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The expression of the endogenous Corticotropin Releasing Factor (CRF) gene was examined in the human neuroblastoma cell line, BE(2)-M17. In this cell line, treatment with retinoic acid induces CRF mRNA transcription. We examined the requirement for the POU transcription factor, Brn-2, for this response. We confirmed that Brn-2 is expressed in retinoic acid-induced BE(2)-M17 cells. Expression of antisense Brn-2 message aborted the retinoic acid induction of CRF transcription. However, over-expression of Brn-2 was not sufficient for CRF expression in the absence of retinoic acid. These experiments support the hypothesis that Brn-2 is an intermediary for retinoic acid-induced CRF expression.

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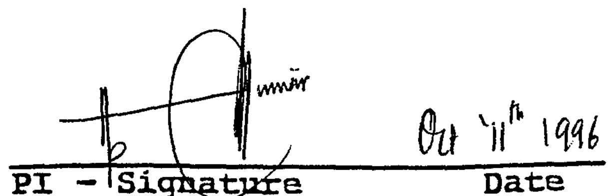

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Foreword

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INTRODUCTION:

Corticotropin Releasing Factor (CRF) is a neuropeptide, synthesized primarily by the paraventricular nucleus of the hypothalamus (PVN) in the mammalian brain (1). Previous studies involving the biosynthesis and secretion of CRF have provided substantial information regarding the role of this molecule in maintaining and modulating neuroendocrine homeostasis (2,3). Although the downstream effects of secreted CRF have been studied at length, the upstream molecular events that affect its temporal and spatial expression patterns are yet to be fully elucidated. Studies using non-neuronal tissue culture models have implicated several ubiquitous transcription factors, including CREB (4), glucocorticoid receptor (5,6) and estrogen receptor (7,8) in modulating CRF gene expression. *In situ* studies with the AP-1 family of transcription factors show circumstantial evidence for a possible role in CRF gene regulation (9,10).

The POU family of transcription factors is expressed in a cell lineage specific pattern and is known to be involved in neuroendocrine pathways. For example, Pit-1 regulates the expression of prolactin and growth hormone (11), and Unc-86 is required for sensory neuron development in *C. elegans* (12). Brn-2, another POU domain transcription factor, has been shown to be involved in retinoic acid mediated neural differentiation of pluripotent embryonal carcinomas (13). *In situ* hybridization studies demonstrate the presence of Brn-2 protein in the PVN (14). Furthermore, transgenic mouse experiments have also shown that null mutants of Brn-2 fail to properly develop neurons that make up the CRF synthesizing population of the PVN (15,16). *In vitro* experiments have shown the presence of Brn-2 binding sites in the 5'-promoter region of the CRF gene (17). In the CV-1 monkey kidney cell line, these sites have been demonstrated to activate the expression of an artificial reporter gene driven by the CRF promoter.

Although a number of studies have given us information on the candidate factors controlling CRF gene expression, detailed molecular analyses are hampered by the lack of a

suitable neuronal system in which the endogenous CRF gene is expressed. Recently, a human neuroblastoma derived cell line, BE(2)-M17, has been shown to express the endogenous CRF gene upon retinoic acid induction (18,19). In the following report, we present experiments performed in this neuronal cell line. Using antisense Brn-2 constructs, we demonstrate the requirement for Brn-2 in retinoic acid induced expression of CRF. In addition to its demonstrated role in hypothalamic development, this suggests that Brn-2 may be required for the expression of the CRF gene in terminally differentiated neurons.

RESULTS

Brn-2 site binding activity is expressed in BE(2)-M17 cells upon induction with retinoic acid.

The BE(2)-M17 cell line, derived from a human neuroblastoma, has been shown to express the endogenous CRF peptide in a time dependent manner upon induction with retinoic acid (19). To ensure this was not a result of gross genomic rearrangements involving the sequences in and around the CRF gene, we performed Southern blots on the genomic DNA from the BE(2)-M17 cell line. The blots were probed with a DNA fragment containing the second exon of the human CRF gene. We did not detect any differences between genomic DNA from BE(2)-M17 and control human leukocyte DNA (data not shown).

In a mouse embryonal carcinoma cell line, P-19, retinoic acid has also been shown to induce the expression of Brn-2, a POU domain transcription factor, while progressing towards a neuronal fate (13). In the same study, the absolute requirement for Brn-2 in neuronal differentation was illustrated by the use of antisense Brn-2 message.

We hypothesized that retinoic acid also induces the expression of Brn-2 in the BE(2)-M17 cell line as a required step in the pathway for expression of the CRF gene. Nuclear extracts were prepared from untreated cells and cells treated with retinoic acid for 3 days. The nuclear extracts were then used for specific gel shift assays in reactions that were normalized for the total amount of protein. An oligonucleotide with a Brn-2 binding site was used as a probe. Parallel experiments were performed with unlabelled Brn-2 binding site oligonucleotides as specific competitors, or with an oligonucleotide containing the CREB binding site as non-specific competitor.

Consistent with the neuronal origin of these cells, endogenous Brn-2 site binding activity is present, even in untreated BE(2)-M17 cells, as evidenced by the gel shift (Fig 1a). This shift is specific, as it is competed by excess unlabelled oligonucleotides (Fig 1a lanes 1 & 2). Moreover,

a similar shift can be obtained using *in vitro* translated Brn-2 protein (Fig 1a lane 7) and hypothalamic nuclear extracts (data not shown). A similar but more robust shift is seen when extracts from retinoic acid treated cells are used. Since the reactions were standardized for the total amount of protein, this suggests that there is more active Brn-2 protein in the induced cells. The shift from the induced cell extracts is also specific as it is competed away by a specific unlabelled competitor, while the shifts are unaffected when a non-specific competitor is used (Fig1a lanes 4, 5 & 6). To control for non-specific changes in the activity of the nuclear extract preparations, gel shifts with labeled CREB oligonucleotide were also performed. While the shifts on the Brn-2 oligonucleotide were induced by the retinoic acid treatment, induced and uninduced extracts had similar binding activity on the CREB oligonucleotide (data not shown).

The expression of Brn-2 was also studied using an RT-PCR method (Fig 1b). We were able to detect low levels of the Brn-2 mRNA in the untreated parental cells (Fig 1b lane 3). It should be noted that this observation was rather inconsistent. The parental cells that were treated with retinoic acid showed visible amounts of Brn-2 transcript (Fig 1b lane 2).

Brn-2 affects expression of CRF reporter genes in retinoic acid treated and untreated cells.

Using the same sense and antisense Brn-2 expression plasmids used by Fujii and Hamada (13), we studied the effect of Brn-2 on transcription from a full length CRF promoter. The reporter plasmid used was constructed from a 8 kb human CRF genomic (20), in which the coding region of the second exon was replaced in-frame by a sequences encoding the firefly luciferase gene (21). This construct was co-transfected with either CMV-Brn-2 (Fig 2a) or PGK-αBrn2 (Fig 2b) into BE(2)-M17 cells and either treated with retinoic acid or left untreated. Various amounts of the Brn-2 expression plasmids were transfected and fusion gene expression was assayed.

In agreement with previous studies demonstrating an increase in CRF mRNA and protein in response to retinoic acid treatment (19, 22), we observed an increase in CRF reporter

gene expression. There is a 6.5 fold increase in expression levels upon retinoic acid treatment (Fig 2a). Furthermore, the retinoic acid treated cells respond to the presence of exogenous Brn-2, rather robustly. A 8-10 fold increase in expression was observed when the amount of transfected CMV Brn-2 was increased to 0.5 ug/well. Further increases in the amount of Brn-2 did not affect fusion gene expression. We postulate this to be due to a limiting factor and/or competition for cellular transcription factors. On the other hand, the untreated cells did not show any marked increase in expression from the CRF-luciferase reporter gene, even on addition of a substantial amounts of CMV Brn-2. This suggests that the presence of Brn-2 alone is not sufficient to mediate the increase in CRF expression seen with retinoic acid treatment.

