are found in the 5' flanking region (bases -408 to -422 of the anticoding strand, TTCACTCACTGTCCT) and in bases 83-97 of intron A (GTTCCTCATTGTCCT). Potential cAMP regulatory elements exist at bases -363 to -374 (anticoding strand, CATACCTCAGAG) and at bases -91 to -80 (CCTGCGTCAGCT), as these sequences resemble part of a cAMP-regulatory element of the rat phosphoenolpyruvate carboxykinase gene (bases -91 to -80, CTTACGTCAGAG) (32). In fact, the sequence -91 to -80 of the rat ppEnk gene is is preserved within a region of the human ppEnk gene recently shown to confer cAMP control of transient gene expression (34).

We suggest that modulation of ppEnk mRNA levels by glucocorticoids and cAMP may occur in normal rat enkephalinergic cells. Possible physiological consequences of such regulation can be envisioned. For example, elevation of glucocorticoids and circulating catecholamines by stress may increase

proenkephalin biosynthesis and subsequent enkephalin production and release, which may modulate the characteristics of the stress response.

After the completion of this work, Naranjo et al. (35) reported that glucocorticoids elevate ppEnk mRNA in bovine chromaffin cells and potentiate an increase in ppEnk mRNA induced by depolarization with veratridine. This interaction between glucocorticoids and a stimulus to secretion and synthesis appears similar to that shown in this paper.

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