Participation of Brn-2 in the expression of the transfected CRF reporter gene was demonstrated using an antisense construct. The retinoic acid treated and untreated cells both show reproducible decreases in luciferase expression on co-transfection of a plasmid that expresses the anti-sense Brn-2 mRNA (Fig 2b). Very small amounts of antisense plasmid were sufficient to produce this change, which is dramatic in the treated cells. This is striking when compared to the levels of CMV Brn-2 sense plasmid construct, considering that the strength of CMV promoters is much higher than PGK promoters.

The relative expression values with co-transfected antisense Brn-2 in the untreated cells were similar to background levels. The expression of CRF/luciferase for the treated cells was always higher than that of untreated cells, over the entire range of anti-sense plasmid concentration. This suggests that in treated cells, either small quantities of functional Brn-2 are present, or there is a Brn-2 independent pathway for retinoic acid mediated expression.

Brn-2 binding sites can mediate both Brn-2 and retinoic acid responses.

To determine whether the induction observed with retinoic acid is specific and to assess the role of Brn-2 and its binding sites in this induction, we evaluated the responses of artificial

promoters and compared these results to a CRF promoter (Fig. 3). We used a 5 kb hCRF reporter plasmid (CRF LΔ2S), a minimal 36 bp promoter (P36 LΔ2S), and the corresponding promoter containing 3 tandem repeats of the native Bm-2 binding site (Multi-3 P36) . The P36 plasmid was not responsive to retinoic acid induction. Similarly, this P36 plasmid was not induced by expression of Bm-2 by co-transfection of the Bm-2 expression vector. In addition the co-transfection of the Bm-2 antisense expression vector did not result in a lower signal for the P36 plasmid. These data serve to exclude the possibility of cryptic or unrecognized retinoic acid response elements or Bm-2 response elements in the plasmid backbone or reporter gene. The Multi-3 plasmid containing Bm-2 binding sites was not only induced by Bm-2 co-transfection and repressed by expression of anti-sense Bm-2, but this plasmid was also induced by retinoic acid. These data indicate that Bm-2 binding sites can mediate transcriptional responses dependent on Bm-2, and, in addition, also confer responsiveness to retinoic acid, even in the absence of sequences identified as canonical retinoic acid receptor binding sites. In contrast, the 5 kb CRF promoter, as shown with the CRF fusion plasmid, is induced by retinoic acid, exhibits an inhibition of basal expression with antisense Bm-2, but is not induced by Bm-2 expression in the absence of retinoic acid. These data demonstrate that isolated Bm-2 binding sites can transfer retinoic acid responses and Bm-2 responses to a minimal promoter. Yet, in the context of the CRF promoter, while antisense Bm-2 produces inhibition of the basal expression level, increased responses to Bm-2 are not seen in the absence of retinoic acid.

Stable transformation of sense and anti-sense Bm-2 constructs affect inducibility by Retinoic Acid.

Three lines of stable transformants of the BE(2)-M17 cell line were made for further analysis. One stably transformed line containing the CMV Bm-2 plasmid, and two transformed cell lines containing either a high copy (MAM-19) or a low copy (MAM-3) of PGK-αBm-2 plasmid were isolated. These transformants, along with the parental BE(2)-M17 cell line, were treated with retinoic acid and were transiently transfected with CRF reporter plasmid and assayed for luciferase expression.

As seen in the dose response profile (Fig 2), the parental BE(2)-M17 shows a 6.5 fold increase in CRF expression with retinoic acid (Fig 4). No major differences were seen in the CMV Brn-2 stable line. The antisense transformants, MAM-3, which has 3 copies of PGK-αBrn2, and MAM-19, which has 19 copies of PGK-αBrn2, show a marked and reproducible drop in activity both in treated and untreated cells. MAM-19 shows virtually no CRF luciferase expression, with only background levels of luciferase activity. This suggests that both basal and retinoic acid induced transcription of CRF are inhibited by the presence of antisense Brn-2 and suggests a possible requirement for Brn-2 transcription factor for even basal expression of CRF. In the MAM-3 and MAM-19 stable transformant cells expressing the antisense Brn-2 construct we did not detect endogenous Brn-2 mRNA. (Fig 1b lanes 4 &5). This could be either due to antisense mechanisms resulting in the degradation of Brn-2 mRNA within the cells, or, alternatively, due to interference of the anti-sense messages with the reverse-transcriptase (RT-PCR) assay.

Endogenous CRF expression in BE(2)-M17 cell lines is affected by Brn-2.

To document the effect of sense and anti-sense Brn-2 expression on the levels of the endogenous CRF mRNA levels, we evaluated CRF mRNA by reverse transcriptase PCR (RT-PCR). Total RNA was isolated from parental BE(2)-M17 cells, that were either left untreated or treated with retinoic acid. RNA was also isolated from stable transformants MAM-3 and MAM-19, after they were grown in the presence of retinoic acid for 3 days. After reverse transcription of the RNA, the resulting cDNA was spiked with the addition of 0.1 ng of plasmid that had the CRF primer binding sites, but generated a distinctly shorter fragment. This was used as an internal control and for quantitation. The samples were then subjected to PCR. An external control for the total amount of RNA included in the reactions was also performed using cyclophilin mRNA as the target sequence (data not shown).

As previously observed (19), we note that the endogenous CRF mRNA is transcribed after induction with retinoic acid. (Fig 5 lanes 1 & 2). When MAM-3 and MAM-19 are treated with

retinoic acid, we still detect the endogenous CRF message but at markedly lower levels, more so for MAM-19 than for MAM-3 (Fig 5 lanes 3 & 4). It is interesting to note that complete inhibition of the endogenous CRF mRNA is not seen even in the high copy stable line MAM-19. Neither of the stable lines express their endogenous CRF gene when not treated with retinoic acid (data not shown). The quantitative data from Fig. 4 and multiple repetitions of the experiment in Fig. 5 are presented in Table 1.

Retinoic acid inducibility is not mediated by the CRE.

To test the hypothesis that the retinoic acid induction of CRF transcription is mediated by the previously identified cAMP response element (CRE) (4), we used a CRF reporter construct with a mutation in the CRE (Fig. 6). Transient transfections were performed in the BE (2)-M17 cell line, both in the absence and presence of retinoic acid. We used a 532 bp proximal hCRF promoter construct as our control (CRF-532), while the mutant had a non-functional CRE in the same context (-532 X-CRE). The mutation alters the CRE at -220 to an Eco RI site. As shown in Fig. 6, both the wild type and mutant reporter plasmids are equally responsive to retinoic acid although, we did notice a slight difference in the basal expression levels between the two constructs. Further, to ascertain a role in retinoic acid response for Protein Kinase A (PKA), a key factor in the cAMP second messenger pathway, we co-transfected an expression vector (RSV-PKI) for PKI along with CRF-532. PKI is a peptide inhibitor of PKA and has been shown to be active in transient transfection assays. The presence of PKI did not affect retinoic acid inducibility but dropped the basal expression levels noticeably (Fig. 6).

DISCUSSION

CRF plays a key role in regulating the daily excursions of glucocorticoid production and in the mammalian response to stress (1-3). The primary site of CRF synthesis is the PVN, where it initiates the regulatory cascade of the HPA axis by controlling the expression and secretion of ACTH from the anterior pituitary. Studies to date have indicated two distinct mechanisms for the control of CRF expression. In the first case, in which CRF expression is steroid sensitive, glucocorticoids act *via* a negative feed back loop. This sensitivity is linked to the circadian rhythm of CRF gene expression and secretion. The second mechanism, in which CRF is insensitive to plasma glucocorticoid levels, is apparent during conditions of stress. In spite of elevated levels of glucocorticoids, CRF expression remains high. The biochemical mechanisms for both of these regulatory pathways remain to be elucidated.

CRF is a single copy gene and the primary sequences of the gene and the peptide product have been determined (20). But detailed molecular studies of CRF expression have been hampered by difficulties involved in obtaining experimental amounts of hypothalamic explants and the lack of a suitable cell line of hypothalamic origin. In this study we present experiments in a cell line of neuronal origin which expresses the endogenous CRF mRNA and peptide upon stimulation with retinoic acid.

Previous studies on CRF gene regulation have examined the effects of various cellular stimuli on the mRNA and peptide expression from exogenously transfected gene constructs in non-native and often non-neuronal cell lines. In one such study, a monkey kidney CV-1 cell line was used with transfected CRF reporter plasmids (17). That study demonstrated the presence of binding sites for Brn-2 in the 5'-promoter proximal region. The identified binding sites mediated transcriptional activation when a plasmid expressing Brn-2 was co-transfected.

Transgenic mice with targeted deletions of the Brn-2 gene have further implicated Brn-2 as a candidate transcription factor in the control of CRF expression (15,16). The Brn-2 null mice

fail to develop and differentiate the hypothalamic region where the CRF neurons reside. This strongly suggests that Brn-2 is required for the differentiation of progenitor neuronal cells in the PVN into CRF-expressing, post-mitotic neurons. Unfortunately, this type of experimental model is unable to determine the role of Brn-2 in the modulation of CRF gene expression in the mature and differentiated, CRF-producing cells.

Brn-2 has previously been shown to participate in the differentiation of P-19 embryonal cells, a model system for neuronal development (13,23). In these cells retinoic acid induces differentiation towards a neuronal fate, an effect conclusively shown to be mediated through Brn-2. The cellular system that we have used in our study is the BE(2)-M17 cell line. The BE(2)-M17 cells are derived from a human neuroblastoma and have previously been shown to express the endogenous CRF peptide upon induction with retinoic acid. Our experiments were designed to test the hypothesis that Brn-2 does indeed affect the expression of CRF in the BE(2)-M17 cell line.

Consistent with this hypothesis, retinoic acid induces the expression of Brn-2. This was demonstrated at both the protein level and mRNA level using electromobility gel shift assays and RT-PCR, respectively. These experiments suggest that, although there is a noticeable increase in the amount of Brn-2 binding activity upon retinoic acid treatment, a constant basal level of Brn-2 activity is present in the untreated cells. Explanations consistent with these observations include the possibility that there is a requirement for Brn-2 above a critical threshold of cellular concentration for CRF expression, or that other factor(s) are needed to complete the pathway to CRF expression.

Experiments with antisense Brn-2 constructs provide the strongest case for Brn-2's role in CRF regulation. Effects of antisense Brn-2 were observed with both the endogenous CRF gene and transfected CRF-reporter genes. Dose response profiles with the antisense Brn-2 in the presence of a CRF-reporter plasmid indicate that even low levels of antisense constructs can markedly inhibit transcription from the CRF promoter. The observed inhibition is apparent even

in the presence of retinoic acid. A similar pattern was observed when the endogenous CRF mRNA was studied in cell lines stably transfected with the antisense Brn-2 construct. CRF mRNA induction, as measured by RT-PCR, was markedly lower in the Brn-2 antisense transfected cell lines when compared to controls. Moreover, the inhibition was dependent on the number of stable insertion events of the antisense construct. These experiments suggest a role for Brn-2 in CRF expression. Another series of dose response experiments evaluating CRF-reporter gene expression in the presence of over-expressed Brn-2 protein indicates that the Brn-2 protein alone cannot account for the increase in expression seen upon induction with retinoic acid.

Numerous recent studies have shown that many transcription factors including the POU proteins, have the ability to interact with other transcription regulators to effect additional control over gene expression (24). These interactions, though specific, may be facilitated (or inhibited) by the DNA sequences that constitute the factor binding sites (25). Moreover, sequences flanking the binding site may also contribute to these interactions (26). In our study, we noticed a difference in the ability of a Brn-2 binding site to activate transcription dependent on the promoter context. Brn-2 binding sites present in the intact CRF promoter are unable to respond to expressed Brn-2 in the absence of retinoic acid. The same Brn-2 binding sequence is transcriptionally active when present in tandem repeats, in the context of a minimal promoter. This hints at the possible influence of the surrounding sequences in the native CRF promoter context on the ability of Brn-2 to bind and/or activate transcription. A possible explanation for this modulation of activity is the binding of other transcription factors to flanking sequences. One such known site is the CRE at -220 bp, which has been shown to be active. We tested this hypothesis by mutating the CRE. We also suppressed the activity of CREB by expressing an inhibitor for protein kinase A, a critical component in the cAMP second messenger pathway. In both the cases, the retinoic acid response was not inhibited, indicating that the CRE and any transcription factors binding to it are not required for the observed retinoic acid response.

Our results suggest two alternate hypotheses. One possibility is that retinoic acid activates Brn-2 expression which in turn activates another transcription factor, a direct regulator

of CRF. We regard this scenario as unlikely, since it does not explain the requirement for retinoic acid even in the presence of Brn-2. Alternatively, retinoic acid activates both Brn-2 and another factor concurrently, with CRF expression being dependent on the presence of both factors. Of course, one candidate for the second transcription factor is a retinoic acid receptor (RAR/RXR). This possibility may be tested by evaluating the time course of CRF induction. Normally, retinoic acid induction of Brn-2 proceeds over a few days. If the cells transfected with a Brn-2 over-expressing plasmid (CMV Brn-2) were induced by retinoic acid, the time required to see the first traces of CRF mRNA should be noticeably reduced compared to controls.

The requirement for Brn-2 in CRF expression may be similar to the requirement for Pit-1, another POU factor, in the expression of prolactin. Pit-1 has been shown to be necessary for both the differentiation and the maintenance of prolactin and growth hormone secreting cells, while playing a direct role in the cell type specific transcription of these genes (11). The interactions of Pit-1 and estrogen receptor in pituitary lactotrophs (27,28) may serve as a model, and suggest that a similar interaction may exist between Brn-2 and a retinoic acid receptor family member in the regulation of CRF (Fig 7). Furthermore, the negative regulation of prolactin by glucocorticoid receptor (29) may similarly be a model for the negative regulation of CRF by glucocorticoid receptor. It was also noted in preliminary observations that reporter constructs with deleted proximal promoter sequences containing the identified Brn-2 binding sites, retained the ability to be induced by retinoic acid. This observation, that distal sequences may play a role in the nuclear receptor response, parallels a similar requirement for regulation of prolactin. The potential existence of all of these types of interactions between POU factors and nuclear receptors in CRF regulation remain an intriguing possibility for future studies.

MATERIALS & METHODS

Cell Lines:

The BE(2)-M17 cell line (18) was a generous gift from Dr. June Beidler (Memorial Sloan-Kettering Cancer Center, NY). The cell line was initially grown at 37 °C in a 5% CO_2 incubator and in MEM/F12 medium supplemented with 15% fetal bovine serum (FBS-Intergen, Purchase, NY), 1X Non-essential amino acids (Mediatech, Washington DC) and penicillin-streptomycin and fungizone (Tissue Culture Support Center, Washington University). The cells were then adapted to a similar media containing 10% FBS and 5% enriched calf serum (ECS-Gemini Bioproducts, Calabasas, CA) instead of 15% FBS. Cells grown in this medium were used for all experiments. The cells were periodically checked for mycoplasma contamination using a PCR assay kit (Stratagene, La Jolla, CA).

Transfections:

Transient transfections were performed using a calcium phosphate (30) in 6 well 35mm plates. Typically, the cells were seeded at 1.25×10^5 cells/ well in 2 ml of growth media on day 1. The actual transfection on day 3 was carried out by replacing the growth medium with DMEM containing 5% FBS+5% ECS and incubated in 10% CO_2 for 4 hrs. Later, 125 ul of transfection mixture containing 2.5 ug of total DNA in N,N-bis (2-hydroxyethyl)-2-aminoethane-sulfonic acid (BES)-buffered saline (BBS)-$CaCl_2$ was dripped onto the medium and the cells were incubated overnight in a 5% CO_2 environment. The following day, the cells were washed with DMEM and fed with growth medium. The transfected cells were then harvested on Day 5 in 150 ul of lysis buffer containing 50 mM Tris-HCl, 50 mM 2(N-morpholino)ethane sulfonic acid (pH 7.8), 1 mM dithiothreitol, and 1% Triton X-100. The lysate was then assayed for luciferase and β-galactosidase activity. Cells were treated with 5 uM retinoic acid using solutions in 95% ethanol, on day 1 after seeding. All subsequent manipulations were carried out in the presence of retinoic acid.

Stable transfections used essentially the same protocol as for transient transfections. The target plasmid DNA was mixed with RSV-Hygromycin DNA (31) in a 10:1 ratio during transfection. Growth medium containing 100 ug/ml of hygromycin B (Sigma Chemicals, St. Louis, MO) was used for selection. Three days post transfection, the wells were washed to remove the dead, untransfected cells. The remaining transformants were trypsinized, transferred to four 150 mm dishes and maintained under selective pressure. The selective medium was replaced every 3 days until the appearance of foci, apparently from a single stably transfected cell.

In experiments co-transfecting varying amounts of Bm-2 or αBm-2 plasmids, the effects of promoter competition were controlled by transfecting constant total amounts of CMV and PGK plasmids. CMV Neo was used as the compensatory plasmid in the case of CMV Bm-2 and PGK-MT, an empty vector, with PGK-αBm-2. In all transfection experiments the total amount of expression plasmids was kept at 1.25 ug/well.

Plasmids and Luciferase Assays:

The human CRF-luciferase reporter plasmids used were as described (21). The sense (CMV Bm-2) and anti-sense (PGK-αBm-2) constructs that were used in these experiments were a precious gift from Dr. H. Fujii (Osaka University, Japan). The plasmids, CMV-Neo and PGK-MT, were used as controls for dose response experiments. PGK-MT was constructed by deleting the αBm-2 fragment from PGK-αBm-2 plasmid. CRF-532 and -532 X-CRE plasmids used in this study were as previously described (21). A plasmid for expressing the specific inhibitory peptide for Protein Kinase A, RSV-PKI (32), was obtained from Dr. Richard Maurer (Oregon Health Sciences University, Portland, OR, USA). To create a reporter containing 3 tandem repeats of the Bm-2 binding site, a partially kinased oligonucleotide duplex from -146 to -107 bp of the hCRF proximal promoter was multimerized in 3 copies using DNA ligase. These multimerized fragments were subcloned in front of the minimal 36 bp promoter, P36 (33). RSV-β-Gal (21) was

used for standardization of transfection efficiency. RSV-Hygromycin was a gift from Dr. H. Elsholtz (University of Toronto, Ontario, Canada)

Luciferase assays were done as previously described (34) in a MonoLight 2010 luminometer from Analytical Luminescence Labs (San Diego, CA). β-galactosidase assays were performed using chlorophenol red β-galactopyranoside (Boeringher Mannheim, Indianapolis, IN) as substrate (35) and read on an Anthos Plate Reader (Anthos Labtec Instruments, Austria).

Determination of the number of stable insertion events:

The hygromycin resistant colonies were picked and individually transferred to 96 well plates, 24 wells plates and 6 well plates sequentially. Representative stocks of 20 resistant picks were frozen in liquid nitrogen and/or were grown in 100mm dishes for DNA isolation. Genomic DNA was prepared as described (36) and was slot blotted on to Magna nylon membranes (MSI, Westboro, MA). A 400 bp Pst I fragment from the Brn-2 coding region was isolated and radiolabelled by random priming with minor modifications to the manufacturer's instructions (Prime It, Stratagene, La Jolla, CA). The membrane was probed and washed, before exposure to storage phosphor screens. A parallel control experiment was performed using human γ-actin as probe. The phosphor storage screens were developed on a PhosphorImager 425B (Molecular Dynamics, Sunnyvale, CA). The signal intensities were quantitated using the ImageQuant 2.0 software (Molecular Dynamics), and the number of stable insertion events was estimated by comparison with the γ-actin signal. A transformed line with a high copy number (MAM-19) of transfected plasmid and another line with a low copy number (MAM-3) were identified for further experiments.

Electromobility Shifts :

Nuclear extracts from retinoic acid treated and untreated cells were prepared as previously described (21). B6/SJL mice (Jackson Labs, MA.) were used as a source for hypothalamic nuclear extracts. The extracts were prepared from tissue isolated and pooled from

the median hypothalamic region of three mice as previously outlined (37). Brn-2 protein was expressed by *in vitro* translation using the TNT T7 reticulocyte lysate system (Promega, Madison, WI). Complimentary DNA oligomers were synthesized, annealed and 3' end-filled with radioactive deoxy-nucleotides and used as the probe in the assay. The final filled in sequence was dTCTGCTCCTGCATAAATCATAGGGCC and has been shown to be a strong Brn-2 binding site (17). The non-specific oligonucleotide used in the competition reactions contained the CREB binding site with the final filled sequence which read dGATCGGATCCGATTGCCTGA-CGTCAGAGAGCAGATCTATCG. The activity of the different nuclear extracts were also checked using radiolabelled CREB oligonucleotide as probe. Equal amounts of total protein, as determined by Lowry's method (38), were used in the binding reactions which were performed as described (21). Unlabelled and filled, duplex oligonucleotide at 125 fold molar excess were used as competitors for protein binding. The binding reactions were resolved on a 4% poly-acrylamide gel in tris-taurine EDTA buffer. The gels were then fixed, dried and exposed to phosphor storage screens and the resulting images were processed on a Molecular Dynamics PhosphorImager.

Reverse-Transcriptase PCR:

RNA was isolated from treated cells as described (39). Total RNA was then used to generate cDNA utilizing the following primers -

Brn-2: Forward - dCGCCGACCTCGGACGACCTG

Reverse - dCCCCAGCTTGAGTTCACTGGACG

CRF: Forward - dCCAAGT-A/C-C-A/G-TTGAGAGACTGA

Reverse - dTTCCCCAGGCGGAGGAAGT

Cyclophilin: Forward - dTTCATCTGCACTGCCAAGAC

Reverse - dAACCCAAAGGGAACTGCAG

SuperScript reverse transcriptase (Life Technologies, Gaithersburg, MD) was used for cDNA synthesis as per manufacturer's instructions. As an internal control and for quantification,

the subsequent PCR reaction was spiked with constant amount of plasmid DNA containing the complimentary sites for the two primers. The PCR product from the spiked plasmid DNA is of a different length when compared to that obtained from the cDNA. An external control was also performed using the cyclophilin transcript as the target sequence. The PCR reactions were performed for 25 cycles as follows:

Bm-2: 95 °C for 30 sec, 64 °C for 30 sec and 72 °C for 2 minutes.

CRF: 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 minutes.

Cyclophilin: 95 °C for 60 sec, 52 °C for 60 sec and 72 °C for 2 minutes.

The PCR reactions were resolved on a 2% agarose gel and photographed with ethidium bromide UV fluorescence using Polaroid 557 film (Polaroid Corp.) The resulting negative was digitized on a Personal Densitometer (Molecular Dynamics) and quantitated using the ImageQuant software. A parallel experiment with known amounts of DNA was photographed and digitized for use as a standard.

Southern Blots:

Genomic DNA was isolated from BE(2)-M17 cells. The DNA was subjected to restriction enzyme digestion and resolved on a 0.7 % agarose gel. The blotting, hybridization and washing was carried out as previously described (31). A 400 bp Pst I fragment from the Bm-2 coding region was isolated and radiolabelled by random priming with minor modifications to the manufacturer's instructions (Prime It Kit, Stratagene, La Jolla, CA). Magna nylon membranes (MSI, Westboro, MA) were used as the hybridization medium. The membranes were exposed to a phosphor storage screen and visualized on the PhosphorImager. Normal human DNA was used as control and was a kind gift of Cris Welling (Internal Medicine, Washington University).

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FIGURE LEGENDS

***Figure 1** - Brn-2 is expressed in BE(2)-M17 cells upon induction with retinoic acid:*

A. The presence of Brn-2 site binding activity was assayed by electromobility shifts. The arrows indicate the specific shifts which are similar to those observed with hypothalamic tissue extracts. The probe used to obtain the shifts was a Brn-2 binding site from hCRF. Lane 1 - probe alone; Lane 2 - untreated BE(2)-M17 cells; Lane 3 - with 125 fold excess specific unlabelled competitor; Lane 4 - Three day retinoic acid treated BE(2)-M17 cells; Lane 5 - with 125 fold excess specific unlabelled competitor; Lane 6 - with 100 fold excess non-specific unlabelled competitor with a CREB binding site, Lane 7 - Translated Brn-2 protein. (+C, with specific unlabelled competitor ; +nsC, with non-specific unlabelled competitor.)

B. The presence of Brn-2 message was confirmed by RT-PCR. The arrow indicates the Brn-2 RT-PCR product. Lane 1 - molecular size ladder; Lane 2 - BE(2)-M17 cells induced with retinoic acid; Lane 3 - untreated BE(2)-M17 cells; Lane 4 - retinoic acid treated, low copy BE(2)-M17 stable transformant of PGK-αBrn-2; Lane -5 retinoic acid treated, high copy BE(2)-M17 stable transformant of PGK-αBrn-2

***Figure 2** - Brn-2 affects expression of CRF-Luciferase reporter in retinoic acid treated and untreated cells:*

Transient transfections were performed with CRF reporter plasmid along with either CMV Brn-2 (A) or PGK-αBrn-2 (B) in varying amounts as indicated. The cells were then harvested and assayed for luciferase as described. The standardized luciferase values are plotted along with the amount of transfected plasmid. (-□-) No retinoic acid; (-O-) treated with retinoic acid. Results are means ± StdDev. of at least 3 experiments.

***Figure 3** - Brn-2 binding sites in isolation are both retinoic acid and Brn-2 responsive:*

Transient transfections were carried out in the BE(2)-M17 cell line. Luciferase reporter constructs used were either a minimal P36 promoter, a P36 promoter with 3 tandem Brn-2 binding sites, or a 5 Kb hCRF promoter. Each of the reporters was examined under 3 different conditions and results are shown as Fold Expression, determined as the ratio of luciferase activity from each treatment versus its corresponding control. Activity in the absence (control) or presence of retinoic acid, open bars; activity with co-transfection of either CMV-Neo (control) or CMV-Brn-2 plasmids, hatched bars; activity with co-transfection of either PGK-MT (control) or PGK-αBrn-2, solid bars. Results are means ± StdDev of 3 experiments.

***Figure 4** - Stable transformation of sense and anti-sense Brn-2 constructs affect inducibility by Retinoic Acid:*

Stable transformants of the indicated plasmids were made in the BE(2)-M17 cell. MAM- 3 and MAM-19 are low and high copy transformants with PGK-αBrn-2. The transformants were then transiently transfected with a CRF reporter plasmid and then either, left untreated or treated with 5 uM retinoic acid. The cells were then assayed for luciferase activity. The luciferase values obtained were then standardized to that of the untreated wild type BE(2)-M17 cells. Results are means ± StdDev of at least 3 experiments.

***Figure 5** - Endogenous CRF expression in BE(2)-M17 cell lines is affected by Brn-2*:

Total RNA isolated from the indicated cells was subjected to RT-PCR. After RT, the reactions were spiked with 0.1ng of a plasmid containing complementary sequences to the PCR primers, to serve as an internal control. The reactions were resolved by electrophoresis. Lane 1 - untreated BE(2)-M17 cells; Lane 2 - retinoic acid treated BE(2)-M17 cells; Lane 3 - retinoic acid treated, low copy BE(2)-M17 stable transformant of PGK-αBrn-2 ; Lane 4 - retinoic acid treated, high copy BE(2)-M17 stable transformant of PGK-αBrn-2 .

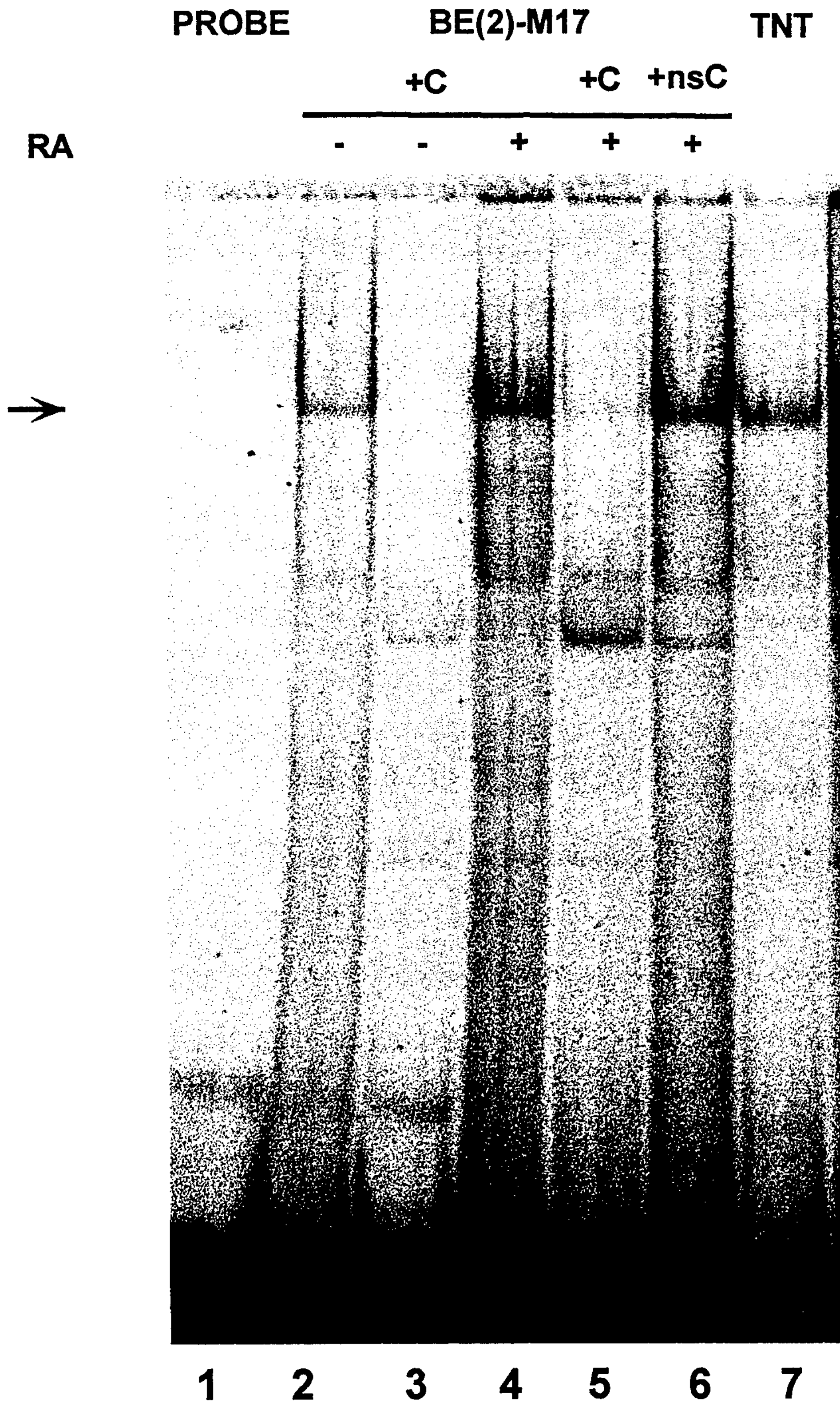
***Figure 6** - Retinoic acid induction is not mediated by the CRE:*

Transient transfections were carried out in the BE(2)-M17 cell line as described. Luciferase reporter constructs used were either a 532 bp CRF promoter (CRF-532) or a similar construct but, with its CRE at (-220) mutated to an Eco RI site (-532 X-CRE). CRF-532 was also co-transfected with PKI, a peptide inhibitor of Protein Kinase A. All the transfections were done, both in the presence (open bars) and absence (solid bars) of Retinoic acid. Results are calculated means ± StdDev of at least 3 experiments and the mean of the luciferase values of CRF-532 in the absence of Retinoic acid was arbitrarily set to 1.

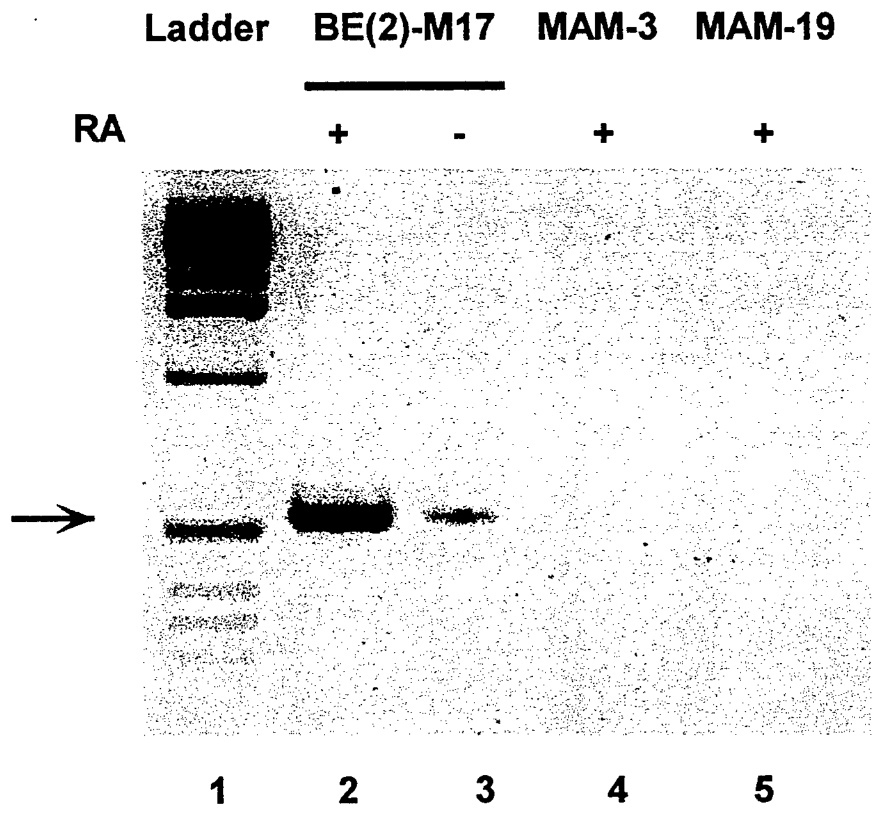
***Figure 7** - Model for a parallel role for Pit-1 and Brn-2 in development:*

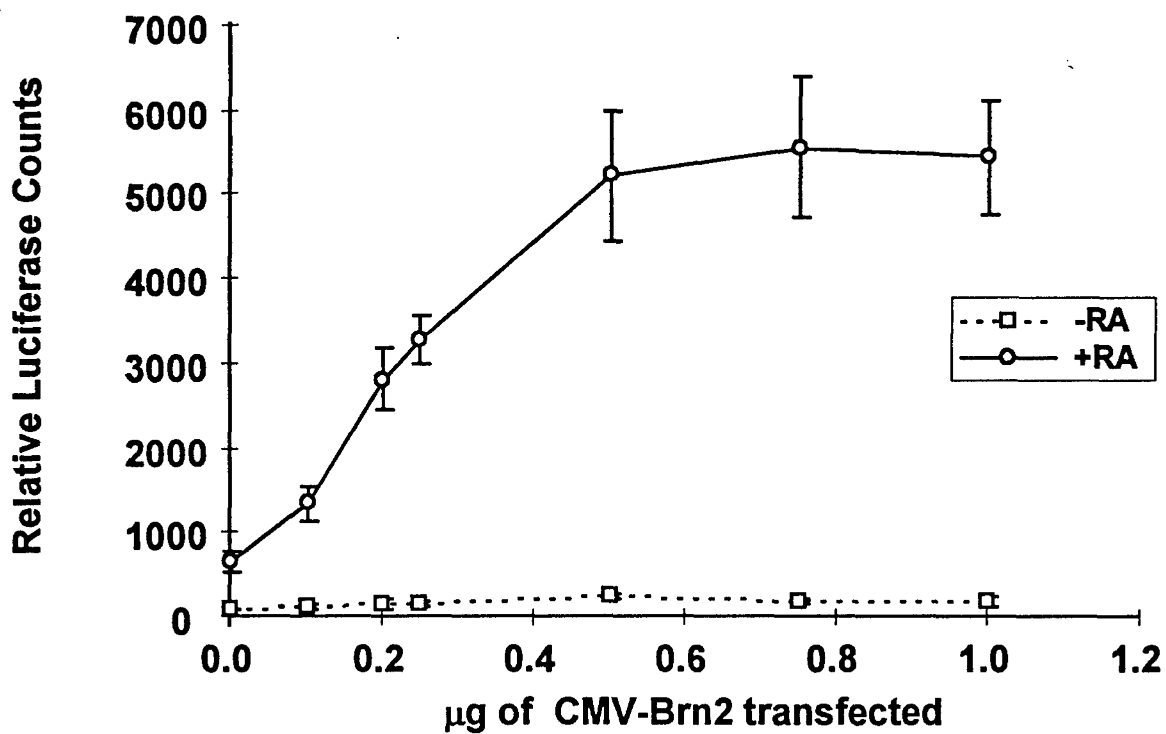
Based on the experiments described in this study, we present a possible model for the role of Brn-2 in CRF neuron development and CRF gene expression. This model reflects the established role of Pit-1 in the development of lactotrophs and expression of the prolactin gene. We further suggest parallel roles for the nuclear receptors, Estrogen Receptor (ER) and a member of the Retinoic Acid Receptor (RAR/RXR) family, along with the documented negative effect of glucocorticoid receptor (GR) on gene transcription in both cases.

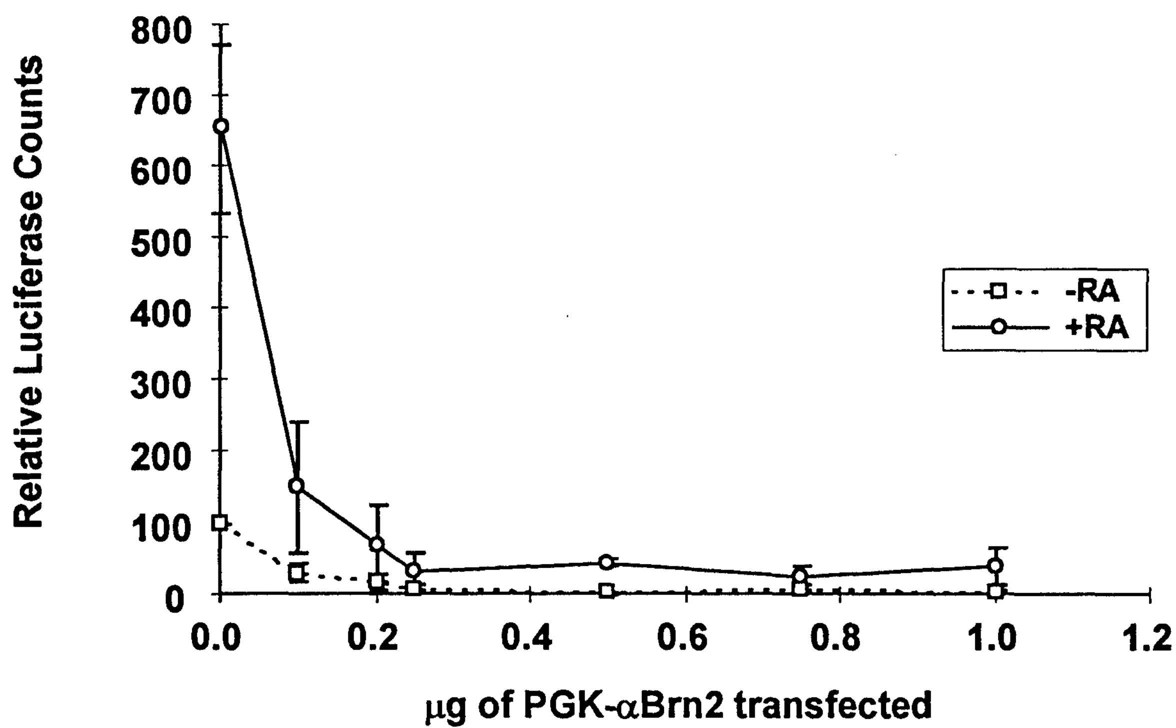
A

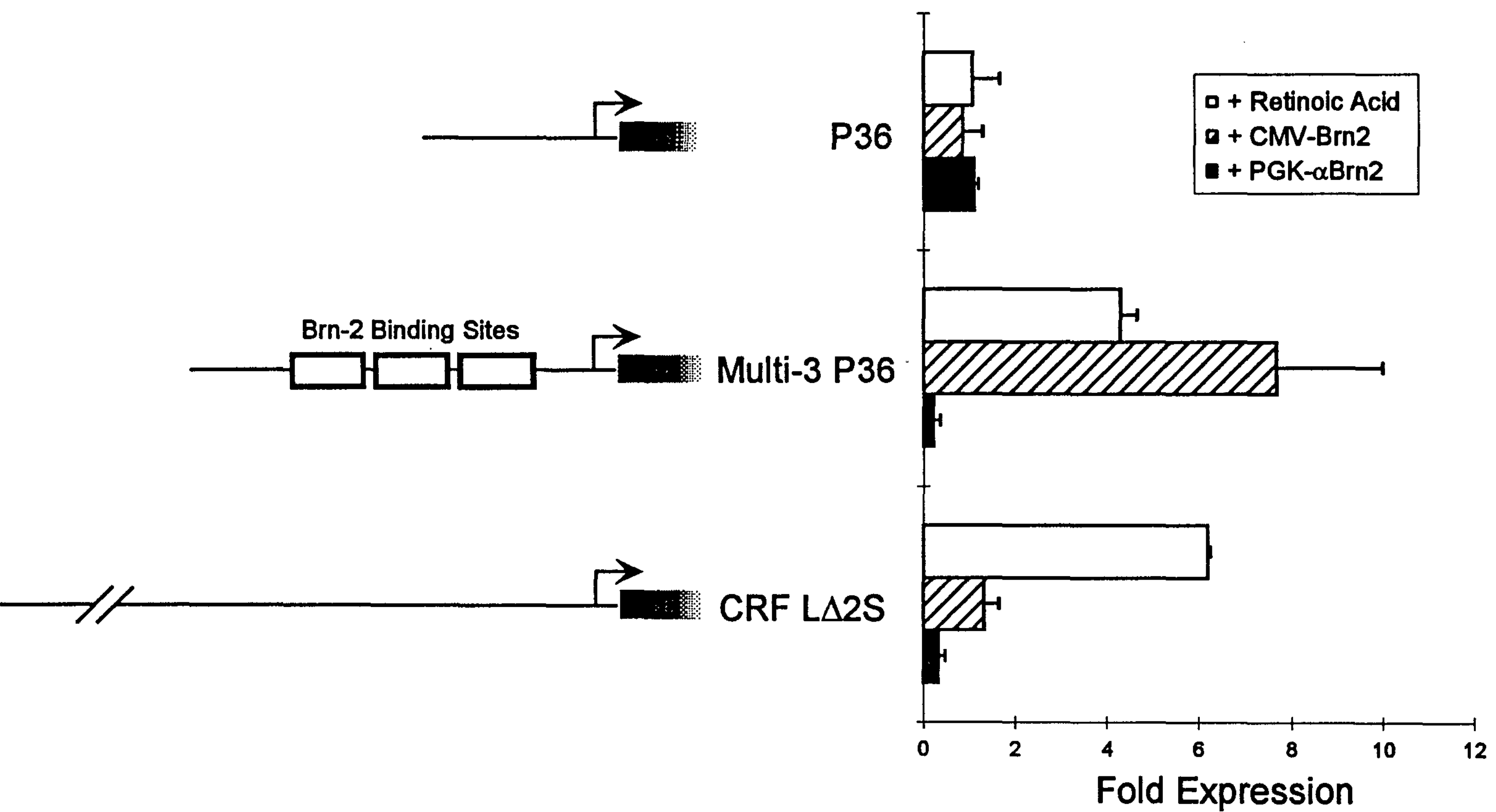


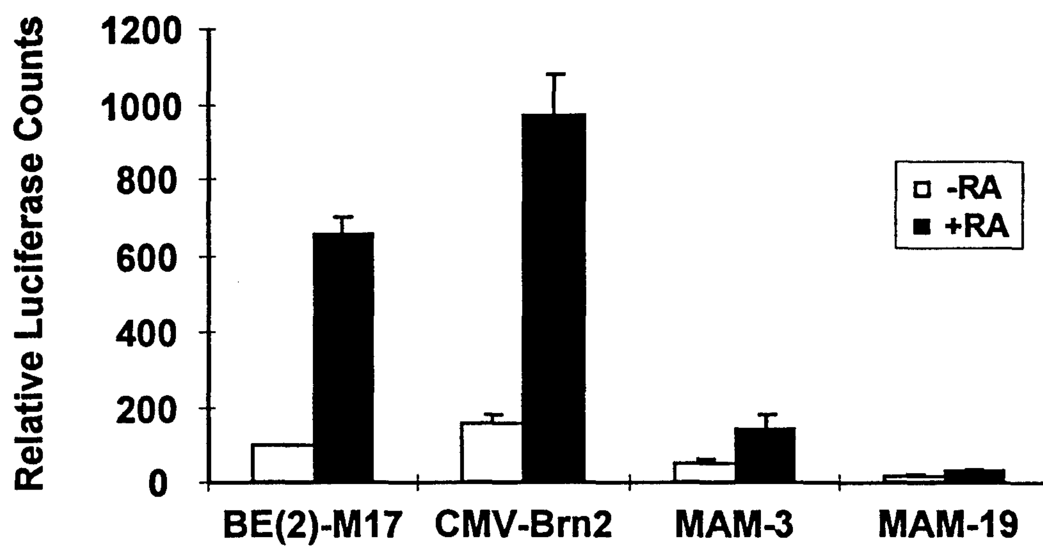
B



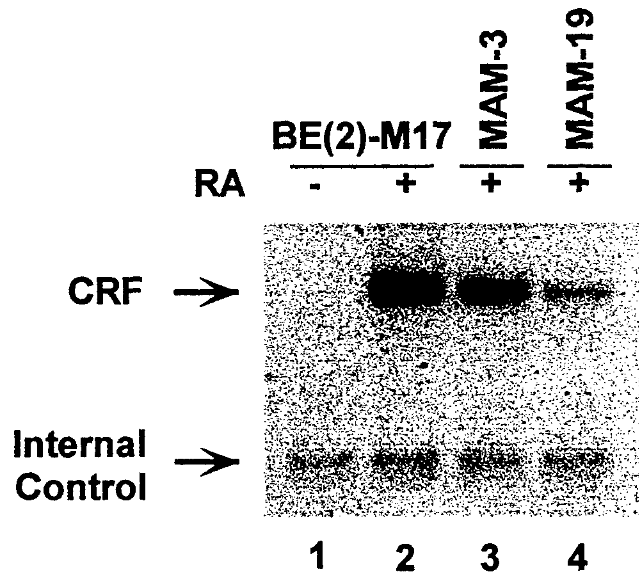

A
Relative Luciferase Counts
7000
6000
5000
4000
3000
2000
1000
0
-RA
+RA
0.0
0.2
0.4
0.6
0.8
1.0
1.2
μg of CMV-Brn2 transfected

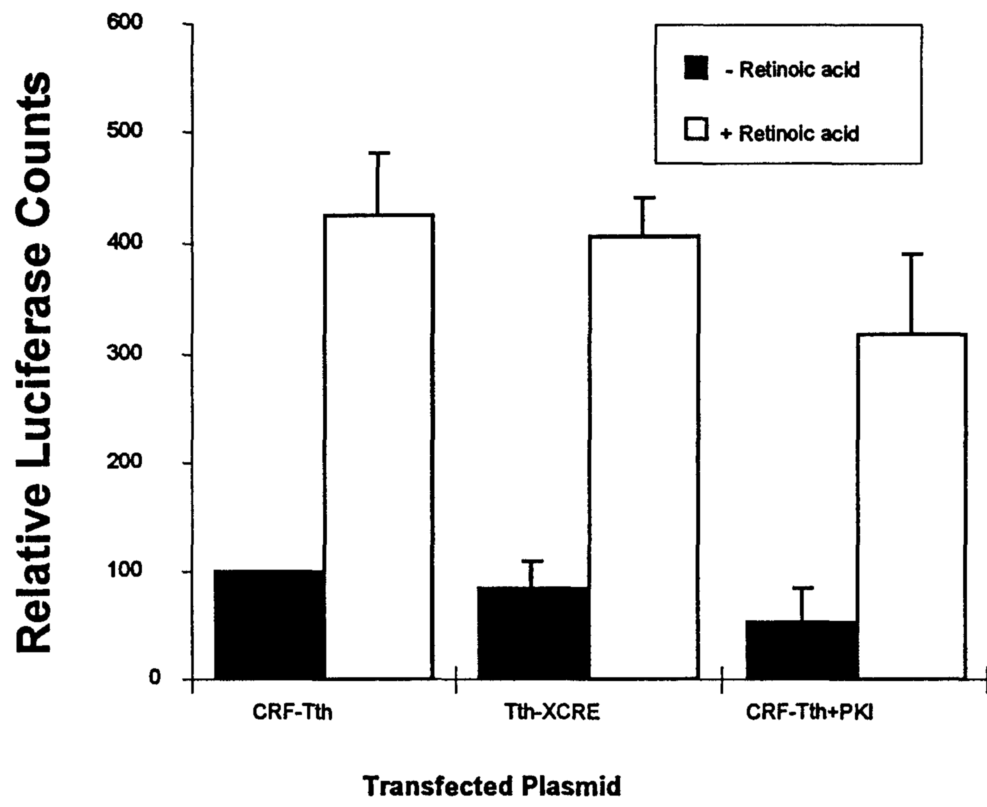

B
Relative Luciferase Counts
800
700
600
500
400
300
200
100
0
-RA
+RA
0.0
0.2
0.4
0.6
0.8
1.0
1.2
μg of PGK-αBrn2 transfected


+ Retinoic Acid
+ CMV-Brn2
+ PGK-αBrn2
P36
Brn-2 Binding Sites
Multi-3 P36
CRF LΔ2S
0
2
4
6
8
10
12
Fold Expression

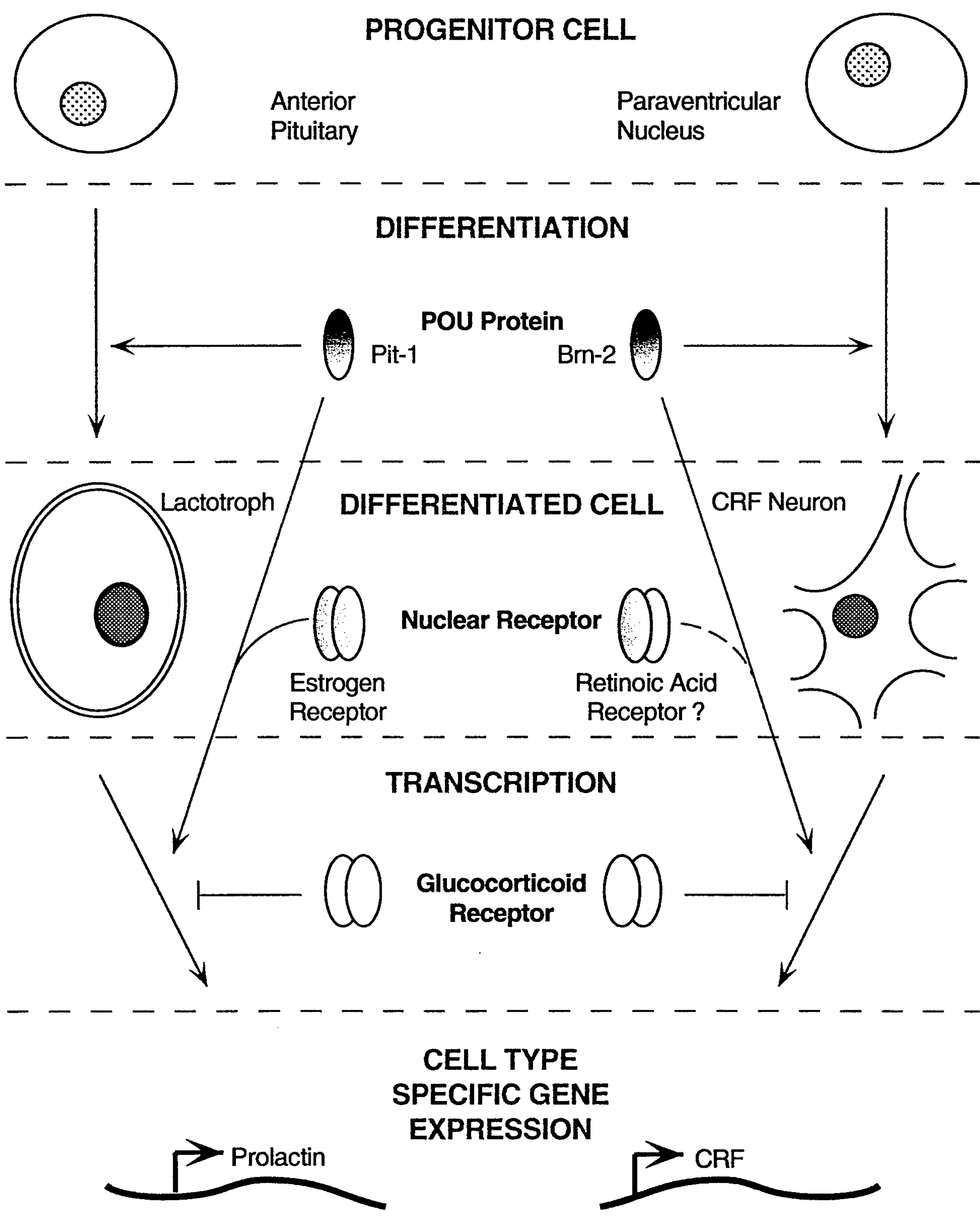


Relative Luciferase Counts
1200
1000
800
600
400
200
0
-RA
+RA
BE(2)-M17
CMV-Brn2
MAM-3
MAM-19


BE(2)-M17
MAM-3
MAM-19
RA
-
+
+
+
CRF
Internal
Control
1
2
3
4



Relative Luciferase Counts
600
500
400
300
200
100
0
- Retinoic acid
+ Retinoic acid
CRF-Tth
Tth-XCRE
CRF-Tth+PKI
Transfected Plasmid



PROGENITOR CELL
Anterior Pituitary
Paraventricular Nucleus
DIFFERENTIATION
POU Protein
Pit-1
Brn-2
DIFFERENTIATED CELL
Lactotroph
CRF Neuron
Nuclear Receptor
Estrogen Receptor
Retinoic Acid Receptor ?
TRANSCRIPTION
Glucocorticoid Receptor
CELL TYPE SPECIFIC GENE EXPRESSION
Prolactin
CRF

Table 1: Quantitation of expression levels of CRF:

Cell line	# of stable insertion events	Transfection		Reverse Transcriptase PCR	
		Expression Levels upon RA induction	Fold Induction with RA	Expression Levels upon RA induction	Estimated Fold Induction with RA *
BE(2)-M17	0	100	6.5±0.8	100±13	>13
MAM-3	3	22±4	3.1±0.3	74±17	>10
MAM-19	19	4±1	2.5±0.3	26±3	>4
+CMV Brn-2	nd	150±17	6.3±1.1	nd	nd

* - Since endogenous CRF mRNA is not detectable in the uninduced cells, the lightest recognizable band from a standard curve was used as the reference point and hence, all folds are presented as greater than the calculated values. The levels of induced expression and fold induction are represented as calculated means ± Std. Dev. ($n \geq 4$).

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REPLY TO
ATTENTION OF:

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2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

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